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## Immunoglobulin D enhances immune surveillance by activating antimicrobial, pro-inflammatory and B cell-stimulating programs in basophils

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### Abstract

Immunoglobulin D (IgD) is an enigmatic antibody isotype that mature B cells co-express with IgM through alternative RNA splicing. We found active T cell-dependent and T cell-independent

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IgM-to-IgD class switching in human upper respiratory mucosa B cells. This process required activation-induced cytidine deaminase and generated local and circulating IgD-producing plasmablasts reactive to respiratory bacteria. Circulating IgD bound to basophils through a calcium-mobilizing receptor that induced antimicrobial, opsonizing, inflammatory and B cell-stimulating factors including cathelicidin, interleukin-1, interleukin-4 and B cell-activating factor BAFF upon IgD cross-linking. By showing dysregulation of IgD class-switched B cells and IgD-armed basophils in autoinflammatory syndromes with periodic fever, our data indicate that IgD orchestrates an ancestral surveillance system at the interface between immunity and inflammation.

## INTRODUCTION

Immunoglobulin D (IgD) has remained an enigmatic antibody class since its discovery more than forty years ago<sup>1</sup>. Because of its spotty presence in mammals and absence in birds, IgD was initially thought to be a recently evolved Ig isotype<sup>2</sup>. By showing that xenopus is orthologous to IgW, an antibody class found in cartilaginous fish and lungfish, previous studies demonstrate that IgD was present in the ancestor of all jawed vertebrates and arose together with IgM at the time of the emergence of the adaptive immune system, approximately 500 million years ago<sup>3</sup>. While IgM remains stable over evolutionary time, IgD shows greater structural plasticity and can be predominantly expressed as a transmembrane or secretory molecule in a species-specific manner<sup>4,5</sup>. One possible interpretation is that IgD has been preserved as a structurally flexible locus to complement the functions of IgM.

IgM and IgD are the first antibody isotypes expressed during B cell ontogeny. Bone marrow B cell precursors acquire surface IgM after assembling heavy (H) and light (L) chain variable region exons from prototypic variable (V), diversity (D) and joining (J) gene segments through an antigen-independent process mediated by recombination activating gene (RAG)-1 and RAG-2 proteins<sup>6</sup>. After leaving the bone marrow to colonize secondary lymphoid organs, B cells acquire surface IgD of the same specificity as surface IgM through alternative splicing of a pre-messenger RNA comprising V(D)J and both heavy chain constant  $\mu$  ( $C_{\mu}$ ) and  $C_{\delta}$  exons<sup>7</sup>. The significance of dual IgM and IgD expression remains unclear, because either isotype largely compensates for the loss of the other<sup>8-10</sup>.

After encountering antigen in secondary lymphoid organs, mature B cells transcriptionally down-regulate surface IgD<sup>11</sup> and thereafter undergo somatic hypermutation (SHM) and class switch DNA recombination (CSR), two Ig gene-diversifying processes that require the DNA-editing enzyme activation-induced cytidine deaminase (AID)<sup>12</sup>. SHM introduces point mutations into  $V_HDJ_H$  and  $V_LJ_L$  exons, thereby providing the structural correlate for selection of high-affinity Ig variants by antigen<sup>13</sup>, whereas CSR substitutes the  $C_{\mu}$  gene with  $C_{\gamma}$ ,  $C_{\alpha}$  or  $C_{\epsilon}$ , thereby generating secondary IgG, IgA and IgE isotypes with the same antigen binding specificity as IgM but additional effector functions<sup>14</sup>. Of note, CSR occurs through either a T cell-dependent (TD) follicular pathway involving engagement of the CD40 receptor on B cells by CD40 ligand (CD40L) on antigen-activated CD4<sup>+</sup> T cells or through a T cell-independent (TI) extrafollicular pathway involving engagement of TACI and BAFF-R receptors on B cells by BAFF and APRIL, two CD40L-related tumor necrosis

factor (TNF) family members released by antigen-activated dendritic cells, macrophages and mucosal epithelial cells<sup>15-21</sup>. Ultimately, antigen-experienced B cells generate antibody-secreting plasma cells and memory B cells<sup>22</sup>. These latter form new plasma cells upon exposure to previously encountered antigens. In general, plasma cell-derived IgG, IgA and IgE antibodies facilitate the elimination of invading pathogens by activating powerful Fc receptors that enhance the phagocytic, cytotoxic and pro-inflammatory functions of various innate immune cells, including granulocytes<sup>23</sup>.

Instead of switching from IgM to IgG, IgA or IgE, some B cells switch to IgD<sup>24</sup>, suggesting that IgD confers some functional advantage over IgM. The resulting IgD<sup>+</sup>IgM<sup>-</sup> plasma cells release highly mutated mono- and polyreactive IgD antibodies mostly containing  $\lambda$  light chains in the blood as well as respiratory, salivary, lacrimal and mammary secretions<sup>1,4,25-28</sup>. Secreted IgD might enhance immune protection by regulating B cell homeostasis and activation. Indeed, IgD-deficient mice have fewer B cells, delayed affinity maturation, and weaker production of IgG1 and IgE, two isotypes highly dependent on the cytokine interleukin-4 (IL-4; <http://www.signaling-gateway.org/molecule/query?afcsid=A001262>)<sup>8,9</sup>. Conversely, mice injected with a goat polyclonal anti-human IgD with potential agonistic activity produce more IgG1 and IgE and show robust IL-4 production by T cells and basophils<sup>29-31</sup>. These latter are a small granulocytic subset that triggers T and B cell responses by releasing IL-4 upon recognizing antigen via pre-bound IgE and IgG<sup>32-35</sup>. Whether IgD also facilitates antigen recognition by basophils remains unknown.

We found that human upper respiratory mucosa B cells generated local and circulating IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts by undergoing C <sub>$\mu$</sub> -to-C <sub>$\delta$</sub>  CSR through both TD follicular and TI extrafollicular pathways involving AID. Circulating IgD interacted with basophils through a calcium-fluxing receptor that induced antimicrobial, opsonizing, inflammatory and immunostimulating factors such as cathelicidin, pentraxin-3 (PTX3), IL-1, IL-4 and B cell-activating factor of the TNF family member (BAFF; <http://www.signaling-gateway.org/molecule/query?afcsid=A000383>). By showing dysregulation of IgD class-switched B cells and IgD-armed basophils in autoinflammatory syndromes, our data indicate that IgD orchestrates an ancestral surveillance system at the interface between immunity and inflammation. This system may not only monitor invasion by respiratory bacteria, but also regulate antibody responses.

## RESULTS

### IgD CSR occurs in the upper respiratory mucosa

Human B cells release IgD in the blood as well as respiratory, salivary, lacrimal and mammary secretions<sup>1,4,25,27</sup>. We utilized immunohistochemistry, light microscopy and flow cytometry to elucidate the geography and phenotype of IgD<sup>+</sup>IgM<sup>-</sup> B cells producing IgD. Unlike intestinal, hepatic, lymph nodal, splenic and hematopoietic tissues, tonsillar and nasal tissues harbored abundant IgD<sup>+</sup>IgM<sup>-</sup> B cells that had topographic and morphologic features distinct from those of conventional IgD<sup>+</sup>IgM<sup>+</sup> B cells (Fig. 1a,b). These latter occupied the follicular mantle but not the germinal center of secondary lymphoid follicles, had a small size, round shape, scant cytoplasm and large nuclei, and expressed either  $\kappa$  or  $\lambda$  light chains on their surface in addition to the pan-B cell molecules CD19, CD20, CD21,

CD22, CD24, CD39, CD40, major histocompatibility complex (MHC) class I and class II molecules, BAFF-receptor (BAFF-R), transmembrane activator and CAML interactor (TACI), and the transcription factor Pax5 (Fig. 1c and Supplementary Fig. 1 online).

In contrast, IgD<sup>+</sup>IgM<sup>-</sup> B cells populated both germinal center and extrafollicular areas, had an ovaloid-elongated shape, medium-large size, abundant cytoplasm containing C<sub>8</sub> IgH chains preferentially coupled with λ light chains, and small and often picnotic eccentric nuclei. Consistent with prior data<sup>24,26,27</sup>, these IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts accounted for up to 20-25% of antibody-forming plasma cells and for about 1.5-5% of total CD19<sup>+</sup> B cells in tonsils. In spite of their plasma cell morphology, only a subset of IgD<sup>+</sup>IgM<sup>-</sup> B cells expressed B lymphocyte-induced maturation protein-1 (Blimp-1) and B cell maturation antigen (BCMA), and virtually none of them expressed CD138 (or syndecan-1), three hallmarks of mature plasma cells<sup>36</sup>. Furthermore, IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts retained surface immunoglobulin as well as retained CD19, CD20, CD21, CD22, CD24, CD39, CD40, MHC class I and class II, BAFF-R, TACI and Pax5, which are usually down-regulated by terminally differentiated plasma cells<sup>36</sup>.

In addition to showing a bias for surface λ light chains, some or all IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts expressed the activation molecule CD5 (ref. 37) the germinal center molecules CD10, CD38, CD77 and Ki-67 (ref. 24), the effector-memory molecule CD27 (ref. 38), as well as the DNA-editing enzyme AID, a hallmark of ongoing class switching<sup>18,19</sup>. Similar IgD<sup>+</sup>IgM<sup>-</sup> blasts were found in the peripheral blood (Supplementary Fig. 1 online), where they accounted for 0.5-1% of circulating CD19<sup>+</sup> B cells. Our data indicate that the upper respiratory mucosa generates local and circulating IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts specialized in IgD production. These cells express a complex phenotype that likely reflects a derivation from multiple follicular and extrafollicular precursors, including germinal center B cells.

