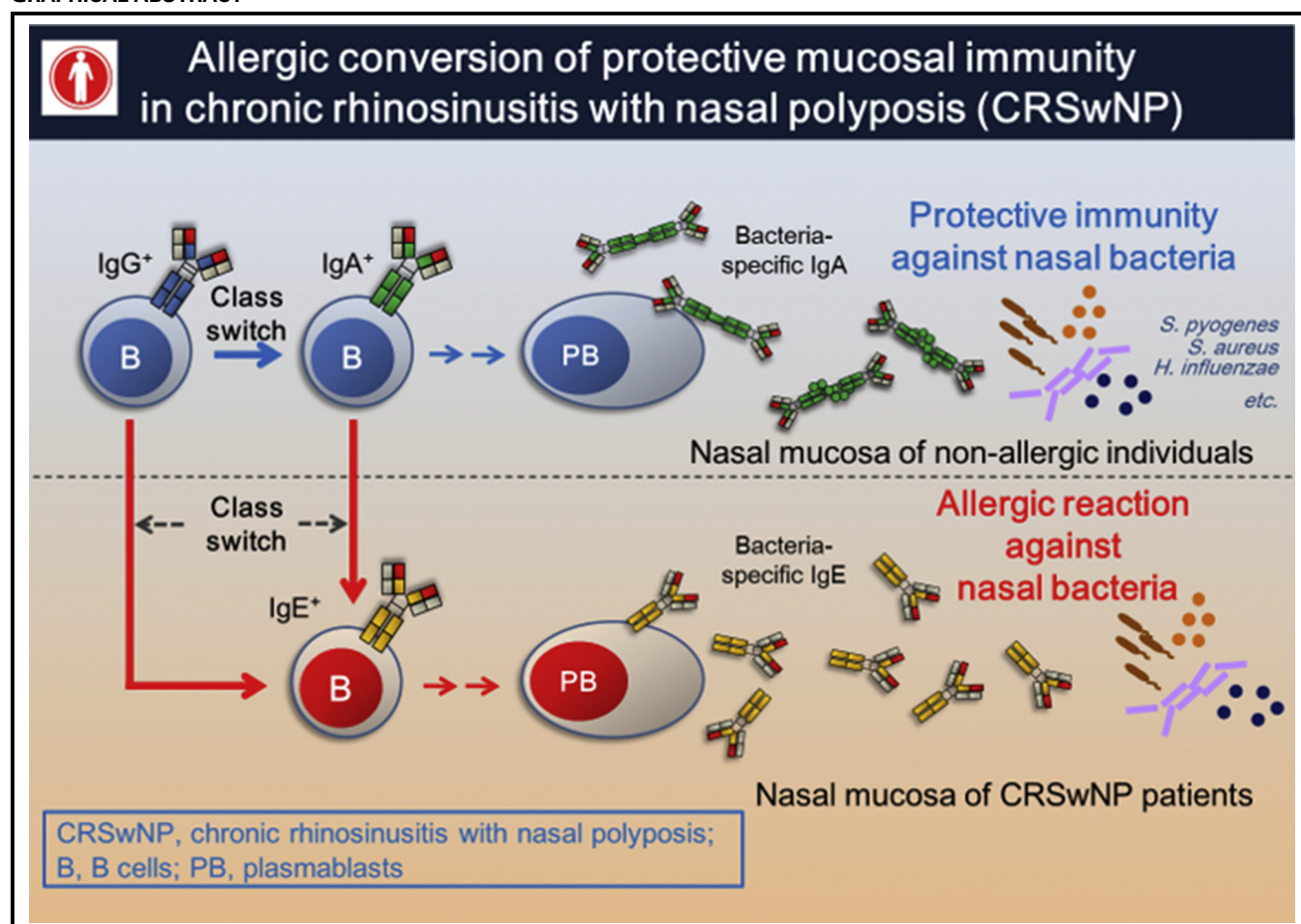


Allergic conversion of protective mucosal immunity against nasal bacteria in patients with chronic rhinosinusitis with nasal polyposis



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GRAPHICAL ABSTRACT



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Background: Chronic rhinosinusitis with nasal polyposis (CRSwNP) is characterized by eosinophilic inflammation and polyposis at the nose and paranasal sinus and a high concentration of IgE in nasal polyps (NPs). The causative antigen and pathogenesis of CRSwNP remain unknown.

Objective: We aimed to identify reactive allergens of IgE antibodies produced locally in NPs of patients with CRSwNP. We also attempted to unravel the differentiation pathway of IgE-producing B cells in NPs.

Methods: IgE reactivity of patients with CRSwNP was investigated by characterizing single cell-derived mAbs. T-cell response against identified allergens was investigated *in vitro*. NP-infiltrating lymphocytes were characterized by using flow cytometry. Immunoglobulins expressed in NPs were analyzed by using high-throughput DNA sequencing for immunoglobulin.

Results: About 20% of isolated IgE antibodies derived from NP-residing plasmablasts specifically recognized surface determinants of nasal bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Haemophilus influenzae*. A T_H2 response against *S pyogenes* was observed in patients with CRSwNP. Flow cytometric analysis revealed sizable germinal center B-like cell and plasmablast subsets expressing IgE on the cell surface in NPs. High-throughput DNA sequencing immunoglobulin analysis highlighted the clonal connectivity of IgE with IgG and IgA₁. The Ie-Cα₁ circle transcript was detected in NPs.

Conclusions: In patients with CRSwNP, nasal bacteria-reactive B cells differentiate into IgE-producing B cells through IgG/IgA₁-IgE class switching, suggesting that allergic conversion of the mucosal response against nasal bacteria underlies disease pathogenesis. (J Allergy Clin Immunol 2019;143:1163-75.)

Key words: Chronic rhinosinusitis with nasal polyposis, IgE, human mAb, high-throughput immunoglobulin analysis, T_H2 inflammation

Chronic rhinosinusitis with nasal polyps (CRSwNP), also known as eosinophilic chronic rhinosinusitis, is characterized by T_H2 inflammation, aggressive eosinophilic infiltration, and tissue remodeling of the nasal mucosa.¹⁻⁴ T_H2 cytokines are robustly produced in nasal polyps (NPs) and contribute to eosinophil maturation, activation, and migration.¹⁻⁴ Activated eosinophils in the nasal mucosa release lipid mediators and tissue-damaging proteases, resulting in polyposis. The pathoetiology of CRSwNP is not firmly defined⁵ and can result from multiple factors. Specifically, it has been suggested that *Staphylococcus aureus*-derived superantigens (staphylococcal enterotoxins [SEs]), such as SEB, might have an etiological role in patients with CRSwNP.⁵⁻⁸ For example, previous studies have reported an increased colonization rate of *S aureus* in NPs of patients with CRSwNP,⁹ and the T-cell receptor Vβ genes targeted by SEB and staphylococcal toxic shock syndrome toxin have been found to be expanded in NPs of patients with CRSwNP.¹⁰ In addition to superantigenic properties, SEB has been reported to induce production of T_H2 cytokines in NP explants.¹¹

Together, these observations indicate that SEs are a prominent factor in CRSwNP pathogenesis. However, other reports have found similar rates of *S aureus* colonization between patients with CRSwNP and those with chronic rhinosinusitis without nasal polyps.^{12,13} Therefore it remains unclear whether CRSwNP is

Abbreviations used

AID:	Activation-induced deaminase
BCL-6:	B-cell lymphoma 6
CMV:	Cytomegalovirus
CRSwNP:	Chronic rhinosinusitis with nasal polyposis
CSR:	Class-switch recombination
CTV:	CellTrace Violet
GC:	Germinal center
HTS:	High-throughput DNA sequencing
ICOS:	Inducible costimulator
LTA:	Lipoteichoic acid
NP:	Nasal polyp
PD-1:	Programmed cell death protein 1
SE:	Staphylococcal enterotoxin
SHM:	Somatic hypermutation
T _{FH} :	Follicular helper T
VH:	Variable region of immunoglobulin heavy chain

caused solely by bacterial superantigens or by specific allergens like other allergic disorders.^{5,14,15}

IgE is another key player in the pathogenesis of CRSwNP, and anti-IgE treatment can mitigate the associated polyposis.^{16,17} Nevertheless, it is not clear which antigens or allergens induce IgE production in patients with CRSwNP. Furthermore, although IgE is thought to be produced mainly by mucosa-infiltrating plasmablasts in NPs,¹⁸ the differentiation process of IgE-producing cells remains to be elucidated. A subset of patients with CRSwNP have ectopic lymphoid follicles in NPs,¹⁹ and evidence of local class-switch recombination (CSR) from IgG to IgE has been reported.^{20,21} Although sequential switching to IgE has been well characterized in mice, in which IgG⁺ germinal center (GC) B cells serve as precursor cells of IgE⁺ B cells in secondary lymphoid tissues,^{22,23} it is not clear at which stage of B-cell differentiation CSR to IgE takes place in NPs of patients with CRSwNP.

In this study we generated and analyzed recombinant mAbs derived from IgE⁺ plasmablasts residing in NPs of patients with CRSwNP, with the goal of identifying their target antigens. We also analyzed IgE repertoires using high-throughput DNA sequencing (HTS) of immunoglobulin transcripts in NPs. Furthermore, we attempted to identify the lymphocyte subsets involved in IgE production. Our study not only identified target antigens of NP-derived IgE but also elucidated the differentiation process of IgE-producing cells in NPs of patients with CRSwNP.

METHODS

Human subjects

Clinical specimens were collected at Osaka University Hospital and Ikeda Municipal Hospital (Osaka, Japan), according to the protocol approved by Osaka University Research Ethics Committee and Ikeda Municipal Hospital Research Ethics Committee. Bilateral NPs were collected from 46 patients with CRSwNP. Diagnosis of CRSwNP was made based on the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 criteria⁵ and the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis.²⁴ All patients with CRSwNP in this study had tissue eosinophilia (≥70/high-powered field). Normal nasal tissues were collected from the turbinate or uncinate tissues of 15 control subjects (anatomic nasal obstruction, sinonasal benign tumors, and bone fracture) during routine

TABLE I. Patients' characteristics

ID	NP8	NP9	NP12	NP23
Age and sex	39 F	77 M	51 F	46 M
Asthma	Yes	Yes	No	Yes
Aspirin intolerance	No	No	No	No
Duration of disease (y)	10	30	2	6
No. of nasal surgeries	2	10	1	6
Blood eosinophils (/mm ³)	713	1021	189	357
Blood eosinophils (%)	8.6	13.8	3.6	5.6
Polyp eosinophils (/HPF [eosinophilic CRS ≥70/HPF])	368	88	93	279
JESREC score (eosinophilic CRS ≥11)	15	15	11	15
Bacterial culture test (Swab)	<i>M catarrhalis</i>	<i>Corynebacterium</i> species	No colony	<i>S aureus</i>
Total serum IgE (IU/mL)	104.7	974.6	214.0	656.0
Total polyp IgE (ng/mL)	44.9	1202.5	547.7	553.5
Specific serum IgE (IU/mL)				
Orchard grass	<0.10	<0.10	<0.10	<0.10
Ragweed	<0.10	<0.10	<0.10	<0.10
Mugwort	<0.10	<0.10	<0.10	<0.10
White birch	<0.10	<0.10	<0.10	<0.10
Cedar pollen	4.75	<0.10	0.99	<0.10
Cat dander	0.12	<0.10	<0.10	<0.10
Dog dander	<0.10	<0.10	<0.10	<0.10
<i>Cladosporium</i> species	0.28	<0.10	<0.10	<0.10
<i>Aspergillus</i> species	0.31	<0.10	<0.10	<0.10
<i>Candida</i> species	0.6	<0.10	<0.10	0.81
<i>Alternaria</i> species	0.34	<0.10	<0.10	<0.10
Cockroach	<0.10	<0.10	<0.10	<0.10
House dust mite (<i>Dermatophagoides pteronyssinus</i>)	NA	<0.10	<0.10	9.07
House dust mite (<i>Dermatophagoides farinae</i>)	16.2	<0.10	<0.10	15.1
SEA	<0.10	0.21	<0.10	<0.10
SEB	<0.10	<0.10	<0.10	<0.10

F, Female; HPF, high-powered field; JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; M, male; NA, not analyzed.

sinonasal surgery. Tonsils were obtained from patients with IgA nephropathy with tonsillitis during routine tonsillectomies. Blood samples were collected from patients with CRSwNP, patients with allergic rhinitis, and healthy donors.

Antibody cloning and expression

Single cell-derived mAbs were generated, as reported previously,²⁵ with minor modifications. IgE⁺ plasmablast-derived mAbs were cloned from 4 NPs randomly selected from among 46 subjects (Table I). IgG₁⁺ plasmablast-derived mAbs were isolated from tonsils of 2 different patients with tonsillitis (see Table E1 in this article's Online Repository at www.jacionline.org). For NP samples, single-cell suspensions were prepared with the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), and then CD45⁺ cells were enriched by using autoMACS (Miltenyi Biotec). Single plasmablasts (CD45⁺CD3⁻CD14⁻CD56⁻CD66b⁻CD19⁺CD20^{lo}CD27^{hi}CD38^{hi}) were isolated into 0.2-mL 96-well PCR plates with the BD FACSAria II (BD Biosciences, San Jose, Calif).

