

ALLERGY

Chronic allergen exposure drives accumulation of long-lived IgE plasma cells in the bone marrow, giving rise to serological memory

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Immunoglobulin E (IgE) plays an important role in allergic diseases. Nevertheless, the source of IgE serological memory remains controversial. We reexamined the mechanism of serological memory in allergy using a dual reporter system to track IgE⁺ plasma cells in mice. Short-term allergen exposure resulted in the generation of IgE⁺ plasma cells that resided mainly in secondary lymphoid organs and produced IgE that was unable to degranulate mast cells. In contrast, chronic allergen exposure led to the generation of long-lived IgE⁺ plasma cells that were primarily derived from sequential class switching of IgG1, accumulated in the bone marrow, and produced IgE capable of inducing anaphylaxis. IgE⁺ plasma cells were found in the bone marrow of human allergic, but not nonallergic donors, and allergen-specific IgE produced by these cells was able to induce mast cell degranulation when transferred to mice. These data demonstrate that long-lived IgE⁺ bone marrow plasma cells arise during chronic allergen exposure and establish serological memory in both mice and humans.

INTRODUCTION

The prevalence and impact of type 1 hypersensitivity reactions, including allergic asthma, atopic dermatitis, life-threatening anaphylaxis, some food/insect/drug allergies, and allergic rhinitis, continue to rapidly increase worldwide (1). Immunoglobulin E (IgE) is a key player in the development and progression of such diseases (2). Although allergen-induced cross-linking of allergen-specific IgE bound to Fcε receptors on effector cells is a trigger for the acute allergic response, the source of IgE serological memory remains elusive.

IgE is the isotype with the lowest abundance and shortest half-life in serum, lasting 2 to 3 days in humans (3) and ~12 hours in mice (4). IgE-producing cells are rarely detected in circulation (5), which presents a substantial challenge for the identification and characterization of these cells. In addition, traditional staining techniques have largely been unreliable in distinguishing IgE class-switched, membrane IgE⁺ cells from cells that bind soluble IgE via the high-affinity IgE receptor, FcεRI, or the low-affinity IgE receptor, FcεRII (CD23). Previous studies have tried to overcome this challenge by stripping IgE from its receptors using low pH (acid wash), by blocking extracellular IgE before intracellular staining, or by using different membrane IgE reporter systems (6–11). Using such detection systems, IgE⁺ B cells have been shown to exit germinal centers (GCs) prematurely and undergo early differentiation to short-lived plasma cells (PCs) during 4-hydroxy-3-nitrophenylacetyl-keyhole limpet hemocyanin (NP-KLH) immunization or helminth infection (8, 9, 11). In contrast to IgG1⁺ cells, short-lived IgE⁺ PCs have been shown to express higher membrane IgE compared with IgE⁺ B cells (9). Signaling through the IgE B cell receptor (BCR) has been shown to induce apoptosis in IgE-switched cells, limiting their life span in secondary lymphoid organs (7). In addition, during NP-KLH immunization, IgE⁺ PCs were undetectable in the bone marrow (BM) (9). Collectively, these data argue against the presence of IgE cellular or serological memory in some murine models.

In contrast, several clinical observations suggest the presence of IgE serological memory in atopic patients. For example, persistent production of serum IgE is observed in allergic patients in the absence of allergen reexposure (12–14). The inadvertent transfer of allergies and detection of allergen-specific IgE after BM transplant from allergic donors also argue for the presence of IgE⁺ BMPCs in atopic individuals (15, 16). In addition, treatments that ablate IgE⁺ B cells and short-lived plasmablasts (e.g., membrane IgE depleting antibody) or prevent class switching to IgE [e.g., interleukin-4 (IL-4)/IL-13 blocking antibodies] reduce serum IgE but are unable to bring serum IgE back to baseline (17–20), indicating the presence of an IgE serological memory source that cannot be efficiently targeted with currently available therapies (20).

The mouse studies using reporter systems cited above relied on antigens delivered by injection with potent adjuvants, and none mimicked natural allergen exposure routes. These circumstances contrast with those required for the emergence and maintenance of allergic diseases, namely, continuous or intermittent exposure to allergen, often delivered by inhalation (e.g., mold, pollen, dust mite, or animal dander). In this study, we used murine models of short-term (4 weeks) and chronic (15 weeks) allergen exposure to study the IgE response in a model that mimics natural routes of environmental allergen exposure and identify the source of allergen-specific IgE memory. Using dual reporter mice that track IgE-producing cells (membrane IgE^{Venus}) and PCs (Blimp-1^{mCherry}), we show that 4-week allergen exposure results in the generation of IgE⁺ B cells and plasmablasts/PCs that mainly reside in secondary lymphoid organs and do not produce allergen-specific IgE that is able to mediate an anaphylaxis response. In contrast, chronic exposure to house dust mite (HDM) extract results in IgE⁺ PCs that primarily arise from sequential class switching of IgG1⁺ B cells, show similar CXCR4 expression to IgG1⁺ PCs, and gradually accumulate in the BM. We also demonstrate that, in contrast to IgE produced from secondary lymphoid organs during short-term allergen exposure, allergen-specific IgE that is produced from BMPCs, in both humans and mice, can cause mast cell degranulation and initiate anaphylaxis.

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RESULTS

PCs accumulate in the BM during chronic allergen exposure

It is well established that the BM provides a niche that allows PCs to survive for long periods of time and that antibody derived from these cells confers IgG serological memory in the absence of naïve or memory B cells (21), for example, the protective response to viral infection in vaccinated individuals. In contrast, the existence of long-lived IgE⁺ PCs in allergy models and their contribution for IgE serological memory has been controversial in previous literature. Some studies in mice have failed to detect IgE⁺ PCs in the BM, and in humans, the presence of IgE⁺ PCs has yet to be convincingly addressed [reviewed in (5)]

To explore this question in mice, we relied on a mouse model that recapitulates features of continual, chronic allergen exposure in humans. Repeated HDM exposure has been shown to elicit several hallmarks of allergic asthma, including airway hyperresponsiveness, lung remodeling, increase in serum IgE and IgG1, and induction of type 2 cytokines and chemokines (22, 23). Accordingly, we exposed mice to HDM intranasally, three times per week either for 4 weeks (4 weeks HDM), to induce type 2 allergic inflammation, or for 15 weeks (15 weeks HDM), to induce mixed type 2 and type 1 inflammation (22, 23).

Overall, PC frequency in the BM increased significantly after 15 weeks of HDM exposure compared with mice exposed to saline (Fig. 1A). This PC accumulation was not observed in mice exposed to HDM for 4 weeks, suggesting that longer exposure to allergens is needed to expand PCs within the BM compartment (Fig. 1A). As an initial step to characterize BMPCs, we purified CD138⁺ PCs, isolated RNA, performed RNA sequencing, and looked for the presence of IgE transcripts. IgE transcripts were detected within sorted BMPCs from mice exposed to HDM for 15 weeks, suggesting that IgE⁺ PCs are present in the BM of mice chronically exposed to allergen (Fig. 1B). Consistent with a PC phenotype, Ig genes were among the most abundantly expressed genes in CD138⁺ cells purified from 15-week HDM BM (Fig. 1B).

CD138 is not unique to BMPCs because it is expressed on epithelial cells, macrophages, dendritic cells [data assembled by the ImmGen consortium (24)], and pre-B and immature B cells in the BM (25). Thus, use of CD138 as a PC marker requires the use of a dump gate (fig. S1A). We looked for potential surface markers that are highly up-regulated in BMPCs. Ly6D, which has been previously identified during early B cell development and late PC differentiation (26, 27), was highly expressed in BMPCs during chronic allergen exposure (fig. S1B). Surface expression of Ly6D was validated on BMPCs, and when combined with CD138, these two markers revealed a distinct population of PCs (fig. S1C). Other receptors that were highly transcribed in our sorted population included known markers of a PC phenotype, including CAMPATH-1 antigen (CD52), major histocompatibility complex II (MHC II) invariant chain (CD74), and B cell maturation antigen (BCMA; TNFRSF17) (fig. S1B).

Our results demonstrated that there are challenges in identifying IgE-producing cells. We have shown that CD138 is not exclusive to BMPCs and have identified additional markers for PCs that could be useful. Given the challenges in tracking IgE-producing cells and the limited available information about whether IgE⁺ PCs express comparable markers as other isotypes, we generated a dual reporter mouse strain to thoroughly track and characterize the source of IgE production in the context of chronic allergen exposure.