### Upper respiratory mucosa B cells undergo IgD CSR *in situ*

B cells express C<sub>H</sub> genes downstream of C<sub>μ</sub> through a CSR reaction that relies on switch (S) regions<sup>14</sup>. Positioned upstream of each C<sub>H</sub> gene and an intronic (I) exon, S regions undergo germline transcription upon exposure of B cells to appropriate stimuli<sup>14</sup>. In addition to yielding I<sub>H</sub>-C<sub>H</sub> transcripts, germline transcription renders S regions substrate for DNA modifications by the CSR machinery, including AID<sup>14</sup>. Processing of these modifications into double-stranded DNA breaks is followed by fusion of these breaks through the non-homologous end joining pathway and looping-out deletion of the intervening DNA<sup>14</sup>. Ultimately, CSR generates post-switch I<sub>μ</sub>-C<sub>H</sub> transcripts as well as an extrachromosomal S-S switch circle (Fig. 1d). Together with AID, switch circles are generally accepted as a marker of ongoing class switching, because they have a short half-life due to their rapid degradation by cellular nucleases<sup>12,14,18,19</sup>. In addition, switch circles do not replicate with the class switched B cell and therefore become rapidly diluted after CSR takes place.

Similar to pigs, sheep and cows<sup>39</sup>, humans have a C<sub>8</sub> gene preceded by a S-like σ<sub>8</sub> region that mediates CSR from C<sub>μ</sub> to C<sub>8</sub><sup>24,40</sup>. Using a nested polymerase chain reaction (PCR)-based strategy, we identified extrachromosomal σ<sub>8</sub>-S<sub>μ</sub> switch circles in B cells from tonsils, but not peripheral blood, spleen, bone marrow, lymph nodes and intestine (Fig. 1e). CSR from C<sub>μ</sub> to C<sub>8</sub> was associated with active C<sub>8</sub> gene transcription, because both the pre-

germinal center IgD<sup>+</sup>IgM<sup>+</sup> B cell line 2E2 and tonsillar follicular mantle IgD<sup>+</sup>IgM<sup>+</sup> B cells contained chimeric germline I<sub>μ</sub>-C<sub>δ</sub> transcripts in addition to conventional germline I<sub>μ</sub>-C<sub>μ</sub> transcripts (Fig. 1f, g).

Although expressing I<sub>μ</sub>-C<sub>μ</sub> transcripts, neither the pre-B IgD<sup>-</sup>IgM<sup>+</sup> cell line Reh nor the germinal center IgD<sup>-</sup>IgM<sup>+</sup> B cell line Ramos contained I<sub>μ</sub>-C<sub>δ</sub> transcripts, suggesting that germline C<sub>δ</sub> gene transcription is a developmentally regulated process beginning at a pre-germinal center stage of mature B cell differentiation. Finally, post-germinal center IgD<sup>+</sup>IgM<sup>-</sup> plasma cells lacked germline I<sub>μ</sub>-C<sub>μ</sub> transcripts, but contained post-switch I<sub>μ</sub>-C<sub>δ</sub> transcripts as a result of S<sub>μ</sub>-to-σ<sub>δ</sub> CSR-induced juxtaposition of the I<sub>μ</sub> exon to C<sub>δ</sub> exons. The identity of σ<sub>δ</sub>-S<sub>μ</sub> and I<sub>μ</sub>-C<sub>δ</sub> amplicons obtained from tonsillar IgD<sup>+</sup> B cells was confirmed by DNA sequencing (Fig. 1g). Together with previous data showing AID expression by a fraction of tonsillar IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts, these findings demonstrate that a subset of B cells actively undergo CSR from C<sub>μ</sub> to C<sub>δ</sub> in the microenvironment of the upper respiratory mucosa.

### IgD CSR requires AID and occurs via multiple pathways

CSR usually involves engagement of CD40 on follicular B cells by CD40 ligand on T cells or engagement of TACI on extrafollicular B cells by BAFF or a proliferation-inducing ligand (APRIL) from innate immune cells<sup>20</sup>. Co-signals from cytokines are also required. IgD<sup>+</sup>IgM<sup>+</sup> B cells induced σ<sub>δ</sub>-S<sub>μ</sub> switch circles, lost surface IgM, and secreted IgD upon exposure to CD40L, BAFF or APRIL plus either IL-15 and IL-21 (<http://www.signaling-gateway.org/molecule/query?afcsid=A001258>) or IL-2 and IL-21 (Fig. 2a-c and Supplementary Fig. 2 online), three cytokines produced by dendritic cells and T cells<sup>16,41,42</sup>. These effects were not due to expansion of IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts present at the onset of the culture, because fresh IgD<sup>+</sup>IgM<sup>+</sup> B cells lacked σ<sub>δ</sub>-S<sub>μ</sub> switch circles (not shown). In addition, CD40L, BAFF or APRIL alone or combined with either IL-2, IL-4, IL-10, IL-13, IL-15 or IL-21 elicited neither loss of surface IgM nor secretion of IgD in spite of being powerful inducers of B cell survival and proliferation (Supplementary Fig. 2 online and not shown). Of note, the specificity of *in vitro* induced σ<sub>δ</sub>-S<sub>μ</sub> switch circles was confirmed by sequencing (not shown).

The IgD-inducing function of CD40L, BAFF and APRIL was further studied in patients with primary immunodeficiencies. *TNFSF5* and *TNFRSF5* gene defects, which impair CD40L and CD40 signaling in patients with hyper-IgM type-1 (HIGM1) and HIGM3 syndromes<sup>43</sup> respectively, led to a reduction of both circulating and follicular IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts, but partially spared extrafollicular IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts (Fig. 2d and Supplementary Fig. 3 online), suggesting that CD40L-CD40 interaction is indispensable for the generation of systemic but not mucosal IgD-producing effector-memory B cells.

*TNFRSF13b* gene defects, which impair TACI signaling in a subset of patients with common variable immunodeficiency (CVID)<sup>44,45</sup>, were also associated with a reduction of circulating IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts. *AICDA* gene defects, which impair AID function in patients with HIGM2 syndrome<sup>43</sup>, caused a reduction of both circulating and follicular IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts. This reduction was linked to a lack of functional AID protein, because B cells from HIGM2 patients neither induced σ<sub>δ</sub>-S<sub>μ</sub> switch circles nor underwent

loss of surface IgD upon exposure to appropriate stimuli (Fig. 2e and Supplementary Fig. 3 online). Of note, HIGM2 as well as HIGM3 syndromes were associated with an increased generation of double-producing IgD<sup>+</sup>IgM<sup>+</sup> plasmablasts at both follicular and extrafollicular mucosal sites, possibly reflecting the activation of a CSR-independent pathway for compensatory IgD synthesis. Thus, IgD CSR requires AID and occurs via both T cell-dependent (TD) and T cell-independent (TI) pathways *in vivo*.

### IgD recognizes respiratory bacteria

Given their origin from the upper respiratory mucosa, IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts may release IgD antibodies that recognize airborne bacteria. Consistent with this possibility, IgD secreted by stimulated IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts bound to the Gram-negative bacteria *Moraxella catarrhalis* and *Haemophilus influenzae* type a and type b (Fig. 2f). As shown by others<sup>46</sup>, IgD also bound the virulence factor *M. catarrhalis* IgD-binding protein (MID, also known as Hag), which facilitates the adhesion of *M. catarrhalis* and *H. influenzae* to mucosal epithelial cells. Furthermore, secreted IgD reacted against lipopolysaccharide (LPS), a key parietal component of Gram-negative bacteria, and capsular polysaccharide (CPS), an essential protective factor for encapsulated bacteria such as *H. influenzae*. Thus, IgD from IgD<sup>+</sup>IgM<sup>-</sup> B cells recognizes respiratory bacteria and their products.

### IgD binds to circulating basophils

Humans have 15-300 µg/ml of circulating IgD<sup>1,27</sup>, which likely originates from IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts entering the bloodstream from inductive sites in the upper respiratory mucosa. To elucidate the function of circulating IgD, we examined its *in vivo* interaction with Fc receptor-blocked peripheral blood leukocytes by flow cytometry. Using a goat polyclonal antibody highly specific for human IgD and with no Fc portion and no IgG cross-reactivity, we found that basophils had abundant surface IgD, whereas T cells, NK cells, monocytes, myeloid dendritic cells, neutrophils, eosinophils and plasmacytoid dendritic cells exhibited no or little surface IgD (Fig. 3a and Supplementary Fig. 4 online). Basophils were consistently positive for surface IgD binding also when stained with a monoclonal anti-IgD (Supplementary Fig. 4 online).

Compared to polyclonal anti-IgD, monoclonal anti-IgD detected less IgD on circulating basophils as well as basophilic KU812 cells incubated with soluble IgD. In addition, monoclonal anti-IgD tententially detected less IgD on circulating neutrophils, although there was inter-individual variability. These results likely reflected the wide range of serum IgD concentrations present in different individuals as well as the fact that monoclonal anti-IgD reagents allow a less complete detection of IgD due to their ability to detect only individual monomorphic C<sub>δ</sub> epitopes and not multiple C<sub>δ</sub> epitopes and conformations as the anti-IgD pAb does<sup>47</sup>. In this regard, transmembrane and secreted IgD preferentially associate with κ and λ light chains, respectively<sup>4</sup>. This phenomenon, known as “IgD paradox”, likely generates conformational differences in C<sub>δ</sub> that further limit the recognition of secreted IgD by anti-IgD mAbs. Of note, one of the monoclonal anti-IgD reagents used in this study failed to effectively stain both soluble IgD bound on KU812 cells and transmembrane IgD on B cells (not shown), suggesting poor fluorochrome conjugation and/or intrinsically weak reactivity against C<sub>δ</sub>.

IgD was detected not only on the surface, but also in the cytoplasm of circulating as well as mucosal basophils (Fig. 3b), suggesting that basophils internalize IgD-bound immune complexes. Mast cells, a mucosal immunocyte functionally related to basophils<sup>48,49</sup>, were also positive for IgD (Supplementary Fig. 5 online). Addition of exogenous monoclonal IgD did not increase the surface IgD binding on basophils, indicating saturation of binding sites by endogenous IgD (Fig. 3c). However, monoclonal IgD could bind to basophils after stripping of endogenous IgD. Finally, addition of increasing amounts of unlabeled monoclonal IgD progressively diminished the binding of labeled monoclonal IgD to basophils stripped of endogenous IgD (Fig. 3d).