After reverse transcription, we amplified IgE heavy chain, Igκ, and Igλ with multiple primers (see Table E2 in this article's Online Repository at www.jacionline.org) in nested PCR.²⁵ Agarose gel electrophoresis confirmed that 15% to 30% of isolated cells expressed IgE. The variable regions of IgE, Igκ, and Igλ were coupled with the constant regions of IgG₁, Igκ, and Igλ, respectively (Fig 1, A) in mammalian expression vectors. The conversion of IgE to IgG enabled us to use secondary antibodies for detection in flow cytometry. HEK293T cells were cotransfected with immunoglobulin heavy and light chain-encoding plasmids.

After a 4-day culture, supernatants were harvested, and antibody concentrations were determined by using a sandwich ELISA. Obtained immunoglobulins were analyzed by using IMGT/V-QUEST (www.imgt.org; see Fig E1, D, in this article's Online Repository at www.jacionline.org).²⁶

For reversion of mutations, predicted germline variable region of immunoglobulin heavy chain (VH) and light chain (VL) of 8B4, 91G9, 23B12, 23E5, 23G2, and 23G9 were custom synthesized and inserted into the corresponding expression vectors for assays shown in Fig 2, C and D, and Fig E1, E. The reverted antibodies were generated as described above.

Reactivity tests

All tested antigens in the assay are shown in Fig E1, A. For the modified IMMULITE 2000 3g Allergy system (Siemens, Munich, Germany), anti-human IgG secondary antibody was used for detection. An ELISA was performed, as described elsewhere.²⁷ For some bacteria, cleared bacterial lysate in PBS containing 0.1% Tween 20 was used. Cell-wall polysaccharides of *Streptococcus pyogenes* were prepared, as described elsewhere.²⁸ Mite antigens, lipoteichoic acid (LTA), and SEs were purchased from Wako (Osaka, Japan), Sigma-Aldrich (St Louis, Mo), and Toxin Technology (Sarasota, Fla), respectively. For bacterial flow cytometry and fluorescent microscopy, bacteria (see Table E3 in this article's Online Repository at www.jacionline.org) were incubated with mAbs at 1 μg/mL in 1% BSA/PBS containing proteinase inhibitors (Roche, Mannheim, Germany) for 15 minutes on ice. After washing, bound antibodies were detected by using Alexa Fluor 647- and Alexa Fluor 488-conjugated anti-IgG secondary antibody (Thermo Scientific, Waltham, Mass) for fluorescence-activated cell sorting and fluorescent microscopy (see Fig E1, B), respectively.

In vitro T-cell stimulation assay

PBMCs from 18 healthy donors and 18 patients with CRSwNP (see Table E4 in this article's Online Repository at www.jacionline.org) were isolated by means of gradient separation with Lymphoprep (STEMCELL

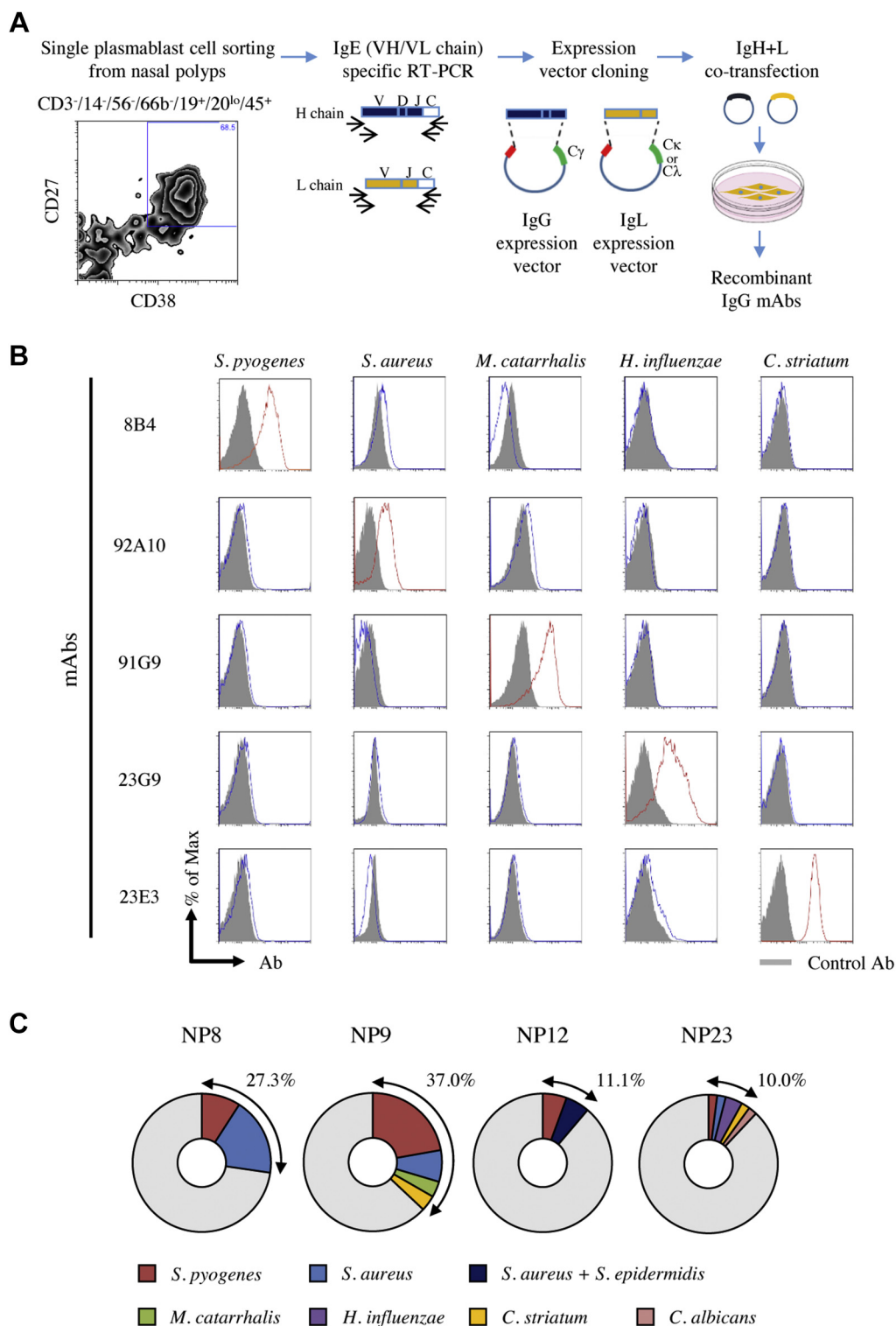


FIG 1. Antibacterial clones isolated from plasmablasts residing in NPs. **A**, Schematic representation of mAb cloning from IgE expressed in NP-residing plasmablasts. **B**, Flow cytometry of bacteria stained with representative antibacterial mAb clones. Gray-shaded area, Control antibody (Ab). **C**, Frequency of nasal bacteria-specific clones in mAbs isolated from NP-residing plasmablasts of the indicated donors (NP8, n = 11; NP9, n = 27; NP12, n = 18; and NP23, n = 50).

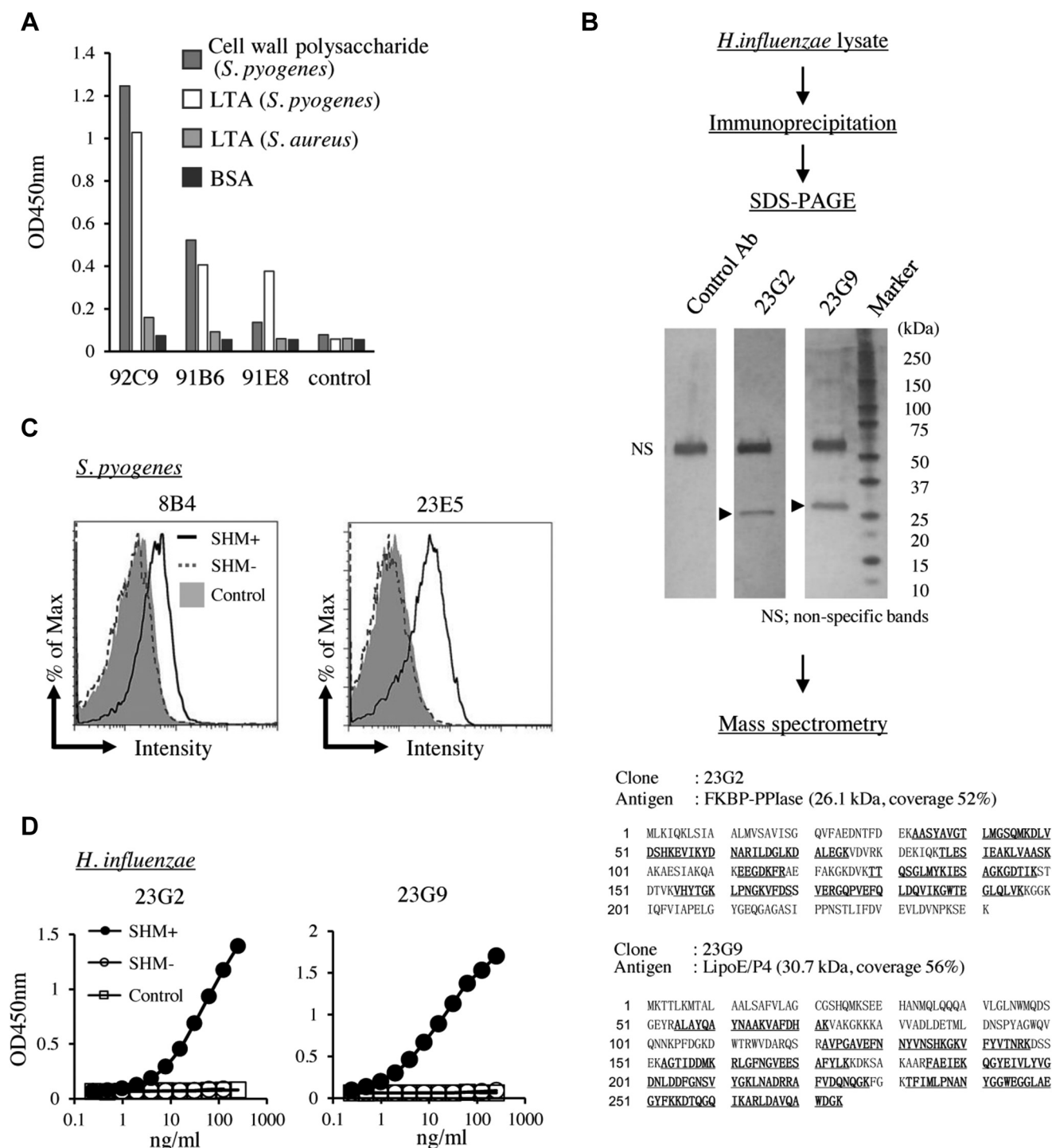


FIG 2. SHM is critical for acquisition of antibacterial reactivity. **A**, Antigen specificity of anti-*S. pyogenes* clones against bacterial cell-wall components in ELISA. **B**, Antigen identification by using mass spectrometry. NS, Nonspecific band. Molecular weight-matched sequences in peptide mass fingerprint are underlined. **C** and **D**, Reduced reactivity of reverted mAbs (SHM-) in fluorescence-activated cell sorting (Fig 2, C) and ELISA (Fig 2, D). SHM+, Mutated antibodies. An unrelated human IgG mAb was used as a control.