Construction and characterization of membrane IgE^{Venus}/Blimp-1^{mCherry} reporter mice

To reliably detect IgE PCs in mice, we generated a dual reporter system that combines two different previously reported strategies to track IgE-producing cells and total PCs (9, 28). To track IgE-switched cells, we generated IgE^{Venus} reporter mice in which the yellow fluorescent protein derivative, Venus, was inserted downstream of the final membrane IgE exon (M2) linked by a viral P2A peptide to enable simultaneous reporter expression with membrane IgE (fig. S2A, top). To monitor PCs, we generated Blimp-1^{mCherry} reporter mice in which the fluorescent reporter, mCherry, was inserted downstream of the PC transcription factor Blimp-1 (*Prdm1*) (fig. S2A, bottom).

To validate that the Venus reporter system can track IgE-switched cells, we purified splenic B cells from IgE^{Venus} mice and treated them with CD40 ligand (CD40L) and IL-4, which together induce class switching of naïve B cells to IgE⁺ and IgG1⁺ cells. After 4 days in culture, IgE class-switched Venus⁺ cells were readily detectable, indicating that the reporter is functional (fig. S2B, top). A similar strategy of in vitro class switching was used to validate mCherry expression in Blimp-1^{mCherry} mice (fig. S2B, bottom). After 4 days in culture, Blimp-1 was up-regulated in ~60% of IgE⁺ cells and ~10% of IgG1⁺ cells (fig. S2C). This confirms a previous finding that, relative to IgG1, IgE BCR signaling promotes Blimp-1 expression and bias toward PC differentiation in IgE⁺ B cells, independent of antigen (9).

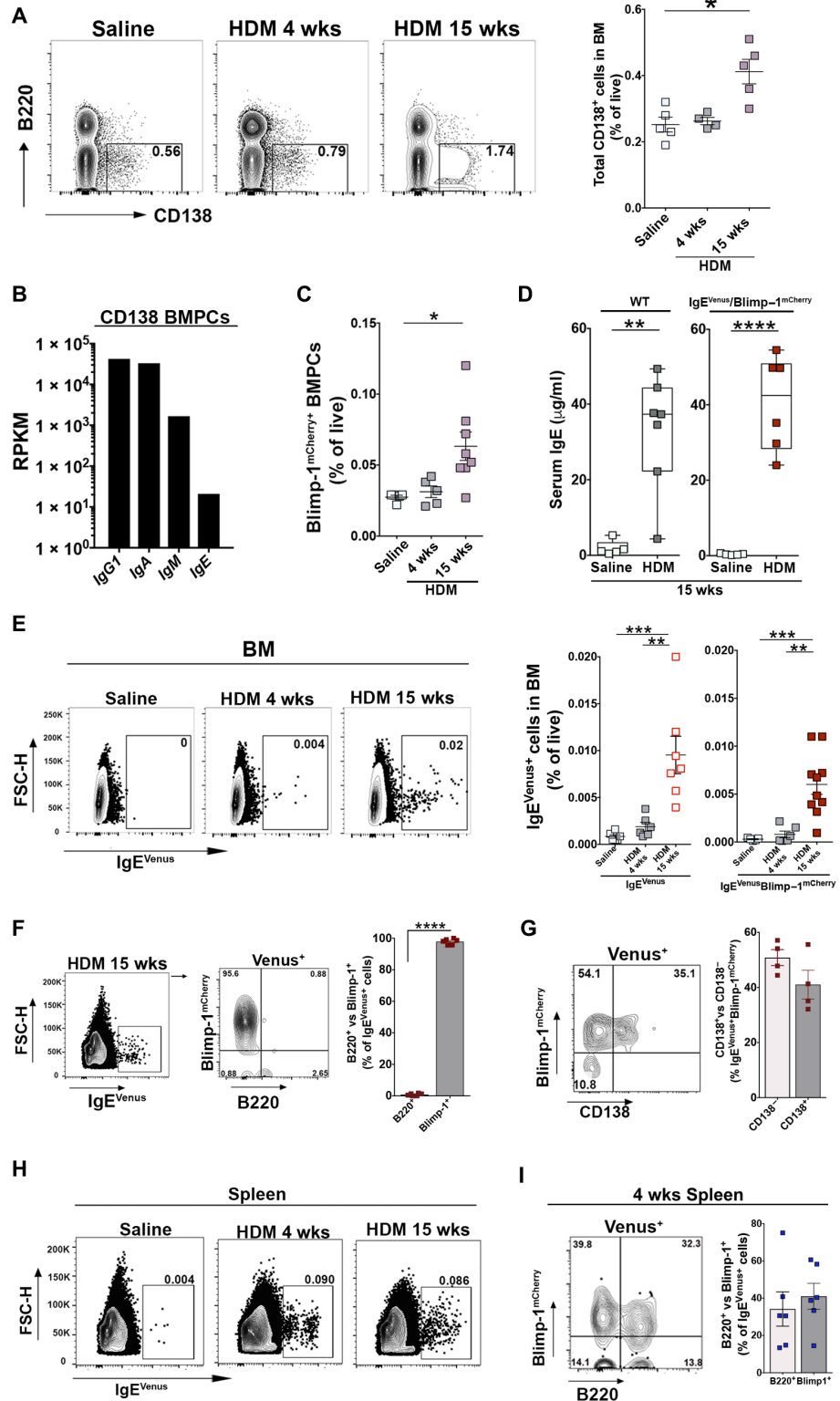
To further characterize these mice and confirm our results from sorted CD138⁺ PCs (fig. S1), we exposed Blimp-1^{mCherry} mice to HDM for 4 or 15 weeks and observed mCherry⁺ PC accumulation in the BM after 15 weeks of HDM exposure (Fig. 1C and fig. S2D). Consistent with a mature PC phenotype, B cell-specific transcription factors and surface receptors were down-regulated in BMPCs, including *Pax5*, *Bcl6*, *Cd19*, and *Fcer2a* (CD23), whereas PC transcription factors, such as *Irf4* and *Prdm1* (Blimp-1), were expressed (fig. S2E).

Having validated these two reporter mouse lines, we combined them to generate mIgE^{Venus}/Blimp-1^{mCherry} double reporter mice, where IgE⁺ PCs would be marked as Venus⁺/mCherry⁺. These three reporter lines (membrane IgE^{Venus} single reporter, Blimp-1^{mCherry} single reporter, and the double reporter mice) were used in the subsequent experiments.

IgE⁺ PCs migrate to the BM during chronic allergen exposure

To track IgE⁺ PCs during chronic allergic inflammation, we treated mIgE^{Venus}/Blimp-1^{mCherry} single and double reporter mice with HDM for 4 or 15 weeks. Serum IgE levels in dual reporter mice exposed to chronic HDM were comparable with serum IgE induced in wild-type (WT) mice by the same HDM exposure (Fig. 1D), confirming that the IgE response induced in the dual reporter mice is comparable with that of WT mice. IgE-Venus⁺ cells accumulated in the BM after 15 weeks of repeated HDM exposure (Fig. 1E), and almost all of the IgE-Venus⁺ cells expressed Blimp-1 and did not express the B cell marker B220 (Fig. 1F), indicating that these cells were mature BMPCs. We noticed that about half of B220[−] Blimp-1⁺ IgE⁺ BMPCs did not express CD138 (Fig. 1G), which has been conventionally used as a marker to identify IgE⁺ PCs in previous studies. These results further support the need for additional surface markers to identify IgE⁺ BMPCs and suggest that IgE⁺ BMPCs quantified from the CD138-gated cells may be an underestimation of the actual frequency, as also evidenced by IgE reads per kilobase, per million mapped reads (RPKM) values from the BM of reporter mice (fig. S2F, compare with Fig. 1B).

Fig. 1. IgE⁺ PCs accumulate in the BM during chronic allergen exposure in IgE^{Venus} and Blimp-1^{mCherry} reporter mice. (A) Representative plots of BMPCs in mice exposed to either saline or HDM for 4 or 15 weeks (wks). Numbers on each plot indicate the percentage of CD138⁺ PCs within a dump⁻ population (left) and quantification as the percentage of live cells (right). **(B)** Ig heavy-chain RNA expression in sorted CD138⁺ BMPCs exposed to HDM for 15 weeks. **(C)** Quantification of total Blimp-1^{mCherry} PCs in the BM of saline or HDM-exposed mice (percentage of live cells). **(D)** Comparison of serum IgE in WT and IgE^{Venus} homozygous/Blimp-1^{mCherry} heterozygous double reporter mice exposed to saline or HDM for 15 weeks. **(E)** Membrane IgE^{Venus}/Blimp-1^{mCherry} single and double reporter mice were intranasally exposed to either saline or HDM for 4 and 15 weeks. Representative dot plots of IgE^{Venus} cells within dump⁻/IgD⁻ population in the BM (left) (refer to fig. S2D for gating). Quantification (shown as the percentage of live cells) of IgE^{Venus} single reporters (center graph) and IgE^{Venus}/Blimp-1^{mCherry} double reporters (right graph). FSC, forward scatter height. **(F)** The frequency of PCs (Blimp-1^{mCherry}+) and B cells (B220⁺ Blimp-1^{mCherry}-) was analyzed within the IgE^{Venus} gate in the BM of 15-week HDM-exposed double reporter mice. **(G)** CD138 expression assessed within the IgE^{Venus}/Blimp-1^{mCherry} IgE PC population in the BM (left) and quantified (right). **(H)** Representative dot plots of IgE^{Venus} cells within dump⁻/IgD⁻ population in the spleen of saline- or HDM-exposed mice (left) and **(I)** the distribution of IgE PCs (Blimp-1^{mCherry}+) and IgE B cells/plasmablasts (B220⁺ Blimp-1^{mCherry}+) (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).