The specific tropism of IgD for basophils and mast cells was confirmed *in vitro* by showing binding of physiological concentrations (from 0.5 to 50  $\mu\text{g/ml}$ ) of polyclonal or monoclonal IgD molecules to the pre-basophilic cell line KU812 and to the mastocytoid cell lines HMC-1 and LAD-2 (Fig. 4a and Supplementary Fig. 6 online). This binding was highly specific, because it was detected with both polyclonal and monoclonal anti-IgD reagents with no cross-reactivity for IgG. Except for the monocytic cell line U937, none of the T, NK and myeloid cell lines tested bound IgD. KU812 and LAD-2 cells up-regulated IgD binding upon exposure to IL-3 and/or IL-4 (Fig. 4b and Supplementary Fig. 7 online), two cytokines involved in basophil and mast cell differentiation<sup>48,49</sup>. In addition to augmenting their IgD-binding activity, IL-3 stimulated KU812 cells to increase surface expression of CD124 (IL-4 receptor) and Fc $\epsilon$ RI (IgE receptor) and decrease expression of CD117 (c-kit receptor), whereas IL-4-stimulated LAD-2 cells increased CD123 (IL-3 receptor) expression. Binding of labeled IgD to KU812 or HMC-1 cells was saturable and could be competed by unlabeled IgD, but not IgG or IgE, whereas IgA had a minimal inhibitory effect (Fig. 4c, d).

Since both IgA and IgD are highly mannose<sup>50</sup>, IgD could utilize mannose to enhance its binding to basophils and mast cells. Accordingly, mannose and mannan slightly inhibited IgD binding to target cells. Finally, IgD binding was abolished by denaturing IgD or by pre-treating target cells with trypsin and papain, but not pepsin. Thus, IgD binds to basophils and mast cells through a protease-sensitive, IL-3 or IL-4-inducible receptor distinct from IgG, IgA and IgE receptors.

### **IgD binding to basophils is evolutionarily conserved**

IgD is an ancestral antibody class expressed by early vertebrates, including teleost fish<sup>3-5</sup>. Knowing that channel catfish (*Ictalurus punctatus*) express both transmembrane and secreted IgD molecules<sup>5</sup>, it was relevant that the peripheral blood of some of these animals contained plasmablast-like IgD<sup>+</sup>IgM<sup>-</sup> B cells expressing transcripts for both transmembrane and secreted C $\delta$  IgH chains, but not for transmembrane and secreted C $\mu$  IgH chains (Supplementary Fig. 8 online). Flow cytometric studies with specific monoclonal antibodies showed catfish also exhibit circulating granular leukocytes armed with surface IgD but not IgM. These IgD<sup>+</sup>IgM<sup>-</sup> granulocytes contained neither transcripts for the T cell antigen receptor nor transcripts for transmembrane and secreted C $\delta$  and C $\mu$  IgH chains and did not react with a specific monoclonal antibody to neutrophils (not shown), suggesting their affiliation to a distinct granulocytic subset. These data suggest that binding of B cell-derived

IgD to granulocytes is part of an evolutionarily conserved and potentially important immune pathway.

### **IgD-activated basophils produce B cell-activating factors**

To elucidate its functions, we cross-linked IgD on basophils with a specific monoclonal anti-IgD in the presence or absence of the basophil maturation factor IL-3 (refs.48,49). Controls were performed with an isotype-matched irrelevant antibody or an antibody to IgE. IgE binds basophils through a high-affinity FcεRI receptor that triggers degranulation and histamine release upon cross-linking<sup>48,49</sup>. Unlike IgE cross-linking, IgD cross-linking elicited neither surface up-regulation of the granular molecule CD63 nor histamine release regardless of the presence of IL-3 (Fig. 5a,b and Supplementary Fig. 9 online). Although less efficient than IgE cross-linking in inducing membrane-bound CD40L and BAFF, IgD cross-linking was more efficient than or as efficient as IgE cross-linking in inducing soluble IL-4, IL-13 and BAFF, membrane-bound APRIL, and soluble IL-8 and CXCL10 (Fig. 5c and Supplementary Fig. 9 online), two chemokines active on monocytes, neutrophils and T cells. These effects were potentiated by IL-3.

Activated basophils usually release mediators by undergoing calcium-dependent degranulation upon IgE cross-linking by antigen<sup>48,49</sup>. Although unable to induce degranulation, IgD cross-linking triggered intracellular calcium fluxes more sustained than those induced by IgE (Fig. 5d). The basophil-stimulating activity of IgD was further confirmed by experiments showing that, in the presence of IgD cross-linking, basophils pre-treated or not with IL-3 acquired the capability of inducing IgM secretion as well as IgG and IgA class switching in B cells (Fig. 5e). Of note, the B cell-licensing function of IgD-cross-linked basophils was largely dependent on BAFF and APRIL, because it was abolished by a soluble TACI-Ig decoy receptor (not shown). As shown by others<sup>32</sup>, IgE cross-linked basophils elicited B cell activation mostly via CD40L (not shown). Thus, IgD stimulates basophils through a calcium-fluxing receptor that induces multiple cytokines, including the B cell-activating factors IL-4, IL-13, BAFF and APRIL.

### **IgD-stimulated basophils produce antimicrobial factors**

In addition to adaptive B cell responses, IgD-activated basophils may trigger innate antimicrobial responses<sup>48,49</sup>. Indeed, IgD-cross-linked basophils induced antimicrobial, opsonizing and alarm-signaling factors<sup>51-53</sup>, including *DEFB103A*, *CAMP*, *SPAG11A/F*, *SPAG11D/G*, *PTX3* and *CRP* transcripts for β-defensin 3, cathelicidin, sperm associated antigen 11 (SPAG11) A/F isoforms, SPAG11 D/G isoforms, PTX3, and C-reactive protein (CRP), respectively (Fig. 6a). In addition, IgD-stimulated basophils released the cathelicidin-derived peptide LL-37 (Fig. 6b). Consistent with these results, supernatants from IgD-cross-linked basophils inhibited the replication of *M. catarrhalis* and *H. influenzae* (Fig. 6c). In contrast, IgE cross-linked basophils had little or no antimicrobial activity. Thus, IgD cross-linking activates powerful antimicrobial, opsonizing and alarm-signaling programs in basophils.



## Basophils are dysregulated in hyper-IgD patients

To elucidate the relationship between IgD and basophils *in vivo*, we analyzed IgD class-switched B cells and IgD-armed basophils from patients with hyper-IgD syndrome (HIDS), TNF receptor-associated periodic fever syndrome (TRAPS), Muckle-Wells syndrome (MWS), and periodic fever-aphthous stomatitis-pharyngitis-cervical adenitis (PFAPA) syndrome. Although characterized by diverse genetic defects, these autoinflammatory disorders share a perturbation of the mechanisms that initiate and control the inflammatory reaction. Such a perturbation causes periodic attacks of fever and tissue damage as well as abnormal IL-1 and TNF release and elevated IgD production<sup>4,54</sup>.

Compared to healthy subjects, HIDS, TRAPS, MWS, and PFAPA syndrome patients had more circulating and mucosal IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts (Fig. 7a,b and Supplementary Fig. 10 online), suggesting that switching from IgM to IgD is augmented in autoinflammatory syndromes. These disorders were also associated with fewer circulating but more mucosal IgD-armed basophils and probably mast cells (Fig. 7c,d and Supplementary Fig. 10 online), suggesting that elevated IgD production enhances mucosal homing and proliferation of basophils and mast cells. Consistent with this possibility, mucosal basophils exhibited signs of hyperactivation, including strong BAFF, APRIL and LL-37 expression.

Knowing that IL-1 $\beta$  and TNF are IgD-inducible pro-inflammatory cytokines involved in autoinflammatory syndromes<sup>54,55</sup>, we wondered whether IgD triggering increases IL-1 and TNF production by basophils. Upon IgD cross-linking, IL-3-treated basophils from healthy subjects released both IL-1 $\beta$  and TNF and this release further increased in the presence of monocytes (Fig. 7e), a cell type heavily involved in inflammation. In contrast, IgE cross-linking had little or no IL-1- and TNF-inducing activity. Overall, our data indicate that IgD and basophils orchestrate an ancestral surveillance system at the interface between immunity and inflammation (Supplementary Fig. 11 online). A dysregulation of this system may contribute to the pathogenesis of autoinflammatory syndromes with periodic fever.

## DISCUSSION

In this study we have reported that human B cells from the upper respiratory mucosa actively class switch from S $_{\mu}$  to  $\sigma_8$ , thereby generating local and circulating IgD antibodies highly reactive to respiratory bacteria. Circulating IgD bound to basophils through a calcium-mobilizing receptor that activated antimicrobial, opsonizing, pro-inflammatory and B cell-stimulating programs upon cross-linking. Both IgD class-switched B cells and IgD-armed basophils were dysregulated in patients with autoinflammatory syndromes and periodic fever, indicating that IgD orchestrates an ancestral surveillance system at the interface between immunity and inflammation.

Human B cells release IgD antibodies in the blood as well as respiratory, salivary, lacrimal and mammary secretions<sup>1,4,25-28</sup>. The regulation and function of these antibodies remain unknown. We found a large fraction of IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts in the upper respiratory mucosa. These plasmablasts originated *in situ* from an active process of S $_{\mu}$ -to- $\sigma_8$  CSR that involved germline I $_{\mu}$ -C $_{\delta}$  transcription, required AID expression, and occurred through either a TD follicular pathway involving engagement of CD40 on B cells by CD40L on T cells or a

TI extrafollicular pathway involving engagement of TACI on B cells by BAFF or APRIL from innate immune cells, possibly including epithelial and dendritic cells<sup>18-20</sup>.