Technologies, Vancouver, British Columbia, Canada). Intracellular cytokine production of T cells after stimulation was measured, as described elsewhere.²⁹ Detailed methods can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

HTS

cDNA was synthesized from NP19- and NP23-derived CD45⁺ lymphocytes (see [Table E5](#) in this article's Online Repository at www.jacionline.org), as described previously.²⁷ The VH regions of each

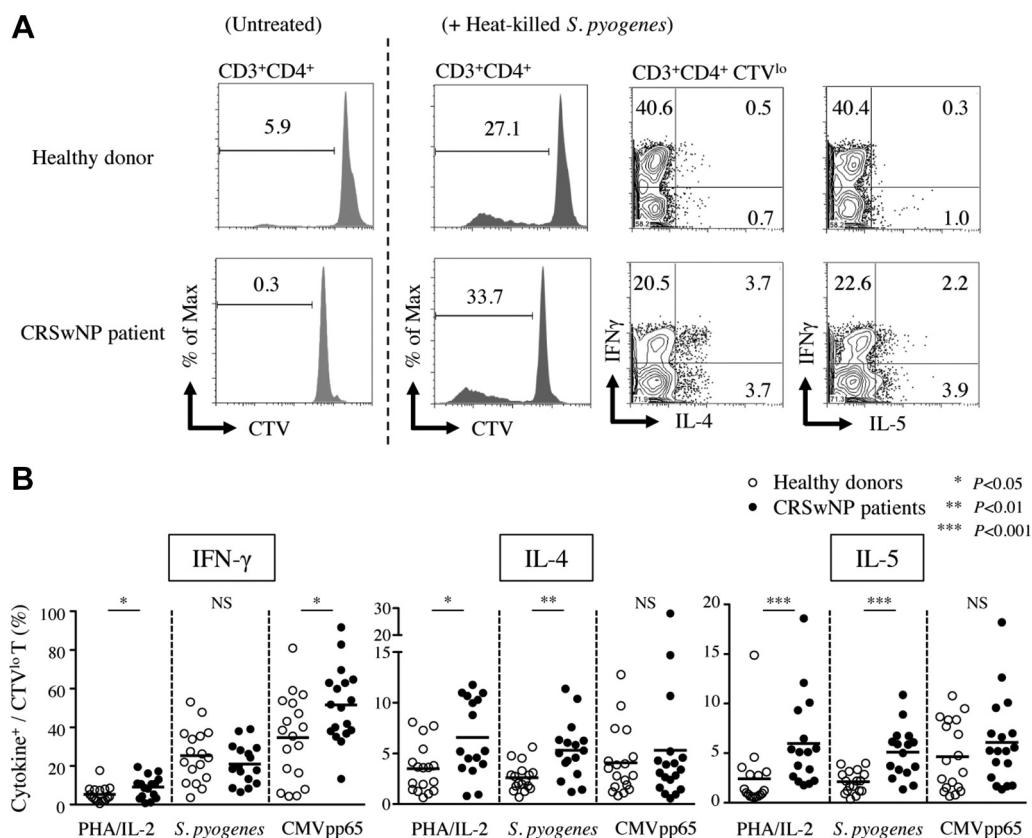


FIG 3. T_H2 response against *S. pyogenes* in patients with CRSwNP. **A**, Representative fluorescence-activated cell sorting histograms and plots of intracellular cytokine expression of CD4⁺ T cells treated with or without bacterial antigens. CellTrace Violet (CTV)-stained PBMCs were stimulated with heat-killed *S. pyogenes* for 7 days. **B**, Frequency of cytokine-producing CTV^{lo}CD4⁺ T cells after stimulation with PHA/IL-2 (healthy subjects, n = 17; patients with CRSwNP, n = 16), *S. pyogenes* (healthy subjects, n = 17; patients with CRSwNP, n = 16), CMV pp65 peptide (healthy subjects, n = 18; patients with CRSwNP, n = 18). **P* < .05, ***P* < .01, and ****P* < .001, Mann-Whitney test. NS, Not significant.

immunoglobulin isotype were separately amplified by using PCR (25–30 cycles) with primers for VH3 and respective isotypes (see Table E2). For MiSeq sequencing, purified amplicons (approximately 500 bp) were subjected to library preparation and paired-end sequencing by using the MiSeq v3 kit (Illumina, San Diego, Calif). Obtained raw sequences were merged with PEAR 0.9.6³⁰ after removal of low-quality sequences. Processing of immunoglobulin gene annotation, clonal gene alignment,³¹ and other analytic methods are described in the Methods section in this article's Online Repository.

Immunoglobulin circle transcripts

The expression of circle transcripts was examined, as described elsewhere,³² with minor modifications. The targeted sites of primers are shown in Fig E2, B, in this article's Online Repository at www.jacionline.org. The sequences of PCR products were verified by using Sanger sequencing (see Fig E2, C). Detailed information can be found in the Methods section in this article's Online Repository.

Statistical analysis

All statistical analyses were performed with Prism 5 software (GraphPad Software, La Jolla, Calif). *P* values were determined by using the Mann-Whitney method. For correlation analysis, Spearman coefficients and *P* values were calculated. Two-tailed *P* values of less than .05 were considered statistically significant.

Further detailed methods can be found in the Methods section in this article's Online Repository.

RESULTS

High frequencies of NP-infiltrating plasmablasts produce IgE antibodies specific for nasal bacteria

To identify targets of IgE produced in NPs of patients with CRSwNP, we generated mAbs derived from IgE expressed in NP-residing plasmablasts of 4 patients (Table I). We amplified variable region sequences of IgE heavy and light chains from single plasmablasts. In the cloning procedure, we ligated VDJ fragments of the IgE heavy chain with the IgG₁ constant (C γ ₁) region to generate recombinant mAbs as IgG (Fig 1, A). We generated 106 mAbs from the 4 patients and investigated their reactivity.

First, using a modified IMMULITE2000 3g Allergy system, we screened mAbs for their reactivity against major inhalant allergens such as pollens, mites, and fungi; however, no clones were reactive to known allergens (see Fig E1, A).

Subsequently, we analyzed the capacity of mAb clones to bind representative nasal bacteria by using flow cytometry (see Table E3). Tested bacteria were selected based on a previously reported human microbiome study.³³ In the assay 10% to 37% of recombinant mAbs generated from these 4 patients reacted with bacterial antigens, including *S. aureus*, *S. pyogenes*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Corynebacterium striatum* (Fig 1, B and C). Additionally, we identified 1 *Candida*

albicans-specific clone (Fig 1, C, and see Fig E1, A and B). Although we found several specific clones reactive to *S aureus*, IgE antibodies specific to SEB or other SEs were not isolated from the 4 patients in contrast to previous reports (see Fig E1, A).^{34,35} We also found that serum titers of anti-*S pyogenes* LTA were greater in patients with CRSwNP than in patients with allergic rhinitis (see Fig E1, C, and Table E6). These results suggest that nasal bacteria are major targets of IgE in patients with CRSwNP.

NP-derived IgE clones specifically recognize bacterial surface structure in a somatic hypermutation-dependent manner

Representative mAb clones were further investigated to determine the identities of their antigens. ELISA for cell-wall components revealed that 3 (92C9, 91B6, and 91E8) of 9 anti-*S pyogenes* clones reacted with cell-wall polysaccharides and/or LTAs derived from *S pyogenes* but not with *S aureus*-derived LTA (Fig 2, A). For the anti-*H influenzae* clones 23G2 and 23G9, the respective antigens were affinity purified from bacterial lysates. Mass spectrometric analysis revealed that the antigens of 23G2 and 23G9 were periplasmic FKBP-type peptidyl-prolyl cis-trans isomerase (FKBP-PPIase) and outer membrane lipoprotein E, respectively (Fig 2, B). Consistent with the results of flow cytometry, the antibacterial clones specifically recognized various bacterial surface structures.

The NP-derived IgE antibodies contained relatively large numbers of mutations, with the exception of antibodies from NP12, which was derived from a patient with CRSwNP for a relatively short period (2 years) in comparison with the others, who had undergone sinus surgery several times during their long disease histories (6-30 years; see Fig E1, D, and Table I). Numbers of VH mutations in IgE antibodies were 25.6 ± 10.6 ($n = 26$), 24.4 ± 12.1 ($n = 70$), and 24.0 ± 10.0 ($n = 63$) in NP8, NP9, and NP23, respectively, exceeding the number in IgG₁ clones isolated from tonsillar plasmablasts, which are representative secondary lymphoid organs (14.7 ± 9.0 [$n = 19$] and 15.5 ± 9.9 [$n = 27$] from 2 patients with tonsillitis; see Table E1). The introduction of reverse mutations completely abolished antibacterial staining of the *S pyogenes*-reactive clones 8B4 and 23E5 (Fig 2, C). Reverse mutants of clones 23G2, 23G9 (anti-*H influenzae*; Fig 2, D), 91G9 (anti-*M catarrhalis*), and 23B12 (anti-*C albicans*; see Fig E1, E) also lost their reactivity to the corresponding bacterial lysates, as determined by means of ELISA. These results indicated that bacteria-reactive IgE is generated through somatic hypermutation (SHM) in the context of specific immune responses against the corresponding bacteria in patients with CRSwNP.

Augmented T_H2 responses against *S pyogenes* in patients with CRSwNP

SHM-dependent specific reactivity implies that antibacterial IgE is generated through T cell-dependent antibody responses specific to nasal bacteria. To further elucidate this dependence, we next investigated *in vitro* T-cell responses against *S pyogenes*. PBMCs were cultured for 7 days in the presence of various stimuli, including heat-killed *S pyogenes*, PHA plus IL-2, cytomegalovirus (CMV) pp65, and heat-killed *C albicans*, and then proliferation and cytokine production of CD4⁺ T cells

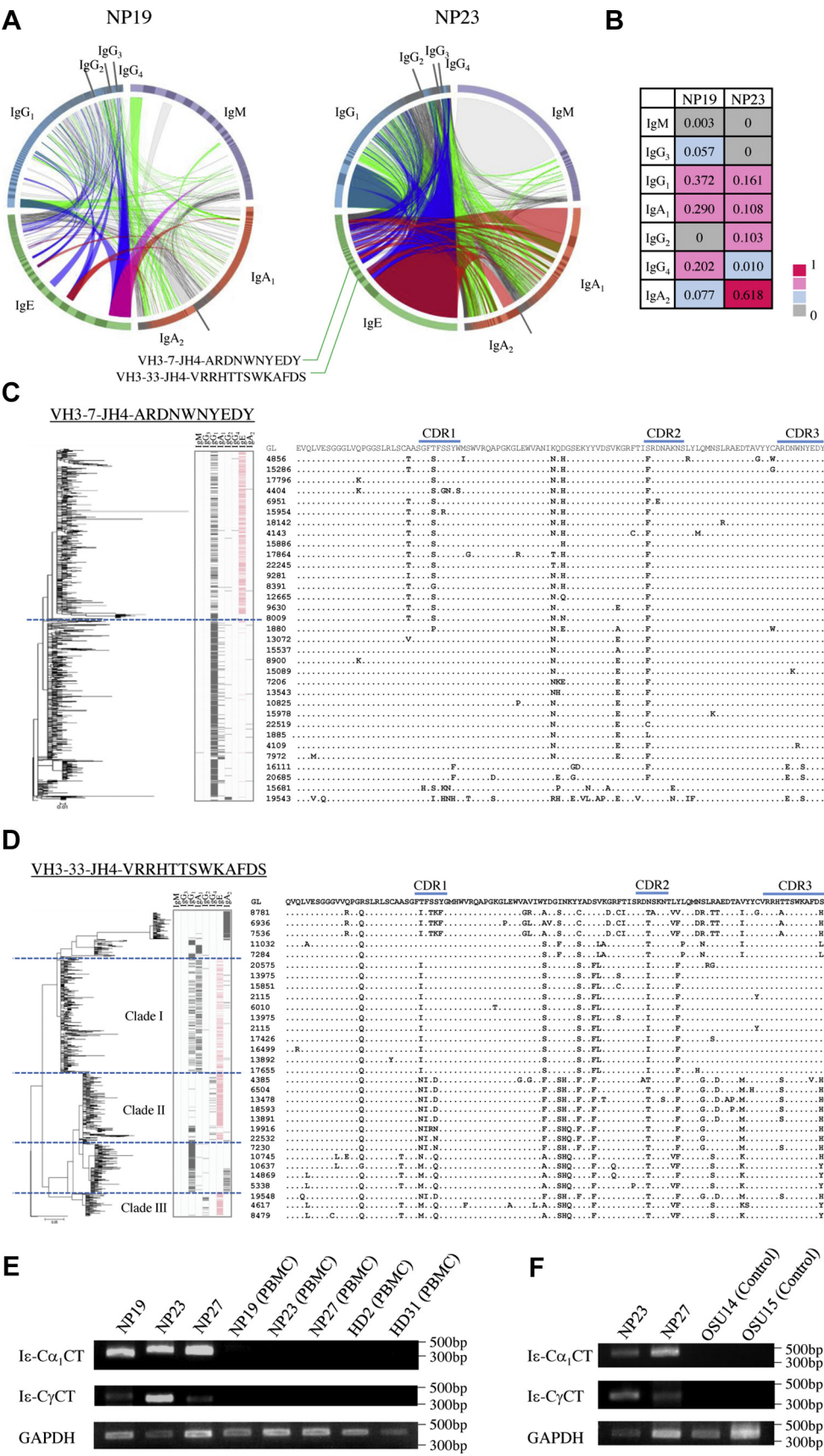
were evaluated by using flow cytometry (Fig 3, A). Significantly greater proportions of IL-4- and/or IL-5-producing T_H cells expanded on polyclonal activation with PHA plus IL-2 and on antigen-specific stimulation with *S pyogenes* in patients with CRSwNP than in healthy subjects (Fig 3, B). IFN- γ ⁺ cells were detected at similar levels in both the healthy and CRSwNP groups in the response against *S pyogenes*, reflecting a typical antibacterial response (Fig 3, B).³⁶ Importantly, the augmented T_H2 response was not observed in patient-derived samples stimulated with other antigens, such as CMV pp65 (Fig 3, B) or *C albicans* (data not shown). These results demonstrated that the immune response against *S pyogenes* in patients with CRSwNP has a substantial T_H2 character in addition to T_H1.