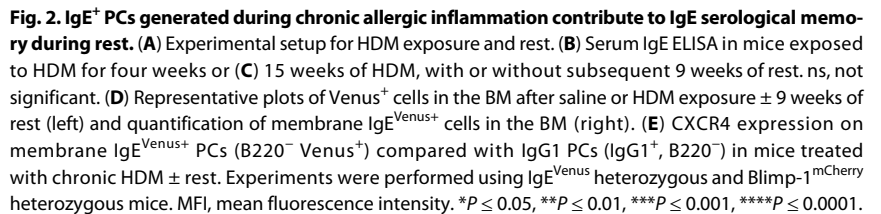
To examine the kinetics of IgE class switching and differentiation into PCs in secondary lymphoid organs, we measured Venus expression in the spleen of HDM-exposed mice and found that mIgE^{Venus} cells could be detected in spleen during short-term (4 weeks) HDM exposure (Fig. 1H). Similar to previous reports with NP-KLH immunization (9), we detected two distinct Venus⁺ populations in spleen, corresponding to IgE⁺ B cells/early plasmablasts (Venus⁺ B220⁺) and IgE⁺ PCs (Venus⁺ Blimp-1⁺B220⁻) (Fig. 1I). Together, these data indicate that short-term (4 weeks) allergen exposure to HDM is sufficient to induce IgE-producing cells in secondary lymphoid organs but not for accumulation in the BM.

IgE⁺ PCs contribute to IgE serological memory

To determine whether IgE⁺ PCs that migrate to the BM during chronic allergic inflammation contribute to IgE serological memory, mIgE^{Venus} reporter mice were exposed to HDM for either 4 or 15 weeks and rested (in the absence of allergen exposure) for an additional 9 weeks (Fig. 2A). Because the half-life of IgE in serum is ~12 hours in mice (4, 29), IgE⁺ plasmablasts are short-lived in secondary lymphoid organs (30, 31), and there is no allergen exposure that would drive de novo IgE production

during rest, we hypothesized that any IgE detected after 9 weeks of rest comes from long-lived IgE⁺ PCs.

The level of serum IgE increased in mIgE^{Venus} reporter mice exposed to HDM for 4 weeks, and these levels decreased after the



The IgE^{Venus+} BMPCs generated after 15 weeks of HDM exposure were maintained after 9 weeks of rest (Fig. 2D), suggesting that this population of cells contributes to the maintenance of IgE levels.

Although CXCR4 expression has been previously linked to migration and retention of BMPCs (32), IgE plasmablasts have been reported to lack CXCR4 expression, possibly explaining their inability to populate the BM (33). In contrast to these observations, IgE⁺ BMPCs generated during chronic allergen exposure expressed CXCR4 at levels comparable to IgG1⁺ PCs, and CXCR4 expression was maintained after the rest period (Fig. 2E). Together, these findings indicate that after continual chronic allergen exposure, IgE PCs accumulate in the BM, express CXCR4 at levels similar to those of IgG1⁺ BMPCs, and can be retained within the BM during a rest phase.

To further define whether these cells persist in the BM beyond the 9-week rest period and continue secreting IgE, we tracked serum IgE levels in mice exposed to HDM for 4 or 15 weeks and subsequently rested for ~6 to 7 months (29 weeks) (Fig. 3A and fig. S3C). In the 4-week HDM exposure model, the low level of serum IgE that we noted after 9 weeks of rest (Fig. 2B) plateaued and was maintained even after 23 weeks in the absence of allergen exposure (fig. S3C), suggesting limited but detectable IgE production that is driven by long-lived IgE⁺ PCs even after short-term allergen exposure. In the 15-week HDM-exposed mice, we observed that IgE levels slightly decline in the initial weeks of rest, and these levels also plateau after ~14 weeks and remain constant through the last time point assayed (29 weeks). These results suggest that, in the initial phase of rest, because short-lived IgE-producing cells disappear and de novo switching of B cells to IgE dissipates, a selected population of long-lived IgE-producing cells persists (Fig. 3A). The level at which the serum IgE plateaus is higher in mice that were exposed to HDM for 15 weeks relative to those exposed for 4 weeks, consistent with a progressive accumulation of long-lived IgE-producing cells over time.

To further characterize the persistence of these IgE PCs, we exposed double reporter mice to HDM for 15 weeks, then treated mice with a single dose of an anti-CD20 antibody or an isotype control antibody, and rested them for an additional 32 weeks (8 months) while bleeding them every 3 to 5 weeks to track serum IgE levels. Anti-CD20, but not isotype control treatment, led to B cell depletion, as confirmed by flow cytometry analysis of circulating B cells 1 week after antibody administration (Fig. 3C). However, because CD20 is not expressed on PCs, anti-CD20 should not affect PCs that are already established. Anti-CD20 treatment had negligible effect on serum IgE levels relative to untreated or isotype control-treated mice (Fig. 3D). This result suggests that at the end of the 15-week HDM treatment, the majority of IgE production comes from PCs and not from B cells and that the reduction observed in the initial weeks of rest is likely due to the loss of preexisting short-lived PCs, whereas the persistent production of IgE in the later time points is driven by long-lived PCs. Consistent with these findings, IgE PCs were present in the BM at comparable frequencies in anti-CD20-treated mice as compared with the control groups at the end of the 32-week rest period, demonstrating that, once established, this long-lived IgE population persists in the BM for a prolonged time (Fig. 3E and fig. S3D). Although anti-CD20-mediated depletion of tissue-resident GC B cells has been reported to be inefficient, recirculating B cells and memory B cells can be eliminated using this approach (34). In addition, at the end of the 32-week rest, no increase in IgE⁺ PCs was detected in the spleens of anti-CD20-treated mice relative to saline mice (fig. S4A), and GC B cells represented a comparable fraction of the B cell pool as compared with saline mice (fig. S4B), indicating that, at the time of harvest, there was no ongoing de novo production of IgE PCs in the spleens of anti-CD20-treated mice. We detected IgE⁺ PCs in the lungs of these mice, suggesting that mucosal sites of allergen exposure can potentially act as additional reservoirs of long-lived IgE⁺ PCs (fig. S4, C to E).

Sequential class switching from IgG1 plays a major role in the chronic IgE response

The findings discussed above demonstrate that IgE⁺ BMPCs arise in significant numbers after chronic HDM exposure, and differential expression of CXCR4 on these cells relative to IgE⁺ plasmablasts

suggests qualitative differences between IgE-producing cells generated during short allergen exposure relative to the IgE-producing cells that accumulate in the BM during chronic allergen exposure. To address potential mechanisms that might contribute to these differences, we focused on a unique feature of IgE⁺ B cells, namely, that they can either arise via a direct class switching pathway from IgM⁺ B cells or from sequential class switching of IgG1⁺ B cells (11).

To directly quantify the frequency of IgE-producing cells that were generated by direct versus sequential class switching during short versus chronic allergen exposure, we used a previously described strategy for measuring IgG1 switch junction remnants (Sy1 switch region sequences) within IgE-switched cells (35). Accordingly, DNA was amplified from pooled spleen of mIgE^{Venus} mice exposed to HDM for 4 weeks or from BM of mice exposed to HDM for 15 weeks using primers for IgM switch region (Sμ) to IgE switch region (Sε) (Fig. 4A). Because the DNA break during class switch recombination occurs at different sites, the first polymerase chain reaction (PCR) leads to multiple products of different sizes (Fig. 4A). The PCR product from both samples was then cloned, and individual colonies were sequenced (Fig. 4A and table S1). Inserts amplified from Sμ and Sε PCR were examined for the presence of switch γ1 remnant sequence by performing an alignment to the 49-base pair (bp) repeat sequence found in IgG1 switch regions (36). In the spleen of mice exposed to HDM for 4 weeks, 12 of the 78 clones (15.3%) contained Sy1 remnant sequences (Fig. 4A and table S1). In contrast, in the BM of mice exposed to HDM for 15 weeks, 53 of 74 IgE switch region colonies screened (71.6%) contained Sy1 remnant sequences, suggesting that most of the IgE⁺ PCs detected in the BM during chronic HDM exposure were generated via sequential class switching from IgG1⁺ cells. To confirm these observations from the alignment data, we amplified individual colonies using a second primer set specific to Sy1 and Sε (Fig. 4B). Sy1 to Sε PCR would amplify ≥1 product/s depending on the length of Sy1 remnant left within the IgE switch region (Fig. 4B, representative gel). Using this PCR strategy, we confirmed the presence of Sy1 remnant sequences in ~75% of IgE switch region colonies screened, consistent with the alignment data.

