IgD-activated mast cells induce IgE synthesis in B cells in nasal polyps

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Background: Although upregulated expression of local IgD has been reported in patients with chronic rhinosinusitis (CRS), its function is unclear.

Objective: We sought to explore the expression and function of soluble IgD in patients with CRS, particularly CRS with nasal polyps.

Methods: IgD levels in sinonasal mucosa were analyzed by using RT-PCR and ELISA. Numbers and phenotypes of IgD^+ cells were studied by means of immunohistochemistry, immunofluorescence, and flow cytometry. HMC-1 cells, a human mast cell line, and mast cells purified from eosinophilic polyps were cultured alone or with naive B cells purified from peripheral blood. The antigen specificity of nasal IgD was investigated by using ELISA.

Results: The mRNA expression of immunoglobulin heavy constant delta gene, numbers of IgD^+ cells, and protein levels of secretory IgD in sinonasal mucosa were increased in patients with CRS with or without nasal polyps compared with control subjects. Numbers of IgD^+ plasmablasts were increased in both eosinophilic and noneosinophilic polyps, whereas numbers of IgD^+ mast cells were only increased in eosinophilic polyps. Cross-linking IgD induced serum preincubated HMC-1 cells and polyp mast cells to produce B-cell activating factor, IL-21, IL-4, and IL-13 and to promote IgM, IgG, IgA, and IgE production from B cells. In eosinophilic polyps expression of those B cell–stimulating factors in mast cells and close contact between mast cells and B cells were found. Moreover, positive correlations of total IgD levels with total IgE levels and eosinophilia and upregulation of specific IgD against house dust mites were discovered in eosinophilic polyps.

Conclusion: IgD-activated mast cells can facilitate IgE production and eosinophilic inflammation in patients with CRS with nasal polyps. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *B cell, chronic rhinosinusitis, eosinophil, IgD, IgE, mast cell, nasal polyps*

Chronic rhinosinusitis (CRS) is a highly prevalent chronic inflammatory disease of the sinonasal mucosa.[1] Clinically, it can be divided into 2 subgroups: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP). It is generally considered that CRSwNP is characterized by T_H2-biased eosinophilic inflammation with local IgE production, whereas CRSsNP is more neutrophilic and type 1/type 17 response dominant.[2-4] However, the immunopathologic features of CRS vary across different geographic areas and populations with distinct racial backgrounds, and about half of Asian patients with CRSwNP do not demonstrate eosinophilic inflammation.[4,5] Current treatment approaches for CRS include medical treatments involving nonspecific suppression of inflammation and endoscopic sinus surgery. Nevertheless, a significant number of patients respond poorly to these treatments, reflecting our limited understanding of the pathogenesis of CRS.

Accumulating evidence has implicated involvement of local immunoglobulin, including IgE, IgG, IgA, and IgD, in the pathogenesis of CRS.[6-8] Compared with other immunoglobulins, IgD remains an enigmatic antibody class.[9,10] IgD is expressed as a transmembrane or secretory molecule in a species-specific manner.[11,12] Transmembrane IgD is one of the major antigen receptor isotypes on the surfaces of human mature naive B cells.[13,14] After encountering antigen, mature B cells transcriptionally downregulate surface IgD,[15] thereafter undergo somatic hypermutation and class-switch recombination of DNA, and differentiate into antibody-secreting plasma cells and memory B cells.[13] In addition to switching from IgM to IgG, IgA, and IgE, some B cells switch to IgD.[16]

Secreted IgD is released into blood, as well as being present in respiratory, salivary, lacrimal, and mammary secretions.[13] Recently, class-switch recombination to IgD and IgD^+IgM^- plasmablasts have been found in the upper airway mucosa.[14] Secreted IgD can afford protection to the respiratory mucosa by binding to pathogenic bacteria, such as *Moraxella catarrhalis* and *Haemophilus influenzae*, and their virulence factors.[14] In addition, secreted IgD participates in B-cell homeostasis and function regulation. Cross-linking of IgD bound to basophils induced release of B cell–stimulating factors, such as B-cell activating factor (BAFF), and promoted IgA, IgG, and IgM production from B cells.[14] However, whether secreted IgD is able to regulate IgE production and how it contributes to pathologic conditions in human diseases remain unclear.

Recently, 2 studies investigated local IgD expression in patients with CRS, and conflicting results were reported.[17,18] Min et al[17]

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Abbreviations used
BAFF: B-cell activating factor
CRS: Chronic rhinosinusitis
CRSsNP: Chronic rhinosinusitis without nasal polyps
CRSwNP: Chronic rhinosinusitis with nasal polyps
HRP: Horseradish peroxidase
IGHD: Immunoglobulin heavy constant delta
NMC: Nasal mucosal mononuclear cell

found increased numbers of mucosal IgD^+ cells in patients with CRSsNP but not those with CRSwNP,[17] whereas Sokoya et al[18] demonstrated enhanced accumulation of IgD^+ cells in both patients with CRSsNP and those with CRSwNP.[18] Nevertheless, more importantly, the specificity and function of IgD have not been explored in those studies.

In this study we studied the expression, specificity, and function of secreted IgD in patients with CRS, especially CRSwNP. We found upregulated mucosal soluble IgD production in both patients with CRSwNP and those with CRSsNP, with the greatest level in patients with eosinophilic CRSwNP. Numbers of IgD^+ mast cells and levels of specific IgD against dust mite antigens were increased, although only in patients with eosinophilic CRSwNP. IgD-activated mast cells induced IgE production from B cells, which potentially contributed to eosinophilic inflammation in patients with CRSwNP.

METHODS

Patients

This study was approved by the Ethics Committee of Tongji Hospital and conducted with written informed consent from every patient. A total of 144 patients with CRSwNP, 32 patients with CRSsNP, and 109 control subjects were enrolled in this study. The diagnosis of CRS was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps.[2] CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of total infiltrating cells, as reported by our previous study.[5] This cutoff was calculated as twice the SD of the mean eosinophil percentage in control subjects.[5] Patients without a history of CRS undergoing nasal surgery for a variety of non-CRS indications were enrolled as control subjects. Polyp tissues from patients with CRSwNP, diseased sinus mucosal samples from patients with CRSsNP, and ethmoid sinus tissues or inferior turbinate tissues from control subjects were obtained during surgery. Because of the limited amount of tissue samples, not all samples were included in every study protocol. Patients' characteristics are summarized in Table E1 in this article's Online Repository at www.jacionline.org, and other additional information is provided in the Methods section in this article's Online Repository at www.jacionline.org.

RT-PCR

Quantitative RT-PCR was performed with specific primers (see Table E2 in this article's Online Repository at www.jacionline.org), as stated elsewhere.[14,19] More information is provided in the Methods section in this article's Online Repository.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence staining were performed, as previously described.[5,7,19] The antibodies used are listed in Tables E3 and E4 in this article's Online Repository at www.jacionline.org. More information is provided in the Methods section in this article's Online Repository.

Mononuclear cell isolation

Sinonasal mucosal samples were dissociated mechanically.[20] Nasal mucosal mononuclear cells (NMCs) from dispersed tissue cells and PBMCs were isolated by means of density gradient centrifugation on Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway), as previously described.[20] More information is provided in the Methods section in this article's Online Repository.

Flow cytometry

NMCs and PBMCs were stained with specific primary antibodies (see Table E5 in this article's Online Repository at www.jacionline.org) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif). More information is provided in the Methods section in this article's Online Repository.

Purification of mast cells and naive B cells

Nasal mast cells from eosinophilic polyps and naive B cells from peripheral blood were isolated from NMCs and PBMCs by means of immunomagnetic cell sorting, respectively.[20,21] The representative results of purification of naive B and mast cells are shown in Fig E1 in this article's Online Repository at www.jacionline.org. More information is provided in the Methods section in this article's Online Repository.

Stimulation of mast cells

HMC-1 cells (ATCC, Manassas, Va), a human mast cell line, were preincubated with human sera from control subjects overnight. Serum incubated HMC-1 cells and isolated polyp mast cells were stimulated with mouse mAb against IgD or IgE.[14,22] After a 16-hour stimulation, supernatants were collected for cytokine measurement.[14,20] More information is provided in the Methods section in this article's Online Repository.

Coculture of mast cells and naive B cells

Peripheral naive B cells were cultured with autologous polyp mast cells or serum preincubated HMC-1 cells, and cells were treated with mAbs against IgD or IgE.[14,21] After an 8-day culture, culture supernatants were harvested for immunoglobulin analysis.[14,20] More information is provided in the Methods section in this article's Online Repository.

Measurement of immunoglobulin and cytokine levels

Total and specific IgE levels were measured by using the ImmunoCAP system (Phadia, Uppsala, Sweden).[7,19] Levels of other immunoglobulins and cytokines were measured by using commercially available ELISA kits or Bio-Plex suspension chip technology.[7,14,19] Lower detection limits for ELISA assays and Bio-Plex suspension chip technology were shown in Table E6 and E7, respectively, in this article's Online Repository at www.jacionline.org. Additional information is provided in the Methods section in this article's Online Repository.

Measurement of antigen-specific IgD levels

A capture ELISA was developed to measure antigen-specific IgD levels.[23] Briefly, goat anti-human IgD antibody–coated polystyrene microtiter plates were incubated with tissue homogenate supernatants. Then biotin-conjugated *Dermatophagoides pteronyssinus* group 1 (Der p 1), *Dermatophagoides farinae* group 1 (Der f 1), or LPS was added to detect the antigen specificity of captured IgD. After washing, streptavidin–horseradish peroxidase (HRP) and substrate tetramethylbenzidine were added. Because there are no available standards for antigen-specific IgD, results are presented as OD_{450} values. No cross-reaction with other immunoglobulins was found for the developed ELISA (see Fig E2 in this article's Online Repository at www.jacionline.org). Inhibition tests confirmed antigen specificity of developed ELISA (see Table E8 in this article's Online Repository at www.jacionline.org). More information is provided in the Methods section in this article's Online Repository.

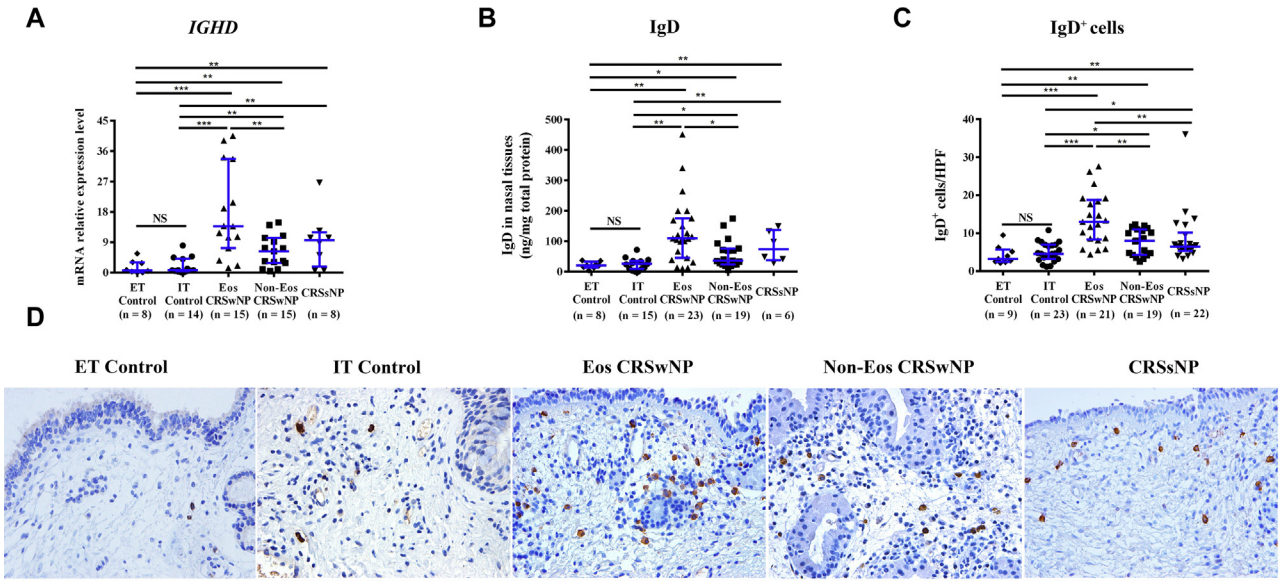

FIG 1. Increased mucosal IgD expression in patients with CRS. **A-C,** Upregulated mRNA expression levels of *IGHD* (Fig 1, *A*), protein levels of secretory IgD (Fig 1, *B*), and numbers of IgD^+ cells (Fig 1, *C*) in diseased sinonasal mucosa in patients with CRSsNP and those with eosinophilic and noneosinophilic CRSwNP. **D,** Representative immunostaining photomicrographs show IgD^+ cells in lamina propria of sinonasal mucosa (original magnification ×400). *Eos CRSwNP*, Eosinophilic CRSwNP; *ET*, ethmoid sinus tissues; *IT*, inferior turbinate tissues; *Non-Eos CRSwNP*, noneosinophilic CRSwNP; *NS*, no significant difference. $*P < .05$, $**P < .01$, and $***P < .001$.

Statistics

Statistical analysis was performed by using SPSS 18.0 software (SPSS, Chicago, Ill). Expression results are presented in dot plots. Symbols represent individual samples; horizontal bars represent medians, and error bars show interquartile ranges. When comparisons were made between groups, the Kruskal-Wallis *H* test was used to assess significant intergroup variability. The Mann-Whitney *U* 2-tailed test was used for between-group comparison. The Spearman rank test was used for correlations. Cell-culture data are expressed as means ± SEMs and analyzed by using the paired Student *t* test. A *P* value of less than .05 was considered significant.

RESULTS

Increased levels of mucosal IgD in patients with CRS

We first detected the mRNA expression of gene immunoglobulin heavy constant delta *(IGHD)* in nasal tissues, which reflects local IgD synthesis, including the transmembrane and secretory type. Compared with control inferior turbinate or ethmoid sinus mucosal samples, mRNA expression of *IGHD* in diseased sinus mucosa was upregulated in patients with CRSsNP and in patients with CRSwNP of both the eosinophilic and noneosinophilic types (Fig 1, *A*). The secretory type of IgD, as detected by means of ELISA, was found to be upregulated in the sinonasal mucosa in patients with all types of CRS (Fig 1, *B*). The immunohistochemistry study revealed enhanced accumulation of IgD^+ cells in the lamina propria of sinonasal mucosa from patients with CRS (Fig 1, *C* and *D*). In contrast, we did not find significant differences in serum IgD levels among different subject groups (see Fig E3 in this article's Online Repository at www.jacionline.org).

Among different types of CRS, eosinophilic CRSwNP demonstrated the highest levels of local IgD expression (Fig 1). Therefore we next focused on the role of IgD in pathophysiology of patients with eosinophilic CRSwNP. Control inferior turbinate and control ethmoid sinus mucosal samples had comparable IgD expression (Fig 1). Given the larger amount of samples generally obtained from inferior turbinates, we used inferior turbinate tissues as a control for immunofluorescence and flow cytometry study thereafter.

Increased numbers of mucosal IgD^+ plasmablasts in patients with CRSwNP

Chen et al[14] previously demonstrated that secreted IgD can bind to mast cells and basophils but not T cells, natural killer cells, monocytes, dendritic cells, or granulocytes through an unidentified calcium-fluxing receptor. IgD^+ cells in sinonasal mucosa might be IgD^+ B cells or "IgD-armed" immune cells.

