Characterization of immunoglobulin E plasma cells that are elevated in the upper airway mucosa of nonatopic patients with chronic rhinosinusitis without nasal polyps

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Background: **The immunologic mechanisms driving inflammation in the upper airways of patients with chronic rhinosinusitis (CRS) are poorly understood. Previous studies have shown that B cells and immunoglobulin E (IgE) levels are elevated in the nasal tissue of patients with atopic chronic rhinosinusitis without nasal polyps (CRSsNP). However, less is known regarding B cell subsets and IgE-producing plasmablasts in nonatopic CRSsNP patients.**

Methods: **Human blood and ethmoid sinus mucosa samples were analyzed from control (n = 6) and nonatopic CRSsNP (n = 11) patients. Tissue samples were evaluated using high-dimensional flow cytometry.**

Results: **A population of IgE antibody secreting cells is significantly increased in situ within inflamed nasal tissue of nonatopic CRSsNP subjects when compared to control nasal tissue and the circulating peripheral blood ($p < 0.05$). This IgE plasma cell population displays ~90% cell surface Ig lambda light chain, is mitotically active (Ki-67^{+}), and displays intracellular IgE expression. The predominant B cell population expressing IgE are plasmablasts (CD38^{high}, CD138^{-}) not typically found in the blood or peripheral tissue of these patients.**

Conclusion: **The nasal mucosa from nonatopic CRSsNP patients demonstrate a significant regional spike in resident in situ IgE plasmablast cells not seen in control nasal tissue or peripheral blood from the same patient. The restricted expression of Ig lambda light chain in this mitotically active IgE plasmablast population supports the hypothesis of aberrant B cell proliferation in the context of CRS. These findings suggest the presence of a unique regional immune microenvironment for B cell priming and/or selection within chronically inflamed airway tissues.** © 2016 ARS-AAOA, LLC.

Key Words:
flow cytometry; FACS; nasal mucosa; PBMC; IgE; B cell; plasmablast; CD38; CD138; upper airway; chronic rhinosinusitis

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Chronic rhinosinusitis (CRS), the most common chronic inflammatory disease in the United States, is estimated to affect 31 million people and imposes annual costs of >$10 billion on the U.S. healthcare system.[1,2] CRS, however, remains a poorly understood upper airway disease process, characterized by persistent recruitment of inflammatory cells to the nasal and sinus epithelium, ultimately leading to tissue inflammation, edema, sinus outflow obstruction, and infections. In more pronounced cases, patients suffer from nasal polyp formation and lung inflammation.[3] Current treatments for CRS are mainly restricted to optimizing surgical procedures and topical medicated rinse delivery to the nose, but improved understanding of the immunologic basis of inflammation in CRS is ultimately critical to disease control and the rational design of new therapeutic options.

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There has been increasing evidence to suggest that local proliferation and activation of B cells may be critical to, and dysregulated in, the pathophysiology of CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).[4–6] In line with this, Chen et al.[7] have noted the unusual presence of immunoglobulin (Ig) delta (D) molecules in the upper airway mucosa as well as tonsillar tissues. Our group has also found the selective presence of IgD+ B cells in the nasal mucosa of CRS patients (data not shown). Taken together, these observations suggest that not only are B cells, and their resultant immunoglobulins (Igs), likely accumulating within the nasal tissues of CRS patients, but that Ig production by B lymphocyte clones may be altered in situ within the upper airways of CRS patients. Furthermore, studies have demonstrated an increase in B cell chemoattractants, B cells (naive, activated, and memory-switched),[5,8] plasma cell numbers, and antigen-specific IgE in the pathogenesis of CRSwNP.[4,5,9–12]

Although a growing body of literature demonstrates that the presence of IgE is also detected in nonatopic CRS patients—a phenomenon referred to as entopy—we are unaware of any studies that characterize the plasma cell population responsible for this IgE production.[13–15] IgE is important to various immune responses and allergic pathogenesis, and the pathways by which IgE is produced and regulated are poorly understood in humans. Recent studies in mice have increased our understanding of the production and the regulation of IgE in vivo.[16–18] However, the study of IgE production and regulation in humans in vivo has been more challenging because this Ig has limited concentrations within the airway tissues sampled. IgE B cells and IgE plasma cells are tightly regulated, and the half-life of this Ig is truncated—20 days vs 2 days for IgE in humans.[17]