It has previously been shown that during antigen-independent IgE class switching or a single immunization with NP-KLH, IgE⁺ plasmablasts undergo limited GC reaction and primarily arise from direct class switching pathway (11), similar to what we see with 4 weeks of HDM exposure in spleen. We show here that during chronic allergen exposure, IgE⁺ PCs that migrate to the BM predominantly arise from sequential class switching of IgG1⁺ cells.

Serum from chronically HDM-exposed mice, but not from 4-week HDM-exposed mice, contains IgE that can induce systemic anaphylaxis

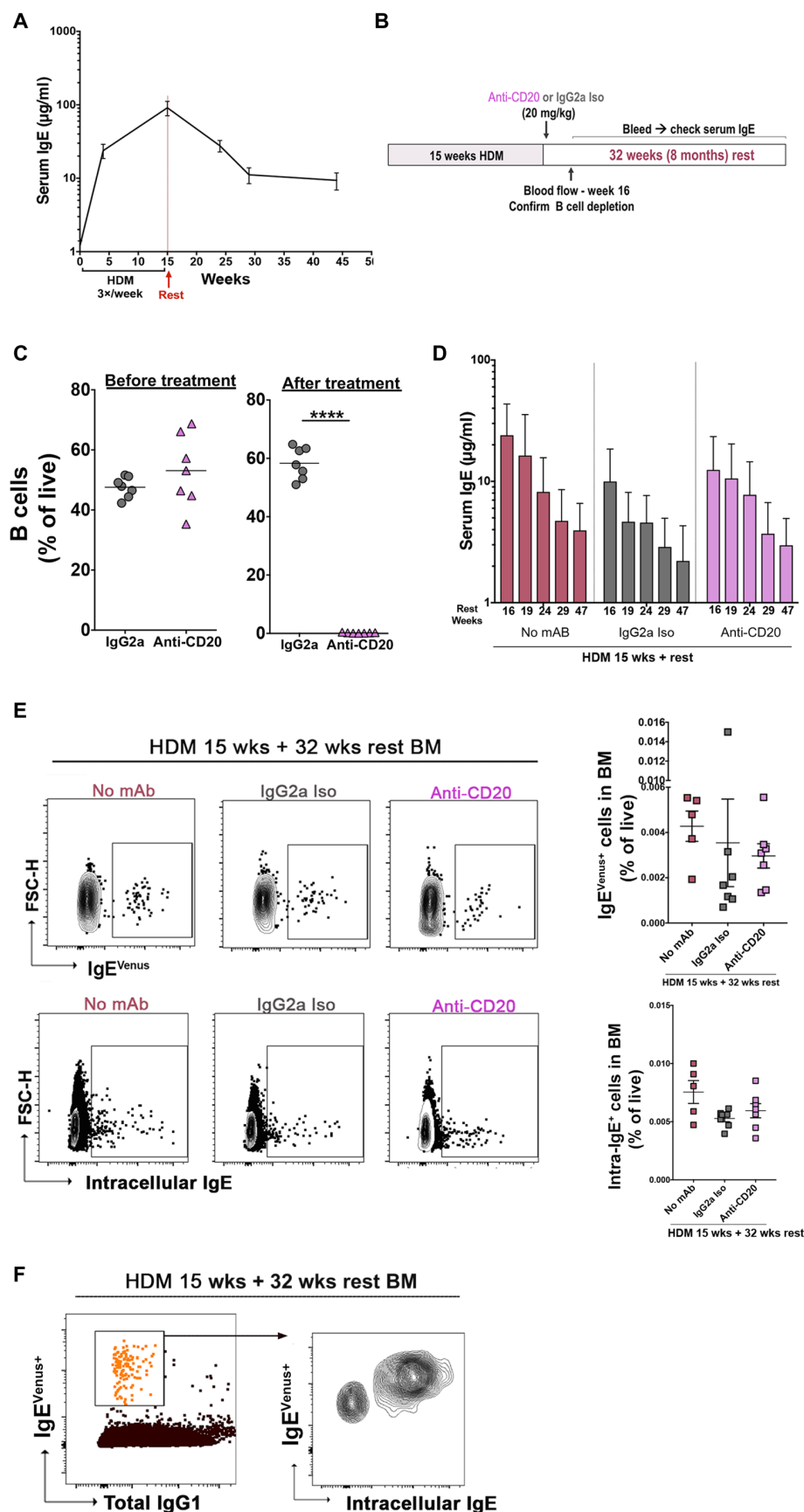
Previous studies have shown that IgE-switched B cells exit GCs prematurely and rapidly differentiate into PCs (9). As a consequence, somatic hypermutation and affinity maturation are blunted, resulting in lower-affinity antibody relative to IgGs that are retained in the GCs for extensive affinity maturation. It has therefore been proposed that the generation of a high-affinity IgE response necessitates an IgG intermediate that is capable of acquiring high affinity before IgE switching (11). Having defined that most of the IgE⁺ BMPCs in chronically allergen-exposed mice come from sequential class switching, we hypothesized that this developmental history might have an important impact on the specificity, affinity, or

Fig. 3. IgE⁺ BMPCs generated during chronic HDM exposure are maintained in the BM after 8 months of rest. (A) Serum IgE ELISA in mice exposed to HDM for 15 weeks and rested for 29 weeks. (B) Experimental setup for HDM exposure and rest $\pm$ anti-CD20 and IgG2a isotype (Iso) control monoclonal antibody (mAb) treatments. (C) Circulating B cell frequency before antibody treatment (left graph) and 7 days after antibody treatment (right graph) in mice exposed to HDM for 15 weeks and treated with anti-CD20 or IgG2a control monoclonal antibody. (D) Serum IgE levels at various time points over the course of a 32-week (8-month) rest period after 15 weeks of HDM exposure. (E) Representative plots of Venus⁺ cells in the BM after HDM exposure and 32-week rest $\pm$ anti-CD20 or IgG2a control monoclonal antibody treatment (top left) or intracellular IgE⁺ cells in the BM (bottom left) and quantification of membrane IgE^{Venus+} cells in the BM (top right) or intracellular IgE⁺ cells in the BM (bottom right). (F) Intracellular IgE staining within BM Venus⁺ cells. **** $P \leq 0.0001$.

overall pathogenicity of the IgE produced by these cells.

To determine the functional relevance and specificity of IgE produced by BMPCs, we used a passive systemic anaphylaxis (PSA) model, where naïve mice were sensitized systemically with serum from either 4- or 15-week HDM-exposed mice or serum from mice rested for 9 weeks after HDM exposure (Fig. 5A and fig. S5). Recipient mice were then challenged systemically with an intravenous injection of Der p 1, a dominant allergen in HDM extract (37), and temperature changes in the mice were measured as a readout of systemic anaphylaxis (38).

Initially, mice were sensitized systemically with serum containing HDM-specific IgE that was diluted twofold (Fig. 5A and fig. S5B). Mice that were sensitized with serum from 4 weeks HDM, or 4 weeks HDM + 9 weeks rest, showed a minor temperature drop upon Der p 1 challenge (Fig. 5A, left graph, and fig. S5B, left graph), similar to control (saline) mice. In contrast, mice sensitized with serum from 15 weeks of HDM exposure had symptoms of severe systemic anaphylaxis ($\sim 6^\circ$ to 8°C drop in core temperature) measured after 30 min of Der p 1 challenge (Fig. 5A, right graph, and fig. S5B, left graph). Similarly, mice that received serum from 15 weeks HDM + 9 weeks rest induced a comparable drop in core body temperature to mice sensitized with serum from 15-week exposed/unrested mice (Fig. 5A and fig. S5B). In addition, plasma histamine levels were significantly increased in these mice 30 min after challenge (Fig. 5B), confirming an ongoing anaphylactic response.