We then phenotyped IgD^+ cells in nasal polyps using immunofluorescence staining and flow cytometry. Using immunofluorescence staining, we found that the numbers of IgD^+CD19^+ B cells in lamina propria were increased in both eosinophilic and noneosinophilic polyps compared with control inferior turbinate tissues, with a further increase in eosinophilic polyps (Fig 2, *A* and *B*). Importantly, most IgD^+CD19^+ B cells were found to be IgM^- and $CD138^-$ but $CD38^+$ (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting that they are plasmablasts.

Using flow cytometry, we confirmed that most polyp IgD^+CD19^+ B cells were IgM^- (Fig 2, *C*), and those IgD^+IgM^- B cells were $CD38^+CD138^-$ plasmablasts with intracellular IgD expression (see Fig E5 in this article's Online Repository at www.jacionline.org), which is consistent with the previous report.[17] We found that the frequencies of total IgD^+ cells, as well as IgD^+IgM^- B cells, in NMCs were increased in both eosinophilic and noneosinophilic polyps, with a more prominent increase in eosinophilic polyps (Fig 2, *D*). IgD^+ plasmablasts accounted for about 46% and 70% of total IgD^+ cells in

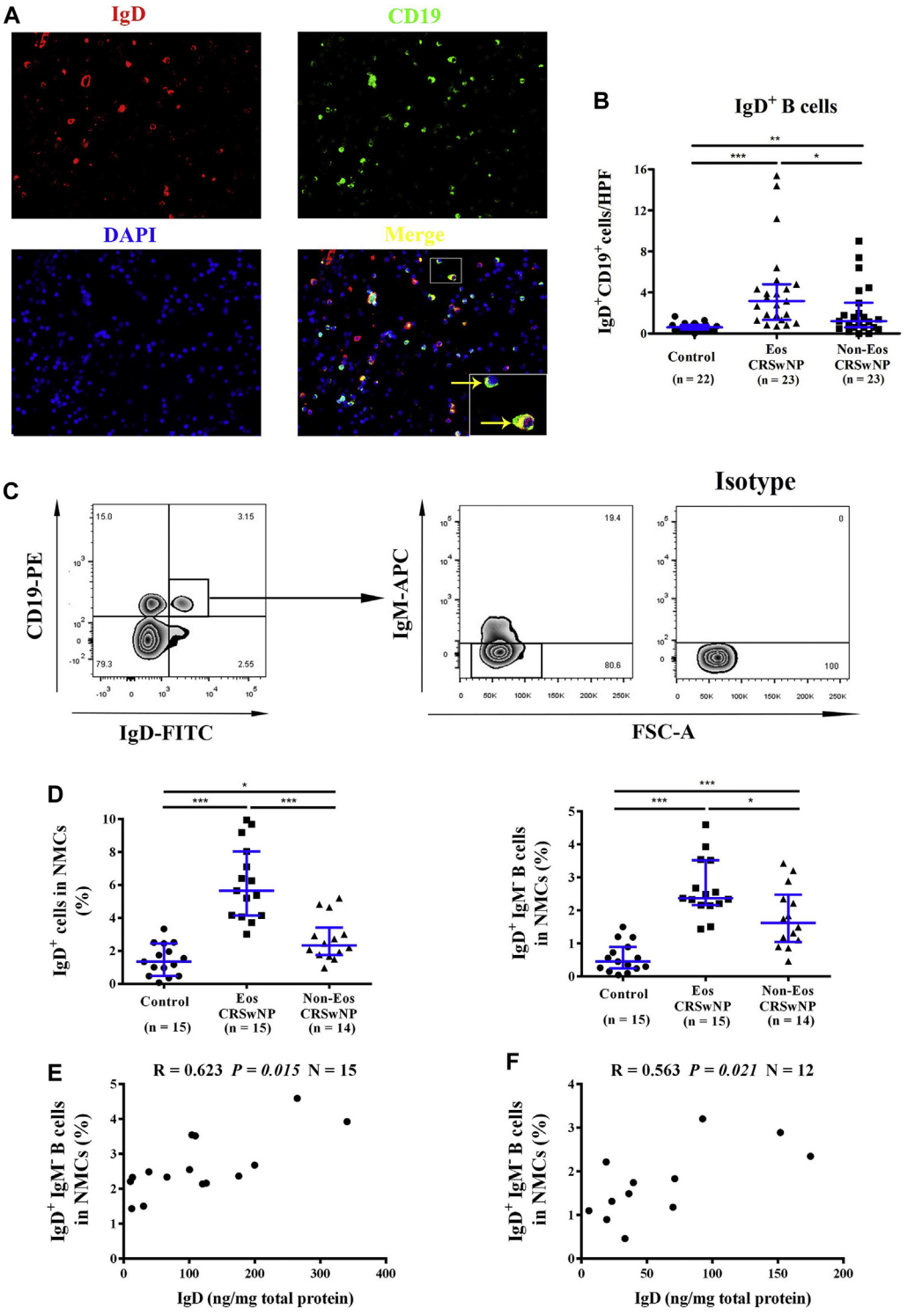

FIG 2. Increased numbers of mucosal IgD^+ B cells in patients with CRSwNP. **A,** Representative immunofluorescence photomicrographs show IgD^+CD19^+ B cells in lamina propria of nasal polyps (original magnification ×400). *Inset* shows a higher magnification of the outlined area. *Arrows* denote positive cells. **B,** Quantification of IgD^+CD19^+ B cells in nasal polyps. **C,** Gating strategy and representative flow plots show that the majority of IgD^+ B cells are IgM^- in patients with nasal polyps. NMCs were analyzed. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *FSC*, forward scatter; *PE*, phycoerythrin; *SSC*, side scatter. **D,** Frequencies of total IgD^+ cells, as well as IgD^+IgM^- B cells, in NMCs in patients with nasal polyps. **E** and **F,** Correlations between mucosal IgD levels and frequencies of IgD^+IgM^- B cells in NMCs in eosinophilic (Fig 2, *E*) and noneosinophilic CRSwNP (Fig 2, *F*). *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

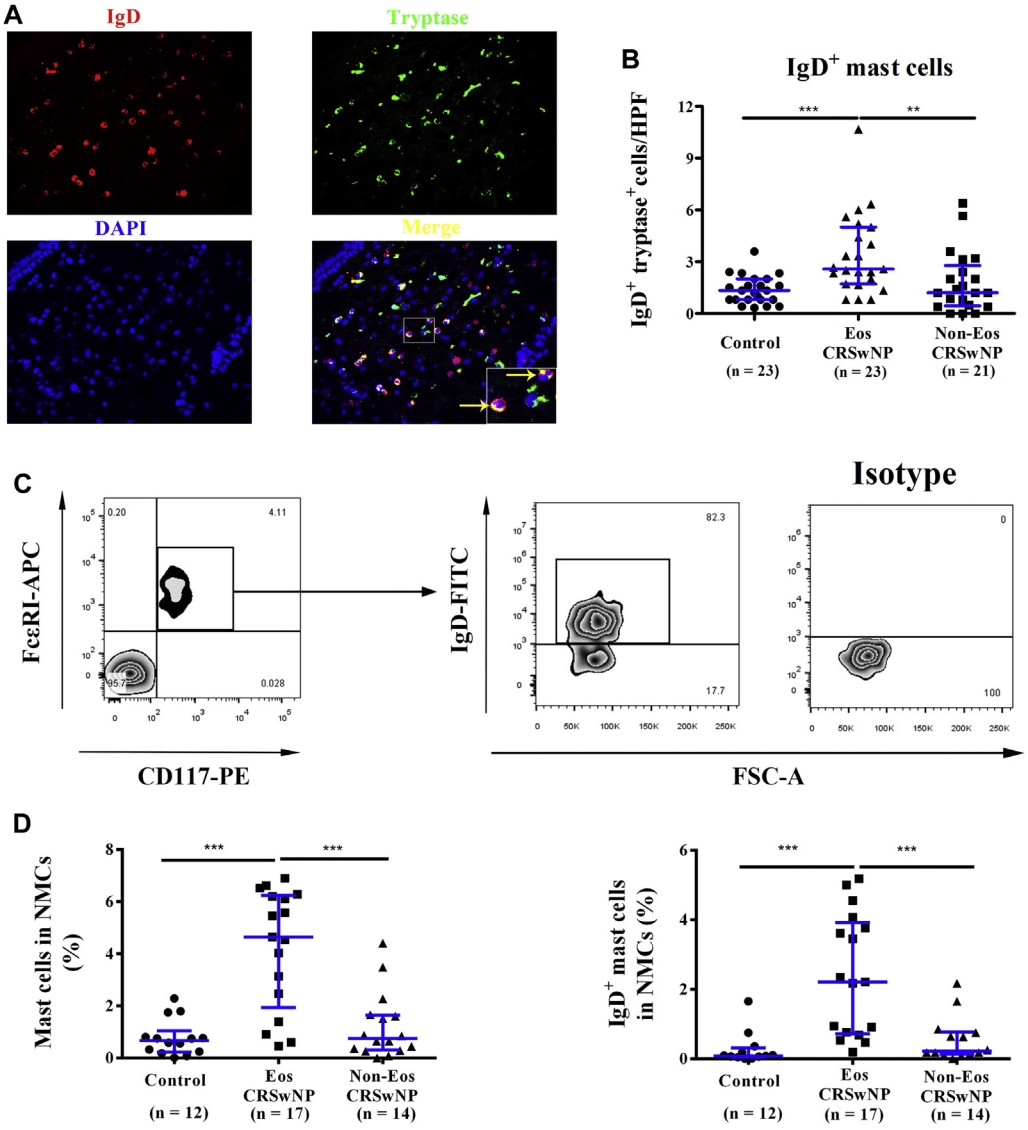

FIG 3. Increased accumulation of mucosal IgD$^+$ mast cells in patients with eosinophilic CRSwNP. **A,** Representative immunofluorescence photomicrographs show IgD$^+$ tryptase-positive mast cells in lamina propria of patients with nasal polyps (original magnification ×400). *Inset* shows a higher magnification of the outlined area. *Arrows* denote positive cells. **B,** Quantification of IgD$^+$ tryptase-positive mast cells in nasal polyps. **C,** Gating strategy and representative flow plots of IgD$^+$ mast cells in nasal polyps. NMCs were analyzed. *APC,* Allophycocyanin; *FITC,* fluorescein isothiocyanate; *FSC,* forward scatter; *PE,* phycoerythrin. **D,** Percentages of total mast cells and IgD$^+$ mast cells in NMCs in nasal polyps. *Eos CRSwNP,* Eosinophilic CRSwNP; *Non-Eos CRSwNP,* noneosinophilic CRSwNP. $^{**}P < .01$ and $^{***}P < .001$.

eosinophilic and noneosinophilic polyps, respectively. We further found that the frequencies of IgD$^+$IgM$^-$ B cells positively correlated with the levels of secretory IgD in both eosinophilic (Fig 2, *E*) and noneosinophilic polyps (Fig 2, *F*), suggesting local production of secretory IgD by IgD$^+$ plasmablasts in sinonasal mucosa. On the contrary, almost all peripheral IgD$^+$ B cells were IgD$^+$IgM$^+$ naive B cells (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org), and no significant difference in frequencies of total IgD$^+$ cells or IgD$^+$IgM$^-$ B cells in PBMCs was discovered among different groups (see Fig E6, *B*).

To validate our immunofluorescence staining and flow cytometric data, we analyzed IgD expression on polyps (see Fig E7, *A*, in this article's Online Repository at www.jacionline.org) and peripheral CD3$^+$ T cells (see Fig E7, *B*). Consistent with the previous report,[14] we did not find IgD expression on CD3$^+$ T cells (see Fig E7).

Increased numbers of mucosal IgD$^+$ mast cells in patients with eosinophilic CRSwNP

Increased accumulation and activation of mast cells have been reported for eosinophilic polyps.[19,24] Our flow cytometric analysis indicated that in addition to B cells, there were other types of IgD$^+$ cells in polyps (Fig 2, *C*). Therefore we investigated the presence of IgD-armed mast cells in sinonasal mucosa. We

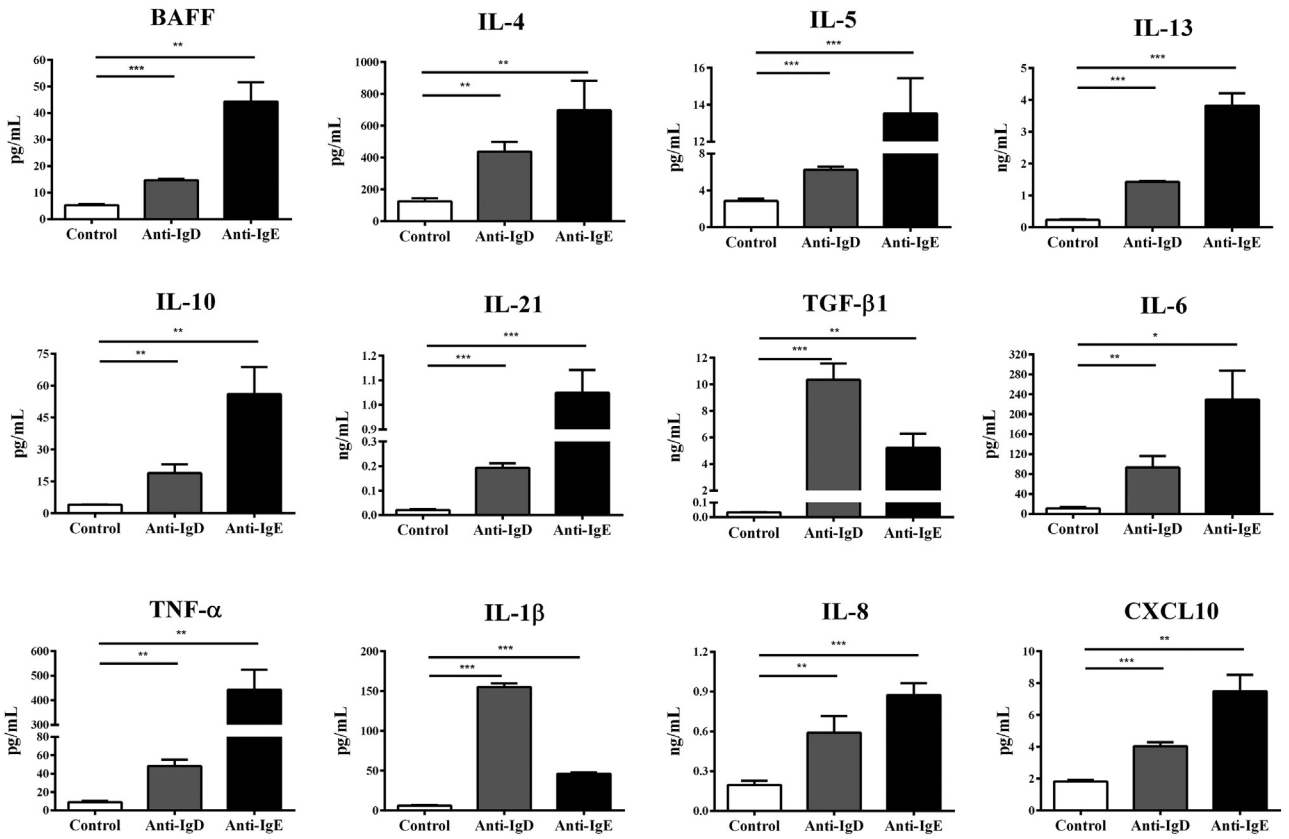

FIG 4. IgD cross-linking activates polyp mast cells to produce B cell–stimulating factor, inflammatory cytokines, and chemokines. Mast cells purified from eosinophilic polyps were treated with anti-IgD, anti-IgE, or control mouse mAb for 16 hours, and levels of mediators in culture supernatants were measured (n = 6). **P* < .05, ***P* < .01, and ****P* < .001.

found that the lamina propria of polyps was infiltrated by IgD^+ tryptase-positive cells (Fig 3, *A*). Numbers of IgD^+ mast cells were increased in eosinophilic polyps compared with noneosinophilic polyps and control tissues, as detected by using immunofluorescence staining (Fig 3, *B*). Consistently, flow cytometric analysis demonstrated increased frequencies of total mast cells and IgD^+ mast cells in NMCs in eosinophilic but not noneosinophilic polyps in comparison with control tissues (Fig 3, *D*). About 56% of mast cells had surface-bound IgD, and mast cells accounted for about 40% of IgD^+ cells in eosinophilic polyps.