The composite ontogeny of mucosal IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts was consistent with the fact that they expressed phenotypic traits usually associated with extrafollicular activated B cells or follicular germinal center B cells. Like these B cell types, mucosal IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts produce both polyreactive and monoreactive IgD antibodies encoded by unmutated and mutated V(D)J genes, respectively<sup>28</sup>, possibly reflecting the need of the upper respiratory mucosa to mount maximally diversified IgD responses for frontline defense purposes. Such IgD responses likely entail the stimulation of specific  $\lambda^+$  B cell precursors by a unique cocktail of mucosal signals comprising IL-21 and IL-2 or IL-21 and IL-15, three cytokines released by dendritic cells and T cells<sup>16,18,41,42</sup>. Together with CD40L, BAFF and APRIL, these cytokines might account for the massive V(D)J gene diversification and oligoclonal expansion of IgD<sup>+</sup>IgM<sup>-</sup> B cells previously observed in tonsils<sup>24,26</sup>. Why these cells are strongly biased for  $\lambda$  light chain expression remains unclear. One possibility is that the formation of fully functional soluble IgD antibodies requires pairing of C $\delta$  IgH with  $\lambda$  light chains.

IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts in the peripheral blood are likely in transit from inductive sites in the upper respiratory mucosa to distant mammary, salivary, lacrimal, respiratory, tubal and auditive sites<sup>27,38,56</sup>. At these effector sites, soluble IgD antibodies might enhance immune protection against local pathogens and commensal bacteria. Indeed, IgD released by stimulated IgD<sup>+</sup>IgM<sup>-</sup> B cells showed strong binding activity against respiratory bacteria and their products, including *M. catarrhalis*, *H. influenzae*, LPS, CPS and MID. Although unable to utilize the polymeric Ig receptor<sup>27</sup>, which translocates polymeric IgA and IgM from the basolateral to the apical surface of epithelial cells, monomeric IgD could reach mucosal surfaces via an alternative epithelial transporter, perhaps similar to that mediating transcytosis of monomeric IgG<sup>27</sup>. Once on the mucosal surface, IgD could take advantage of its poor complement-inducing activity to mediate immune exclusion in a non-inflammatory fashion<sup>4</sup>. Alternatively, IgD may confine its defensive functions to the subepithelial area of the upper respiratory tract.

In addition to mediating mucosal immunity, IgD may enhance systemic immunity by 'educating' circulating innate effector cells as to the antigenic composition of the upper respiratory tract. Indeed, we detected abundant IgD on basophils, but not on other leukocytes, except B cells. In the presence of IgD cross-linking, basophils acquired the ability to inhibit the growth of respiratory bacteria, presumably through the induction of  $\beta$ -defensin 3, SPAG11 isoforms, PTX3, CRP and cathelicidins. These antimicrobial and opsonizing factors also have immunostimulating and alarm-inducing activity<sup>51-53</sup>. Thus, IgD-armed basophils may function as circulating sentinels capable of triggering rapid innate and adaptive immune responses upon sensing pathogens from the upper respiratory tract.

The primordial nature of this immune surveillance system was confirmed by the presence of both IgD-producing (IgM-negative) plasmablasts and IgD-armed granulocytes in channel catfish. That IgD must mediate some important and evolutionarily conserved functions complementary to those of IgM was also suggested by the presence of double-producing

IgD<sup>+</sup>IgM<sup>+</sup> plasmablasts in patients with HIGM syndromes. This compensatory pathway for IgD production could explain why serum IgD is conserved or even elevated in HIGM and other primary immunodeficiencies resulting from an overall or selective impairment of CSR, including CVID and selective IgA deficiency<sup>4,25,27</sup>.

Consistent with the B cell-activating, antibody-inducing and basophil-stimulating properties of IgD and anti-IgD *in vivo*<sup>29,30,57</sup>, we found that basophils up-regulated the production of IL-4, IL-13, BAFF and APRIL upon IgD cross-linking *in vitro*. This effect correlated with the acquisition of B cell-licensing functions, including the capability to elicit IgM secretion as well as IgG and IgA class switching. In addition to promoting TI humoral immunity, IL-4 and IL-13 from IgD-activated basophils may initiate TD humoral immunity by triggering the formation of T<sub>H</sub>2 (CD4<sup>+</sup> T helper type-2) cells with B cell-helper activity<sup>34,35</sup>. Thus, it is likely that basophils respond to IgD-reactive pathogens by eliciting not only innate, but also adaptive immune responses. Such responses would take place in both systemic and mucosal districts. Indeed, while earlier work shows migration of basophils to draining lymph nodes<sup>34</sup>, we detected basophils in the upper respiratory mucosa, particularly in the presence of inflammation.

Our finding that IgD-stimulated basophils release an obligatory B cell survival factor such as BAFF would provide a mechanistic explanation to prior studies showing that IgD deficiency leads to a contraction of the peripheral B cell compartment in mice<sup>8,58</sup>. As suggested by the poly- and autoreactivity of a large fraction of IgD antibodies<sup>28</sup>, basophils could undergo tonic or intermittent release of BAFF upon sensing circulating foreign or autologous antigens through pre-bound IgD. The ensuing receptor-dependent internalization of antigen-IgD complexes might account for the cytoplasmic accumulation of IgD observed in *ex vivo* isolated basophils.

In addition to basophils, IgD bound to mast cells through an IL-4-inducible calcium-fluxing receptor distinct from IgG, IgA and IgE receptors. Indeed, basophil-like and mast cell-like lines pre-treated with IgG, IgA and IgE retained IgD binding activity. A minimal but reproducible inhibition was induced by IgA, the only antibody isotype together with IgD exhibiting C<sub>H</sub> region-associated O-linked carbohydrates<sup>50</sup>, which may therefore contribute to the binding of IgD to its receptor sites. The basophilic cell line KU812 increased IgD binding in response to IL-3, a mast cell-derived cytokine that enhances basophil activation and recruitment to lymphoid and mucosal effector sites<sup>48,49</sup>. Since IL-3 also enhances basophil activation by IgD, basophils and mast cells may form an IgD-mediated IL-3-dependent axis for the amplification of both systemic and mucosal immune responses.

The interaction of IgD with basophils and mast cells was sensitive to the proteolytic activity of trypsin and papain, but not to that of pepsin. Previous studies show that papain initiates IgE responses by inducing IL-4 release from basophils through an enzymatic mechanism<sup>34</sup>. One possibility is that IgD and papain interact with similar receptors on basophils. Importantly, IgD clearly stimulates basophils through a pathway distinct from that induced by IgE. Indeed, in spite of eliciting IgE-like intracellular calcium fluxes, IgD cross-linking did not induce degranulation and histamine release. Furthermore, IgD was less effective than IgE cross-linking in up-regulating surface CD40L, a powerful class switch-inducing

factor<sup>32</sup>. Conversely, IgD was generally more effective than IgE cross-linking in inducing antimicrobial, immunostimulating and pro-inflammatory factors such as IL-1 $\beta$ , TNF, IL-8 and CXCL10.

The pro-inflammatory function of IgD was further suggested by the analysis of autoinflammatory syndromes, a group of disorders characterized by periodic attacks of fever and inflammation as well as exaggerated IL-1 $\beta$  and IgD production<sup>4,54</sup>. We found more class-switched IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts and more mucosal IgD-armed basophils in patients with HIDS, TRAPS, MWS or PFAPA syndrome. In these subjects, mucosal basophils could enhance inflammation by releasing IL-1 $\beta$  and LL-37 in response to microbial antigens with IgD-binding activity. Consistent with this possibility, periodic fever is often triggered by infections or immunization, particularly in HIDS patients<sup>54</sup>. Similar IgD-binding antigens could also increase basophil expression of B cell-activating and class switch-inducing factors such as CD40L, BAFF and APRIL. This effect could account for the elevated serum IgG and IgA titers often observed in individuals with HIDS, TRAPS, MWS and PFAPA syndrome<sup>4,54</sup>. Finally, the fact that IgD predominantly exerts its activity as a basophil-bound antibody could explain why periodic attacks of fever and inflammation do not predictably correlate with serum IgD in patients with HIDS<sup>54</sup>.

In summary, human B cells may produce IgD to instruct basophils as to the antigenic composition of the upper respiratory tract. This evolutionarily conserved immune surveillance system would not only monitor systemic invasion by airborne pathogens, but also regulate B cell homeostasis, antibody production and inflammation.

## METHODS

### Fresh cells

Peripheral blood mononuclear cells from buffy coats of healthy subjects were purchased at the New York Blood Center. Additional mononuclear cells were isolated from the peripheral blood of patients with primary immunodeficiencies, mastocytosis or autoinflammatory syndromes. Tonsillar and pulmonary mononuclear cells were obtained from tissue specimens of patients undergoing resection of hypertrophic tonsils or lung neoplastic lesions at New York Presbyterian Hospital-Weill Cornell Medical Center. The Institutional Review Board of Weill Medical College of Cornell University approved the use of blood, tonsil and lung specimens for this study, and patients provided informed consent. IgD<sup>+</sup>IgM<sup>+</sup> B cells and CD14<sup>+</sup> monocytes were magnetically sorted from peripheral blood and tonsillar mononuclear cells as reported<sup>16</sup>. In the experiments involving induction of  $\sigma_8$ -S $\mu$  switch circles and loss of surface IgM, peripheral blood IgD<sup>+</sup>IgM<sup>+</sup> B cells were depleted of antigen-experienced plasmablasts using CD27-targeting microbeads (Miltenyi Biotec). For morphological studies, IgD<sup>+</sup>IgM<sup>+</sup> and IgD<sup>+</sup>IgM<sup>-</sup> B cells were FACSorted from tonsillar mononuclear cells. Granulocytes were separated from peripheral blood mononuclear cells using Histopaque-1119 and Histopaque-1077 double gradients (Sigma). Untouched basophils were purified by negative selection from peripheral blood mononuclear cells using a Basophil Isolation Kit (Miltenyi Biotec). Mast cells were enriched from lung mononuclear cells using a biotin-conjugated AER-37 monoclonal antibody (mAb) to Fc $\epsilon$ RI (eBiosciences) and anti-biotin microbeads Miltenyi Biotec). Catfish peripheral blood

lymphocytes were obtained and stained as reported<sup>5,59</sup>. Catfish IgM and IgD were detected with 9E1 and 7D11 mAbs, respectively.