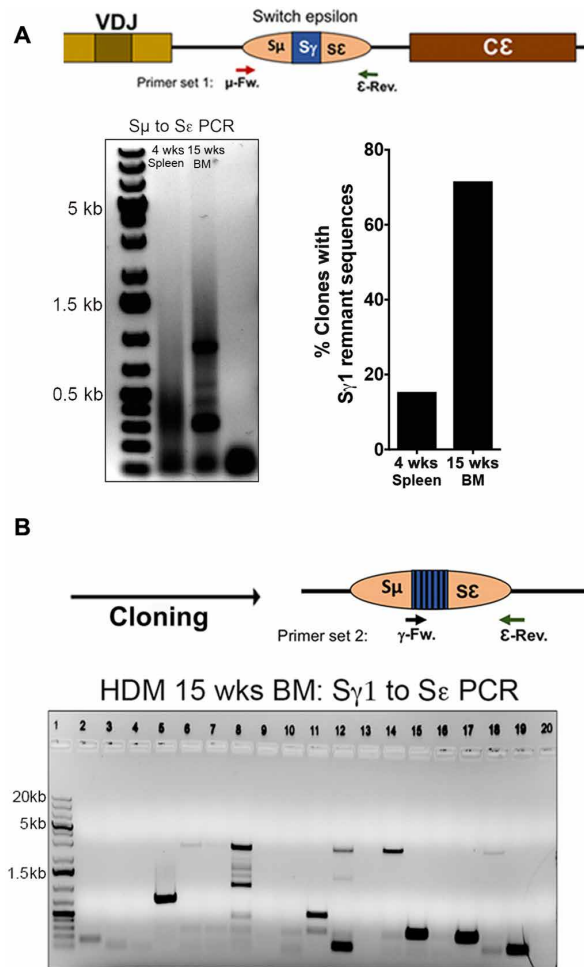


Fig. 4. IgE⁺ PCs that migrate to the BM are primarily derived from sequential class switching of IgG1⁺ cells. (A) Quantification of the percentage of sequential class switching (presence of IgG1 remnants) within IgE-switched cells in the spleen of mice exposed to HDM for 4 weeks or the BM of mice exposed to HDM for 15 weeks (refer to table S1). VDJ, variable-diversity-joining. (B) The presence of S γ 1 remnant sequence within IgE clones in the BM of 15-week HDM-exposed mice was confirmed by PCR using primers specific to S γ 1 repeat region and S ϵ . Image shows representative gel from one experiment. The presence of ≥ 1 band indicates that the IgE-switched clone contains IgG1 remnants and was derived from sequential class switching of an IgG1⁺ cell.

To determine whether the increase in systemic anaphylaxis after 15 weeks of HDM exposure was due to higher IgE concentration (~ 3 -fold higher IgE in 15 weeks HDM relative to 4 weeks HDM; fig. S5A), we normalized the total amount of IgE used to sensitize the mice to 500 ng across all sera that were injected. Consistent with the previous data, only serum derived from chronic HDM exposure (± 9 weeks rest) induced systemic anaphylaxis (fig. S5B, right graph). These data suggest qualitative changes to the IgE pool after chronic allergen exposure that enable IgE pathogenicity.

It has previously been shown that both IgE and IgG are capable of inducing systemic anaphylaxis in mice (39). Thus, to further confirm these qualitative differences between the IgE generated in short-term versus chronic HDM exposure, we used a model that is IgE and mast cell dependent, namely, the passive cutaneous anaphylaxis (PCA) mouse model. The PCA model assesses type 1 hypersensitivity and measures local IgE-mediated mast cell activation-induced vascular

permeability in tissue (40). In these studies, mice were passively sensitized intradermally in the ear with sera normalized to 25 ng of IgE and subsequently challenged with an intravenous injection of Der p 1 diluted in Evans blue dye. Vascular permeability induced by mast cell degranulation was then monitored by Evans blue leakage into ear tissue. Consistent with the PSA results, significant mast cell degranulation could only be induced when mice ears were sensitized with serum from 15 weeks of HDM exposure (Fig. 4C, left graph) or 15 weeks HDM + rest (Fig. 5C, right graph). These findings demonstrate that, in contrast to short allergen exposure, IgE produced during chronic HDM exposure is allergen specific and can drive local and systemic anaphylaxis.

IgE⁺ PCs can be detected in the BM of allergic patients

The presence of IgE BMPCs and their contribution to the progression of allergy in patients are currently not well understood (5). To characterize IgE PCs in human BM, we obtained BM aspirates along with matched serum samples from five allergic donors and two age-matched controls with no history of allergy. The allergen-specific IgE profile of the donors was determined by ImmunoCAP, an *in vitro* diagnostic assay that detects allergen-specific IgE in human samples, and showed that one donor was only allergic to cats; the second was allergic to cats, dogs, HDM, and mold; the third was allergic to cats, dogs, and mold; the fourth donor was only allergic to olive; and the fifth donor was allergic to olive, grass, and mold (fig. S6A). This test also confirmed that sera from the two nonallergic donors were not reactive to any allergens tested.

To reliably detect IgE-producing cells in human BM, we adopted a previously described method where, in addition to gating out irrelevant or contaminating cell types [T cells (CD3⁺), myeloid cells (CD11b⁺), basophils, and other Fc ϵ R1 α -expressing cells (Fc ϵ R1 α ⁺ and CD123⁺)], surface IgE was saturated with an unlabeled anti-IgE antibody, followed by intracellular IgE staining with the same antibody clone that is fluorescently labeled (9). Using this staining method, intracellular IgE⁺ cells were detected in the BM of all allergic individuals but not in nonallergic controls (Fig. 6A).

We further confirmed the PC phenotype of these cells by staining the cells with several PC markers. Compared with naïve B cells, human BMPCs expressed significantly higher levels of the defined PC markers BCMA (TNFRSF17), IL-6R, and CD27 on their surface (gating strategy in fig. S6B). In contrast, expression of IL-4R α and surface IgG was down-regulated on BMPCs compared with IgG B cells. The expression of all of these surface markers was comparable between allergic and nonallergic individuals (Fig. 6B).

When cultured *in vitro*, IgE⁺ PCs from the BM of the three cat-allergic donors produced detectable levels of IgE (Fig. 6C). IgE secretion was detectable by enzyme-linked immune absorbent spot (ELISpot) after 24 hours in culture (fig. S6C). This time frame is insufficient for *de novo* PC differentiation, thus confirming a pre-existing population in the BM that readily secretes IgE in culture. After 8 days in culture, the secreted IgE levels mirrored the donor-to-donor variability observed in the allergic donor sera (compare Fig. 6, C and D, left graphs), where cells from cat-allergic donor 2 produced the highest amount of IgE and cells from cat-allergic donor 1 produced the lowest. IgE was not detected in serum or BM supernatant of the two nonallergic donors, whereas total IgG levels were comparable across all donors (Fig. 6, C and D, right graphs), independent of allergy status. Moreover, ImmunoCAP scores of BM supernatants from the three allergic donors confirmed cat dander reactivity of