IgD-activated HMC-1 cells produce B cell–stimulating factors

IgD cross-linking induced production of B-cell activating factors from IgD-armed basophils.[14] Given the significant accumulation of IgD-armed mast cells and upregulated local production of IgE in eosinophilic polyps,[19] we explored whether IgD cross-linking is able to activate mast cells and therefore contribute to local IgE production in eosinophilic polyps. Because we were unable to get commercially available IgD protein for cell culture, we incubated HMC-1 cells, a human mast cell line, with human normal serum. After incubation, about 18% and 7% of HMC-1 cells had surface-bound IgD and IgE, respectively (see Fig E8 in this article's Online Repository at www.jacionline.org). Cross-linking prebound IgD-and IgE-activated HMC-1 cells to produce B cell–stimulating factors, including BAFF, IL-4, IL-5, IL-13, IL-10, IL-21, TGF-β1, and IL-6; proinflammatory cytokines, including TNF-α and IL-1β; and IL-8 and CXCL10, 2 chemokines acting on monocytes, neutrophils, and T cells (see Fig E9 in this article's Online Repository at www.jacionline.org). We did not directly compare the efficiency of IgE and IgD cross-linking because different percentages of IgD^+ and IgE^+ HMC-1 cells were generated after serum incubation. IgD and IgE cross-linking did not activate HMC-1 cells without serum preincubation (data not shown). In addition, we found that anti-IgD antibody did not activate HMC-1 cells preincubated with IgE only (data not shown), confirming the specificity of anti-IgD antibody.

IgD-activated polyp mast cells produce B cell–stimulating factors

We next studied the effect of IgD cross-linking on polyp mast cells. Previous studies showed that mast cells in eosinophilic polyps also had surface-bound IgE.[19] Similar to the findings in HMC-1 cells, we found that both IgD and IgE cross-linking induced production of B cell–stimulating factors, proinflammatory cytokines, and chemokines from mast cells purified from eosinophilic polyps (Fig 4). Again, because the proportions of IgD^+ and IgE^+ mast cells in total purified polyp mast cells were different, we did not compare the efficiency of IgE and IgD cross-linking. Differing from IgE, IgD cross-linking did not

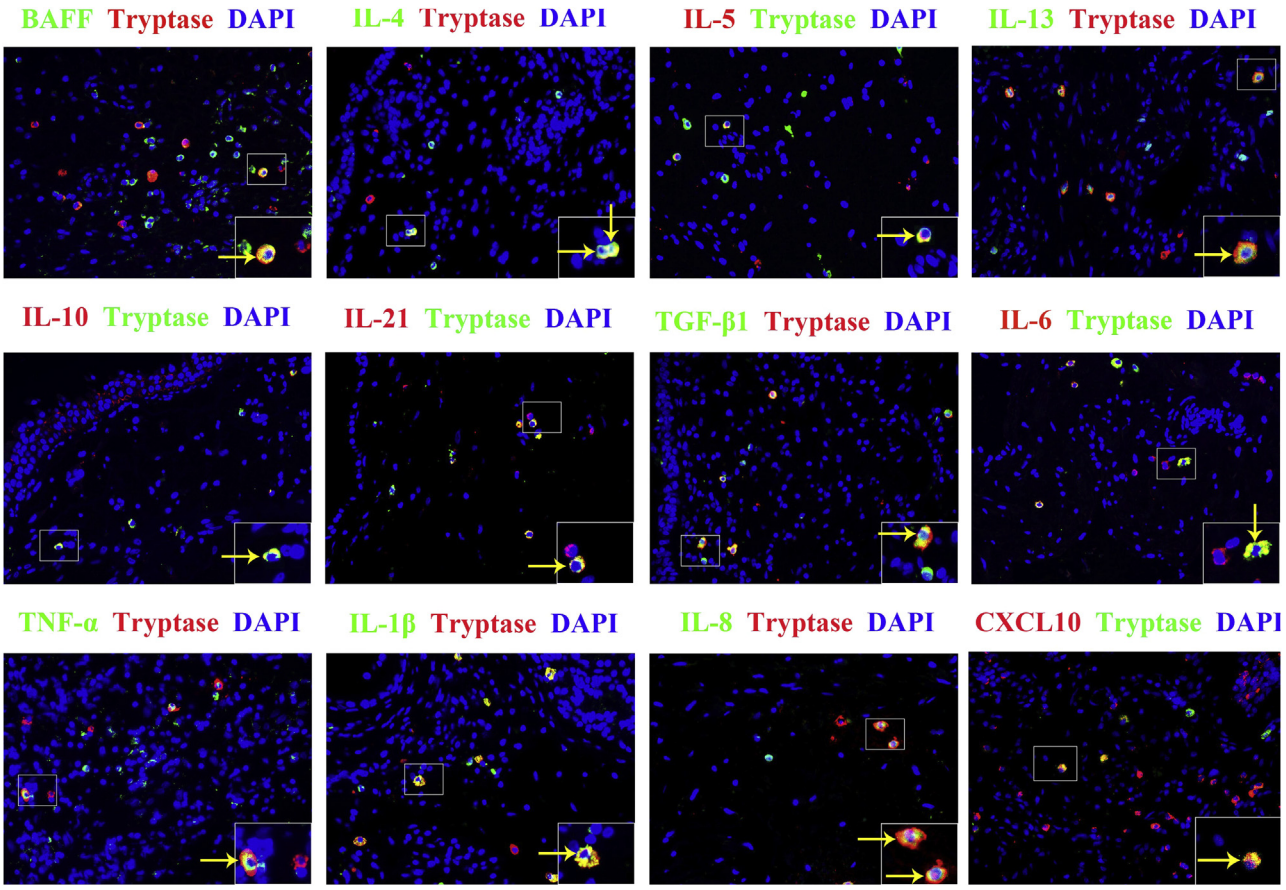

FIG 5. Mast cells are a cellular source of B cell–stimulating factor, inflammatory cytokines, and chemokines. Representative immunofluorescence photomicrographs are shown. Original magnification ×400. *Insets* show a higher magnification of the outlined area. *Arrows* denote positive cells. *DAPI*, 4′-6-Diamidino-2-phenylindole dihydrochloride.

induce mast cell degranulation (data not shown), which is consistent with the finding of basophils.[14] Furthermore, we found that mast cells in eosinophilic polyps had expression of BAFF, IL-4, IL-5, IL-13, IL-10, IL-21, TGF-β1, IL-6, TNF-α, IL-1β, IL-8, and CXCL10 *in vivo* by using immunofluorescence staining (Fig 5).

IgD-activated mast cells induce immunoglobulin production from B cells

Given the capacity of releasing B cell–stimulating factors by IgD-activated mast cells, we investigated whether IgD-activated mast cells were able to regulate B-cell antibody production. We found that IgD and IgE cross-linking upregulated the capacity of serum-incubated HMC-1 cells (see Fig E10 in this article's Online Repository at www.jacionline.org) and polyp mast cells (Fig 6) to induce production of nonswitched IgM, as well as isotype class-switched immunoglobulins, including IgG_1, IgG_3, IgG_4, IgA, and IgE, from B cells. We did not detect IgE production in the condition with anti-IgE treatment and IgG_2 production in all conditions because of the possible interference caused by experimental antibodies.

Interestingly, in polyp tissues we observed close contact between mast cells and B cells, which provided structure basis for the regulation of B cells by mast cells *in vivo* (Fig 7, *A*). Consistent with our previous studies,[19,20] we demonstrated increased local total IgE levels, but not serum total IgE levels, in patients with eosinophilic CRSwNP compared with those with noneosinophilic CRSwNP and control subjects (see Fig E11, *A* and *B*, in this article's Online Repository at www.jacionline.org). Moreover, there was no correlation between total IgE levels in tissues and those in sera in patients with eosinophilic CRSwNP (see Fig E11, *C*), suggesting local production of IgE in eosinophilic polyps. In addition, we found significant correlations of total IgD levels with total IgE levels and eosinophil numbers in eosinophilic polyps (Fig 7, *B*). Furthermore, we revealed significant associations between IgD^+ mast cells and total IgE levels and eosinophilia in patients with eosinophilic polyps (Fig 7, *C*).

Polyp IgD recognizes aeroallergens and bacterial products

Chen et al[14] previously reported that IgD bound to the gram-negative bacteria *M catarrhalis* and *H influenzae* and reacted to LPS and capsular polysaccharide.[14] Our previous study indicated that aeroallergens, particularly house dust mites, can induce local IgE production in patients with eosinophilic polyps.[7,19,20] Therefore we tested whether nasal mucosal IgD had antigen specificity against LPS and house dust mites,

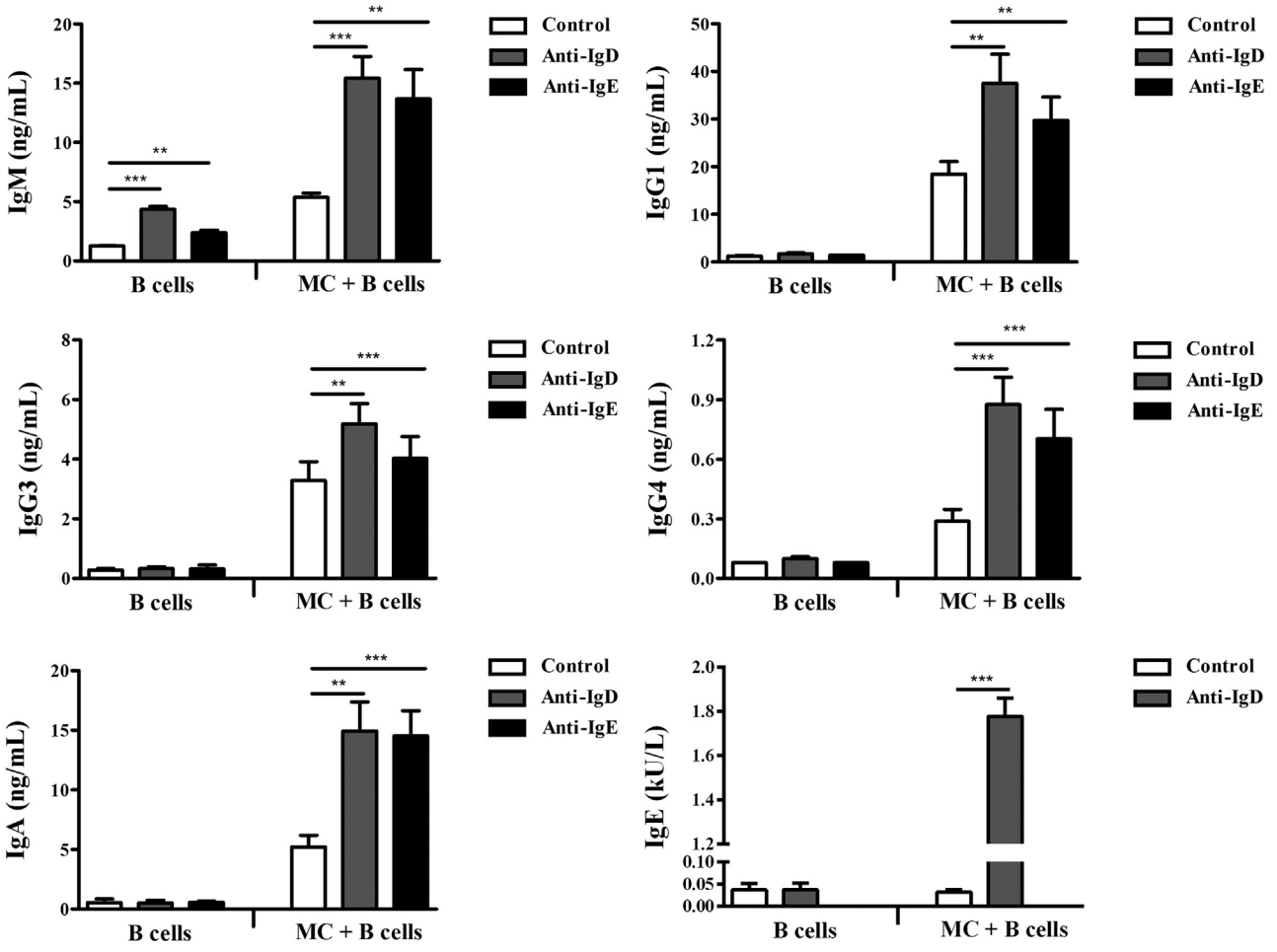

FIG 6. IgD-activated polyp mast cells *(MC)* induce immunoglobulin production from B cells. Mast cells purified from eosinophilic polyps were cocultured with autologous peripheral naive B cells and treated with anti-IgD, anti-IgE, or control mouse mAb for 8 days, and immunoglobulin levels in culture supernatants were measured (n = 5). IgG_2 levels in all conditions and IgE levels in anti-IgE treatment conditions were not analyzed because of the possible interference caused by experimental antibodies. $^{**}P < .01$ and $^{***}P < .001$.

as determined by using a capture ELISA. We found that levels of LPS-specific IgD were upregulated in both patients with eosinophilic and those with noneosinophilic polyps, whereas the levels of Der f 1– and Der p 1–binding IgD were only increased in patients with eosinophilic polyps, but not noneosinophilic polyps, compared with control tissues (Fig 8). Consistent with previous reports,[19] we found that levels of *Dermatophagoides pteronyssinus*– and *Dermatophagoides farinae*–specific IgE in polyp tissues, but not those in serum, were upregulated in patients with eosinophilic CRSwNP compared with patients with noneosinophilic CRSwNP and control subjects (see Fig E12, *A-D*, in this article's Online Repository at www.jacionline.org). Again, no correlation between local and serum specific IgE against house dust mites was found (see Fig E12, *E* and *F*), further supporting local IgE production independent of systemic atopy in patients with eosinophilic CRSwNP.[8,19,22] In addition, we did not find a significant association between local Der p 1–specific IgD and *D pteronyssinus*–specific IgE or between local Der f 1–specific IgD and *D farinae*–specific IgE in patients with eosinophilic polyps (see Fig E12, *G* and *H*).