A major focus of IgE production in the upper airway of humans has been to determine whether IgE can be produced locally in inflamed mucosal tissues in allergic diseases such as asthma and allergic rhinitis.[19] Previous studies have provided strong evidence that local tissue production of antigen-specific IgE and IgE class switching in upper and lower airway mucosal tissues occurs in a variety of atopic disease processes, including allergic rhinitis.[2,12] Gevaert et al.[20] demonstrated secondary lymphoid tissue organization associated with plasma cells and follicular structures associated with B and T cells in nasal polyp tissue; IgE was found in conjunction with both of these lymphoid accumulations. These studies suggest that local IgE production is not only present, but potentially an important factor in driving or maintaining sinonasal inflammation. By contrast, investigations of local IgE in nasal polyp tissue in patients without atopy have introduced the hypothesis that *Staphylococcus aureus* superantigens "colonize" the nasal cavity. Bachert et al.[21] have shown that IgE antibodies to *S. aureus* enterotoxin are detectable in nasal polyp tissue, suggesting a link between *S. aureus* antigens or superantigens and CRSwNP in both atopic and nonatopic patients.

We sought to more critically discriminate the subpopulations of IgE-producing B cells that may be present within the resident intranasal tissues of nonatopic CRSsNP patients compared to immune cells present from upper airway tissues in control samples, and those found in the circulating central blood compartment.

Patients and methods

Patient tissue collection and processing

This study was conducted at the Stanford University Medical Center with approval of the Institutional Review Board for Human Research (IRB# 18981). All patients provided written, informed consent at the Stanford Sinus Center. For this work, only 2 diagnostic patient cohorts were assessed: control and CRSsNP (Table 1). Ethmoid sinus nasal tissue specimens, including ethmoid sinus, were obtained during routine endoscopic nasal and sinus surgery. All patients included in this study meet the diagnostic criteria for CRS as defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force.[22] Control tissue specimens were obtained from endoscopic transsphenoidal pituitary surgery for nonsecretory adenomas or skull-base tumor surgery for benign neoplasms both without subjective or radiographic evidence of CRS. Peripheral blood samples were collected on the day of surgery as per protocol. All samples were placed on ice and transported immediately from the operating room (OR) to the laboratory for processing. Kruskal-Wallis 1-way analysis was used to determine statistical differences in B cell subsets in the nasal tissue and blood of control and nonatopic CRSsNP patients. A sample size of 10 patients for the nonatopic CRSsNP was needed to yield an $\alpha = 5\%$.

Exclusion criteria for tissue acquisition included patients less than 18 years of age; patients diagnosed with diabetes, immune disorders, or pregnancy; patients with immunosuppressive agents preceding surgery or currently taking immunosuppressive drugs; and patients with allergic fungal sinusitis or cystic fibrosis were also excluded from this study.

The nonatopic status for both cohorts, control and CRSsNP, was based on undetectable levels of IgE in PBMCs, and patient history from electronic medical records. Patients selected for this study denied any history of local or systemic allergic symptoms in their medical history.

Upper airway nasal tissue and PBMC immunophenotyping using flow cytometry (fluorescence-activated cell sorting)

PBMCs were enriched on Ficoll gradients using standard protocols.