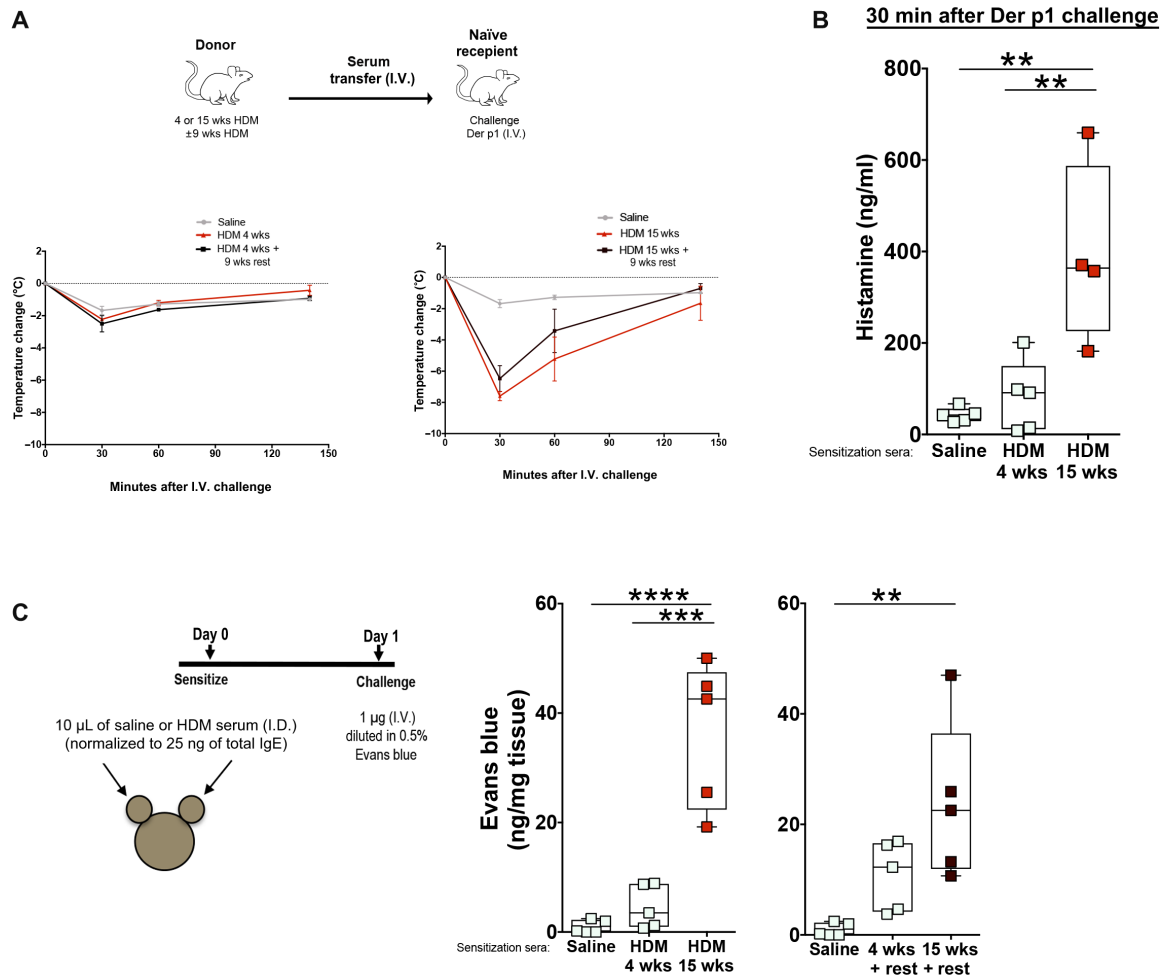


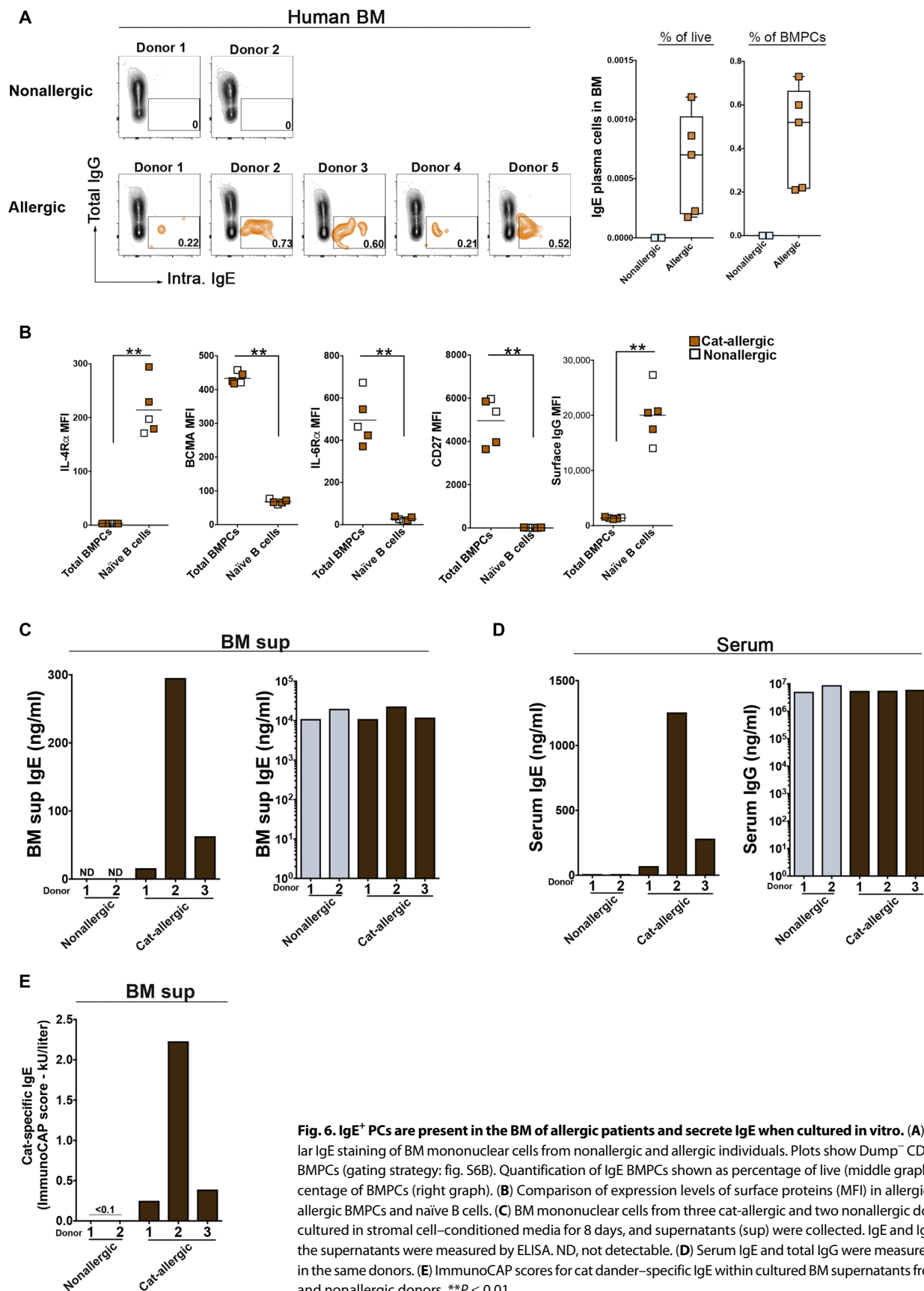
Fig. 5. IgE⁺ PCs secrete Der p 1-specific IgE that can induce mast cell degranulation and systemic anaphylaxis. (A) Groups of naïve mice received an intravenous (I.V.) injection of serum from mice that were exposed to saline, 4 weeks, or 15 weeks HDM ± rest allowing allergen-specific IgE to bind FcεRI-expressing cells systemically. After 24 hours, basal core temperature measurements were taken for all mice, followed by intravenous challenge with Der p 1 allergen. Core temperature change relative to basal temperature is shown as a readout for systemic anaphylaxis. (B) Histamine levels in the plasma of mice 30 min after Der p 1 challenge. (C) PCA (mast cell degranulation) was assayed by intradermal (I.D.) injection of the same sera as (A) from saline- or HDM-exposed mice. After 24 hours, the mice were challenged intravenously with Der p 1 diluted in 0.5% Evans blue dye. Evans blue dye was extracted from ear tissue and measured spectrophotometrically. Plot shows Evans blue dye extravasation in the tissue quantified as nanograms of Evans blue per milligram of tissue as a measure of local mast cell degranulation. ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

the IgE produced by these BMPCs (Fig. 6E). These data demonstrate that IgE-secreting PCs are present in the BM of allergic patients in frequencies that correlate with their serum IgE levels.

IgE derived from the BM of allergic patients can drive mast cell degranulation in FcεR1α humanized mice

One limitation to modeling the human IgE response in mice is that human IgE does not bind mouse FcεR1α, the high-affinity IgE receptor that mediates anaphylactic responses (41). To circumvent this species specificity issue, we generated FcεR1α humanized mice (*Fcer1a^{hu/hu}*), in which the full mouse *Fcer1a* coding region was replaced with human *FCER1A* coding sequence (Fig. 7A). In contrast to previously generated transgenic mice (42), these knock-in mice replace the entire mouse *Fcer1a* gene with human *FCER1A* and preserve regulatory elements that control expression levels (and, thus, thresholds of stimulation that will trigger anaphylaxis). The surface expression of human FcεR1α in these mice was confirmed on splenic basophils (Fig. 7B). In vivo local mast cell-driven anaphy-

laxis was also confirmed in these mice using the PCA model (Fig. 7C) in which groups of WT or *Fcer1a^{hu/hu}* mice received an intradermal injection in the ear with a cocktail of two Fel d 1 [major cat allergen (43)]-specific human IgE antibodies or an irrelevant IgG antibody (negative control) into the right and left ears, respectively (Fig. 7C). After 24 hours, the mice were challenged by intravenous injection of Fel d 1 diluted in Evans blue dye. Mast cell degranulation was observed in *Fcer1a^{hu/hu}* mice, but not in WT mice (Fig. 7C), demonstrating functionality of the mice and IgE: FcεR1α engagement and activation. Similar results were obtained using the PSA model (fig. S7A), further confirming successful humanization of FcεR1α. To further confirm that the PCA response was driven by IgE in serum, we performed a PCA challenge sensitizing with human cat-allergic donor serum in WT (non-FcεR1α humanized) mice, where human IgE would not be able to bind to mouse FcεR1α. No response was observed in WT mice when they were sensitized with the human sera, confirming that serum IgE is the driver of the PCA response in the FcεR1α humanized mice (fig. S7B).