### Cell lines

The T cell lines Jurkat and Molt-3, the B cell lines Reh, 2E2 and Ramos, the myeloid cell lines THP-1, U937 and HL60, and the pre-basophilic cell line KU812 were cultured in complete RPMI 1640 medium (Gibco). The T cell line HuT78 was cultured in complete Iscove's DMEM (Mediatech). The NK cell line NKL was cultured in complete RPMI 1640 medium further supplemented with 1% non-essential amino acids (Mediatech) and 200 U/ml IL-2. The NK cell line NK-92 was cultured in MEM- $\alpha$  medium with 2 mM glutamine, 1.5 mg/ml NaHCO<sub>3</sub>, 0.1 mM  $\beta$ -mercaptoethanol, 0.02 mM folate, 0.2 mM inositol, 12.5% FBS and 12.5% horse serum. The mastocytoid cell line LAD2 (from A. Kirshenbaum and D. Metcalfe, National Institutes of Health) was cultured in serum-free Stem Pro-34 medium (Invitrogen) containing 100 ng/ml of stem cell factor (Peprotech). The mastocytoid cell line HMC-1 was cultured in Iscove's DMEM (Mediatech) with 10% FBS, 2 mM glutamine, 0.02%  $\alpha$ -thioglycerol and antibiotics.

### Tissues and immunohistochemistry

Frozen and paraffin-embedded tissue sections from tonsils, nasal cavities, intestine, liver, lymph nodes, bone marrow and spleen from healthy subjects or patients with primary immunodeficiencies or autoinflammatory syndromes were stored at  $-80^{\circ}\text{C}$  and  $25^{\circ}\text{C}$ , respectively. Sections were blocked with a saturating concentration of purified human IgG and stained for immunohistochemical analysis with various combinations of antibodies (Supplementary Table 1 online) as described previously<sup>18</sup>. Primary antibodies with irrelevant binding activity and appropriate secondary reagents were utilized to test the specificity of tissue stainings as previously described<sup>33</sup>. The proportion of IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts in different organs was determined by sequentially staining tissue sections with a polyvalent goat polyclonal antibody (pAb) to human Igs (Cappel), an Alexa Fluor 647-conjugated secondary pAb to goat Igs, a fluorescein-conjugated goat F(ab')<sub>2</sub> pAb to human IgD (Southern Biotech), and a biotin-conjugated goat F(ab')<sub>2</sub> pAb to human IgM. Nuclei were visualized with DAPI, 4',6-diamidino-2'-phenylindole dihydrochloride (Boehringer Mannheim). The following formula was used: number of cytoplasmic IgD<sup>+</sup>IgM<sup>-</sup> B cells / number of cytoplasmic Ig<sup>+</sup> B cells  $\times$  100. Sections adjacent to those analyzed for IgM, IgD and total Ig were stained with 7G3 mAb to CD123. IgD<sup>+</sup> cells with no clear plasmacytoid morphology, but with granular cytoplasm, lobated nucleus and/or CD123 expression were scored as basophils and therefore excluded from the analysis. Follicular mantle IgD<sup>+</sup>IgM<sup>-</sup> B cells were also excluded from the analysis. These B cells have bright IgD and dim IgM expression due to the higher stability and less rapid turnover of V(D)J-C $\delta$  transcripts compared to V(D)J-C $\mu$  transcripts<sup>4</sup>. Pseudocolor images were composed using Metamorph 7.5 (Molecular Device) and edited using Photoshop CS2 (Adobe).

### Patients

HIGM patients had various *TNFSF5*, *AICDA* or *TNFRSF5* gene mutations impairing the function of CD40L, AID and CD40, respectively<sup>43</sup>. CVID patients had a heterozygous

C104R mutation in the *TNFRSF13b* gene. This mutation prevents the binding of BAFF and APRIL to TACI44,45. The MWS patient had a heterozygous C1043T mutation in the *NALP3* gene. This mutation enhances the activity of cryopyrin, a key component of the inflammasome<sup>54</sup>. TRAPS patients had heterozygous G173A, G362A, or T123G mutations in the *TNFRSF1A* gene. These mutations prevent cleavage of the extracellular domain of TNF receptor type-1, thereby impairing feedback inhibition of the pro-inflammatory cytokine TNF by its soluble decoy receptor<sup>54</sup>. HIDS patients had a homozygous G1129A mutation or compound G632A and G1129A mutations of the *MVK* gene, which encodes mevalonate kinase. The mechanism by which these mutations cause inflammation remains unknown. PFAPA patients had no molecular diagnosis, except one, who was reported to have a heterozygous mutation in the *MEFV* gene encoding pyrin (also known as marenstrin), a negative regulator of the inflammasome<sup>54</sup>.

## Bacteria

*M. catarrhalis* BAA-1425, *H. influenzae* type-a 9006, and *H. influenzae* type-b 9795 (American Type Cell Culture) were cultured according to the manufacturer's instructions.

## Cultures and reagents

Cultures were performed in complete RPMI medium supplemented with 10% (volume/volume) bovine serum. Purified B cells were incubated with CD40L (Amgen), 500 ng/ml; BAFF (Alexis), 500 ng/ml; APRIL MegaLigand (Alexis), 500 ng/ml; IL-2 (Peprotech), 100 ng/ml; IL-10 (Peprotech), 50 ng/ml; IL-15 (Sigma), 50 ng/ml; and IL-21 (R&D Systems), 100 ng/ml. Cultures involving HIGM2 specimens were carried out with peripheral blood mononuclear cells instead of purified B cells due to the scarce amount of blood available. In these cultures, B cell activation was optimized by using 5 µg/ml goat F(ab')<sub>2</sub> pAb to human IgM (Southern Biotech). Purified basophils with Fc receptors already blocked during the purification step were incubated with or without 30 ng/ml of IL-3 (Peprotech) for 4 h. These basophils were stimulated with 5 µl of anti-mouse IgG-coated microbeads (Miltenyi Biotec) mixed with one of the following antibodies: a control IgG2a mAb with irrelevant binding activity (Santa Cruz Biotechnology), 25 µg/ml; a mouse IgG2a IA6-2 mAb to IgD (BD Biosciences), 25 µg/ml; or a mouse IgG2a G7-18 mAb to IgE (BD Biosciences), 2 µg/ml. Basophils stimulated with equal amounts of microbeads only were included as an additional control. Basophil-B cell co-cultures were set up by incubating IgD<sup>+</sup>IgM<sup>+</sup> B cells with basophils activated as described above for 1 h (1:1 ratio). In these co-cultures, basophils were extensively washed to eliminate excess mAb to IgD or IgE before B cell addition.

## Flow cytometry

Cells were incubated with an Fc blocking reagent (Miltenyi Biotec) or saturating concentrations of purified human IgG and stained at 4 °C with appropriate combinations of pAbs and mAbs to various surface antigens (Supplementary Table 1 online). 7-AAD was routinely used to exclude dead cells from the analysis. All gates and quadrants were drawn to give 1% total positive cells in the sample stained with control antibodies. Events were acquired on a FACS Calibur or BD LSR II (BD Biosciences) and analyzed by FlowJo (Tree

Star). Tonsillar IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts were identified within an electronic gate containing CD19<sup>+</sup> mononuclear cells.

### Enzyme-linked immunosorbent assay (ELISA) and cytometric bead array

IgD (Bethyl Laboratories), histamine (Neogen Corporation), IL-1 $\beta$ , IL-4, IL-13, APRIL and TNF (Bender Medsystems) were measured by ELISAs as instructed by the manufacturer. IL-8 and CXCL10 were measured using Human T<sub>H</sub>1–T<sub>H</sub>2 Cytometric Bead Array (BD Biosciences). IgM, IgG, IgA and BAFF were detected by standard ELISAs as described<sup>16</sup>, whereas IgE was measured by ELISA using a mAb to human IgE (G7-18, BD Biosciences) as capture antibody and a biotinylated mAb to human IgE (G7-26, BD Biosciences) as detection antibody. LL-37 was measured by ELISA using a mouse mAb to LL-37 (3D11, Hycult Biotechnology) as capture antibody and a rabbit pAb to LL-37 (PANATecs GmbH) as detection antibody, followed by a pAb to rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Human LL-37 peptide (PANATec GmbH) was used to generate a standard curve. Supernatants from basophil-containing cultures were centrifuged at 18,000 g for 20 min twice and adsorbed with mouse IgG before using them for ELISAs.

### Genomic PCR, Southern blotting, cloning and sequencing

Active CSR from IgM to IgD was determined by PCR amplifying extrachromosomal  $\sigma_8$ -S $_{\mu}$  reciprocal DNA recombination products instead of ligated chromosomal S $_{\mu}$ - $\sigma_8$  DNA products, which could be inflated by the proliferation of the class-switched B cells. Genomic DNA was extracted using the QIAamp DNA Mini Kit.  $\sigma_8$ -S $_{\mu}$  switch circles were amplified from genomic DNA using elongase (Invitrogen) and a  $\sigma_8$  sense primer 5'-TCATCATTGCCAGATGCTAGGGCT-3' coupled with an S $_{\mu}$  antisense primer 5'-TGAGTGCCCTCACTACTTGCGTCCCG-3' under the following conditions: initial denaturation for 30 s at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C, and extension for 7 min at 68 °C, and a final extension for 15 min at 68 °C. Products were resolved in a 1% agarose gel, transferred overnight onto nylon membranes, and hybridized with two <sup>32</sup>P-labeled probes specific to the 3' portion of S $_{\mu}$  and the 5' portion of  $\sigma_8$  respectively. To further verify the specificity of PCR-amplified products, a second PCR was performed under the same conditions using the  $\sigma_8$  sense primer 5'-TCATCATTGC CCAGATGCTAGGGCT-3' coupled with a nested S $_{\mu}$  antisense primer 5'-CAGACTGTCATGGCTATCA GGGGTGGCGGGG-3'. PCR products longer than 1.5 kb were purified, ligated into pCR2.1 TOPO vectors, and transformed into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen). Plasmids in selected colonies were purified and sequenced. Sequences were aligned with human genomic DNA sequences to identify regions spanning both  $\sigma_8$  and S $_{\mu}$ .