DISCUSSION

The functional relevance of IgD in human diseases is poorly studied. In this study we showed that the numbers of IgD^+IgM^- plasmablasts and levels of secretory IgD were increased locally, but not systemically, in both patients with CRSsNP and those with CRSwNP, indicating a localized IgD response in patients with CRS. Upregulation of local IgD production in patients with CRSsNP has been reported previously.[17,18] Min et al[17] found that local soluble IgD levels were greater in those with preoperative antibiotic use or pathogenic bacteria presence, suggesting a potential involvement of IgD in immune response to bacterial infection in patients with CRSsNP. However, regarding local IgD production in patients with CRSwNP, inconsistent results have been reported.[17,18] In this study, using several complimentary techniques, we demonstrated increased production of secretory IgD in Chinese patients with patients with CRSwNP. Interestingly, patients with eosinophilic CRSwNP demonstrated the highest levels of local IgD. These findings drove us to explore the role of soluble IgD in the pathogenesis of eosinophilic CRSwNP.

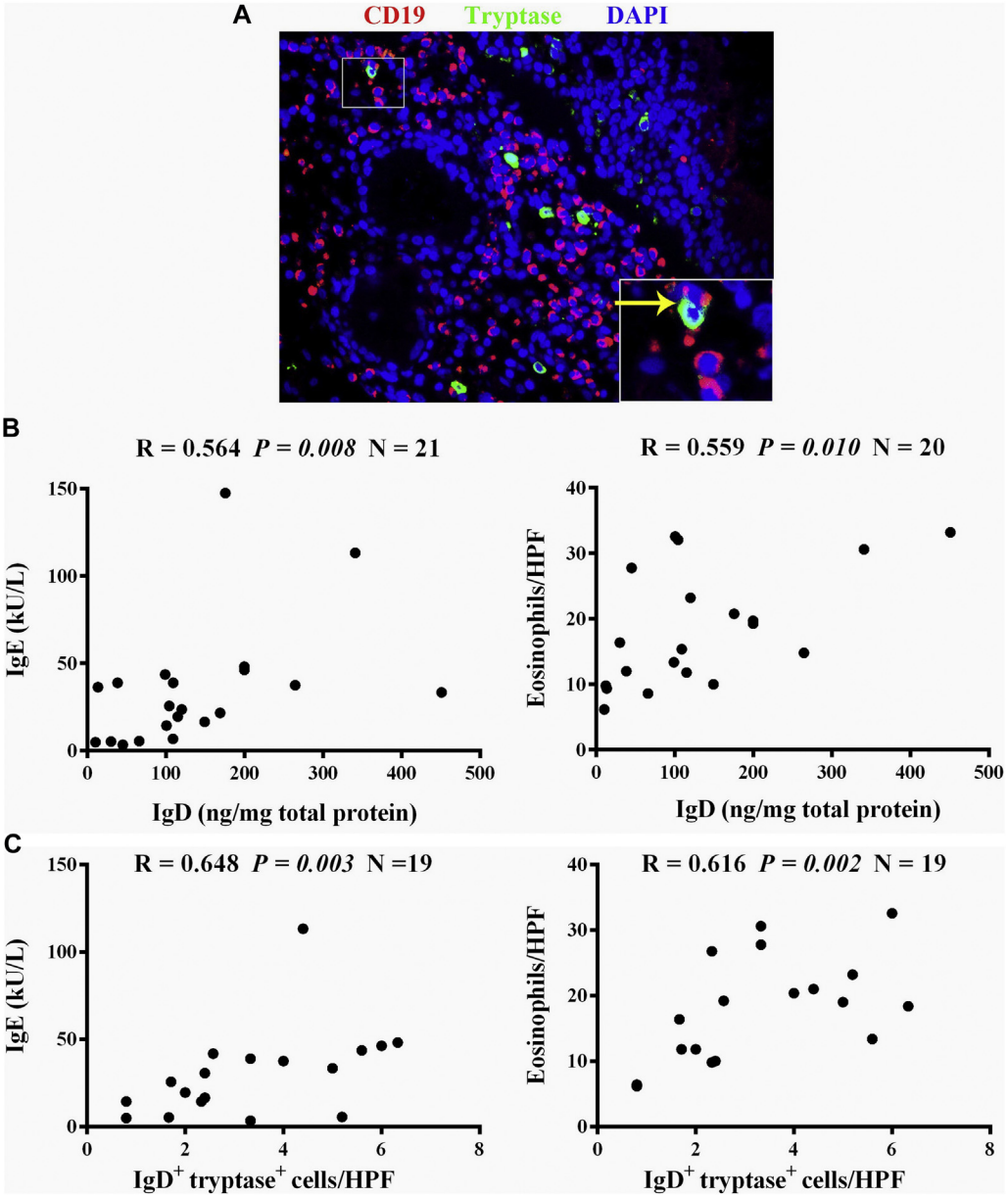

FIG 7. Local total IgD levels correlate with local total IgE levels and eosinophilia in patients with eosinophilic CRSwNP. **A,** Representative photomicrographs show close contact between $CD19^+$ B cells and tryptase-positive mast cells (original magnification ×400). *Inset* shows a higher magnification of the outlined area. *Arrow* denotes close contact. *DAPI,* 4′-6-Diamidino-2-phenylindole dihydrochloride. **B,** Correlations of total IgD levels with total IgE levels and eosinophilia in eosinophilic polyps. **C,** Correlations of IgD^+ mast cells with IgE levels and eosinophilia in eosinophilic polyps. *HPF,* High-powered field.

In addition to IgD^+IgM^- plasmablasts, we found that IgD-bound mast cells constitute the second largest population of IgD^+ cells in patients with eosinophilic polyps. Mast cells play key roles in host defense, tissue repair, and allergic inflammation.[25] Cross-linking IgE bound to mast cells by corresponding antigens can lead to mast cell activation, which correlates with eosinophilia in nasal polyps.[19,24] Nevertheless, the regulatory effect of IgD on mast cells is unknown. In this study, for the first time, we found that IgD cross-linking activated IgD-armed mast cells to release B cell−stimulating factors, which are crucial for the activation, proliferation, and class-switch recombination of B-cell DNA. These findings suggest a "B cell−licensing" function of mast cells with cross-linked IgD.

We further provided several additional lines of novel evidence for the regulatory function of IgD-activated mast cells on B-cell antibody production, especially IgE, in patients with eosinophilic CRSwNP. First, in addition to the *in vitro* finding of production of B cell–stimulating factors by IgD-activated polyp mast cells, we demonstrated that mast cells in patients with eosinophilic polyps had the expression of B cell–stimulating factors *in vivo*.

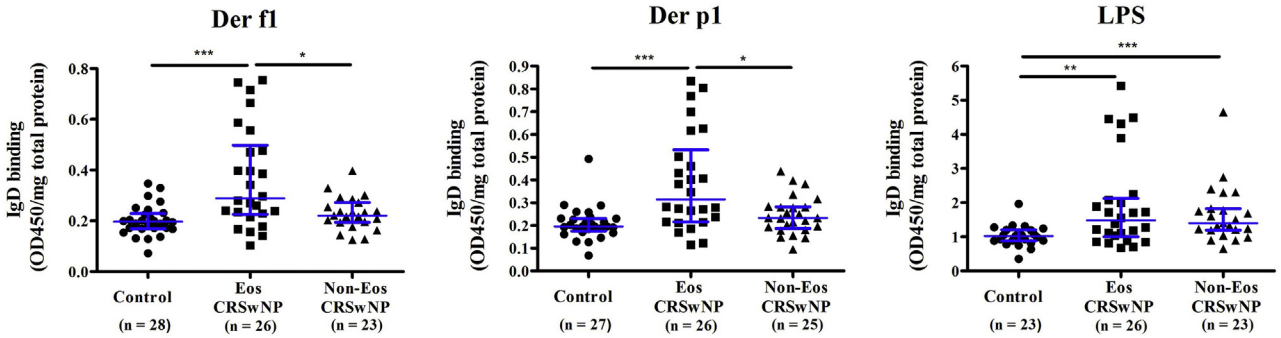

FIG 8. Local specific IgD binding to LPS and house dust mite antigens. A capture ELISA was developed to detect specific IgD binding to Der p 1, Der f 1, and LPS in tissue homogenates. Results are presented as OD_{450} and normalized to total protein levels of tissue homogenates. *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP. $*P < .05$, $**P < .01$, and $***P < .001$.

Second, we found that IgD-activated mast cells isolated from eosinophilic polyps not only induced IgM, IgA, and IgG but also IgE production from B cells. Although we did not perform blocking experiments, the B cell–licensing function of IgD-activated mast cells was likely dependent on self-secreted BAFF and cytokines important for immunoglobulin class-switching, such as IL-21, IL-4, and IL-13. A proliferation-inducing ligand and CD40 ligand also play an important role in B-cell antibody production.[26] In this study we did not find robust changes of these molecules on HMC-1 cells after IgD cross-linking (data not shown); however, we could not preclude the involvement of these molecules in mast cell and B-cell interaction. Local IgA and IgG were reported to be upregulated in both patients with eosinophilic and those with noneosinophilic CRSwNP; however, local IgE levels were only found to be increased in patients with eosinophilic CRSwNP.[7,20] Upregulation of local IgE irrespective of the presence of atopy and the presence of the markers of class-switch recombination to IgE, including ε germline gene transcript and ε circle transcripts in nasal polyps, strongly suggest local IgE production in nasal polyps.[8,22,27,28] In this study the increase in total and specific IgE levels in polyps, but not in serum, and the lack of association between serum and polyp IgE levels in patients with eosinophilic CRSwNP confirmed a localized IgE production. Local IgE levels are associated with tissue eosinophilia, asthma comorbidity, and disease relapse after surgery in patients with CRSwNP.[8,29,30] Therefore the regulation of IgE production by IgD-armed mast cells is of particular importance for the pathogenesis of eosinophilic CRSwNP.

Third, interestingly, we found close contacts between mast cells and B cells in patients with eosinophilic polyps, providing a structural basis for interaction between B cells and mast cells *in vivo*.

Fourth, the positive associations of IgD and IgD^+ mast cells with IgE and eosinophilia in eosinophilic polyps further support the regulatory function of IgD on local IgE production and subsequent eosinophilic inflammation in patients with CRSwNP. In addition to secreting B cell–stimulating factors, we found that IgD-activated mast cells simultaneously produced inflammatory cytokines and chemokines, underscoring an inflammatory role of IgD beyond regulating B cells in patients with CRSwNP.

The specificity of nasal IgD has not been studied previously. Soluble IgD can enhance immune protection against local pathogens and commensal bacteria.[13,14] Although there is not a definitive conclusion regarding the role of bacteria in CRS pathogenesis, a number of studies show that the microbiome of the nasal cavity differs significantly between patients with CRS and control subjects.[31,32] Multiple microbial products, including LPS, are major triggers of inflammation.[33,34] In the present study we found that IgD with binding activity to LPS was upregulated in both patients with eosinophilic and those with noneosinophilic CRSwNP, indicating that IgD can contribute to the immunity to bacterial infection not only in patients with CRSsNP, as previously suggested,[17] but also in patients with CRSwNP. Our pervious study indicated that polyp IgE can be induced by house dust mites, the most common aeroallergens in our area.[7]

Serum total IgD and specific IgD levels against pollens have been reported to be upregulated in atopic patients.[23,35] In this study we discovered an upregulation of specific IgD against Der p 1 and Der f 1 in eosinophilic, but not noneosinophilic, polyps, suggesting that in addition to IgE response, a localized IgD response to common aeroallergens was elicited during the development of eosinophilic CRSwNP. Nevertheless, more investigations are required to understand the relationship between soluble IgD production and allergen sensitization and bacterial infection in sinonasal mucosa. In the present study we could not directly demonstrate the effect of house dust mites in IgD-mediated response in eosinophilic polyps because of our technique limitations; however, it might be speculated that house dust mites cross-link IgD and activate mast cells in patients with eosinophilic CRSwNP. In this study we did not find an association between dust mite–specific IgE and IgD levels, indicating that IgD-activated mast cells more likely induce polyclonal B-cell activation through B cell–stimulating factors. Obviously, the complex interregulation of different immunoglobulins in patients with CRSwNP is an interesting topic for future investigations.

In conclusion, in this study we demonstrated an enhanced localized IgD response in both patients with CRSsNP and those with CRSwNP potentially induced by bacteria or aeroallergens. IgD-activated mast cells can facilitate local IgE production and exaggerate eosinophilic inflammation in patients with CRSwNP (see Fig E13 in this article's Online Repository at www.jacionline.org). Blocking IgD represents a potential novel therapeutic strategy to alleviate local IgE production and eosinophilic inflammation in patients with CRSwNP. Nevertheless, IgD likely has different pathologic roles in patients with CRSsNP and those with noneosinophilic CRSwNP, which deserves further studies.

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Clinical implications: Therapeutic strategies that target IgD-mediated mast cell activation might be helpful to suppress local IgE production and eosinophilic inflammation in patients with CRSwNP.

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METHODS

Patients

This study was approved by the Ethics Committee of Tongji Hospital of Huazhong University of Science and Technology and was conducted with written informed consent from every patient. A total of 75 patients with eosinophilic CRSwNP, 69 patients with noneosinophilic CRSwNP, 32 patients with CRSsNP, and 109 control subjects were included in this study. The diagnosis of CRS was made according to the current European Position Paper on Rhinosinusitis and Nasal Polyps.[E1] CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of total infiltrating cells, as reported in our previous study.[E2] Patients without a history of CRS and undergoing nasal surgery for a variety of non-CRS indications (eg, septoplasty, skull base tumor, obstructive sleep apnea, and facial fracture repairs) were enrolled as control subjects. Atopic status was evaluated by using the skin prick test to a standard panel of aeroallergens common in our region and/or ImmunoCAP (Phadia, Uppsala, Sweden) to detect IgE antibodies against common inhalant allergens.[E3] The diagnosis of asthma was made based on Global Initiative for Asthma guidelines.[E4]

Oral glucocorticoids and intranasal steroid sprays were discontinued at least 3 months and 1 month before surgery, respectively. None had received antileukotrienes or immunotherapy.

Subjects who had fungal sinusitis, cystic fibrosis, vasculitis, antrochoanal polyps, primary ciliary dyskinesia, gastroesophageal reflux disease or an acute upper respiratory tract infection within 1 month of entering the study were excluded from the study. None of the patients had a history of aspirin sensitivity. Polyp tissues from patients with CRSwNP, diseased sinus mucosal samples from patients with CRSsNP, and ethmoid sinus tissues or inferior turbinate mucosal tissues from control subjects were obtained during surgery. Because of the limited amount of tissue samples, not all samples were included in every study protocol. Demographic characteristics of patients are summarized in Table E1.