Nasal and sinus tissue collected intraoperatively was mechanically separated using small surgical scissors and digested in a solution of collagenase type IV and DNAse

TABLE 1. Summary of demographic information of included patients

Patient group (n)	Age (years) mean	Gender (M:F)	Alcohol (Y:N)	Smoking (Y:N)	Race/ethnicity	Repeat FESS (Y:N)	Asthma	LMS median (range)[a]	Allergic rhinitis by testing
Control (n = 6)	48	1:5	4:2	5:0	1 AA; 3 W; 1 H; 1 N/A	0:6	0/6	0	0/6
CRSsNP (n = 11)	45	6:5	9:2	3:8	2 A; 5 W; 4 H	4:7	0/11	6 (3–10)	0/11

[a]LMS for assessment of chronic rhinosinusitis.
A = Asian; AA = African American; CRSsNP = chronic rhinosinusitis without nasal polyps; H = Hispanic; LMS = Lund-Mackay score; M:F = male:female; W = white; Y:N = yes:no.

at 37°C for 1 hour. Nasal tissue solution was then washed in phosphate-buffered saline (PBS) and resuspended in Roswell Park Memorial Institute (RPMI) culture medium solution before subject to a 16G needle pressurized chamber and passage through a 40-μm cell strainer to increase the number of singlet cells in solution. Specimens were also treated with ammonium-chloride-potassium (ACK) red blood cell lysis buffer. Single-cell suspensions of both PBMCs and nasal tissue were now available for antibody staining.

Surface marker profiling

The gating strategy to identify B cell subsets is illustrated in Figure 1. For surface staining, 50 to 100 μL of PBMCs and nasal tissue single-cell suspensions were preincubated on ice for 15 minutes with anti-CD32 monoclonal antibody (mAb) (BD Biosciences, San Jose, CA) to block FcγRII/III receptors. To exclude dead (ie, aqua amine–positive) cells, LIVE/DEAD® Fixable Aqua Amine Stain was used (Invitrogen, Carlsbad, CA). Single-cell suspensions were then incubated with fluorochrome-conjugated mAb in a 14-color staining combination (Table 2) per manufacturer recommendations. These antibodies include: fluorescein isothiocyanate (FITC)-labeled anti-CD43; phycoerythrin (PE)-labeled anti-CD138; Alexa Fluor-700–labeled Anti-IgD; PE Cy5-labeled Anti-CD20; Pacific Orange–labeled Anti-CD3; Pacific Orange–labeled Anti-CD14; Pacific Orange–labeled Anti-CD16; PerCp Cy5.5-labeled Anti-IgM; H7 allophycocyanin (H7APC)-labeled Anti-CD 27; CD38-labeled Cy7PE; Biotin-labeled Anti-light chain lambda; and APC-labeled Anti-IgE. Antibodies were purchased from either Invitrogen or BD Biosciences. Cells were then washed and stained again on ice for 15 minutes with streptavidin Qdot 605 (Invitrogen) to reveal biotin-coupled antibodies. After staining, cells were washed with excess PBS–ethylenediamine tetraacetic acid (EDTA), centrifuged (490 *g*, 5 minutes, 4°C) and the supernatant was removed. Upon resuspension in 100 μL of PBS-EDTA (2.5 mmol final), cells were fixed with 2 mL of 1× Lyze/Fix PhosFlow (BD Biosciences) for 30 minutes on ice, in the dark before acquisition on the flow cytometer.

Flow cytometry data acquisition and analysis

Cells were analyzed on Stanford fluorescence-activated cell sorting (FACS) facility instruments LSRII, equipped with 4 lasers (535, 488, 633, 405 nm), 2 light-scatter detectors (yielding forward-scatter and side-scatter data) and 18 fluorescent detectors (BD Biosciences). Acquisition of 500K events was performed at the same flow rate and was controlled using the DiVa software (BD Biosciences).[23] Compensation was performed using single-stained beads during postacquisition analysis in the FlowJo software (Treestar, Ashland, OR), as described.[24] Data were collected for 0.2×10^7 to 1×10^7 cells. To distinguish autofluorescent cells from cells expressing low levels of individual surface markers, we established upper thresholds for autofluorescence by staining samples with fluorescence-minus-1 (FMO) control stain sets in which a reagent for a channel of interest is omitted.[25]

Results

Single-cell suspensions of human nasal and peripheral blood were taken directly from patients undergoing endoscopic sinus surgery for treatment of CRSsNP and control patients undergoing endoscopic sinonasal interventions, and then analyzed by high-dimensional (12 color, 14 parameter) FACS. Six control and 11 CRSsNP patients were included in this study. Based on undetectable IgE levels in patients' PBMCs and patient history, no patient enrolled in the study had a pertinent history of allergy. Of the recorded patient demographic information, the smoking status between both patient cohorts was disparate, with 100% in the control group vs 38% in the CRSsNP group endorsing smoking. Although a potential confounding factor, the smoking status of both cohorts did not appear to impact IgE plasmablast expression.