### RT-PCR

Active germline C $_{\delta}$  gene transcription and post-switch juxtaposition of chromosomal I $_{\mu}$  and C $_{\delta}$  exons were evaluated through the analysis of chimeric I $_{\mu}$ -C $_{\delta}$  transcripts in pre-switched and post-switched B cells, respectively. Total RNA was extracted using the QIAamp DNA Mini Kit and RNeasy Mini Kit (Qiagen) and cDNA was synthesized as previously described<sup>16</sup>. I $_{\mu}$ -C $_{\delta}$  transcripts were amplified from cDNA using Taq polymerase with an I $_{\mu}$

sense primer 5'-GTGATTAAGGAGAAACACTTTGAT-3' and a C<sub>8</sub> antisense primer 5'-CTGGCCAGCGGAAGATCTCCTTCTT-3' under the following conditions: initial denaturation for 5 min at 94 °C, followed by 25 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C and extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C. Bands were detected as described above, using <sup>32</sup>P-labeled probes specific for I<sub>μ</sub> and C<sub>8</sub> exons. RT-PCR products were cloned and sequenced to identify regions spanning both I<sub>μ</sub> and C<sub>8</sub>.

### QRT-PCR

Total RNA was extracted from basophils using TRIzol (Invitrogen). cDNA synthesis and QRT-PCR were performed using various primer pairs (Supplementary Table 2 online) as described<sup>18</sup>.

### Calcium flux assay

Basophils ( $2.5 \times 10^6$ ) were resuspended in 1 ml of phosphate buffer (pH 7.4) solution containing 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid (EDTA). Cells were loaded with 5 μM Fura-3/AM (Sigma) for 30 min at 37 °C. Excess Fura-3/AM was removed by washing cells twice in phosphate buffer solution. Cells were then stimulated as reported in the text. Intracellular Ca<sup>2+</sup> dynamics was monitored following the addition of stimulants in a plate reader with a 336 nm laser and a 495 nm filter. Readings were recorded every 40 seconds.

### IgD binding assay

Polyclonal or monoclonal IgD antibodies purified from the plasma of healthy individuals or multiple myeloma patients (Athens Research & Technology) were incubated at the indicated concentrations with various cell types ( $1 \times 10^5$ ) for 20 min at 4 °C. Binding assays were performed with a concentration of IgD within or below the physiological range of serum IgD concentrations (15-300 μg/ml). Cells were then washed, stained with fluorescein-conjugated F(ab')<sub>2</sub> pAb to IgD (Southern Biotech), washed and analyzed by flow cytometry. This F(ab')<sub>2</sub> antibody had no cross-reactivity to other immunoglobulin classes (Supplementary Figure 6 online) and could not bind to Fc receptors due to the lack of its Fc portion. Cells stained with this pAb to IgD in the absence of purified IgD were used as background control (Supplementary Figure 6 online). Alternatively, cells were stained with fluorescein-conjugated mouse IA6-2 or IgD26 mAbs to IgD.

### Competitive IgD binding assays

Monoclonal IgD purified from the plasma of a multiple myeloma patient was labeled with Alexa Fluor 488 using a Microscale Protein Labeling kit (Molecular Probes) and dialyzed in phosphate buffer (pH 7.4) solution overnight to remove excess fluorochrome. Freshly isolated peripheral blood basophils were stripped of bound endogenous IgD by incubation in an acidic sodium citrate buffer (40 mM sodium citrate, 140 mM NaCl, pH 3.0) for 3 min. Then, cells were quickly spun down to remove the acidic buffer and PBS was added to the cells to neutralize the pH. Basophils stripped of endogenous bound IgD were incubated with 0, 100 or 500 μg/ml unlabelled monoclonal IgD, followed by 40 μg/ml Alexa Fluor 488-



labelled monoclonal IgD. After incubation, cells were washed and analyzed by flow cytometry. 7-AAD was used to exclude dead cells.

### Statistical analysis

Values were expressed as mean  $\pm$  standard error of the mean (s.e.m.). Statistical significance was assessed by a one-tailed unpaired Student's *t*-test.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

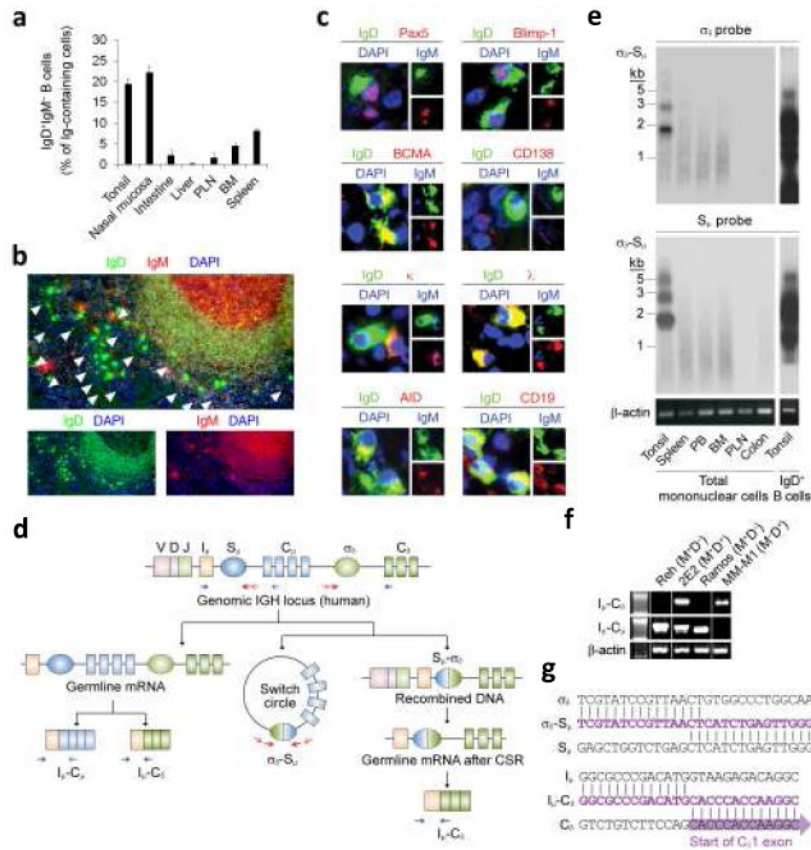
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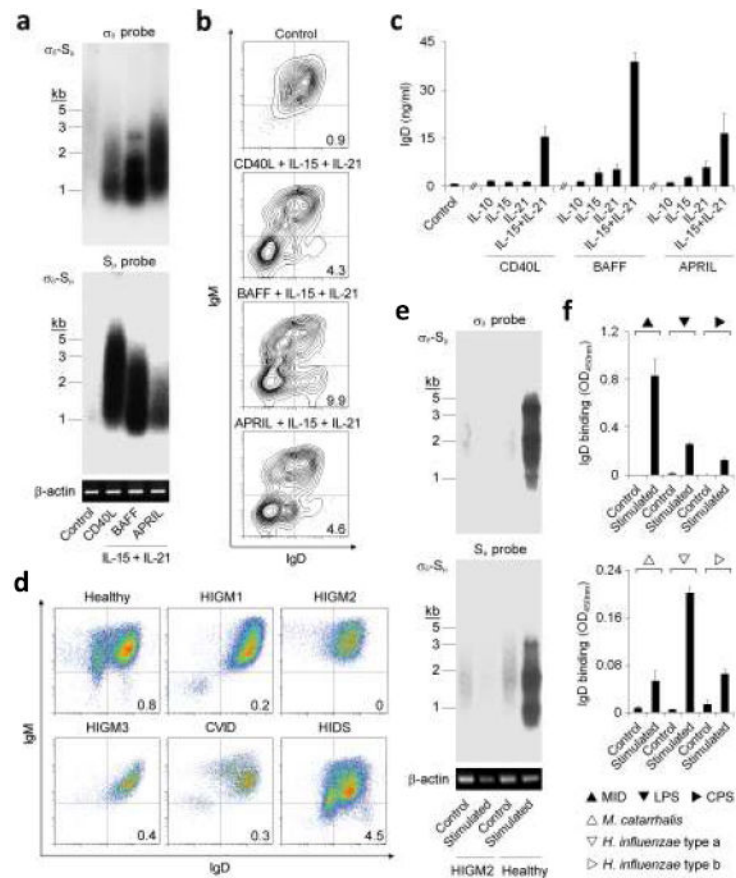
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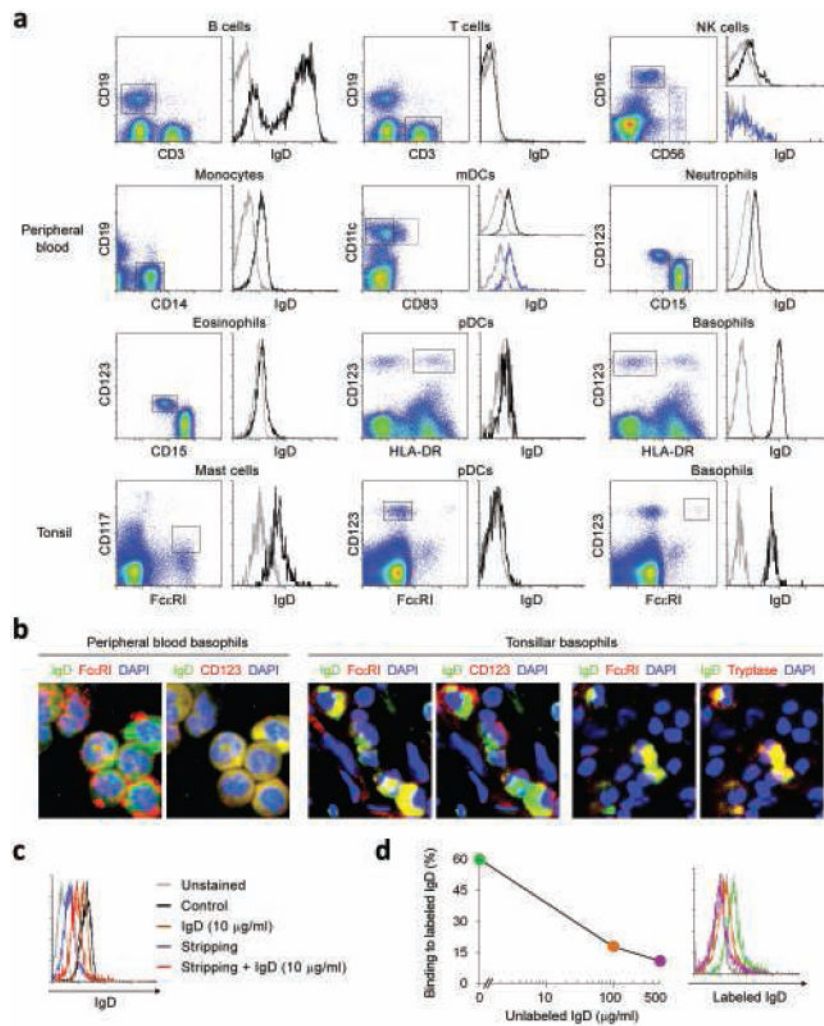
**Figure 1. Upper respiratory mucosa B cells generate IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts by undergoing C<sub>μ</sub>-to-C<sub>δ</sub> CSR *in situ***