Histology study

Paraffin-embedded sections (4 μm) of human sinonasal mucosa were stained with hematoxylin and eosin to determine the general pathologic features of tissues. Eosinophils and total inflammatory cells per high-powered field were evaluated.[E2]

RT-PCR analysis of *IGHD*

As mentioned elsewhere,[E2,E5] total RNA from tissue was extracted by using TRIzol (Invitrogen Life Technologies, Carlsbad, Calif) and treated with DNase (TaKaRa Biotechnology, Dalian, China) to avoid interference of genomic DNA. Then RNA was reverse transcribed to cDNA by using the Prime Script RT Reagent Lit (TaKaRa Biotechnology). Real-time PCR of *IGHD* was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany) with the SYBR Premix Ex Taq Kit (TaKaRa Biotechnology), with specific primers listed in Table E2.[E2,E5] The specificity of primers for analysis of *IGHD* was confirmed by using the sequencing PCR product. cDNA equivalent to 50 ng of total RNA was used, and real-time PCR was performed on StepOnePlus (Applied Biosystems, Foster City, Calif). Amplification was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, specific annealing temperature for 10 seconds, and 72°C for 15 seconds. After PCR, a melting curve was constructed by increasing the temperature from 65°C to 95°C, with a temperature transition rate of 0.1°C/s. Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method.[E2,E5] An inferior turbinate sample from a control subject was used as a calibrator. β-Glucuronidase *(GUSB)* was used as a housekeeping gene for normalization of gene expression. No template sample was used as a negative control.

Immunohistochemistry and immunofluorescence

Paraffin-embedded sections (4 μm) were deparaffinized and rehydrated and then subjected to heat-induced antigen retrieval by using Target Retrieval Solution (Dako, Carpinteria, Calif). The 3% hydrogen peroxidase was used to inhibit endogenous peroxidase, and 5% BSA was used to block nonspecific binding. For immunohistochemistry, sections were stained with a specific primary antibody to IgD at a proper dilution (Table E3), and the streptavidin-biotin-peroxidase complex detection method was used (Boster Biotechnology, Wuhan, China). Color development was achieved with 3′, 3′-diaminobenzidine.[E2] For immunofluorescence staining, tissue sections were incubated with specific primary antibodies at proper dilutions (Table E3) overnight at 4°C. After washing, sections were incubated with fluorescence-labeled secondary antibodies (Table E4) for 1 hour at room temperature. If necessary, sections were stained with 4′-6-diamidino-2-phenylindole dihydrochloride at room temperature for 5 minutes. Species- and subtype-matched antibodies were used as controls. Human tonsillar tissues were used as positive controls for IgD staining.

NMC and PBMC isolation

Sinonasal mucosa samples were dissociated mechanically, as previously stated.[E6] The resulting cell suspensions were filtered 2 times through the mesh of a 40-μm cell strainer (BD Biosciences, San Jose, Calif). NMCs were isolated from dispersed tissue cells by means of density gradient centrifugation on Lymphoprep, as previously described.[E6] PBMCs were also isolated by means of density gradient centrifugation on Lymphoprep.[E6]

Flow cytometry

Isolated NMCs and PBMCs were first stained with the Fixable Viability Stain 700 (BD Biosciences) as a live/dead discriminator.[E7] Cells were stained with appropriate combinations of antibodies to various surface antigens at 4°C for 30 minutes.[E8,E9] Species- and subtype-matched antibodies were used as controls. For intracellular staining of IgD or tryptase, after surface staining, cells were fixed and permeabilized with the BD Cytofix/Cytoperm Kit (BD Biosciences). Cells were then stained with mouse mAb against human IgD or rabbit polyclonal antibody against tryptase for 30 minutes at 4°C. If necessary, cells were incubated with secondary antibodies for 30 minutes at 4°C. The detailed information of primary and secondary antibodies used is listed in Table E5. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). All gates and quadrants were drawn to result in 1% or less of total positive cells in the sample stained with control antibodies. Data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Purification of mast cells and naive B cells

Peripheral naive B cells and polyp mast cells were isolated from PBMCs and NMCs by means of immunomagnetic cell sorting, respectively. NMCs and PBMCs were obtained, as mentioned above. B cells were isolated from the low-density fraction of PBMCs, and naive $CD19^+IgD^+$ B cells were isolated by means of depletion of nonnaive B cells through use of the Naïve B Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol.[E6] Purity of $CD19^+IgD^+IgM^+$ naive B cells was greater than 98%. The viability of purified peripheral naive B cells was greater than 97%. Nasal mast cells were purified from NMCs from eosinophilic polyp tissues through removal of monocytes by means of plastic adherence at 37°C for 90 minutes and removal of $CD3^+$ cells (Miltenyi Biotec) and positive selection of $CD117^+$ (c-kit^+; Miltenyi Biotec) cells by using magnetically activated cell sorting, as previously described.[E9,E10] The purity of purified nasal mast cells was greater than 98%, and the viability was greater than 95%. Representative results of purification of naive B cells and nasal mast cells are shown in Fig E1.

Stimulation of mast cell line and polyp mast cells

HMC-1 cells (ATCC), a human mast cell line, were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, Mass) with 10% (vol/vol) FBS (Gibco) and 1% (vol/vol) penicillin/streptomycin (Guge Biotechnology, Wuhan, China) in a 5% CO_2-humidified atmosphere at 37°C. Before stimulation, HMC-1 cells (1×10^7 cells) were preincubated

with human serum (15 mL) from control subjects for 16 hours. After incubation, about 18% and 7% of HMC-1 cells had surface-bound IgD and IgE, respectively (Fig E8). Serum preincubated HMC-1 cells were seeded onto 12-well plates at a density of 4×10^5 cells/well, and polyp mast cells were cultured at a density of 5×10^4 cells/well in U-bottom 96-well plates in RPMI 1640 medium (Gibco) supplemented with 10% (vol/vol) BSA (Gibco) and 1% (vol/vol) penicillin/streptomycin (Guge Biotechnology). Cultured HMC-1 cells and polyp mast cells were stimulated with mouse mAb against IgD (25 μg/mL; IgG_{2a}; IA6-2; BD Biosciences), with mouse mAb against IgE (2 μg/mL; IgG_{2a}; G7-18; BD Biosciences) as a positive control and control mouse mAb with the same isotype and irrelevant binding activity (25 μg/mL; IgG_{2a}; sc-3878; Santa Cruz Biotechnology, Dallas, Tex) as a negative control.[E8,E9] After 16 hours of stimulation, culture supernatants were collected for cytokine detection.

Coculture of mast cells and naive B cells

Peripheral naive B cells were cultured alone or with autologous polyp mast cells (5×10^4 cells/well) or human serum preincubated HMC-1 cells (4×10^5 cells/well; 1:1 ratio) in RPMI 1640 medium (Gibco) with 1% (vol/vol) penicillin/streptomycin (Guge Biotechnology), 10% heat-inactivated FBS (HyClone, Thermo Scientific, Beijing, China), BAFF (500 ng/mL; BD Biosciences), and staphylococcal enterotoxin B (1 μg/mL; Millipore, Merck KGaA, Darmstadt, Germany) for 8 days in 96-well plates in a 5% CO_2-humidified atmosphere at 37°C.[E6,E8,E9] Mast cells were stimulated with mAb against IgD or IgE, as mentioned above. Culture supernatants were harvested for immunoglobulin analysis.

Measurement of immunoglobulins, cytokines, and chemokines

Tissue homogenates were generated, as previously described.[E5] Briefly, every 0.1 g of tissue was added by 1 mL of 0.9% sodium chloride solution containing protease inhibitor cocktail (Guge Biotechnology). Tissues were then homogenized on ice. Tissue homogenates were generated, and human serum was centrifuged at 3000 rpm for 10 minutes at 4°C. Supernatants were harvested and stored at −80°C for further analysis. Protein levels of IgD in tissue homogenates and serum, various cytokines in supernatants of HMC-1 cell culture, and TGF-β1 in supernatants of nasal mast cell culture were detected by using commercially available ELISA kits.[E6,E8] Lower detection limits for ELISA assays were shown in Table E6. Cytokines, except TGF-β1, in supernatants of nasal mast cell culture and IgG_1, IgG_3, IgG_4, IgM, and IgA in culture supernatants were measured by using Bio-Plex suspension chip technology (R&D systems, Minneapolis, Minn).[E6,E8] Lower detection limits for Bio-Plex suspension chip technology were shown in Table E7. Total and specific IgE levels in tissue homogenates, sera, and cell-culture supernatants were detected by using the ImmunoCAP system (Phadia, Uppsala, Sweden), and the lower detection level is 0.1 kU/L.[E5] In culture supernatants IgG_2 levels in all conditions and IgE levels in the condition with anti-IgE antibody treatment were unable to be detected because of the possible cross-reactivity caused by experimental antibodies.

Measurement of antigen-specific IgD

A capture ELISA was developed to measure antigen-specific IgD.[E11] Briefly, polystyrene microtiter plates were coated with 100 μL/well affinity-purified goat anti-human IgD antibody (Alpha Diagnostic International, San Antonio, Tex) or goat IgG isotype control (Alpha Diagnostic International) at a concentration of 10 μg/mL in 0.05 mol/L coating buffer at room temperature for 1 hour. After washing 5 times with washing buffer, plates were blocked with 200 μL/well blocking buffer overnight. After removing the blocking buffer and washing 5 times with washing buffer, 100 μL/well of tissue homogenates or PBS (negative control) was added and incubated for 2 hours. After washing, 100 μL/well of biotin-conjugated Der p 1 (10 μg/mL), biotin-conjugated Der f 1 (10 μg/mL), biotin-conjugated LPS (10 μg/mL; InvivoGen, San Diego, Calif), 1% BSA (negative control), or HRP-conjugated goat anti-human IgD antibody (1 μg/mL; positive control) were added and incubated for another 2 hours. After that, 100 μL of streptavidin-HRP was added and incubated for 1 hour. Plates were subsequently incubated with 100 μL/well of tetramethylbenzidine substrate buffer for 30 minutes. OD_{450} was determined by using an automated microtiter plate reader (Thermo Fisher Scientific). Because there are no standards available for antigen-specific IgD, results are presented as OD_{450} values and normalized to total protein levels of tissue homogenates. Der p 1 (Prospec, East Brunswick, NJ) and Der f 1 (Prospec) were conjugated with biotin by using a Lightning-Link Rapid Biotin Conjugation Kit (Innova Biosciences, Cambridge, United Kingdom).[E12] HRP-conjugated goat anti-human IgA, IgM, IgG, and IgE antibody at 1 μg/mL were used as cross-reaction controls. No cross-reaction was found (Fig E2). The polystyrene microtiter plates, buffers, and all goat anti-human immunoglobulin antibodies were obtained from Bethyl Laboratories (Montgomery, Tex).

Antigen specificity of the assay was first examined with an inhibition test by using the same antigens without biotin conjugation.[E12] Briefly, after incubating with tissue homogenates (100 μL/well) for 1 hour at room temperature, the plates were incubated with corresponding Der p 1 (Prospec), Der f 1 (Prospec), and LPS (InvivoGen) without biotin conjugation at a concentration of 10 μg/mL, 100 μg/mL, 1 mg/mL, or diluting buffer. As to nonspecific inhibition of antigen-specific IgD binding to corresponding allergen, LPS (1 mg/mL; InvivoGen) was used to inhibit antigen-specific IgD binding to Der f 1 and Der p 1. Nonspecific inhibition tests for antigen-specific IgD binding to LPS were carried out in the same manner by using Der p 1 (1 mg/mL; Prospec). Inhibition tests confirmed antigen specificity of developed ELISA, and results are shown in Table E8.

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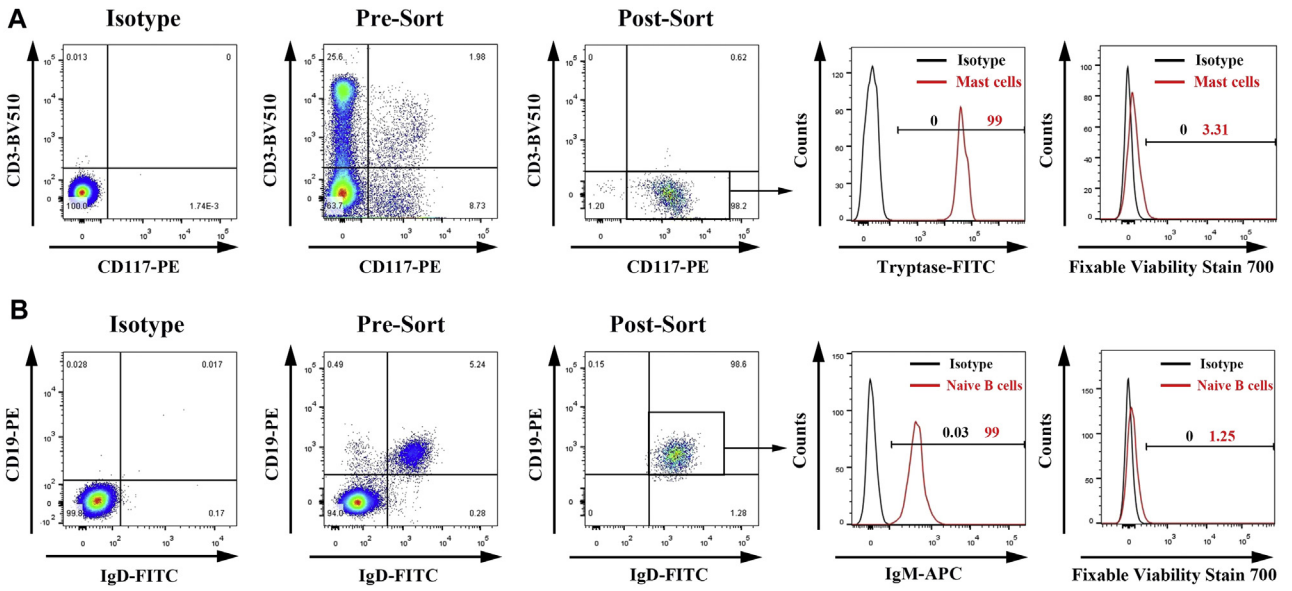

FIG E1. Representative results of mast cell **(A)** and naive B-cell **(B)** purification. Purity and viability of isolated mast cells and naive B cells are shown. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate.

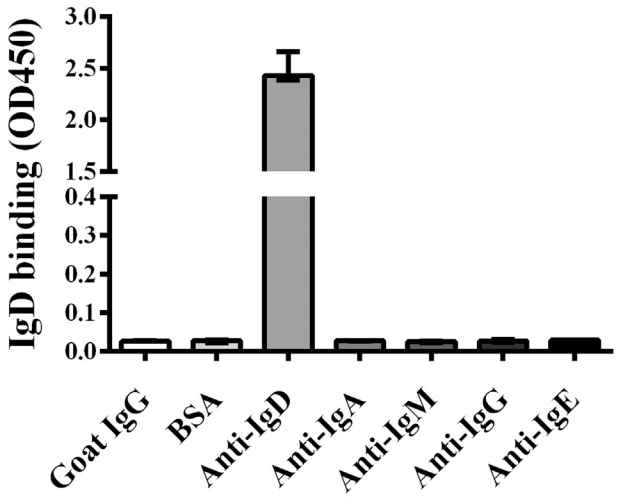

FIG E2. No cross-reaction with IgA, IgG, IgM, or IgE was found for the developed capture ELISA of IgD. *Goat IgG*, Isotype control for coated anti-IgD antibody. After adding tissue homogenates, HRP-conjugated goat anti-human IgD antibody was added. BSA (1%) was used as a negative control, and HRP-conjugated goat anti-human IgA, IgM, IgG, and IgE antibodies were used as cross-reaction controls. There was no cross-reaction found (n = 4).