Statistically significant increases ($p < 0.05$) were noted in various B cell subsets (Table 3), especially the naive B lymphocyte population ($CD20^+CD19^+CD43^-$) in the resident sinus tissues acquired from nonatopic CRSsNP patients compared to nasal tissue acquired from control patients. Statistically significant increases ($p < 0.05$) were noted in activated B lymphocyte populations ($CD20^+CD19^+CD43^+$) in the nasal mucosa of

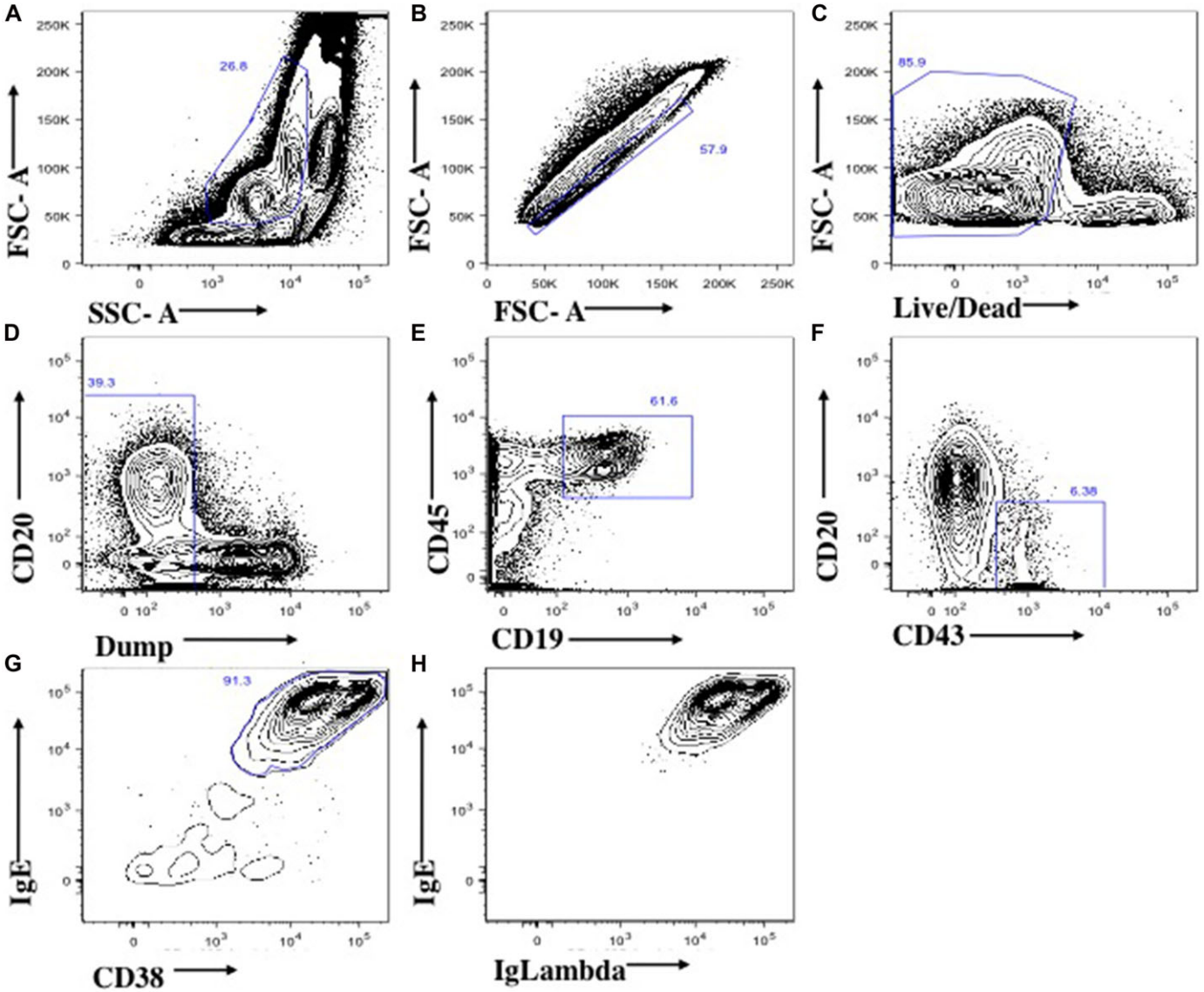

FIGURE 1. IgE plasmablast gating strategy. Cellular events of interest are highlighted within blue gates. IgE plasmablasts demonstrate almost exclusive expression of cell surface Ig Lambda. Ig = immunoglobulin. FSC-A = Forward Scatter Area; SSC-A = Side Scatter Area; Dump = Unwanted cells which collectively including monocytes (CD14), NK cells (CD16) and T cells (CD4/8); Ig = immunoglobulin. Letters A-H indicate th intended order of FACS images.

nonatopic patients with CRSsNP when compared to activated B-cell populations in the nasal tissue from controls. A significant increase ($p < 0.05$) was also noted in memory-switched B-cells (IgD^{-} IgM^{-} $CD20^{+}CD19^{+}CD27^{+}$), and-plasmablast ($CD19^{+}CD20^{+}CD27^{high}CD38^{high}CD138^{neg}$) populations in nasal tissue of nonatopic CRSsNP patients when compared to control nasal samples. Furthermore, IgE plasmablast populations within the nasal tissue of nonatopic CRSsNP patients demonstrated a significant increase ($p < 0.01$) when compared to peripheral blood samples from the same individual patient as well as control nasal tissue (data not shown).