NP-derived IgE antibodies share reactivity with not only IgG but also IgA antibodies

To gain insight into the generation of IgE⁺ B cells and their relationship with other isotype-expressing B cells, we amplified VH3 transcripts, the most prevalent in human VH genes, from infiltrating lymphocytes of 2 NP samples (NP19 and NP23) and analyzed them by using HTS. Because separated PCRs were performed for IgM, IgG, IgA, and IgE to avoid generation of cross-isotype chimeric products, coverage and depth of the sequences in each isotype were unequal. Considering the abundance of immunoglobulin transcripts in plasmablasts, the obtained reads are likely to be dominated by sequences from plasmablasts rather than B cells. Verified sequences of each immunoglobulin isotype were classified into clonal lineages based on the combination of VH and complementarity determining region 3 sequences. In the NP IgE transcripts, 55 and 71 clonal lineages comprising 108,130 and 139,284 sequences were obtained from NP19 and NP23, respectively (see Tables E7 and E8 in this article's Online Repository at www.jacionline.org). Sequences belonging to the 3 and 4 largest IgE lineages covered more than 80% of the total sequences in NP19 and NP23, respectively, indicating that the IgE repertoires in NPs were highly oligoclonal (see Fig E2, A).

To evaluate clonal overlapping between IgE and other isotypes, we combined all immunoglobulin transcripts and classified them into clonal lineages. It appeared that many clonal lineages of IgE were connected with IgG and IgA but not IgM (Fig 4, A). Connectivity to IgG or IgA was observed in 34.0% and 87.0% of the IgE clonal lineages from NP19 and NP23, respectively. The presence of overlapped lineages between different isotypes indicates class-switching to IgE within lineages after accumulation of somatic mutations in NPs.

To predict immediate neighbors of IgE lineages, the degree of connectivity between IgE and other isotypes was evaluated based on numbers of identical and nearly identical VH sequences across different isotypes within the same lineages and expressed as a connectivity ratio between 0 and 1, as defined in the Methods section in this article's Online Repository. In this analysis only the dominant connectivity of IgE with other isotypes in each lineage was used to simplify the evaluation of connectivity. In this evaluation the reported counts were given through a much stricter threshold than the analysis shown in Fig 4, A, where all connections were reported, and the distance between VDJ sequences across isotypes in the same lineages was not considered. In NP19 IgE clonal lineages were more related to IgG₁, IgG₄, and IgA₁ lineages with ratios of 0.372, 0.202, and 0.290, respectively



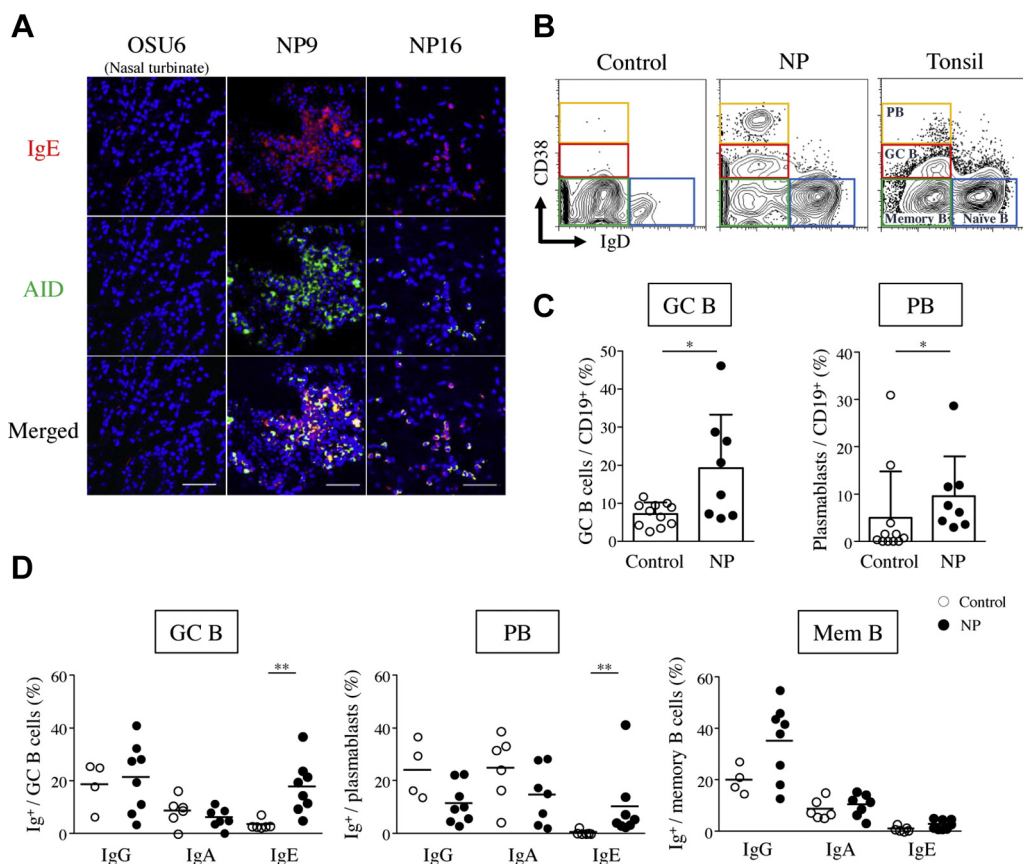


FIG 5. A large proportion of GC B-like cells express IgE in NPs of patients with CRSwNP. **A**, Representative immunofluorescence images of nasal tissues from control nonallergic subjects (OSU6) and NPs (NP9 and NP16). Red, IgE; green, AID; blue, 4', 6-diamidino-2-phenylindol. Scale bars = 50 μ m. **B**, Gating strategy of naive (blue), memory B (green), GC B-like (red), and plasmablast (orange) subsets defined by tonsillar B cells. Plots shown are cells after 7AAD⁻CD45⁺CD19⁺ gating. **C**, Frequencies of GC B-like cells (GC B) and plasmablasts (PB) in control nasal tissues and NPs (control tissue, n = 11; NPs, n = 8). **D**, Immunoglobulin isotypes expressed on the GC B-cell, plasmablast, and memory B-cell (Mem B) subsets (control tissue, n = 4-6; NPs, n = 7-8). * P < .05 and ** P < .01, Mann-Whitney test.

(Fig 4 B). A similar result was obtained from immunoglobulin repertoires of NP23, in which IgE clones were particularly connected to IgA₂ at a high ratio (connectivity ratio, 0.618; Fig 4, B). Thus IgE antibodies can largely share antigen specificity not only with IgG subclasses but also with IgA in NPs.

The phylogeny of 2 representative clonal lineages of NP23 facilitated understanding of the relationship between mutational diversification and CSR (Fig 4, C and D). The trees were rooted on germline sequences of the V, D, and J combination, and therefore sequences at the ends of branches have more mutations. In both cases IgG₁ and other IgG subclass sequences had substantial diversity produced by a large number of mutations, some of which

were shared by most sequences in each lineage. This consistency suggests that these clonal lineages were selected based on antigen reactivity. Notably, each phylogenetic clade consisted of a different combination of immunoglobulin classes. In the lineage of VH3-7-JH4-ARDNWNEDY, most of the IgE and IgA₁ sequences were found in distinct clades with higher and lower degrees of mutations, respectively, whereas IgG₁ sequences were spread across the clades (Fig 4, C). Clade-dependent distributions of immunoglobulin isotypes and their combinations were more evident in the lineage of VH 3-33-JH4-VRRHTTSWKAFDS (Fig 4, D). Particularly in the clades containing IgE, clade I was largely shared by IgG₁, IgA₁, and

FIG 4. NP-residing IgE⁺ B cells share reactivity with not only IgG⁺ but also IgA⁺ B cells. **A**, Circos plots of clonal lineages in IgM, IgG, IgA, and IgE expressed in NPs. Areas with different colors represent proportions of individual clonal lineages. The same lineages in different immunoglobulin isotypes are connected. Locations of the lineages shown in Fig 4, C and D, are marked on IgE lineages of NP23. **B**, Connectivity ratio of IgE with other immunoglobulin isotypes. The close proximity of IgE to other isotypes was evaluated based on the number of identical and nearly identical sequences across isotypes in the same lineages and expressed as a connectivity ratio that ranged from 0 to 1, as described in the Methods section in this article's Online Repository. **C** and **D**, Phylogenetic trees of 2 representative clonal lineages from NP23. **E** and **F**, Detection of κ -C α 1 and κ -C γ circle transcripts. HD2 and HD31, Healthy donors (Fig 4, E); OSU14 and OSU15, control tissues (nasal turbinate) from nonallergic patients (Fig 4, F). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

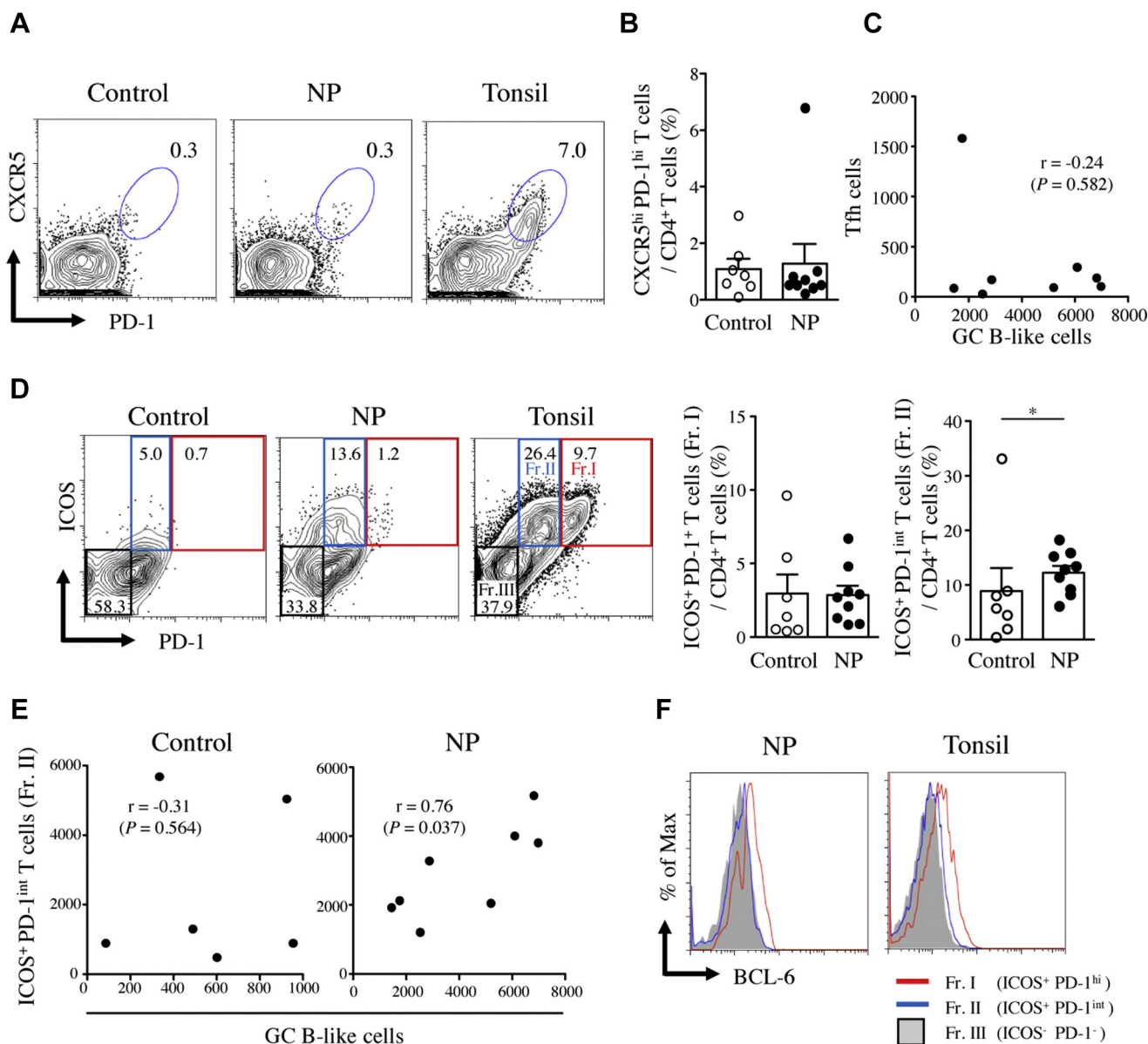


FIG 6. ICOS⁺PD-1^{int}CD4⁺ T cells but not conventional T_{FH} cells are correlated with GC B-like cells in NPs. **A**, Representative fluorescence-activated cell sorting plots of CD4⁺ T cells from control nasal tissues, NPs, and tonsils. Plots show cells after gating of 7AAD⁻CD45⁺CD19⁻CD4⁺. **B**, Frequencies of CXCR5^{hi}PD-1^{hi} T_{FH} cells (control tissue, n = 7; NPs, n = 9). * $P < .05$. **C**, Frequencies of GC B-like and T_{FH} cells in NPs (per one million CD45⁺ cells). **D**, ICOS and PD-1 expression in CD4⁺ T cells from indicated samples. Plots shown are cells after gating of 7AAD⁻CD45⁺CD4⁺. Fr. I, ICOS⁺PD-1^{hi} cells; Fr. II, ICOS⁺PD-1^{int} cells; and Fr. III, ICOS⁻PD-1⁻ cells. Frequencies of Fr. I and Fr. II are summarized in the graphs (control tissue, n = 7; NPs, n = 9). **E**, Frequencies of ICOS⁺PD-1^{int}CD4⁺ T cells (Fr. II) and GC B-like cells in control tissues and NPs (per one million CD45⁺ cells). **F**, Representative histograms of BCL-6 expression in Fr. I to Fr. III from Fig 6, D.