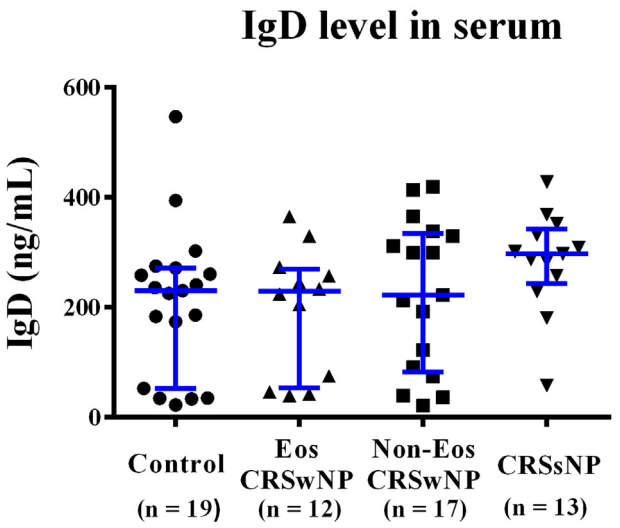

FIG E3. No significant difference in serum secretory IgD levels among different study groups. *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP.

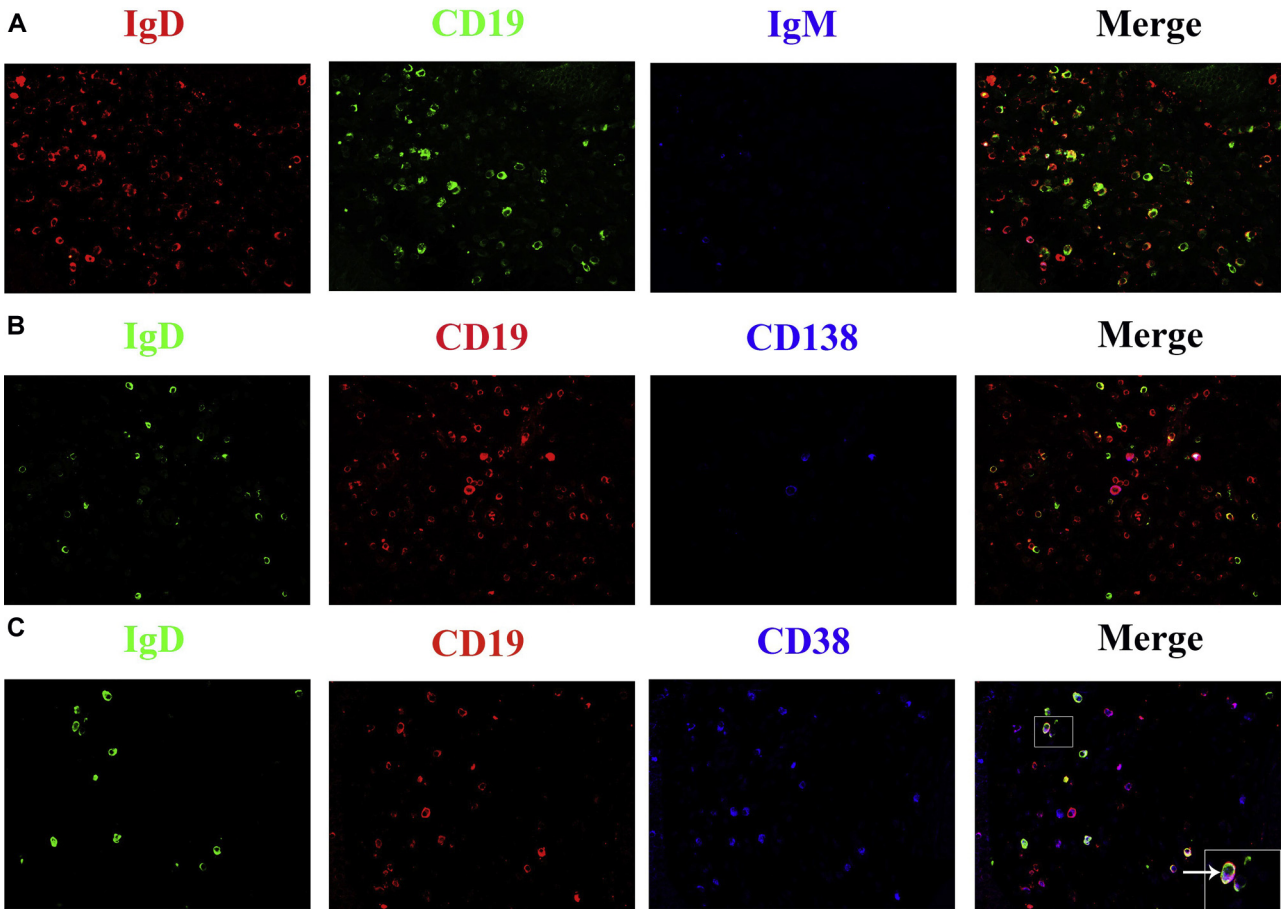

FIG E4. The majority of polyp IgD^+ B cells are IgM^-, $CD138^-$, and $CD38^+$ plasmablasts detected by means of immunofluorescence. Polyp tissue sections were stained with different combinations of 3 primary antibodies. Representative photomicrographs are shown (original magnification ×400). *Inset* shows a higher magnification of the outlined area. *Arrow* denotes positive cells.

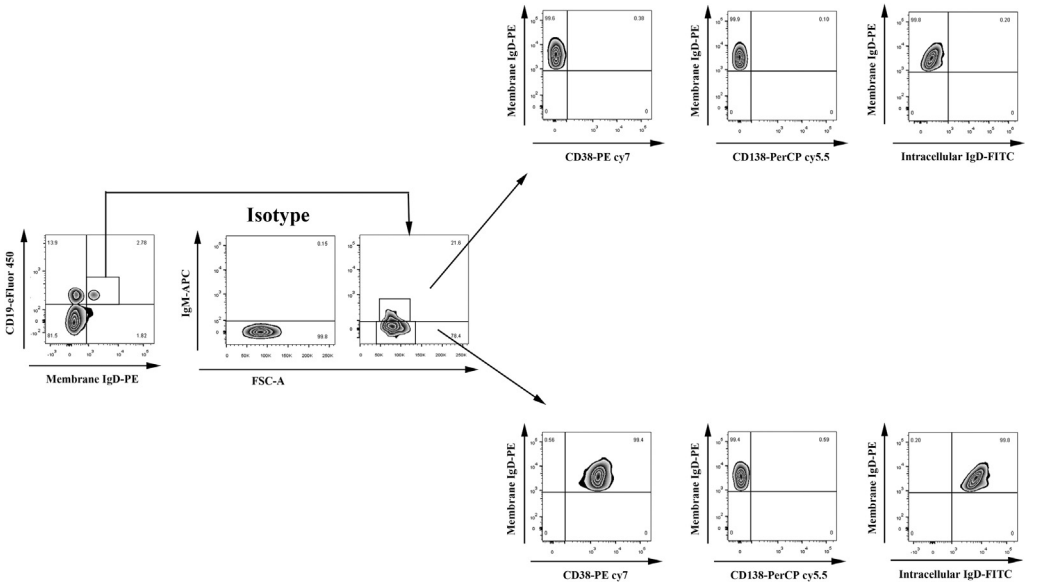

FIG E5. Representative flow plots show that polyp $IgD^+IgM^-CD19^+$ B cells are plasmablasts expressing CD38 and intracellular IgD but not CD138. In contrast, polyp IgD^+IgM^+ naive B cells do not express CD38 or intracellular IgD. *APC,* Allophycocyanin; *FITC,* fluorescein isothiocyanate; *FSC,* forward scatter; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

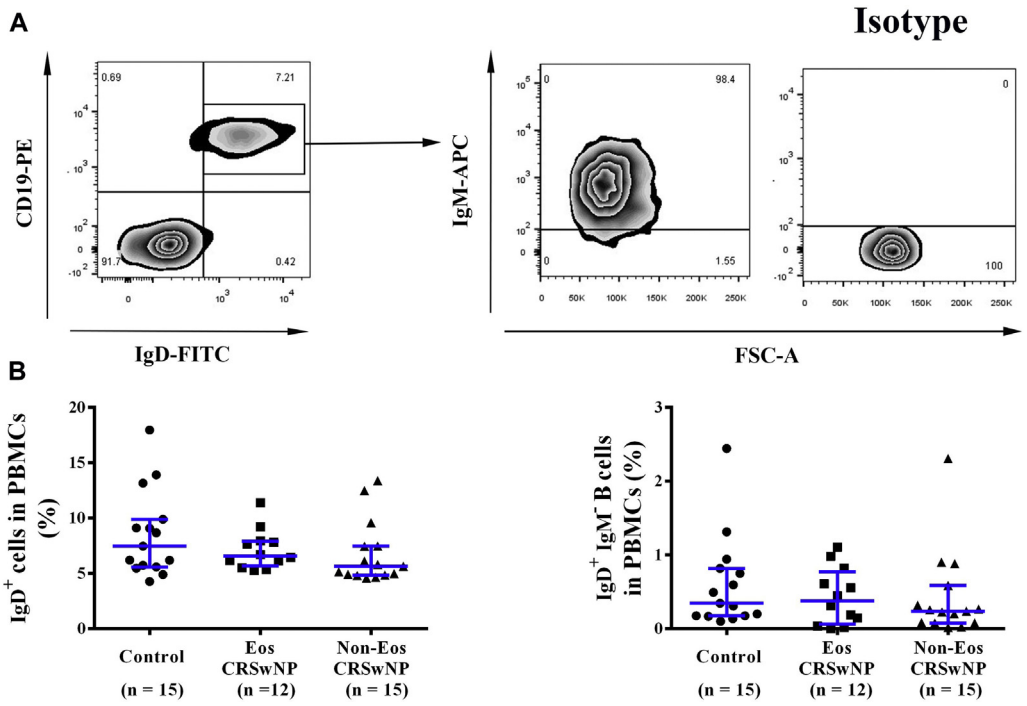

FIG E6. No difference in peripheral blood IgD^+ cells among different study groups. **A,** Representative flow plots show that the majority of peripheral IgD^+CD19^+ B cells are IgM^+ naive B cells. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *FSC*, forward scatter; *PE*, phycoerythrin. **B,** Percentages of total IgD^+ cells, as well as IgD^+IgM^- B cells, in PBMCs did not differ significantly among different study groups. *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP.

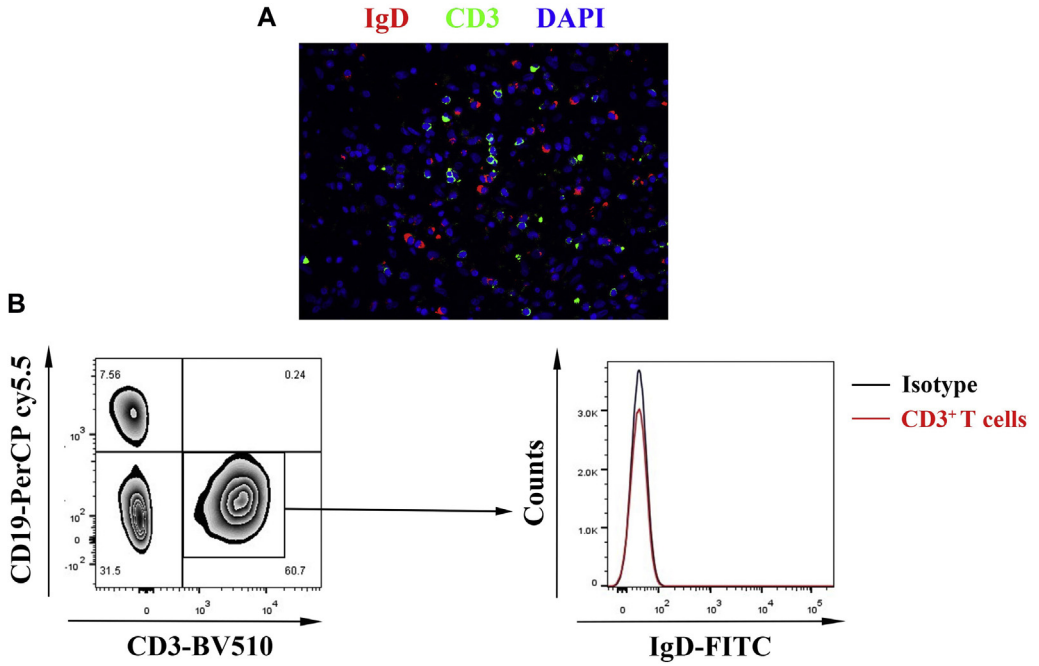

FIG E7. No binding of secretory IgD to the surfaces of $CD3^+$ T cells. **A,** Representative photomicrograph shows no colocalization of IgD and CD3 in nasal polyps (original magnification ×400). *DAPI*, 4′-6-Diamidino-2-phenylindole dihydrochloride. **B,** Representative flow plots show that peripheral blood $CD3^+$ T cells do not have surface-bound IgD. *FITC*, Fluorescein isothiocyanate; *PerCP*, peridinin-chlorophyll-protein complex.

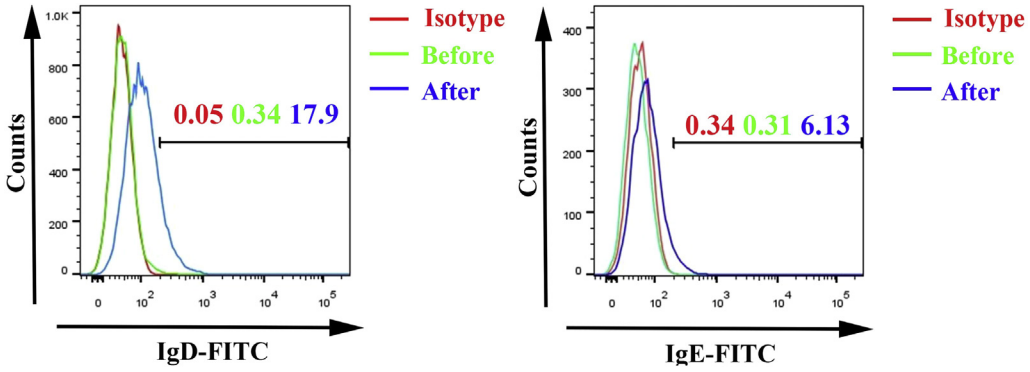

FIG E8. Generation of HMC-1 cells with surface-bound IgD and IgE by means of incubation with serum from healthy control subjects. After incubation, about 18% and 7% of HMC-1 cells had surface-bound IgD and IgE, respectively. Representative flow plots are shown. *FITC*, Fluorescein isothiocyanate.