(a) Proportion of IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts in various tissues calculated by immunofluorescence as described in Methods. PLN, peripheral lymph nodes; BM, bone marrow. (b) Tonsil tissue stained for IgD (green) and IgM (red). DAPI (blue) counterstains nuclei. Arrowheads point to IgD<sup>+</sup>IgM<sup>-</sup> plasmablasts. Original magnification  $\times 10$ . (c) Representative IgD<sup>+</sup>IgM<sup>-</sup> B cells from tonsils stained for IgD (green), Pax-5, Blimp-1, BCMA, CD138,  $\kappa$ ,  $\lambda$ , AID or CD19 (red), and IgM or DAPI (blue). (d) Diagram of CSR from S<sub>μ</sub> to σ<sub>δ</sub>. Germline I<sub>μ</sub>-C<sub>μ</sub> and I<sub>μ</sub>-C<sub>δ</sub> transcripts, σ<sub>δ</sub>-S<sub>μ</sub> switch circles, and post-switched I<sub>μ</sub>-C<sub>δ</sub> transcripts are shown. Arrows indicate primers. (e) Southern blots of σ<sub>δ</sub>-S<sub>μ</sub> switch circles PCR amplified from mononuclear cells of various tissues and hybridized with σ<sub>δ</sub> or S<sub>μ</sub> probes. Rightmost lane shows a typical multi-banded σ<sub>δ</sub>-S<sub>μ</sub> smear in enriched tonsillar IgD<sup>+</sup> B cells. Kb, kilobases. (f) Germline I<sub>μ</sub>-C<sub>μ</sub> transcripts and germline or post-switch I<sub>μ</sub>-C<sub>δ</sub> transcripts in Reh IgD<sup>-</sup>IgM<sup>+</sup> pre-B-like cells, 2E2 pre-germinal center IgD<sup>+</sup>IgM<sup>+</sup> B cells, Ramos germinal center-like IgD<sup>-</sup>IgM<sup>+</sup> B cells, and MM-M1 IgD<sup>+</sup>IgM<sup>-</sup> plasma cells. Genomic β-actin is a loading control. (g) Representative σ<sub>δ</sub>-S<sub>μ</sub> and I<sub>μ</sub>-C<sub>δ</sub> DNA sequences from tonsillar IgD<sup>+</sup>IgM<sup>-</sup> and IgD<sup>+</sup>IgM<sup>+</sup> B cells, respectively. Panel a summarizes 1 of 3 experiments (bars indicate s.e.m.), whereas panels b, c, d, f, g and h show 1 of 5 experiments yielding similar results.



**Figure 2.  $C_\mu$ -to- $C_\delta$  CSR occurs through both TD and TI pathways, requires AID, and leads to the production of IgD antibodies that bind to respiratory bacteria**

(a)  $\sigma_S-S_\mu$  switch circles from circulating IgD<sup>+</sup>IgM<sup>+</sup> B cells stimulated with or without CD40L, BAFF or APRIL plus IL-15 and IL-21 for 4 d. Genomic  $\beta$ -actin is a loading control. Kb, kilobases. (b, c) Flow cytometric analysis of surface IgD and IgM and ELISA of secreted IgD from circulating IgD<sup>+</sup>IgM<sup>+</sup> B cells stimulated as in a for 7 d. Control indicates medium alone. (d) Percentage of circulating IgD<sup>+</sup>IgM<sup>-</sup> B cells in a healthy donor and patients with *TNFSF5* (HIGM1), *AICDA* (HIGM2), *TNFRSF5* (HIGM3), *TNFRSF13b* (CVID) or *MVK* (HIDS) gene defects. (e) Induction of  $\sigma_S-S_\mu$  switch circles in circulating mononuclear cells from a HIGM2 patient and a control healthy donor incubated with medium alone (control) or BAFF, IL-15 and IL-21 (stimulated) for 4 d. An anti-IgM antibody was added to optimize the activation and expansion of IgD<sup>+</sup>IgM<sup>+</sup> B cells. (f) Binding of secreted IgD to MID, CPS, LPS, *M. catarrhalis*, and *H. influenzae* type a or type b as determined by ELISA. IgD secretion was obtained by incubating circulating IgD<sup>+</sup>IgM<sup>+</sup> B cells with or without BAFF, IL-15 and IL-21 for 7 d. Panels a, b, d and e show 1 of 5 experiments yielding similar results, whereas panels c and f summarize 3 experiments (bars indicate s.e.m.; \*,  $p < 0.05$ ).



### Figure 3. IgD binds to basophils and mast cells *in vivo*

(a) Flow cytometric analysis of IgD on circulating or tonsillar CD19<sup>+</sup>CD3<sup>-</sup> B cells, CD19<sup>-</sup>CD3<sup>+</sup> T cells, CD16<sup>high</sup>CD56<sup>low</sup> or CD16<sup>low</sup>CD56<sup>high</sup> NK cells, CD19<sup>-</sup>CD14<sup>+</sup> monocytes, CD11c<sup>+</sup>CD83<sup>-</sup> or CD11c<sup>+</sup>CD83<sup>+</sup> myeloid dendritic cells (mDCs), CD15<sup>+</sup>CD123<sup>-</sup> neutrophils, CD15<sup>+</sup>CD123<sup>low</sup> eosinophils, CD123<sup>+</sup>HLA-DR<sup>+</sup> or CD123<sup>+</sup>FcεRI<sup>low</sup> plasmacytoid dendritic cells (pDCs), CD123<sup>+</sup>HLA-DR<sup>-</sup> or CD123<sup>+</sup>FcεRI<sup>+</sup> basophils, and CD117<sup>+</sup>FcεRI<sup>+</sup> mast cells. Gray and black histograms depict control unstained cells and IgD, respectively. A F(ab')<sub>2</sub> pAb was used to detect IgD. Dead cells were excluded using 7-AAD staining. (b) Immunofluorescence analysis of circulating and tonsillar basophils stained for IgD (green) and FcεRI, CD123 or tryptase (red). DAPI (blue) counterstains lobated nuclei of basophils. A F(ab')<sub>2</sub> pAb was used to detect IgD. (c) Flow cytometry of IgD levels on circulating basophils before (black histogram) and after exposure to exogenous monoclonal IgD (10 μg/ml, brown histogram), treatment with acidic buffer (blue histogram), and treatment with acidic buffer followed by addition of exogenous IgD (red histogram). A F(ab')<sub>2</sub> pAb was used to detect IgD. Unstained basophils were used as control (gray histogram) and dead cells were excluded using 7-AAD staining. (d) Binding of labeled monoclonal IgD to circulating basophils stripped of endogenous IgD and

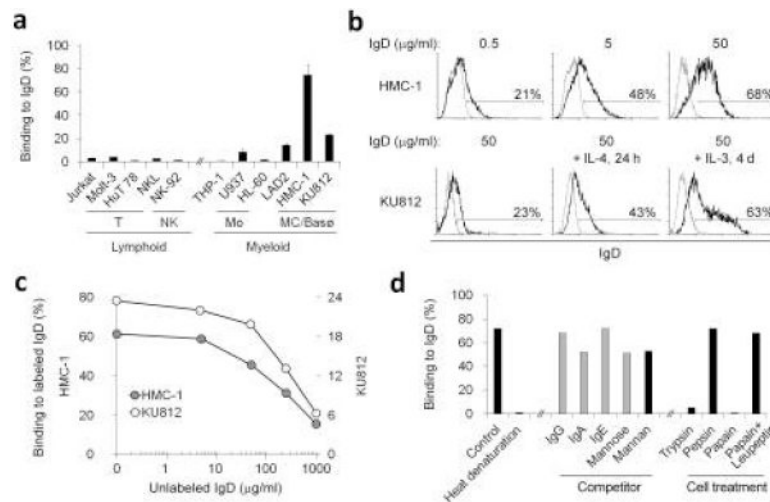
incubated with 0  $\mu\text{g/ml}$  (green histogram), 100  $\mu\text{g/ml}$  (orange histogram) or 500  $\mu\text{g/ml}$  (purple histogram) of unlabelled monoclonal IgD. A  $\text{F(ab')}_2$  pAb was used to detect IgD. Unstained basophils were used as control (gray histogram) and dead cells were excluded using 7-AAD staining. Panels a-d show 1 of 5 experiments yielding similar results.