IgE, whereas clade II was composed of IgG₁, IgG₄, and IgE, and clade III was composed of IgG₂ and IgE. These results indicate that each phylogenetic clade of repertoires independently undergoes switching to different isotypes in the same clonal lineage. Collectively, these findings raise the possibility that IgE⁺ B cells might have differentiated from IgG⁺ and IgA₁⁺ B cells through sequential switching.

To confirm ectopic sequential switching in NPs, we performed RT-PCR to detect circle transcripts of the immunoglobulin locus, which are transient RNA products transcribed from excision

products of CSR (see Fig E2, B).³⁷ Consistent with earlier studies,^{20,21} the Iε-Cγ circle transcripts were detected in NP-derived cDNA (Fig 4, E). Additionally, we detected Iε-Cα₁ circle transcripts in NPs (see Fig E2, C). These circle transcripts were absent in PBMCs of patients with CRSwNP and healthy subjects (Fig 4, E). It is noteworthy that we could not detect the Iε-Cγ or Iε-Cα₁ circle transcript in control subjects (Fig 4, F). These results demonstrated that IgE⁺ B cells are generated not only from IgG⁺ B cells but also from IgA⁺ B cells through CSR in NPs.

A large proportion of GC B-like cells express IgE on the cell surface in NPs

As previously reported, we observed IgE⁺ cells in immunostaining of NP tissues (NP9 and NP16) but not in those of normal nasal tissues (OSU6 in Fig 5, A). Interestingly, a large portion of IgE⁺ cells also expressed activation-induced deaminase (AID) in NP tissues (Fig 5, A).

We next characterized B-cell subsets in NPs. Based on the gating strategy defined by tonsillar B cells (Fig 5, B, and Tables E9 and E10), we found that approximately 19% and 10% of NP-derived B cells exhibited the IgD⁺CD38^{int} GC B-cell and IgD⁺CD38^{hi} plasmablast phenotypes, respectively, in flow cytometry (Fig 5, B and C). Intracellular staining ensured that the GC B-like population in NPs expressed B-cell lymphoma 6 (BCL-6) at a level similar to that in tonsillar GC B cells (see Fig E3, A, in this article's Online Repository at www.jacionline.org). The frequencies of both GC B-like and plasmablast subsets in NPs from patients with CRSwNP were statistically greater than those in nasal tissues of control subjects (Fig 5, C). About 18% and 10% of GC B-like and plasmablast subsets, respectively, expressed IgE in NPs. In contrast, IgE⁺ cells were hardly detected in all B-cell subsets of normal nasal mucosa, although substantial frequencies of IgG⁺ and IgA⁺ B cells were observed (Fig 5, D). Unlike GC B-like cells or plasmablasts, IgE⁺ cells were almost absent in the memory B subset of NPs (<3%; Fig 5, D). The detected IgE molecules were apparently synthesized in these cells but not captured by CD23, as reflected by the observation that these subsets did not express CD23 (see Fig E3, B). Therefore IgE class-switching takes place in GC B-like cells, and the resultant cells differentiate into IgE⁺ plasmablasts but not memory B cells in NPs.

GC B-like cells are correlated with inducible costimulator-positive programmed cell death protein-intermediate CD4⁺ T cells but not with the conventional follicular helper T-cell subset in NPs

Follicular helper T (T_{FH}) cells play a critical role in GC reactions. Unexpectedly, however, the frequencies of typical T_{FH} cells (CXCR5^{hi} programmed cell death protein 1 [PD-1]^{hi}) were extremely low in NPs and normal nasal tissue from control subjects (Fig 6, A and B). In agreement with mouse studies,³⁸ abundances of T_{FH} cells and GC B cells were highly correlated in tonsils (see Fig E3, C). In contrast, no correlation was observed between the number of NP-derived CXCR5^{hi}PD-1^{hi} T_{FH} and GC B-like cells (Fig 6, C), indicating that typical T_{FH}-GC B interaction does not occur in NPs. NPs harbored higher proportions (13.0% ± 3.4%) of inducible costimulator (ICOS)⁺ PD-1^{int} CD4⁺ T cells (Fr. II in Fig 6, D) than nasal tissues of control subjects (Fig 6, D). Interestingly, frequencies of Fr.II CD4⁺ T cells were well correlated with those of GC B-like cells in NPs but not in control subjects (Fig 6, E). This ICOS⁺PD-1^{int} population did not represent conventional T_{FH} cells because the cells lacked BCL-6 expression (Fig 6, F). These results suggest that the T-cell requirement for ectopic GCs in NPs might differ from that in conventional GCs in secondary lymphoid organs.

DISCUSSION

In this study we found that a relatively high frequency of IgE antibodies produced in NPs of patients with CRSwNP reacted specifically with nasal bacteria, including *S aureus*, *S pyogenes*,

and *H influenzae*. Although diverse bacteria have been observed in the nasal cavity,^{33,39} only limited species and serotypes of bacteria were tested here. Thus we assume that IgE antibodies derived from NPs contain much higher frequencies of antibacterial antibodies than we identified. Our flow cytometric and biochemical studies revealed that the antigen-specific mAbs derived from IgE⁺ plasmablasts recognized bacterial surface structures. In accordance with the presence of IgE specific to *S pyogenes*, enhanced *S pyogenes*-specific T_H2 responses were observed in patients with CRSwNP. Furthermore, our HTS immunoglobulin analysis revealed that IgE repertoires in NPs were oligoclonal. On the basis of these findings, we conclude that IgE in NPs is a product of specific antibody responses against various nasal bacteria but not polyclonal responses induced by superantigens.

The results of the HTS immunoglobulin analysis and our detection of circle transcripts indicate that sequential IgE switching from IgA₁, as well as IgG, occurred locally in NPs of patients with CRSwNP. Inhabitation or invasion by nasal bacteria is assumed to induce mucosal immune responses, particularly IgA responses, in the paranasal sinuses of nonallergic healthy subjects, as previously reported in mouse models.^{40,41} Indeed, substantial frequencies of GC-like B cells and plasmablasts expressed IgG and IgA but not IgE in nasal mucosa from our control subjects. Notably, some mAb clones isolated in this study recognized bacterial antigens, such as *S pyogenes*-derived polysaccharide and *H influenzae*-derived lipoprotein E, which induce protective immune responses and have been considered to represent potential vaccine candidates.^{42,43} Taking these findings into account, we hypothesize that in patients with CRSwNP, protective antibacterial antibody responses can be aberrantly converted into allergic responses through skewed class-switching toward IgE.

It has been reported that nonpathogenic coagulase-negative staphylococci are most frequently isolated from the sinus tissues of patients with CRS.⁴⁴ Among our antibacterial IgE clones, only one clone turned out to react with coagulase-negative *S epidermidis*. Further studies will be required to determine whether pathogenic bacteria preferentially induce IgE production in patients with CRSwNP.

In local tissues sequential switching to IgE can take place in 2 B-cell subsets: memory B cells and ectopic GC B cells. Our flow cytometric analysis detected significantly greater frequencies of BCL-6⁺ GC B-like cells in NPs than in tonsils. Approximately 18% of NP-derived GC B-like cells expressed IgE, which was barely detectable in the memory B-cell subset.

These observations suggest that IgE class-switching from IgG and IgA₁ occurs at the stage of GC B-like cells in NPs. Such IgE-switched B cells can quickly differentiate into plasmablasts but not memory B cells, as previously shown in mouse studies.^{45,46} Our HTS data and a large number of mutations in immunoglobulins indicated that antigen-experienced IgG⁺ and IgA₁⁺ B cells acquire somatic mutations through GC-like reactions in NPs and then switch their B-cell receptors to IgE during long disease histories of CRSwNP.

Previous work reported increased numbers of T_{FH} (CXCR5^{hi}PD-1^{hi}CD4⁺) cells and correlation of the abundance of these cells with local IgE levels in NPs of Chinese patients with CRSwNP.⁴⁷ However, T_{FH} cells were barely detected (approximately 1%) in our analysis. Alternatively, we found that in NPs GC B-like cells correlated not with typical T_{FH} cells

but with ICOS⁺PD-1^{int}BCL-6⁻CD4⁺ T cells with lower expression of CXCR5. In a previous study PD-1 expression was correlated with IL-5 and nasal computed tomographic scan scores of patients with CRSwNP,⁴⁸ implying a possible role of PD-1 for T_H2 response and disease pathology. Increased numbers of bacteria-specific T_H2 cells in PBMCs of patients with CRSwNP indicate that a pathogenic T_H2 subset might facilitate ectopic GC reaction and aberrant IgE CSR in the absence of typical T_{FH} cells.

It has been unclear whether specific allergens or antigens trigger CRSwNP. This study showed that nasal bacteria are candidate causative allergens of this disease. We further elucidated the developmental pathway of allergen-reactive B cells in local tissues. Our findings will provide not only insight into pathogenesis but also clues for developing novel therapies of CRSwNP. Our findings might also be applicable to identifying pathogenic T_H cells involved in this disease, as well as understanding the pathogenesis of other allergic diseases.

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Key messages

- IgE antibodies specific for nasal bacteria are produced in polyps of patients with CRSwNP.
- Antigen-specific IgE antibodies originate from polyp-residing IgG⁺ and IgA₁⁺ GC B-like cells.
- The antibacterial response can be converted to T_H2 inflammation in the nasal mucosa of patients with CRSwNP.

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METHODS

Serum LTA IgE ELISA

Sera were obtained from 22 patients with CRSwNP and 22 patients with allergic rhinitis as control subjects (Table E6). ELISA plates were coated with 0.5 µg of LTA derived from *S pyogenes* or *S aureus* per well. LTA-coated ELISA plates were blocked with 1% BSA in PBS before incubation of serum (1:300 diluted) for 2 hours at room temperature. Plates were washed with PBS containing 0.1% Tween 20. Bound antibodies were detected with biotin-conjugated anti-human IgE (KPL, Gaithersburg, Md) and streptavidin-horseradish peroxidase (BD) in a reaction with One-Step Ultra TMB-ELISA reagent (Thermo Scientific). The reaction was stopped by addition of sulfuric acid, and OD at 450 nm was measured. In each serum sample the background reactivity against BSA was subtracted from the OD value of LTA.

Identification of bacterial antigens by using mass spectrometry

Protein A-purified clones 23G2 and 23G9 were immobilized on AminoLink Plus Coupling Resin (Thermo Scientific). *H influenzae* was lysed in PBS containing 1% Triton X-100, 1% BSA, and proteinase inhibitor cocktail (Sigma-Aldrich). After sonication and high-speed centrifugation, the cleared lysate was incubated with resin overnight at 4°C. After extensive washing with PBS containing 1% Triton X-100, bound antigens were eluted with 0.1 mol/L glycine-HCl (pH 2.4) and separated on SDS-PAGE. The acrylamide gel was stained with the MS silver staining kit (Wako), and specific bands were cut out. For mass spectrometry, fragmented peptides were analyzed by using liquid chromatography-mass spectrometry on an AXIMA Resonance instrument (Shimadzu, Kyoto, Japan). Peptide fingerprinting for protein identification was performed with Mascot software (Matrix Science, London, United Kingdom). *H influenzae* FKBP-type PPIase and lipoprotein E genes were amplified by using PCR from the genomic DNA of *H influenzae* strain Kam350, which was kindly provided by Dr Akeda (Osaka University). The FKBP-type PPIase and lipoprotein E amplicons were subcloned into pet30b bacteria expression vector (Novagen, Madison, Wis) and pGEX-KG (ATCC, Manassas, Va), respectively, and the resultant constructs were transduced into *Escherichia coli* BL21 (Thermo Scientific). Cell lysates of transfectants or purified recombinant protein was used for ELISA with mAbs 23G2 and 23G9.