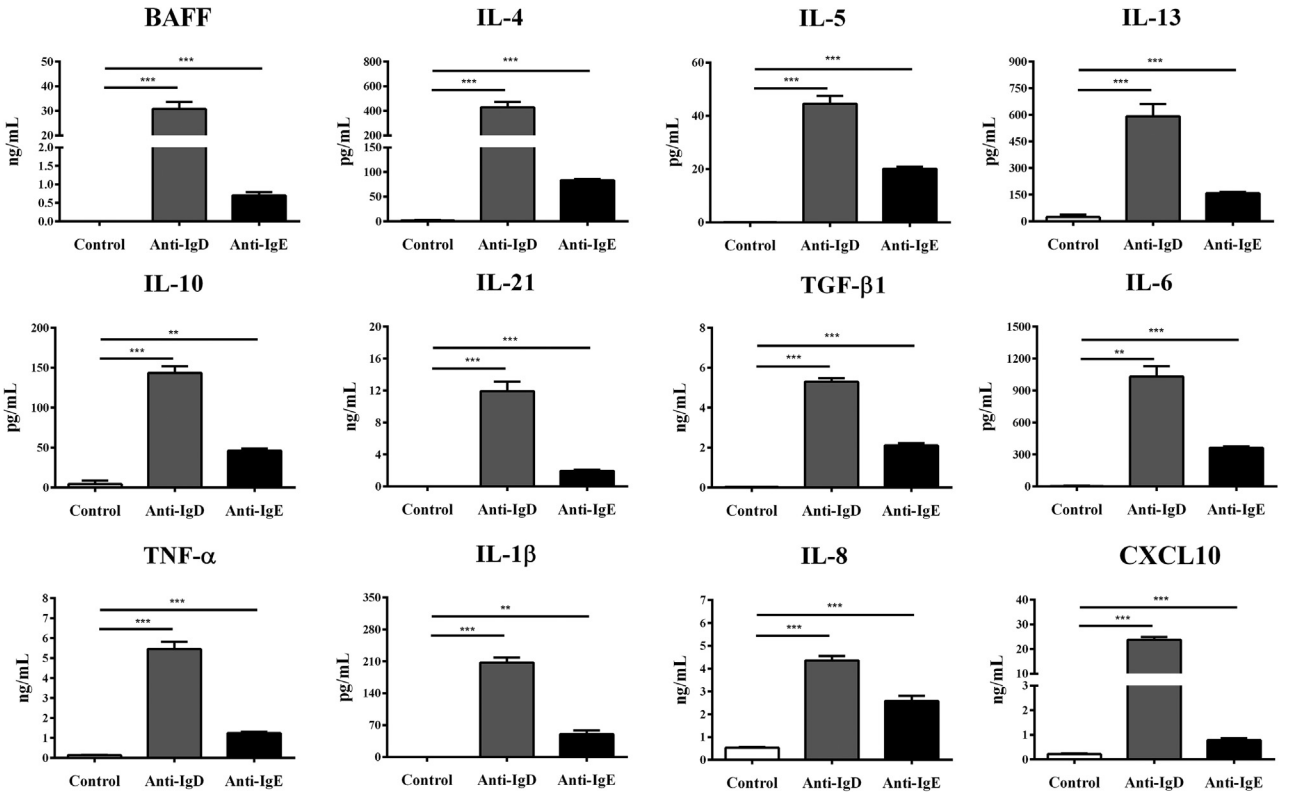

FIG E9. IgD cross-linking activates serum preincubated HMC-1 cells to produce B cell−stimulating factors, cytokines, and chemokines. Serum preincubated HMC-1 cells were treated with anti-IgD, anti-IgE, or control mouse mAb for 16 hours, and levels of mediators in culture supernatants were measured (n = 8). $^{**}P < .01$ and $^{***}P < .001$.

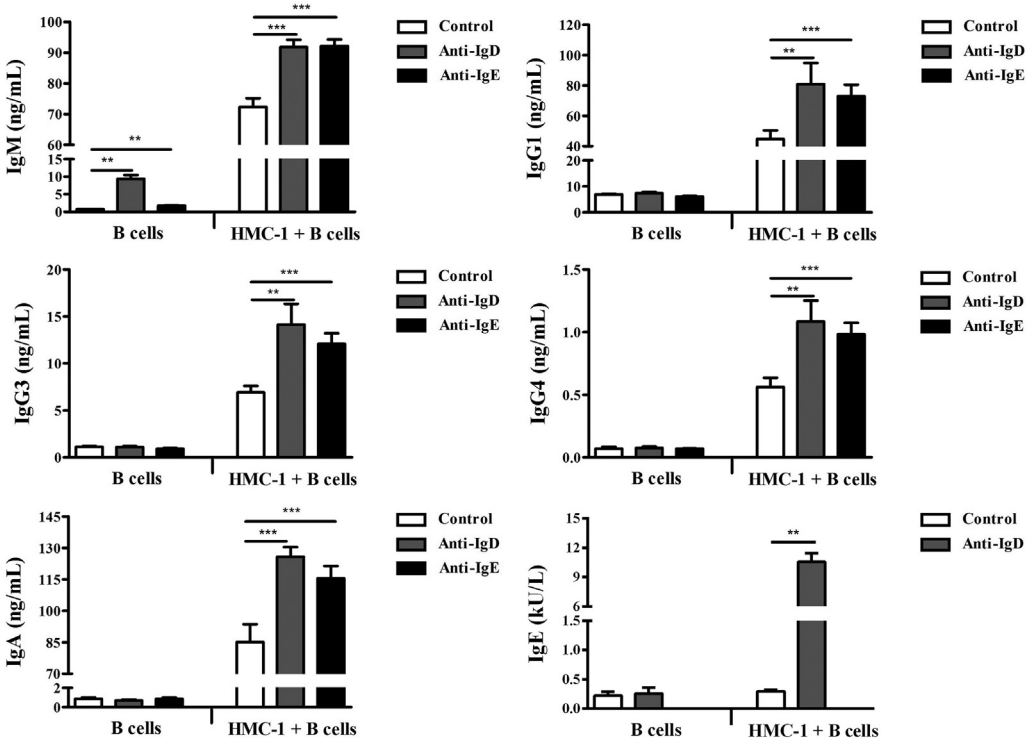

FIG E10. IgD-activated HMC-1 cells induce immunoglobulin production from naive B cells. Serum preincubated HMC-1 cells were cocultured with peripheral naive B cells and treated with anti-IgD, anti-IgE, or control mouse mAb for 8 days, and immunoglobulins in culture supernatants were measured (n = 8). IgG_2 levels in all conditions and IgE levels in anti-IgE treatment conditions were not analyzed because of possible interference caused by experimental antibodies. $^{**}P < .01$ and $^{***}P < .001$.

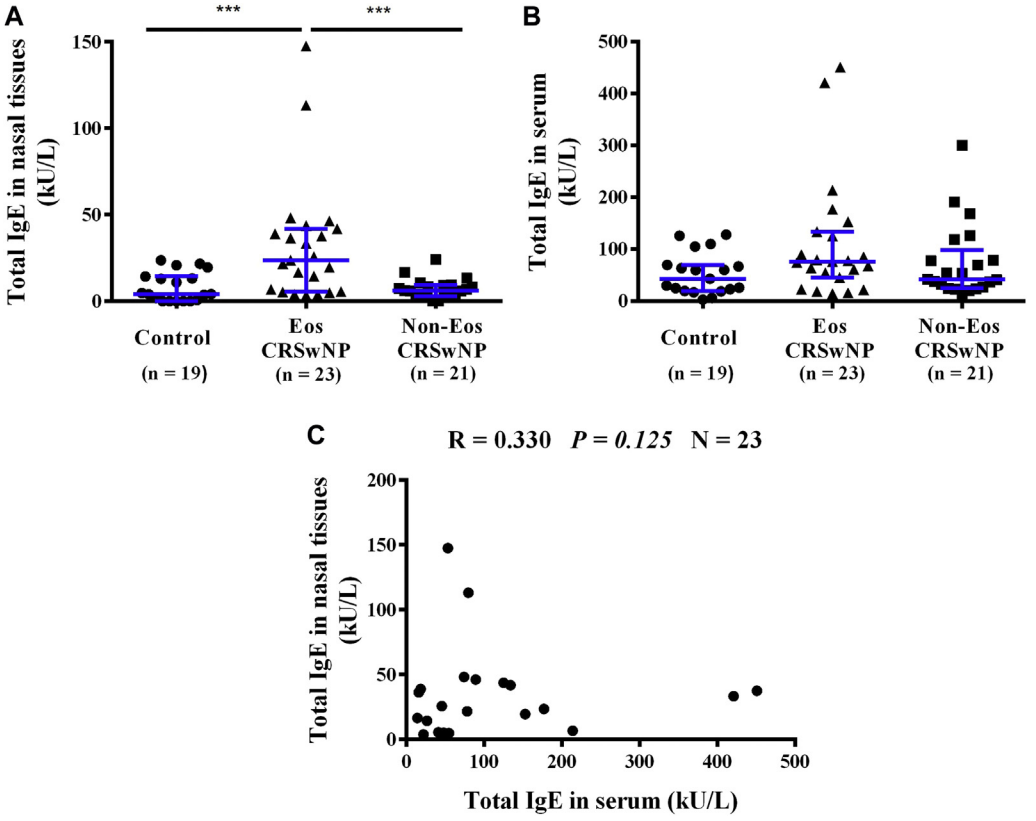

FIG E11. Increased local, but not serum, total IgE levels in patients with eosinophilic CRSwNP. **A,** Increased local total IgE levels in patients with eosinophilic polyps compared with those with noneosinophilic polyps and control inferior turbinate tissues. **B,** No difference in serum total IgE levels among different study groups. **C,** No correlation between local and serum total IgE levels in patients with eosinophilic CRSwNP. *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP. ***$P < .001$.

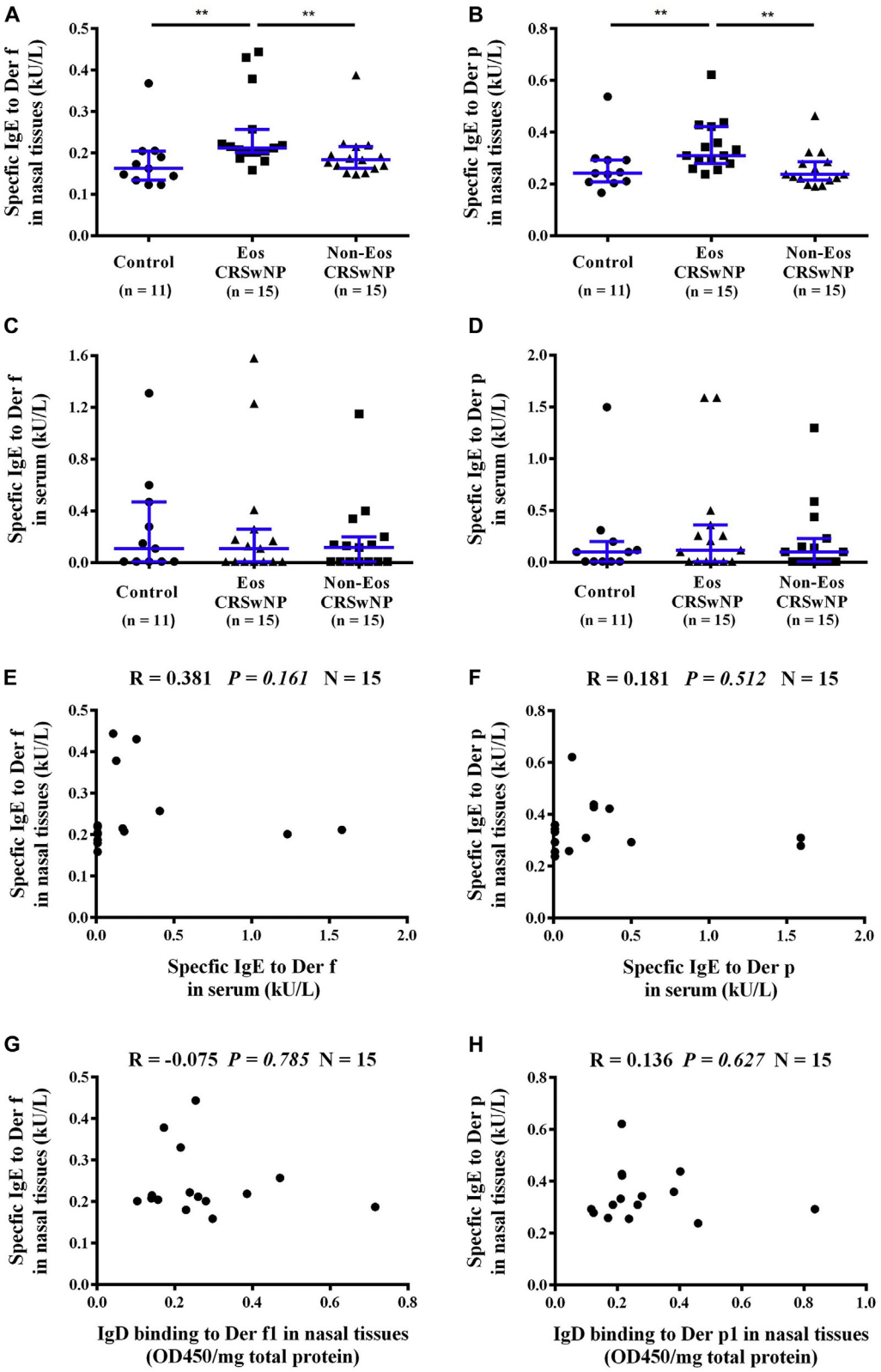

FIG E12. Correlations between antigen-specific IgD and IgE levels. **A** and **B,** Levels of *D farinae*–specific (Fig E12, *A*) and *D pteronyssinus–specific* (Fig E12, *B*) IgE levels were upregulated in patients with eosinophilic polyps compared with those with noneosinophilic polyps and control tissues. **C** and **D,** No difference in *D farinae*–specific (Fig E12, *C*) and *D pteronyssinus–specific* (Fig E12, *D*) IgE levels in sera among different study groups. **E** and **F,** No correlation between local and serum *D farinae*–specific (Fig E12, *E*) or *D pteronyssinus–specific* (Fig E12, *F*) IgE levels in patients with eosinophilic CRSwNP. **G** and **H,** No significant association between local Der f 1–specific IgD and *D farinae*–specific IgE levels (Fig E12, *G*) or between local Der p 1–specific IgD and *D pteronyssinus–specific* IgE levels (Fig E12, *H*) in eosinophilic polyp tissues. *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP. $^{**}P < .01$.