Further gating analysis of nonatopic CRSsNP nasal tissue (Fig. 1) revealed an IgE plasmablast population ($CD19^{+}CD20^{+}CD27^{high}CD38^{high}CD138^{neg}CD43^{+}IgM^{neg}$ $IgD^{neg}IgE^{+}$). This prominent population of plasmablast cells (~6% of total B cells) display an exceptionally high expression (~90%) of lambda light chains compared to memory B cells found in the inflamed nasal tissue of the same patient (Fig. 2). These activated, IgE-secreting cells are below the limits of detection in the circulating peripheral blood from patients afflicted with CRS and nasal tissue samples from control patients (data not shown). This in situ IgE plasmablast population in the nasal tissue of nonatopic CRSsNP patients is $Ki\text{-}67^{+}$, signifying mitotic activity within this effector cell population (Fig. 3).

Discussion

The inflammatory basis of CRS disease is not fully understood. Moreover, there remains a paucity of knowledge with regard to B cell and immunoglobulin involvement

TABLE 2. Individual stain set for outlined experiments

Marker	Function/cell identified	Channel
Live/dead dye	Dead cell debris exclusion	Aqua amine
CD3	T cell exclusion marker	Pacific orange
CD14	Monocyte exclusion marker	Pacific orange
CD16	NK exclusion marker	Pacific orange
CD45	Pan leukocyte marker (except platelets and red blood cells)	Pacific blue
CD19	B lineage cell inclusion; pro-B cell through the mature B-cell stages (except terminally differentiated plasma cells)	BV786
CD20	Resting and activated B lymphocytes (lost prior to differentiation into plasma cells)	Cy5PE
CD27	Memory/activation	Cy7APC
CD38	Differentiation/plasmablast	Cy7PE
CD138	Differentiation/plasma cell	PE
CD43	Activated B and plasma cells	FITC
IgD	Surface immunoglobulin isotype	AF-700
Light chain lambda	Small polypeptide component of Igs on B cells and in serum	BV605
IgE	Surface immunoglobulin isotype	APC
IgM	Surface immunoglobulin isotype	PerCP Cy5.5

APC = allophycocyanin; FITC = fluorescein isothiocyanate; Ig = immunoglobulin; NK = natural killer; PE = phycoerythrin, AF = AlexFluor; BV = Brilliant Violet.