In vitro T-cell stimulation assay

PBMCs of 18 healthy donors and 18 patients with CRSwNP (Table E4) were isolated by means of gradient separation with Lymphoprep (STEMCELL Technologies). Washed PBMCs were stained with CTV (Thermo Scientific) before stimulation. Stained cells were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% FBS. For stimulation, the medium contained heat-killed *S pyogenes* serotype M1 (incubated for 30 minutes at 95°C, 2×10^6 CFU/mL), CMV pp65 peptide (Miltenyi Biotec), or PHA plus IL-2 (15 µg/mL and 1000 U/mL, respectively). On day 7, cells were further stimulated with TPA and ionomycin (10 and 500 ng/mL, respectively; Sigma-Aldrich) for 5 hours in the presence of GolgiPlug (BD) for 3 hours. Cells were then stained for surface markers (Table E10), fixed, and permeabilized with Cytotfix/Cytoperm (BD) and then stained with anti-CD4 antibody (RPA-T4) and anti-cytokine antibodies (Table E10). Cytokine production in proliferating CD4⁺ T cells (CTV^{lo}CD3⁺CD4⁺ cells) was detected by using the BD FACSCanto.

HTS analysis

After removal of low-quality reads (Phred quality score < 30), MiSeq reads were merged by using PEAR 0.9.6.^{E1} For VDJ gene and clone assignment, merged reads shorter than 300 bp were omitted from the analysis. The length and quality of reads were analyzed by using an in-house python script with the Biopython module.^{E2} The igblastn tool of IgBLAST 1.7.0 was used for VDJ gene assignments.^{E3} Nonproductive reads and those with assigned V and J gene lengths shorter than 200 and 30 bp, respectively, were omitted from further analysis. Complementarity determining region 3 sequences of the same length that shared V-J gene use were grouped and then clustered into

clones by using CD-HIT4.7,^{E4} with 85% as a sequence identity threshold. For IGHC gene assignment, reference germline sequences from the IMGT database^{E5} were used. Reads of greater than 70 bp after removal of variable regions were assessed. We made a database of the reads using the makeblastdb tool of BLAST+ 2.6.0^{E6} and used blastn with default settings to find the best-matched IGHC gene for each read. For evaluation of connectivity between IgE and other isotypes, unique sequences of variable regions from each isotype were used. Distances between each pair of sequences within a clone were calculated by using MAFFT, version 7.310.^{E7} Here we used globalpair—distout—retree 0 arguments to generate a hat2 file. The number of sequences with a distance of less than 0.1 were counted for each sequence and then summed for each isotype. We picked the isotype with the largest number of identical or nearly identical sequences and calculated the number of lineages with connection to a given isotype (N_i) and the number of total IgE lineages, which had any connection to other isotypes (N_t). We defined the connectivity ratio (CR) to a given isotype as follows:

$$CR = N_i/N_t.$$

Detection of immunoglobulin circle transcripts

cDNA isolated from 3 NP samples, 2 normal nasal tissues, and PBMCs of 3 patients with CRSwNP and 2 healthy donors (Table E5) was subjected to nested PCR with primers^{E8} described in Table E2. Correct amplification was confirmed by subcloning of the amplicons into pCR2.1 (Thermo Scientific), followed by Sanger DNA sequencing.

Tissue section staining

For immunofluorescence staining, NPs (NP9 and NP16 [Table 1 and Table E5]) and control tissues were fixed with 2% paraformaldehyde; incubated in 15% sucrose/PBS overnight, followed by additional incubation in 30% sucrose/PBS for 24 hours at 4°C; and embedded in OCT compound (Sakura Finetek, Torrance, Calif). Frozen OCT-embedded tissues were sliced into 10-µm sections, blocked with PBS-TB (0.1% Triton X-100, 1% BSA, and 5% FBS), and stained with antibodies against IgE and AID (Table E10). After washing, sections were mounted with Vectashield (Vector Laboratories, Burlingame, Calif) and observed on a FluoView FV1000 confocal laser microscope (Olympus, Center Valley, Pa). Pseudocolored images were converted into TIFF files by using Olympus FV software.

Flow cytometry

Lymphocytes derived from total of 21 NPs, 12 control nasal tissue samples, and 8 tonsils (Table E9) were stained for the surface markers listed in Table E10. For intracellular staining of BCL-6, cells were fixed and permeabilized with Cytotfix/Cytoperm (BD) and then stained with anti-BCL-6 antibody. Stained cells were measured with the BD FACSCanto II, and obtained data were analyzed by using FlowJo software (TreeStar, Ashland, Ore).

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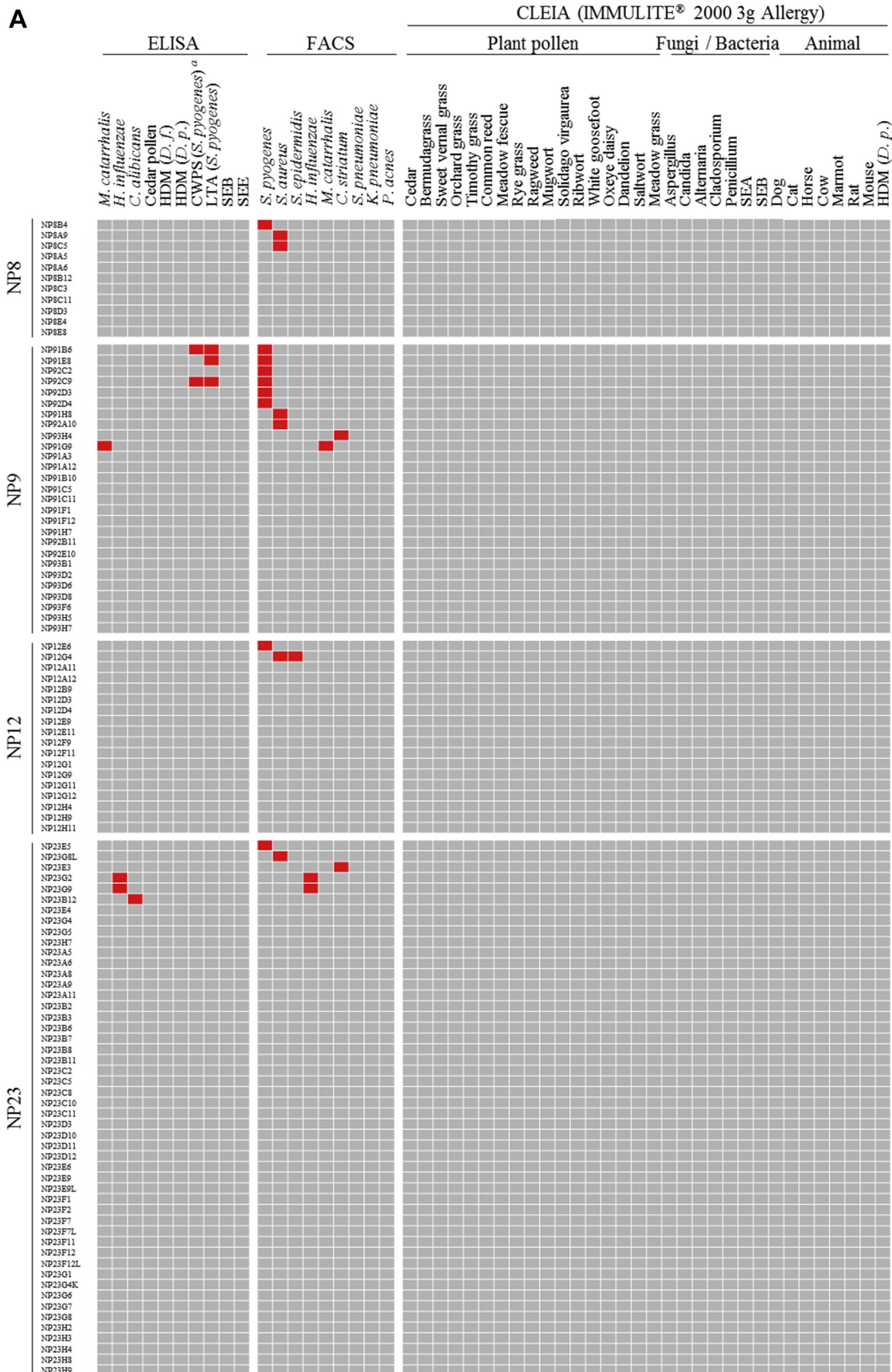


FIG E1. Characterization of IgE derived from patients with CRSwNP. **A**, Summary of reactivity tests of mAb clones. Reactive antigens were colored red. **B**, Fluorescent staining of *C. albicans* by clone 23B12 or control antibody. Bar = 10 μ m. **C**, Serum IgE relativities against LTA derived from *S. pyogenes* (patients with allergic rhinitis [AR], n = 22; patients with CRSwNP, n = 22). **D**, Numbers of mutated nucleotides in VH regions of isolated IgE clones from NPs (NP8, n = 26; NP9, n = 70; NP23, n = 63; and NP12, n = 26) and IgG₁ clones

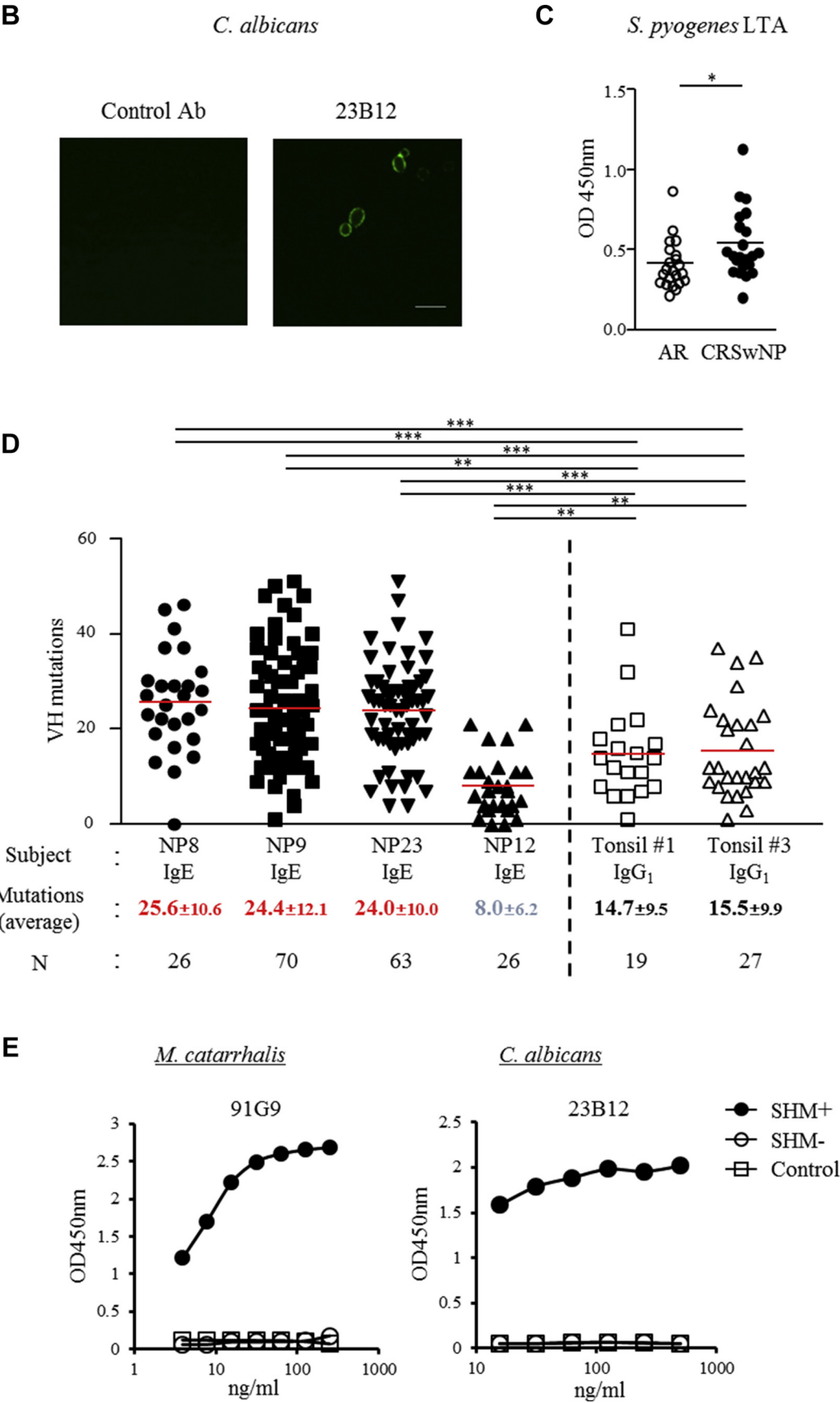


FIG E1. (Continued).

derived from tonsillar plasmablasts from patients with IgA nephropathy (Tonsil #1, n = 19; Tonsil #3, n = 27). **E**, Antibacterial clones were SHM dependent. Reactivity of anti-*M. catarrhalis* (91G9) and anti-*C. albicans* (23B12) clones with mutations (SHM+) or nonmutated germline antibodies (SHM-) was examined in ELISAs with the respective bacterial antigens. **P* < .05, ***P* < .01, and ****P* < .001.