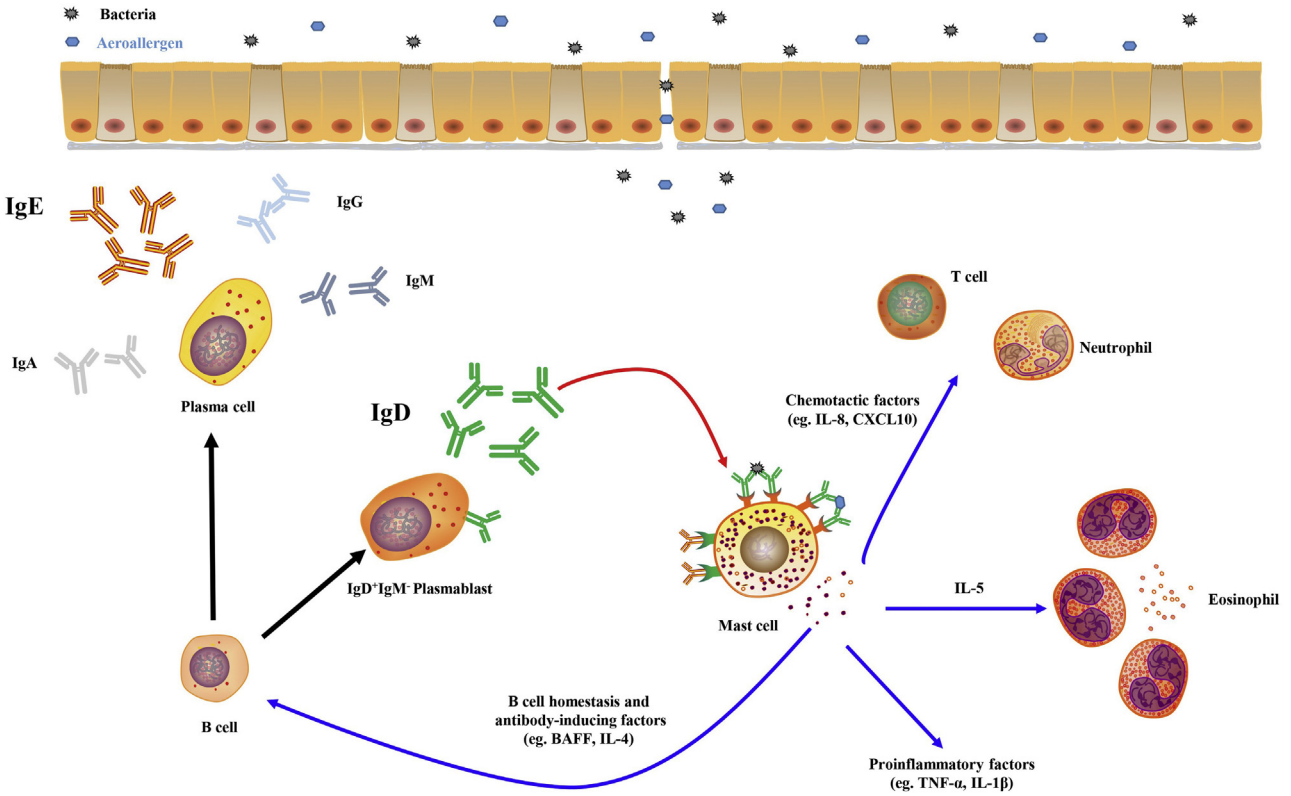

FIG E13. Sketch map shows the contribution of IgD-activated mast cells in the pathologic process of eosinophilic CRSwNP. Soluble IgD is produced by mucosal $IgD^{+}IgM^{\circ}$ plasmablasts in response to bacteria and aeroallergens in the nasal cavity. Cross-linking of IgD bound to mast cells leads to production of inflammatory cytokines (eg, TNF-α, IL-1β, and IL-5) and chemokines (eg, IL-8 and CXCL10) and B-cell hemostasis and antibody-inducing factors (eg, BAFF, IL-4, and IL-13), which facilitate IgE production from B cells and subsequent eosinophilic inflammation in polyps.

TABLE E1. Patients' demographic characteristics

	Control subjects	Patients with Eos CRSwNP	Patients with Non-Eos CRSwNP	Patients with CRSsNP	*P* value
Total subjects enrolled	109	75	69	32	
Methodology used					
Immunohistochemistry and immunofluorescence					
Subject no.	29	25	27	22	
Male sex	18 (62%)	16 (64%)	17 (63%)	13 (59%)	.988
Age (y)	28 (21-51)	48 (26-54)	38 (20-51)	32 (21-50)	.513
Patients with atopy	5 (17%)	9 (36%)	7 (26%)	3 (14%)	.250
Patients with AR	0 (0%)	6 (24%)	4 (15%)	1 (5%)	**.024**
Patients with asthma	0 (0%)	3 (12%)	1 (4%)	0 (0%)	.068
RT-PCR					
Subject no.	14	16	15	8	
Male sex	8 (57%)	9 (56%)	9 (60%)	5 (63%)	.991
Age (y)	36 (23-52)	39 (26-58)	43 (21-51)	48 (23-54)	.760
Patients with atopy	1 (7%)	4 (25%)	2 (13%)	0 (0%)	.307
Patients with AR	0 (0%)	3 (19%)	2 (13%)	0 (0%)	.354
Patients with asthma	0 (0%)	1 (6%)	0 (0%)	0 (0%)	1.000
Flow cytometry					
Subject no.	36	39	38	—	
Male sex	23 (64%)	24 (62%)	25 (66%)	—	.927
Age (y)	43 (24-53)	40 (25-52)	37 (26-46)	—	.704
Patients with atopy	3 (8%)	8 (21%)	7 (18%)	—	.311
Patients with AR	0 (0%)	6 (15%)	5 (13%)	—	.055
Patients with asthma	0 (0%)	4 (10%)	1 (3%)	—	.078
ELISA					
Subject no.	39	35	39	19	
Male sex	25 (64%)	23 (66%)	27 (69%)	11 (58%)	.861
Age (y)	35 (21-50)	42 (28-52)	39 (23-50)	29 (38-47)	.325
Patients with atopy	2 (5%)	7 (20%)	7 (18%)	3 (16%)	.257
Patients with AR	0 (0%)	6 (17%)	5 (13%)	2 (11%)	.080
Patients with asthma	0 (0%)	4 (11%)	1 (3%)	1 (5%)	.083
Cell-culture study					
Subject no.	—	11	—	—	
Male sex	—	8 (73%)	—	—	—
Age (y)	—	32 (25-41)	—	—	—
Patients with atopy	—	2 (18%)	—	—	—
Patients with AR	—	2 (18%)	—	—	—
Patients with asthma	—	0 (0)	—	—	—

For continuous variables, results are expressed as medians and interquartile ranges. Categorical variables are summarized by using percentages. Boldface indicates $P < .05$.
AR, Allergic rhinitis; *Eos CRSwNP*, eosinophilic chronic rhinosinusitis with nasal polyps; *Non-Eos CRSwNP*, noneosinophilic chronic rhinosinusitis with nasal polyps.

TABLE E2. Primers used for quantitative RT-PCR analysis

Primer	Sequence	Annealing temperature (°C)	Expect product size (bp)
IGHD	(Forward) 5′- CAGAGTCCATCAAAAACCCATGC -3′	62	133
	(Reverse) 5′-CAGACCTCACCCCAAACCTAC-3′		
GUSB	(Forward) 5′-GACACGCTAGAGCATGAGGG-3′	60	121
	(Reverse) 5′-GGGTGAGTGTGTTGTTGATGG-3′		

GUSB, β-Glucuronidase.

TABLE E3. Primary antibodies used in immunohistochemistry and immunofluorescence

Antibody	Application	Species	Concentration	Clone ID	Reference	Source
BAFF	IF	Rabbit	1:100	Polyclonal	bs-2431R	Biosynthesis biotechnology (Beijing, China)
CD19	IF	Mouse	1:100	UMB103	ZM-0038	Zhongshan Golden Bridge Biotechnology (Beijing, China)
CD19	IF	Rabbit	1:50	EP169	ZA-0569	Zhongshan Golden Bridge Biotechnology
CD19	IF	Mouse	1:50	BT51E	ZM-0038	Zhongshan Golden Bridge Biotechnology
CD38	IF	Mouse	1:100	SPC32	ZM-0422	Zhongshan Golden Bridge Biotechnology
CD38	IF	Rabbit	1:50	polyclonal	bs-0980R	Biosynthesis biotechnology
CD138	IF	Rabbit	1:50	EP201	ZA-0584	Zhongshan Golden Bridge Biotechnology
IgD	IHC, IF	Goat	1:100	Polyclonal	2030-01	SouthernBiotech (Birmingham, Ala)
IgM	IF	Mouse	1:100	Polyclonal	bs-0345M	Biosynthesis Biotechnology
IgM	IF	Rabbit	1:100	Polyclonal	ZA-0450	Zhongshan Golden Bridge Biotechnology
IL-1β	IF	Rabbit	1:100	Polyclonal	bs-6319R	Biosynthesis biotechnology
IL-4	IF	Rabbit	1:100	Polyclonal	RB22885	Abgent (San Diego, Calif)
IL-5	IF	Rabbit	1:100	Polyclonal	bs-1318R	Biosynthesis Biotechnology
IL-5	IF	Mouse	1:100	QS-5	ab25034	Abcam (Cambridge, United Kingdom)
IL-6	IF	Rabbit	1:100	Polyclonal	bs-0781R	Biosynthesis Biotechnology
IL-8	IF	Rabbit	1:100	Polyclonal	bs-0780R	Biosynthesis Biotechnology
IL-10	IF	Rabbit	1:100	EPR1114	ab133575	Abcam
IL-13	IF	Rabbit	1:100	Polyclonal	bs-0560R	Biosynthesis Biotechnology
IL-21	IF	Mouse	1:100	14k5H3	NBP2-27336SS	Novus Biologicals (Littleton, Colo)
CXCL10	IF	Goat	1:100	Polyclonal	AF-266-NA	R&D Systems (Minneapolis, Minn)
TGF-β1	IF	Rabbit	1:100	Polyclonal	bs-0103R	Biosynthesis Biotechnology
TNF-α	IF	Rabbit	1:100	Polyclonal	bs-2150R	Biosynthesis Biotechnology
Tryptase	IF	Rabbit	1:100	EPR9522	ab151757	Abcam
Tryptase	IF	Mouse	1:100	AA1	ab2378	Abcam

IF, Immunofluorescence; *IHC*, immunohistochemistry.

TABLE E4. Secondary antibodies used in immunofluorescence

Antibody	Concentration	Clone	Reference	Source
IFKine Red donkey anti-mouse IgG	1:100	Polyclonal	A24411-1	Abbkine (Wuhan, China)
IFKine Green donkey anti-mouse IgG	1:100	Polyclonal	A24211-1	Abbkine
DyLight 405 donkey anti-mouse IgG	1:100	Polyclonal	715-475-150	Jackson ImmunoResearch (West Grove, Pa)
IFKine Red donkey anti-rabbit IgG	1:100	Polyclonal	A24421-1	Abbkine
IFKine Green donkey anti-rabbit IgG	1:100	Polyclonal	A24221-1	Abbkine
Alexa Fluor 405 donkey anti-rabbit IgG	1:100	Polyclonal	ab175651	Abcam (Cambridge, United Kingdom)
IFKine Red donkey anti-goat IgG	1:100	Polyclonal	A24431-1	Abbkine
IFKine Green donkey anti-goat IgG	1:100	Polyclonal	A24231-1	Abbkine

TABLE E5. Antibodies used in flow cytometry

Antigen fluorophore	Manufacturer	Clone ID	Source	Isotype	Dilution
CD3-BV510	BioLegend (San Jose, Calif)	OTK3	Mouse	IgG_1, κ	1:20
CD19-PerCP cy5.5	eBioscience (San Jose, Calif)	SJ25C1	Mouse	IgG_1, κ	1:20
CD19-eFluor 450	eBioscience	SJ25C1	Mouse	IgG_1, κ	1:20
CD19-PE	BD PharMingen (San Jose, Calif)	HIB19	Mouse	IgG_1, κ	1:20
CD38-APC	eBioscience	HIT2	Mouse	IgG_1, κ	1:20
CD38-PE cy7	eBioscience	HB7	Mouse	IgG_1, κ	1:20
CD117-PE	BD PharMingen	YB5.B8	Mouse	IgG_1, κ	1:20
CD138-PerCP cy5.5	BioLegend	DL-101	Mouse	IgG_1, κ	1:20
FcεRI-APC	eBioscience	AER-37(CRA1)	Mouse	IgG_{2b}, κ	1:20
IgD-APC	BioLegend	IA6-2	Mouse	IgG_{2a}, κ	1:20
IgD-FITC	SouthernBiotech (Birmingham, Ala)	Polyclonal	Goat	IgG $F(ab')_2$	1:20
IgD-FITC	eBioscience	IA6-2	Mouse	IgG_{2a}, κ	1:20
IgD-PE	eBioscience	IA6-2	Mouse	IgG_{2a}, κ	1:20
IgE-FITC	eBioscience	Ige21	Mouse	IgG_1, κ	1:20
IgM-APC	BD PharMingen	G20-127	Mouse	IgG_1, κ	1:20
Tryptase	Abcam (Cambridge, United Kingdom)	Polyclonal	Rabbit	IgG	1:50
Alexa Fluor 488 donkey anti-rabbit IgG H&L	Antgene (Wuhan, China)	Polyclonal	Donkey	IgG	1:50

APC, Allophycocyanin; *BV*, Brilliant Violet; *cy*, cyanine; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein 501 complex.

TABLE E6. Detection limits for ELISA assays

Target	Detection limit	Manufacturer
IgD	7.8 ng/mL	Bethyl Laboratories (Montgomery, Ala)
BAFF	0.23 pg/mL	Multi Sciences (Hangzhou, China)
CXCL10	3.50 pg/mL	Multi Sciences
IL-1β	0.15 pg/mL	Multi Sciences
IL-4	0.48 pg/mL	Multi Sciences
IL-5	0.76 pg/mL	Multi Sciences
IL-6	0.37 pg/mL	Multi Sciences
IL-8	0.78 pg/mL	Multi Sciences
IL-10	0.59 pg/mL	Multi Sciences
IL-13	0.9 pg/mL	Multi Sciences
IL-21	11.99 pg/mL	Multi Sciences
TGF-β1	3.36 pg/mL	Multi Sciences
TNF-α	0.42 pg/mL	Multi Sciences

TABLE E7. Detection limits for the Bio-Plex assay

Target	Detection limit	Manufacturer
BAFF	1.01 pg/mL	R&D Systems (Minneapolis, Minn)
CXCL10	1.18 pg/mL	R&D Systems
CXCL8	1.8 pg/mL	R&D Systems
IL-1β	0.8 pg/mL	R&D Systems
IL-4	9.3 pg/mL	R&D Systems
IL-5	0.5 pg/mL	R&D Systems
IL-6	1.7 pg/mL	R&D Systems
IL-10	1.6 pg/mL	R&D Systems
IL-13	32.4 pg/mL	R&D Systems
IL-21	0.869 pg/mL	R&D Systems
TNF-α	1.2 pg/mL	R&D Systems
IgG_1	0.205 pg/mL	Bio-Rad Laboratories (Hercules, Calif)
IgG_3	0.008 pg/mL	Bio-Rad Laboratories
IgG_4	0.086 pg/mL	Bio-Rad Laboratories
IgM	0.067 pg/mL	Bio-Rad Laboratories
IgA	0.863 pg/mL	Bio-Rad Laboratories

TABLE E8. Inhibition of antigen-specific IgD binding by corresponding and noncorresponding antigens

Antigen-specific IgD	Corresponding/noncorresponding antigen	10 μg/mL	100 μg/mL	1 mg/mL
Der p 1–specific binding	Der p 1	44.0 ± 4.2	72.6 ± 6.2	93.9 ± 2.1
	LPS			No inhibition observed
Der f 1–specific binding	Der f 1	39.2 ± 4.2	78.2 ± 3.1	94.5 ± 1.7
	LPS			No inhibition observed
LPS-specific binding	LPS	45.8 ± 1.5	87.0 ± 1.5	96.4 ± 0.8
	Der p 1			No inhibition observed

Percentage inhibition is expressed as means ± SEMs (n = 5).