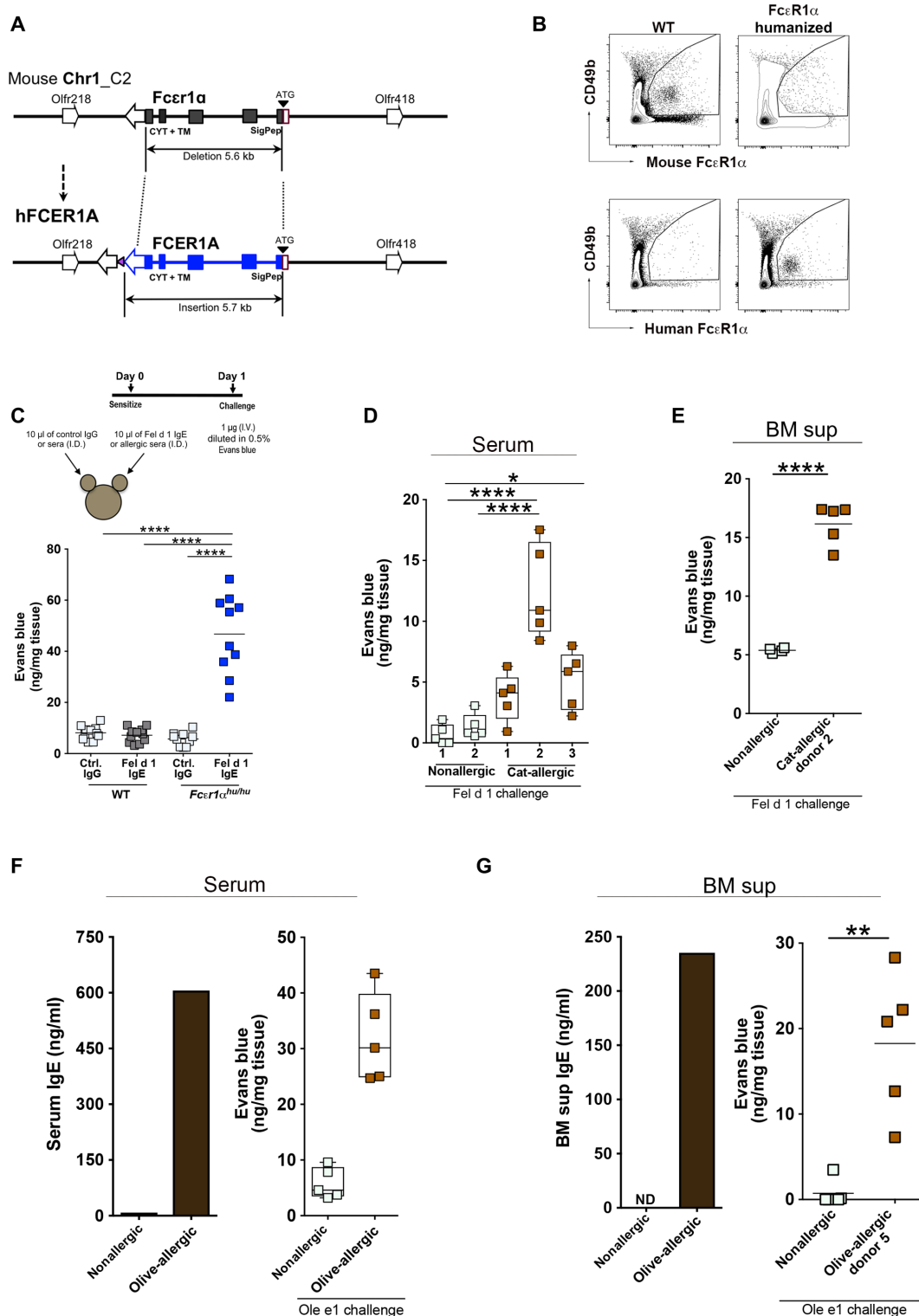


Fig. 7. IgE PCs in the BM of allergic patients secrete allergen-specific IgE that can drive mast cell degranulation in FcεR1α humanized mice. (A) Humanization strategy for the full coding sequence of FcεR1α. (B) Splens were harvested from WT or *FcεR1α*^{hu/hu} mice, and single cell suspensions were stained with antibodies for the basophil marker CD49b and either mouse (top plots) or human (bottom plots) FcεR1α. (C) PCA response of WT or *FcεR1α*^{hu/hu} mice sensitized with an intradermal injection with a cocktail of two allergen-specific human IgE antibodies or an irrelevant IgG antibody (negative control). (D to G) Ears of *FcεR1α*^{hu/hu} were sensitized by intradermal injection of sera from nonallergic or cat-allergic donors (D), BM supernatant from nonallergic or cat-allergic donors (E), sera from nonallergic or olive-allergic donors [(F), right graph], or BM supernatant from nonallergic or olive-allergic donors [(G), right graph]. Plots show Evans blue dye extravasation as nanograms of Evans blue per milligram of tissue. Left graphs on (F) and (G) show IgE levels in serum and BM supernatant in olive-allergic and nonallergic donors, respectively. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

Subsequently, serum from each of the three cat-allergic donors, as well as the two nonallergic controls, was individually used to sensitize ears of *Fcεr1a^{hu/hu}* mice in the PCA model. Upon challenge with Fel d 1, the ears that were sensitized with any of the three cat-allergic donor sera showed Evans blue dye extravasation (Fig. 7D), whereas no Evans blue extravasation was observed in the ears sensitized with either of the two nonallergic donor sera. Donor-to-donor variation was observed among the three donors, and the number of IgE⁺ BMPCs in the individual donors correlated with the levels of serum IgE and the magnitude of the PCA response (Figs. 6D and 7D). In particular, donor 2, which had the highest frequency of IgE cells in the BM (Fig. 6A), also showed the highest level of serum and BMPC-derived IgE (Fig. 6, C and D) and induced the greatest PCA response (Fig. 7D). This donor was also the most polyallergic, being allergic to cats, dogs, HDM, and mold.

To directly demonstrate that the IgE secreted by BMPCs in atopic patients is allergen specific, we performed a PCA assay using supernatant from in vitro cultures of the BM of cat-allergic donor 2 (Fig. 7E). Ears sensitized with supernatant from the allergic donor, and not the nonallergic control, showed robust mast cell degranulation (Fig. 7E). We further investigated whether IgE⁺ BMPCs from donors with different allergies besides cat also produced pathogenic IgE capable of inducing anaphylaxis. For this, we sensitized *FcεR1α* humanized mice with serum from an olive-allergic patient and challenged the mice with Ole e1, the primary allergen in olive pollen (44). Comparable with the data obtained with cat-allergic sera, IgE contained in the serum of the olive-allergic donor was also capable of driving mast cell degranulation in response to Ole e1 (Fig. 7F). Moreover, we also cultured the BM from this patient, and the IgE contained in the supernatant from this culture was capable of sensitizing mice and driving anaphylaxis in response to Ole e1 (Fig. 7G). Together, these findings directly demonstrate that human IgE⁺ PCs reside in the BM of atopic patients and secrete allergen-specific IgE of sufficient affinity to initiate an anaphylactic response.

DISCUSSION

In this study, we used HDM allergen exposure to compare the features of IgE-producing cells during a short-term (4 weeks) and long-term (15 weeks) allergic response. We demonstrate that short-term HDM exposure results in the generation of IgE⁺ B cells and PCs that mainly reside in secondary lymphoid organs (spleen) and produce IgE that is unable to induce robust mast cell degranulation in response to allergen. In contrast, long-term exposure to HDM leads to the generation of IgE⁺ PCs that primarily arise via sequential class switching of IgG1⁺ cells, express CXCR4 at levels similar to those in IgG1⁺ PCs, populate the BM, provide serological memory to the allergen, and produce allergen-specific IgE that can drive local and systemic anaphylaxis. We find that IgE⁺ PCs also reside in the lung and persist after B cell depletion and 8 months of rest, indicating that this is an additional reservoir of long-lived IgE⁺ PCs contributing to serological memory. The kinetics with which IgE-producing cells arise and persist in the lung and other mucosal sites of allergen exposure warrants further investigation.