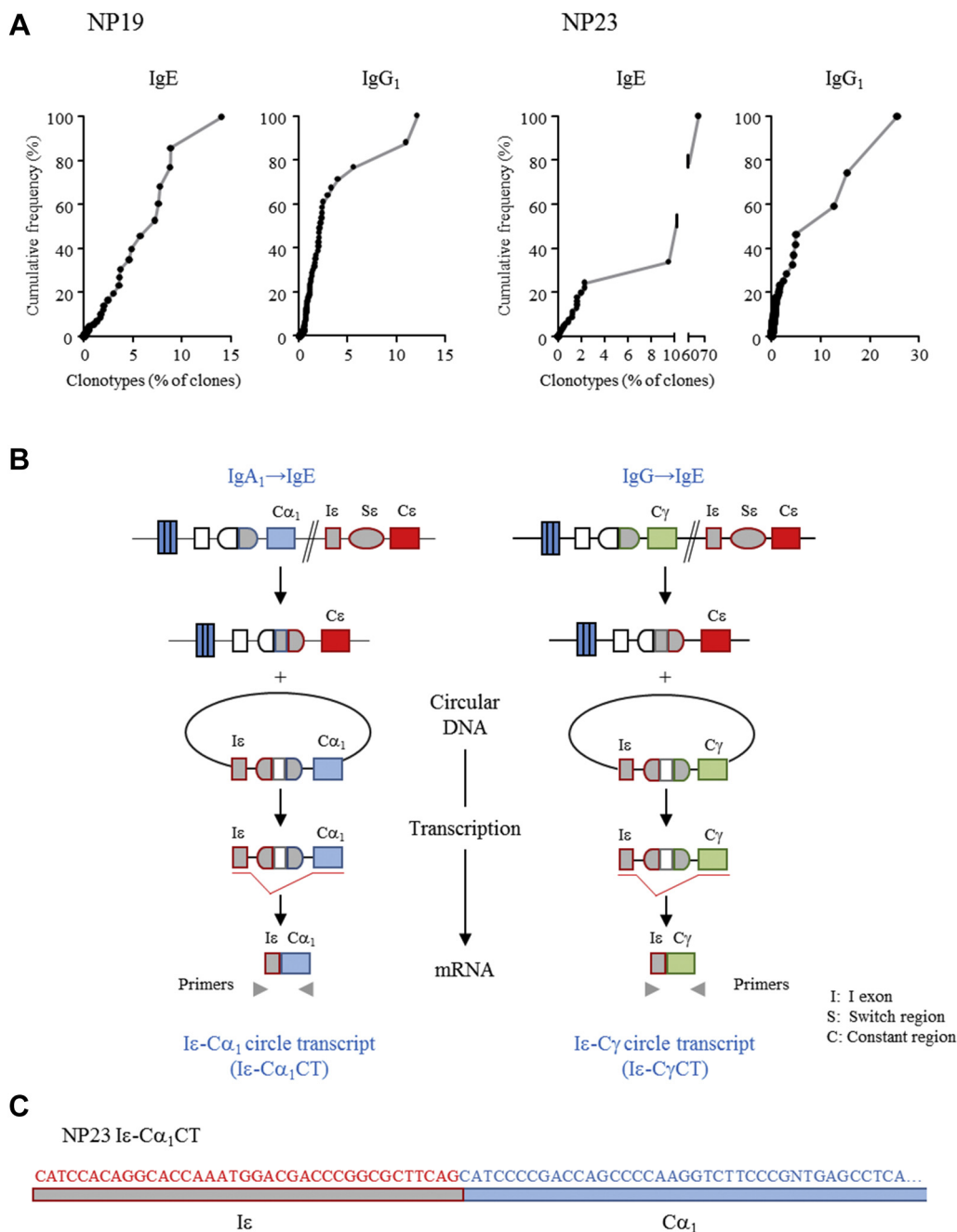


FIG E2. HTS analysis of immunoglobulin repertoires and circle transcripts expressed in NPs. **A**, Cumulative frequencies of immunoglobulin clonotypes in NP-derived IgE and IgG₁ repertoires. The *x-axis* and *y-axis* represent percentages of clonal lineages and cumulative percentages of sequences, respectively. **B**, Schematic diagram of immunoglobulin circle transcripts. **C**, Nucleotide sequence of the Iε-Cα₁ circle transcript expressed in NP23.

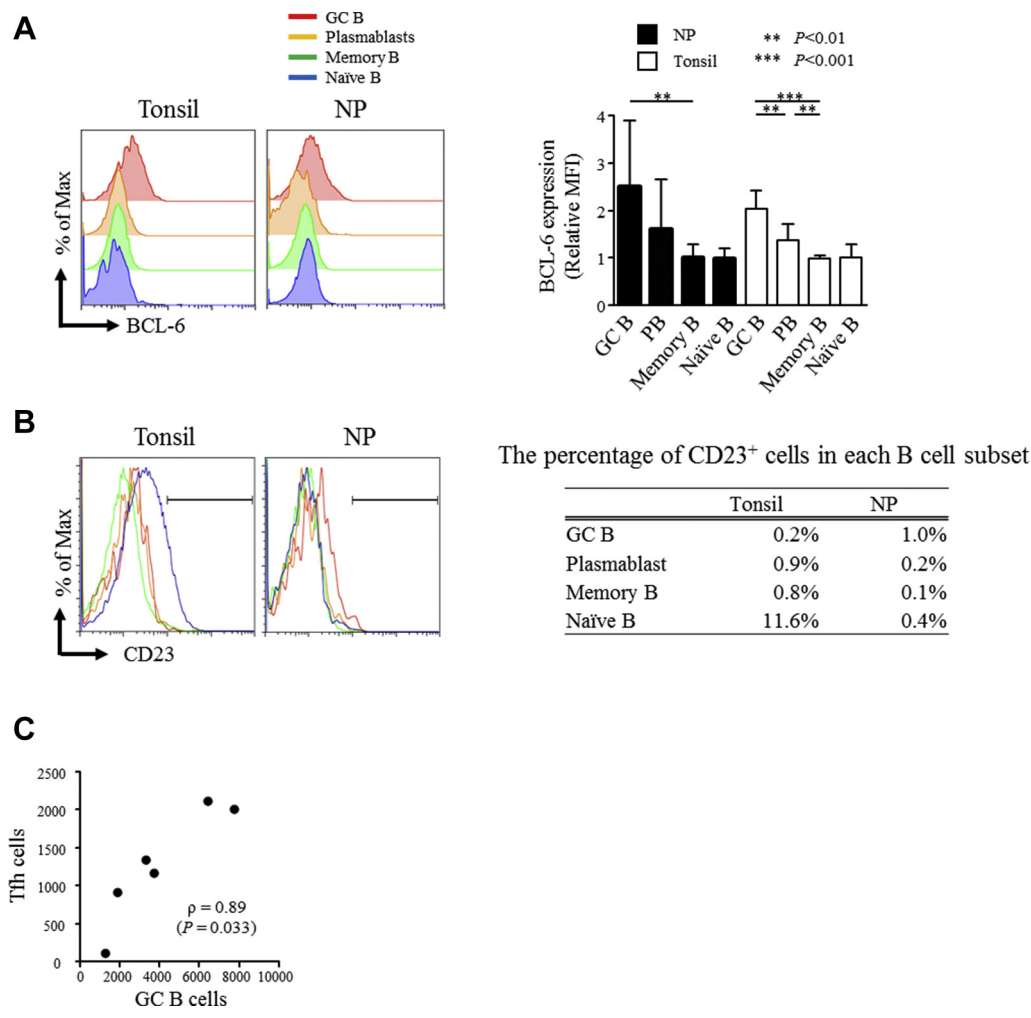


FIG E3. Flow cytometry of tonsillar and NP-infiltrating lymphocytes. **A**, BCL-6 expression of B cells in tonsils and NPs. Representative histograms (*left*) and relative mean fluorescence intensities of BCL-6 in indicated B-cell subsets normalized to those of naïve B cells (*right*; NPs, n = 6; tonsillar tissue, n = 8). ***P* < .01 and ****P* < .001, Mann-Whitney test. **B**, B cells and plasmablasts (PB) in NPs did not express CD23. Representative histograms of B-cell subsets in tonsils and NPs from 2 independent experiments. **C**, Numbers of tonsillar GC B and T_{FH} cells were correlated. Numbers in one million CD45⁺ cells were plotted. Correlation was evaluated by using the Spearman rank correlation method.

TABLE E1. Patients’ characteristics (tonsil donors; [Fig E1, D](#))

ID	T1	T3
Age and sex	22 M	21 M
Disease	IgAN	IgAN
Eosinophils (cells/mm ³)	68	244
Eosinophils (%)	1.5	2.8
CRE (mg/dL)	0.83	0.78
eGFR (mL/min/1.73 m ²)	99.3	106.3

CRE, Creatinine; *eGFR*, estimated glomerular filtration rate; *IgAN*, IgA nephropathy; *M*, male.

TABLE E2. PCR primers used in this study

Primer ID	Sequence (5'-3')	Description
VH1 (2859)	ACAGGTGCCCCACTCCCAGGTGCAG	IgE 1st PCR Fw
VH3 (2860)	AAGGTGTCCAGTGTGARGTGCAG	IgE 1st PCR Fw
VH4/6 (2861)	CCCAGATGGGTCTCTGTCCCAGGTGCAG	IgE 1st PCR Fw
VH5 (2862)	CAAGGAGTCTGTTCCGAGGTGCAG	IgE 1st PCR Fw
VH2 (2863)	GGACATACTTTGTTCCACGCTC	IgE 1st PCR Fw
3'huCe	AGACGCTGAAGGTTTTGTGTGTCGACCC	IgE 1st PCR Rv
VH1-Not1 (2864)	GGGGCGGCCCGCCGCCCCTCCCAGGTGCAGCTGGTGCAG	IgE 2nd PCR Fw
VH3-Not1 (2865)	GGGGCGGCCCGCCGCTCTTGTCCCAGGTACCTTGAAGGAG	IgE 2nd PCR Fw
VH4/6-Not1 (2866)	GGGGCGGCCCGCCGCTCCAGTGTGAAGTGCAGCTGGTGGAG	IgE 2nd PCR Fw
VH5-Not1 (2867)	GGGGCGGCCCGCCGCTCTGTCCCAGGTGCAGCTGCAGGAG	IgE 2nd PCR Fw
VH2-Not1 (2868)	GGGGCGGCCCGCCGCTGCAGCTGGTGCAGTCTGGAGCAGAG	IgE 2nd PCR Fw
JH1/2/4/5-Sal1 (2871)	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG	IgE 2nd PCR Rv
JH3-Sal1 (2872)	TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG	IgE 2nd PCR Rv
JH6-Sal1 (2873)	TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG	IgE 2nd PCR Rv
Vκ1/2 (2875)	ATGAGGSTCCCYGCTCAGCTGCTGG	Igκ 1st PCR Fw
Vκ3 (2876)	CTCTTCCTCCTGCTACTCTGGCTCCCAG	Igκ 1st PCR Fw
Vκ3 (2877)	ATTCTCTGTTGCTCTGGATCTCTG	Igκ 1st PCR Fw
Cκ-Rv (2444)	ACTCAGGAGCAGGTGGGGGCACTTCTCCCT	Igκ 1st PCR Rv
Igk-Not_Vk1-5	GGGCGGCCCGCCGACATCCAGATGACCCAGTC	Igκ 2nd PCR Fw
Igk-Not_Vk1-9	GGGCGGCCCGCCGTACATTCAGACATCCAGTTGACCCAGTCT	Igκ 2nd PCR Fw
Igk-Not_Vk1D-43	GGGCGGCCCGCCCATCCGGATGACCCAGTC	Igκ 2nd PCR Fw
Igk-Not_Vk2-24	GGGCGGCCCGCCGATATTGTGATGACCCAGAC	Igκ 2nd PCR Fw
Igk-Not_Vk2-28	GGGCGGCCCGCCGATATTGTGATGACTCAGTC	Igκ 2nd PCR Fw
Igk-Not_Vk2-30	GGGCGGCCCGCCGATGTTGTGATGACTCAGTC	Igκ 2nd PCR Fw
Igk-Not_Vk3-11	GGGCGGCCCGCCGTACATTCAGAAATTGTGTTGACACAGTC	Igκ 2nd PCR Fw
Igk-Not_Vk3-15	GGGCGGCCCGCCGAAATAGTGATGACGCAGTC	Igκ 2nd PCR Fw
Igk-Not_Vk3-20	GGGCGGCCCGCCGTACATTCAGAAATTGTGTTGACGCAGTCT	Igκ 2nd PCR Fw
Igk-Not_Vk4-1	GGGCGGCCCGCCGACATCGTGATGACCCAGTC	Igκ 2nd PCR Fw
3'-Xho-hu-Ck	AGAGGCAGCTCGAGATTTCAACTG	Igκ 2nd PCR Rv
VI1 (2879)	GGTCCTGGGCCCAGTCTGTGCTG	Igλ 1st PCR Fw
VI2 (2880)	GGTCCTGGGCCCAGTCTGCCCTG	Igλ 1st PCR Fw
VI3 (2881)	GCTCTGTGACCTCCTATGAGCTG	Igλ 1st PCR Fw
VI4/5 (2882)	GGTCTCTCTCSCAGCYTGTGCTG	Igλ 1st PCR Fw
VI6 (2883)	GTTCTTGGGCCAATTTATGCTG	Igλ 1st PCR Fw
VI7 (2884)	GGTCCAATTTCYAGGCTGTGGTG	Igλ 1st PCR Fw
VI8 (2885)	GAGTGGATTCTCAGACTGTGGTG	Igλ 1st PCR Fw
CI1-Rv (2432)	GCTCCCGTGGGTGGGGTTAGAGTTGGGAAC	Igλ 1st PCR Rv
CI2-Rv (2436)	CTCCCGTGGTGGGGGGTGAGGGTTGAGAAC	Igλ 1st PCR Rv
CI3-Rv (2440)	CTCCCGTGGTGGGGGGTGAGGGTTGAGAAC	Igλ 1st PCR Rv
IgI-Not_VL1	ATGGCGGCCCGCATCCTGGGCCAGTCTGTGCTGACKCAG	Igλ 2nd PCR Fw
IgI-Not_VL2	ATGGCGGCCCGCATCCTGGGCCAGTCTGCCCTGACTCAG	Igλ 2nd PCR Fw
IgI-Not_VL3	ATGGCGGCCCGCATCTGTGACCTCCTATGAGCTGACWCAG	Igλ 2nd PCR Fw
IgI-Not_VL4/5	ATGGCGGCCCGCATCTCTCTCTCSCAGCYTGTGCTGACTCA	Igλ 2nd PCR Fw
IgI-Not_VL6	ATGGCGGCCCGCATCCTTGGGCCAATTTATGCTGACTCAG	Igλ 2nd PCR Fw
IgI-Not_VL7/8	ATGGCGGCCCGCATCCAATTTCYAGRCTGTGGTGAOYCAG	Igλ 2nd PCR Fw
3'Xho-Cl	CTCCTACTCGAGGGYGGGAACAGAGTG	Igλ 2nd PCR Rv
IgM RV	CAGCCAACGGCCACGCTGCTC	HTS IgM Rv
IgG int	GTTCGGGGGAAGTAGTCCTTGAC	HTS IgG Rv
IgE RV	GCCCAGAGTCACGGAGGTGGC	HTS IgE Rv
IgA RV	GCGAYGACCACGTTCCCATCT	HTS IgA Rv
IeF1	GGGAGCTGTCCAGGAACCCGACAGAGC	IeCα/IeCγ 1st Fw
IeF2	GGCCACACATCCACAGGC	IeCα/IeCγ 2nd Fw
IgG common Rv	CCTTGGGTTTTGGGGGGAA	IeCγ-CT Rv
IgA1+2 constant Rv	TTCTGAACCTAAGAGCAGGTCTCTCGAG	IeCα-CT 1st Rv
IgA1-PCR	GAGATGGGGTAGGTGGAGTTGA	IeCα-CT 2nd Rv