TABLE 3. Cell surface marker signatures for B cell subsets[8]

Name	Type	Phenotype	Function
Mature naive		IgD^{+} $CD27^{-}$ $CD10^{-}$ $CD38^{+/low}$	Precursor to GC, memory, ASCs
Memory	IgM only	IgD^{-} IgM^{+} $CD27^{+}$ $CD38^{+}$	Rapid Ig secretion to past seen antigens
	Switched	IgD^{-} IgM^{-} $CD27^{+}$	B cells undergoing maturation
ASC	Plasmablast	$CD19^{+}$ $CD20^{+}$ $CD27^{high}$ $CD38^{high}$ $CD138^{neg}$ $Pax5^{+}$	Early antibody secreting cell
ASC	Plasma cell	$CD19^{-}$ $CD20^{-}$ $CD27^{high}$ $CD38^{high}$ $CD138^{+}$ $Pax5^{-}$	Final stage antibody secreting cell

ASC = antibody-secreting cell; GC = germinal center; Ig = immunoglobulin.

in CRS. Of particular interest is the unexpected presence and role of an immunoglobulin (IgE), virtually synonymous with allergy-type responses, that are found to be elevated in nonatopic CRSsNP nasal tissue. Our interest was to investigate the role of B cell subsets in the nasal tissue of nonatopic CRSsNP patients, with particular interest in an IgE antibody-secreting cell. Improved understanding of B cell involvement in CRS will hopefully further our knowledge to identify potential therapeutic targets for CRS patients refractory to available treatment modalities.

Past studies suggest that CRS constitutes an inflammatory response restricted to the nasal mucosa.[6] Some of our findings, which have been demonstrated by previous investigators,[5] show a significant increase in B cell populations (activated B cells, memory switched, and plasmablast) in the upper airway of CRS patients, but not in nasal tissue from controls, or the peripheral blood of patients afflicted with CRS. This supports the notion that CRS is a local inflammatory occurrence. However, definitive evidence demonstrating B cell activation and immunoglobulin production occurring in situ in the nasal mucosa of CRS has yet to be shown. This gap in the literature led to our investigation to characterize a unique plasmablast population, an IgE plasmablast, which we characterize here in the nasal tissue of nonatopic CRSsNP patients. The presence of this plasmablast population in the nasal tissue of nonatopic CRSsNP patients—which is below detectability in the peripheral blood of patients with CRSsNP and from the nasal tissue of control patients—contributes to previous work suggesting that immunoglobulin production in an inflammatory environment such as CRS may occur locoregionally. This provides another context for the function of IgE in contrast to the hypothesis that IgE production is a systemic one.[9,10] Additionally, although IgE involvement has been investigated in the context of CRS,