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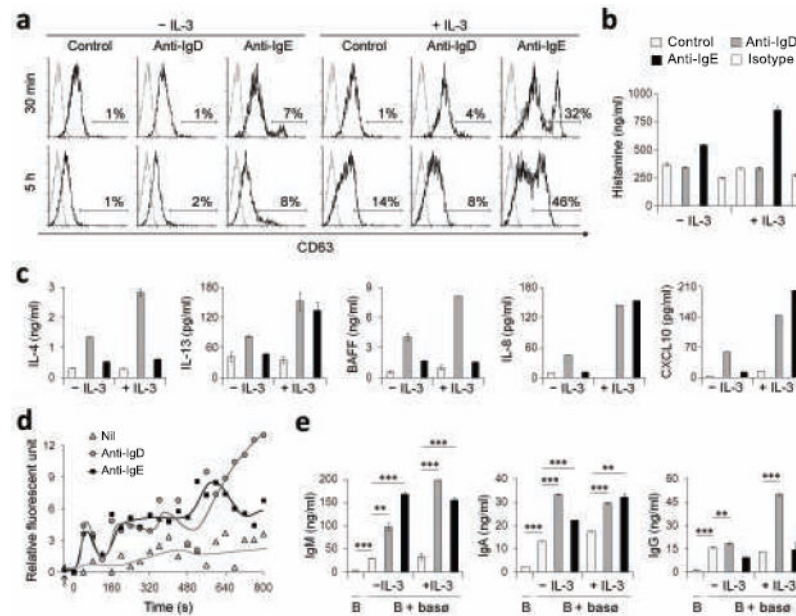
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**Figure 4. IgD binds to basophilic and mast cell lines *in vitro***

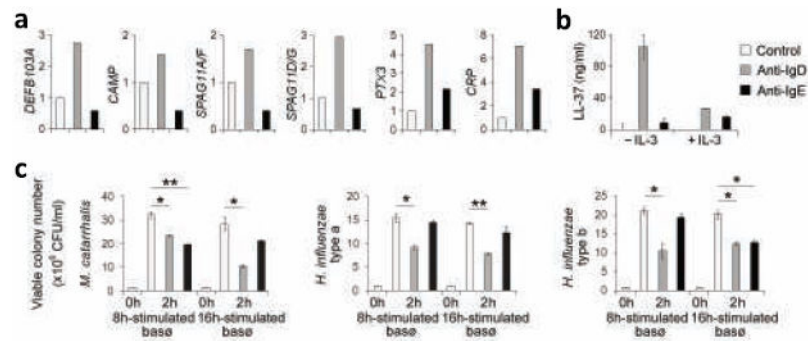
(a) Binding of monoclonal IgD (50  $\mu\text{g/ml}$ ) to various lymphoid and myeloid cell lines. A  $\text{F(ab')}_2$  pAb was used to detect IgD. Mo, MC and Bas $\phi$  indicate monocytic, mast cell and basophilic cell lines, respectively. (b) Binding of increasing amounts (0.5, 5 and 50  $\mu\text{g/ml}$ ) of monoclonal IgD to the mast cell line HMC-1 and binding of monoclonal IgD (50  $\mu\text{g/ml}$ ) to the basophilic cell line KU812 before and after treatment with IL-4 for 1 d and with IL-3 for 4 d. Control indicates medium alone. A  $\text{F(ab')}_2$  pAb was used to detect IgD. Gray histogram depicts background fluorescence of cells that were not incubated with monoclonal IgD. (c) Binding of fluorochrome-conjugated monoclonal IgD to HMC-1 or KU812 cells in the presence of increasing amounts (0, 5, 50, 250 or 1000  $\mu\text{g/ml}$ ) of unlabeled monoclonal IgD. (d) Binding of untreated or denatured monoclonal IgD (50  $\mu\text{g/ml}$ ) to HMC-1 cells pre-incubated or not with IgG, IgA, IgE, mannose, mannan, trypsin, pepsin, papain, or papain plus leupeptin. A  $\text{F(ab')}_2$  pAb was used to detect IgD. In all the experiments dead cells were excluded using 7-AAD staining. Panel a summarizes 3 experiments (bars indicate s.e.m.), whereas panels b-d show 1 of 3 experiments yielding similar results.





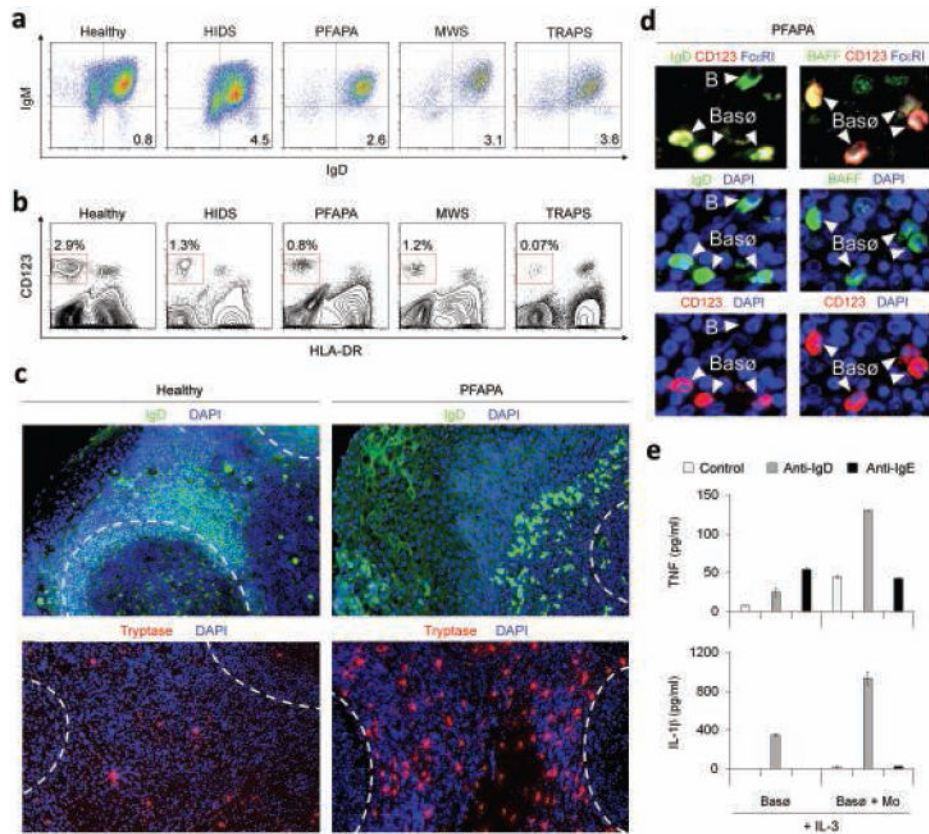
**Figure 5. Basophils release immunostimulating and pro-inflammatory factors upon IgD cross-linking**

(a) Flow cytometry of CD63 on basophils exposed to microbeads alone (control), microbead-bound monoclonal anti-IgD, or microbead-bound monoclonal anti-IgE for 30 min or 5 h in the presence or absence of IL-3. (b) ELISA of histamine from basophils exposed to microbeads alone (open bar), microbead-bound monoclonal anti-IgD (gray bar), microbead-bound monoclonal anti-IgE (black bar), or microbead-bound isotype-matched control monoclonal antibody (striped bar) for 30 min in the presence or absence of IL-3. (c) ELISA of IL-4, IL-13 or BAFF from basophils exposed to microbeads alone (open bar), microbead-bound monoclonal anti-IgD (gray bar), or microbead-bound monoclonal anti-IgE (black bar) for 16 h in the presence or absence of IL-3. IL-8 and CXCL10 were measured after 48 h. (d) Intracellular  $\text{Ca}^{2+}$  levels of basophils treated as in c. The arrow indicates addition of the cross-linking reagent and 0 sec indicates the start of the kinetic measurement. (e) IgM, IgA and IgG production by peripheral blood  $\text{IgD}^+\text{IgM}^+$  B cells exposed for 7 d to basophils treated as in c. Panels a and d show 1 of 3 experiments yielding similar results, whereas panels b, c and e summarize 3 experiments (bars indicate s.e.m.; \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ).



**Figure 6. Basophils release antimicrobial factors upon IgD cross-linking**

(a) QRT-PCR of *DEFB103A*, *CAMP*, *SPAG11A/F*, and *SPAG11D/G* transcripts from basophils exposed to microbeads alone (control, open bar), microbead-bound monoclonal anti-IgD (gray bar), or microbead-bound monoclonal anti-IgE (black bar) for 6 h. *PTX3* and *CRP* transcripts were measured after 16 h. mRNAs were normalized to *ACTB* mRNA. (b) ELISA of LL-37 from basophils stimulated as in a in the presence or absence of IL-3 for 8 h. (c) Growth of *Moraxella catarrhalis* and *Haemophilus influenzae* type-a and type-b upon 2-h exposure to culture supernatants from basophils stimulated as in a for 8 h or 16 h. CFU, colony forming unit. Panels a-c summarize 3 experiments (bars indicate s.e.m.; \*,  $p < 0.03$ ; \*\*,  $p < 0.02$ ; \*\*\*,  $p < 0.01$ ).



**Figure 7. Increased IgD class-switched plasmablasts and IgD-armed basophils in inflamed tissues from patients with periodic fever syndromes**

(a, b) Flow cytometric analysis of circulating IgD<sup>+</sup>IgM<sup>-</sup> B cells and CD123<sup>+</sup>HLA-DR<sup>-</sup> basophils in a healthy subject and hyper-IgD patients with *MVK* (HIDS), unknown (PFAPA), *NALP3* (MWS) and *TNFRSF1A* (TRAPS) gene defects. Numbers indicate percentage of IgD<sup>+</sup>IgM<sup>-</sup> B cells in CD19<sup>+</sup> B cells and of CD123<sup>+</sup>HLA-DR<sup>-</sup> basophils in mononuclear cells. (c) Immunofluorescence analysis of a tonsil tissue specimen from healthy individual or a PFAPA syndrome patient stained for IgD (green), tryptase (red) and DAPI (blue). Dashed lines demarcate lymphoid follicles. (d) BAFF (green) expression by basophils co-stained for IgD (green), CD123 (red) and FcεRI (blue). Arrowheads show both IgD<sup>+</sup>CD123<sup>-</sup> plasmablasts (B) and IgD<sup>+</sup>CD123<sup>+</sup> basophils (Bas $\phi$ ). (e) ELISA of TNF and IL-1 $\beta$  from basophils cultured with IL-3 and microbeads alone (control, open bar), microbead-bound monoclonal anti-IgD (gray bar) or microbead-bound monoclonal anti-IgE (black bar) for 48 h in the presence or absence of monocytes. Panels a-d show 1 of 3 experiments yielding similar results, whereas panel e summarizes 3 experiments (bars indicate s.e.m.).