Fw, Forward; Rv, reverse.

TABLE E3. Origin and strain of bacteria and fungi used in reactivity tests (Fig 1)

Bacteria and fungi	Serotype	Strain	Source
<i>Corynebacterium striatum</i>		OSA001	*
<i>Haemophilus influenzae</i>	NTHi	Kam350	†
<i>Haemophilus influenzae</i>	NTHi	No.1	†
<i>Haemophilus influenzae</i>	NTHi	No.2	†
<i>Haemophilus influenzae</i>	Type B	GB3291	†
<i>Haemophilus influenzae</i>	Type B	ATCC10211	†
<i>Haemophilus influenzae</i>	Type B	ATCC51907	†
<i>Klebsiella pneumoniae</i>		ATCC13883	†
<i>Moraxella catarrhalis</i>		ATCC25238	‡
<i>Propionibacterium acnes</i>		ATCC6919	
<i>Staphylococcus aureus</i>		ATCC25904	†
<i>Staphylococcus epidermidis</i>		OSA002	*
<i>Streptococcus pneumoniae</i>		RIMD3122021	†
<i>Streptococcus pyogenes</i>	M1	RIMD3213008	†
<i>Streptococcus pyogenes</i>	M3	SSI-1	§
<i>Aspergillus fumigatus</i>			*
<i>Candida albicans</i>			*

NTHi, Nontypeable *Haemophilus influenzae*.

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||RIKEN BioResource Center.

TABLE E4. Donors of blood lymphocytes used in T-cell stimulation assay (Fig 3)

	Patients with CRSwNP	Healthy donors
Sample no.	18	18
Mean age (y [minimum-maximum])	59.1 (31-77)	35.3 (21-50)
Sex	10 M/8 F	13 M/5 F
Asthma (%)	9 (50)	0 (0)
Aspirin intolerance (%)	2 (11.1)	0 (0)
Eosinophils (cells/mm ³)	596 (18-1763)	NA
Eosinophils (%)	9.2 (0.2-23.6)	NA
Total serum IgE (IU/mL)	261.7 (23.2-1039)	NA

F, Female; *M*, male; *NA*, not analyzed.

TABLE E5. Donors of nasal tissues, PBMCs, or both used in HTS, RT-PCR, and section staining (Figs 4 and 5, A)

Disease	Patients with CRSwNP				Patients with other nasal diseases*			Healthy control subjects	
Sample	NPs and PBMCs				Nasal tissue			PBMCs	
ID	NP16	NP19	NP23	NP27	OSU6	OSU14	OSU15	HD2	HD31
Age/sex	73/F	68/M	46/M	55/F	71/M	63/M	34/F	41/M	31/F
Asthma	No	No	Yes	Yes	No	No	No	No	No
Aspirin intolerance	No	No	No	No	No	No	No	No	No
Duration of disease (y)	20	3	6	35	n/a	n/a	n/a	n/a	n/a
No. of surgeries	2	1	6	2	n/a	n/a	n/a	n/a	n/a
Blood eosinophils (cells/mm ³)	591	501	357	2424	290	430	117	NA	NA
Blood eosinophils (%)	10.0	8.8	5.6	25.3	5.2	5.9	1.7	NA	NA
Polyp eosinophils (/HPF)	245	136	279	288	NA	NA	NA	NA	NA
JESREC score (eosinophilic CRS ≥11)	15	15	15	17	n/a	n/a	n/a	n/a	n/a
Total serum IgE (IU/mL)	114.0	1039.0	656.0	42.4	NA	NA	NA	NA	NA
Total polyp IgE (IU/mL)	NA	NA	553.5	80.0	NA	NA	NA	NA	NA
Specific serum IgE (IU/mL)									
Orchard grass	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
Ragweed	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
Mugwort	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
White birch	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
Cedar pollen	0.54	0.99	<0.10	<0.10	NA	NA	NA	NA	NA
Cat	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
Dog	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
<i>Cladosporium</i> species	<0.10	0.17	<0.10	<0.10	NA	NA	NA	NA	NA
<i>Aspergillus</i> species	<0.10	0.25	<0.10	<0.10	NA	NA	NA	NA	NA
<i>Candida</i> species	<0.10	0.13	0.81	<0.10	NA	NA	NA	NA	NA
<i>Alternaria</i> species	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
Cockroach	<0.10	<0.10	<0.10	<0.10	NA	NA	NA	NA	NA
<i>Dermatophagoides pteronyssinus</i>	<0.10	0.19	9.07	<0.10	NA	NA	NA	NA	NA
<i>Dermatophagoides farinae</i>	<0.10	0.18	15.1	<0.10	NA	NA	NA	NA	NA
SEA	<0.10	0.18	<0.10	<0.10	NA	NA	NA	NA	NA
SEB	1.65	0.41	<0.10	<0.10	NA	NA	NA	NA	NA

F, Female; HPF, high-powered field; JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; M, male; NA, not analyzed; n/a, not applicable.

*OSU6, Sinonasal inverted papilloma; OSU14, anatomic obstruction; OSU15, frontal sinus osteoma.

TABLE E6. Donors of blood samples tested in LTA-IgE ELISA (Fig E1, C)

	Patients with CRSwNP	Patients with AR
Sample no.	22	22
Mean age (y [minimum-maximum])	60.0 (29-77)	60.1 (26-80)
Sex	15 M/7 F	15 M/7 F
Asthma (%)	11 (50)	0 (0)
Aspirin intolerance (%)	4 (18.2)	0 (0)
Eosinophils (cells/mm ³)	578 (18-1267)	250 (72-945)
Eosinophils (%)	8.6 (0.2-17.9)	4.0 (1.1-13.0)
Total serum IgE (IU/mL)	409.1 (11.0-1346)	194.8 (11.3-1358)

AR, Allergic rhinitis; F, female; M, male.

TABLE E7. Summary of HTS analysis of NP19

Immunoglobulin class	IgM	IgA		IgG				IgE
		IgA ₁	IgA ₂	IgG ₁	IgG ₂	IgG ₃	IgG ₄	
Cell no. (CD45 ⁺)	1,000,000	1,000,000		1,000,000				1,000,000
Total reads (verified)	56,701	36,287	25,041	7,810	3,044	193	1,194	108,130
Clonal lineage	60	211	140	74	45	6	24	55
Clonal lineage shared by IgE	2 (3.33%)	11 (5.21%)	11 (7.86%)	12 (16.2%)	7 (15.6%)	2 (33.3%)	10 (41.7%)	—

TABLE E8. Summary of HTS analysis of NP23

Immunoglobulin class	IgM	IgA		IgG				IgE
		IgA ₁	IgA ₂	IgG ₁	IgG ₂	IgG ₃	IgG ₄	
Cell no. (CD45 ⁺)	1,000,000	1,000,000		1,000,000				1,000,000
Total reads (verified)	189,670	46,690	18,896	30,974	6,745	143	5,536	139,284
Clonal lineage	53	69	72	101	73	21	48	71
Clonal lineage shared by IgE	7 (13.2%)	11 (5.21%)	41 (56.9%)	48 (47.5%)	36 (49.3%)	10 (47.6%)	30 (62.5%)	—

TABLE E9. Donors of lymphocytes analyzed by using flow cytometry (Figs 5 and 6)

	Patients with CRSwNP	Patients with other nasal diseases	IgAN w/tonsillitis
Sample	NP	Nasal tissue	Tonsil
Sample no.	21	12	8
Mean age (y [minimum-maximum])	53.2 (30-73)	59.2 (25-79)	30.6 (20-45)
Sex	11 M/10 F	9 M/3 F	6 M/2 F
Asthma (%)	13 (61.9)	1 (8.3)	0 (0)
Aspirin intolerance (%)	5 (23.8)	0 (0)	0 (0)
Eosinophils (cells/mm ³)	594 (18-1267)	191 (79-497)	138 (0-360)
Eosinophils (%)	9.5 (0.2-17.9)	3.2 (0.8-5.6)	1.9 (0.0-5.2)
Total serum IgE (IU/mL)	394.4 (16.0-1346)	NA	NA

F, Female; *IgAN*, IgA nephropathy; *M*, male; *NA*, not analyzed.

TABLE E10. Antibodies used in flow cytometry and section staining

Antigen	Conjugates	Clone name	Manufacturer
AID	Biotin	mAID-2	eBioscience, San Diego, Calif
Bcl-6	Alexa Fluor 488	K112-91	BD, San Jose, Calif
CD3	PE	OKT3	Tonbo Biosciences, San Diego, Calif
CD3	PECy7	UCHT1	Tonbo Biosciences
CD4	FITC	RPA-T4	Tonbo Biosciences
CD4	FITC	OKT4	Tonbo Biosciences
CD8	Violet Fluor 450	OKT8	Tonbo Biosciences
CD11b	Biotin	ICRF44	BD
CD14	PE	M5E2	BioLegend, San Diego, Calif
CD14	PE/PerCP-Cy5.5	M5E2	BioLegend
CD19	APC-Cy7	SJ25C1	BD
CD20	APC	2H7	BioLegend
CD23	APC	EBVCS-5	BD
CD27	FITC	M-T271	BD
CD38	Violet Fluor 450	HIT2	Tonbo Biosciences
CD45	PECy7	H130	Tonbo Biosciences
CD45	Biotin	H130	BioLegend
CD56	PE	B159	BD
CD66b	PE	G10F5	BioLegend
CD66b	APC	G10F5	eBioscience
CD185 (CXCR5)	APC	MU5UBEE	eBioscience
CD278 (ICOS)	FITC	C398.4A	BioLegend
CD279 (PD-1)	PB	EH12.2H7	BioLegend
IL-4	PE	MP4-25D2	Tonbo Biosciences
IL-5	APC	TRFK5	BioLegend
IFN- γ	APCCy7	4S.B3	BioLegend
IgA	Biotin	G20-359	BD
IgD	PE/PECy7	IA6-2	BD
IgE	PE	MHE-18	BioLegend
IgG	FITC/APC	G18-145	BD

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein complex.