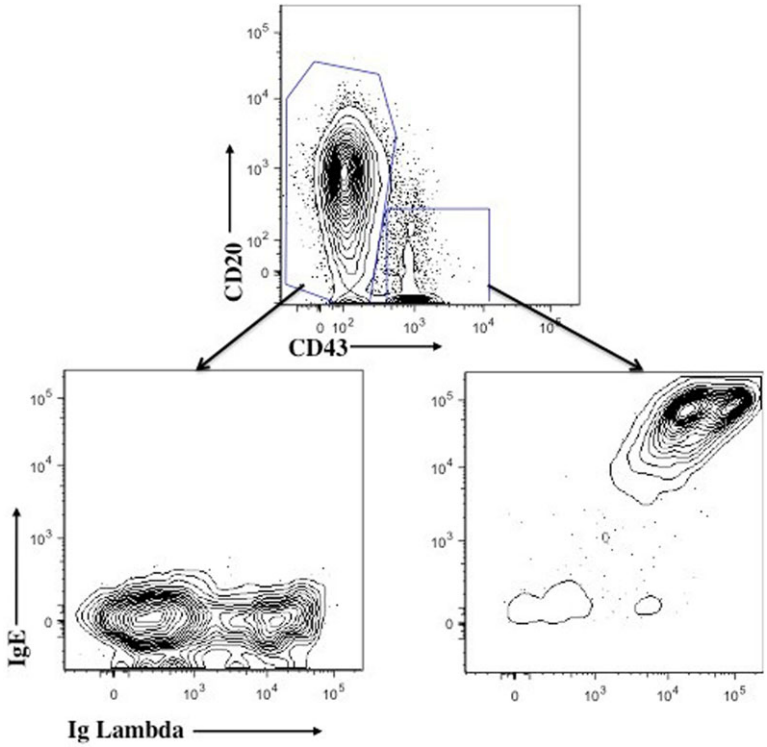

FIGURE 2. CD43^{+}/IgE^{+} plasmablasts in nonatopic CRSsNP nasal mucosal tissue express mostly lambda light chain. CRSsNP = chronic rhinosinusitis without nasal polyps; Ig = immunoglobulin.

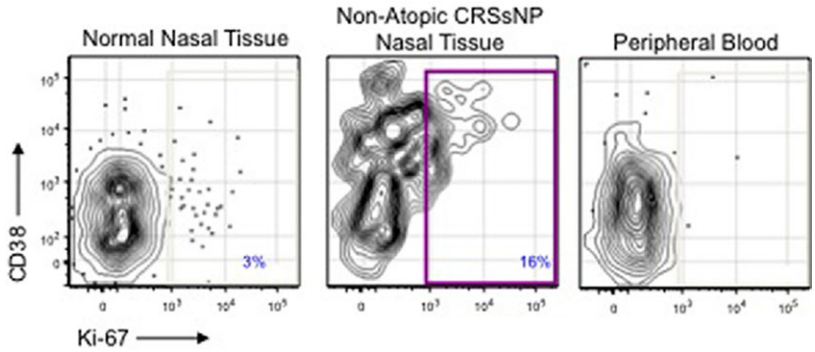

FIGURE 3. IgE plasmablasts in nasal mucosal tissue of nonatopic CRSsNP patients are actively dividing. CRSsNP = chronic rhinosinusitis without nasal polyps; Ki-67 = intracellular stain to detect proteins associated with mitotic activity.

it has been mostly from an atopy standpoint and not in nonatopic patients.[12,26–28] IgE involvement in the inflammatory process of nonatopic patients suffering from CRSsNP provides an additional perspective on the role of IgE in upper airway chronic inflammatory process.

Our findings herein demonstrate that various subpopulations of B cell precursors are exclusively present in the nasal mucosal tissue samples taken from CRS subjects, but not from control, nondiseased nasal specimens. Of the plasmablasts present in nonatopic CRSsNP patients, IgE plasmablasts, display almost exclusive (~90%) lambda light chain expression. The distortion of kappa:lambda light expression in humans (usually 2:1 kappa:lambda) found on this IgE plasmablast population, in conjunction with Ki-67^{+} mitotic activity, together suggest that this B cell population is abnormally proliferating and possibly contributing to the pathogenesis of CRS. However, further definitive studies are needed to elucidate the mechanism that promotes selective expression of lambda chain.

Conclusion

In summary, our characterization of a plasmablast population expressing IgE in the diseased upper airway tissue from nonatopic CRSsNP patients, which is essentially absent from normal nasal tissue sites studied, suggests a unique immunoregulatory microenvironment in the upper airway epithelium of patients afflicted with nonatopic CRSsNP.

Here, we expose the need for a more comprehensive characterization of IgE-expressing plasma cells in the context of nonatopic CRSsNP patients, with an ultimate goal of determining whether the presence of these cells correlates with clinical disease phenotypes. Such information will provide the basis for determining what role locally secreted IgE may play in the pathogenesis of CRS, and whether detection of these cells may have either diagnostic significance or translational relevance. The findings reported here, reflecting work completed during a relatively early stage of our flow cytometric studies, are representative of findings from work we have since completed with a substantially larger number of samples and with larger numbers of cells analyzed per sample. In this recent latter work, we have found that the IgE isotype plasmablasts identified here are frequently accompanied by small numbers of plasmablasts expressing other immunoglobulin isotypes.

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