The presence and relevance of long-lived IgE⁺ BMPCs in maintaining allergic memory have been debated in recent studies. After *Nippostrongylus brasiliensis* infection or NP-KLH challenge, IgE⁺ PCs were detected in lymph nodes transiently, followed by a marked

decline by day 17 after immunization (9). IgE⁺ PCs were undetectable in the BM at this time point, suggesting that IgE-switched cells predominantly differentiated into short-lived plasmablasts that mainly resided in secondary lymphoid organs (9). Their inability to reach the BM was attributed to their short half-life in secondary lymphoid organs, because NP-KLH challenge in *Eu-Bcl2-22* transgenic mice, a system that extends the life span of short-lived PCs by overexpression of the antiapoptotic protein B cell lymphoma 2, led to a ~20- to 30-fold increase in the frequency of IgE⁺ PCs in lymph nodes and a subsequent detection of these cells in the BM (9). In other studies, signaling downstream of the IgE BCR, which involves the Syk-BLNK-Jnk-p38 signaling pathway, was shown to induce apoptosis in IgE-switched cells and was implicated in shortening their life span within secondary lymphoid organs (7, 31). It is speculated that the combination of these restrictions, together with low expression of chemokine receptors required for BM homing [e.g., CXCR4 (33)], limits the ability of IgE⁺ PCs to migrate to the BM and become long-lived PCs that maintain serological memory (9, 30, 31, 33). Other reports found that IgE-producing cells can be detected in the BM after primary and secondary *N. brasiliensis* infection (8, 10), but these cells were not thoroughly characterized and their longevity in the BM or contribution to IgE serological memory was not addressed. IgE⁺ PCs have also been detected in the BM after ovalbumin (OVA) or peanut immunization, albeit in low numbers, and although they suggested that these BMPCs are capable of contributing to long-term production of serum IgE, they also proposed that the most relevant source of IgE memory lies predominantly in IgG1 memory B cells that can sequentially switch to IgE upon rechallenge (8, 45). Earlier literature, in contrast, has highlighted the importance of IgE serological memory in allergy models. One report showed the presence of X-irradiation-resistant IgE production after immunization with dinitrophenyl-KLH (DNP-KLH) or DNP-OVA (46). Persistent production of OVA-specific IgE after repeated low-dose inhalation of aerosolized OVA has also been reported, and this IgE was shown to be resistant to radiation and cytostatic drugs (14, 47).

It is challenging to compare different models that generate IgE⁺ PCs, because the amount of IgE⁺ PCs induced and the kinetics of IgE⁺ PC migration would likely vary with different allergens, routes of sensitization, and differences in the genetic background of the mice used. The discrepancy between the studies outlined above could also be explained by the primary readouts [e.g., enzyme-linked immunosorbent assay (ELISA) versus flow cytometry methods] used to quantify long-lived IgE⁺ PCs, as well as the length of the study to characterize these cells. In early studies that report persistent presence of long-lived IgE⁺ PCs in murine models, IgE production was tracked for ~1 year in serum of irradiated mice by ELISA (14), which would allow quantification of IgE secreted from the extremely low number of long-lived PCs that are present in different survival niches (e.g., BM, spleen, and lung). The number of IgE⁺ PCs with such models may not be sufficient to be detected by flow cytometry, which would explain how recent studies have not captured these cells using different IgE reporter systems. PCs can secrete ~10⁸ Ig molecules per cell per hour (48), suggesting that even at extremely low numbers, long-lived PCs would be capable of maintaining life-long IgE serological memory.

Our data provide evidence to propose a unifying model of how allergy memory arises with allergen exposure (fig. S8). Consistent with previous reports using short-term immunizations, we found that 4 weeks of HDM exposure predominantly generated IgE⁺ plasmablasts

that reside in secondary lymphoid organs, and when these mice were rested in the absence of allergen exposure, serum IgE was significantly reduced, likely indicating that the majority of this IgE comes from short-lived PCs. Nonetheless, we were still able to detect low levels of circulating serum IgE above basal levels in control mice, even after a 23-week rest phase. This suggests that a few long-lived IgE⁺ PCs are generated during short-term allergic inflammation, albeit at a much lower frequency compared with repeated/continuous exposure to the same allergen. In contrast, during a chronic response, the number of long-lived IgE⁺ PCs generated increases over time with persistent exposure to the allergen, allowing accumulation of these cells that become traceable in the BM. A recent report proposed that IgE⁺ PCs are preferentially displaced from the BM, with a half-life of ~8.5 weeks in the BM compared with ~33.5 weeks for IgG1⁺ PCs (45). However, this half-life calculation assumes a linear rate of decrease in both serum IgE and IgE⁺ BMPCs and does not reflect the fact that the decrease plateaus over time. We now report that after an initial decrease in serum IgE in the initial weeks of rest, the levels of serum IgE and of IgE⁺ BMPCs plateau and are preserved for at least 8 months and likely for the entire life span of the mouse. These data suggest that the initial decrease in serum IgE is due to the loss of short-lived IgE⁺ PCs, whereas some IgE⁺ clones are selected and retained in the BM indefinitely where they continue secreting IgE.

Although our data show a quantitative impact of long-term allergen exposure on the number of BMPCs that can sustain IgE production, we also find a remarkable change in the quality of the IgE generated during a chronic response. We find that, during chronic HDM exposure, IgE⁺ PCs primarily arise from sequential class switching of IgG1⁺ GC B cells and/or IgG1⁺ memory B cells. The consequences of this developmental history of IgE⁺ PCs are twofold: First, sequential switching may allow IgE⁺ PCs to retain some features of IgG1⁺ cells, such as surface markers or chemokine receptors (e.g., CXCR4), that increase their odds of homing to the BM. Consistent with this notion, previous reports have proposed that long-lived BMPCs are primarily derived from affinity-matured GC B cells (49). Second, unlike directly switched IgE⁺ B cells, which exit GCs prematurely and have limited capacity to undergo somatic hypermutation and increase affinity (8, 9, 11, 30, 50), IgG1⁺ B cells are capable of remaining in GCs and undergoing extensive affinity maturation, and memory IgG1⁺ B cells can further be recruited to the GC upon rechallenge to undergo additional rounds of affinity maturation (51). Sequential switching from IgG1 to IgE thus enables high-affinity IgG1⁺ clones to give rise to higher-affinity IgE⁺ clones (50), relative to those generated by direct switching from naïve IgM⁺ B cells. We show that IgE only generated after chronic allergen exposure, but not after a short-term exposure, is capable of driving local or systemic anaphylaxis. This difference in the quality of the IgE was evident even when mice were sensitized with equal amount of serum IgE derived from short- or long-term allergen-exposed mice (Fig. 5). We also find evidence of increased frequency of sequential class switching in the spleen of chronically HDM-exposed mice relative to mice with short-term HDM exposure. Together, these data suggest that during chronic allergen exposure, the pool of high-affinity IgG1⁺ intermediates that serve as precursors of IgE⁺ PCs gradually increases, thus enabling the production of higher-affinity IgE⁺ PCs and/or expansion of allergen-specific IgE⁺ clones. As a consequence, after chronic allergen exposure, most of IgE⁺ PCs come from IgG1⁺ intermediates, contrasting with models of antigen-independent B cell responses, short-term immunizations (one dose of NP-KLH), or

parasite infections, where most of the IgE-producing cells are generated via direct switching, undergo limited rounds of affinity maturation, and produce low-affinity IgE (11). Note that by 15 weeks of HDM exposure, the pattern of elevated cytokines found in circulation is different from that found at 4 weeks. In addition, extensive lung remodeling is observed at 15 weeks but not at 4 weeks (22). It is also therefore possible that differences in availability of cytokines and chemokines at 4 weeks versus 15 weeks of HDM exposure may play an important role in the ability of IgE⁺ PCs cells to develop, migrate to the BM, and persist there.

In humans, numerous clinical observations suggest the existence of long-lived IgE⁺ BMPCs in atopic patients. For example, immediate anaphylaxis can be induced by allergens or drugs (e.g., penicillin) years after the initial sensitization, and even in the absence of allergen reexposure, persistent allergen-specific IgE production is maintained in allergic patients (12–14). Accidental transfer of allergies and allergen-specific IgE production after BM transplants from atopic donors also suggests that IgE⁺ PCs exist in the BM of allergic patients (15, 16). Therapeutic agents that target IgE class switching, such as IL-4 and/or IL-13 blockade, are unable to lower serum IgE back to baseline (17–20). Quilizumab, an antibody that targets the membrane-proximal (M1') domain of human IgE, has been shown to efficiently target IgE⁺ B cells and short-lived PCs in patients but was unable to deplete IgE⁺ BMPCs (19). Unexpectedly, serum IgE in quilizumab-treated patients was only reduced by ~20 to 30%, suggesting that >70% of IgE is produced by long-lived IgE⁺ PCs (19).

One lingering question about modeling IgE responses in mice is how faithfully the findings represent what happens in humans. In this study, we demonstrate that IgE⁺ PCs reside in the BM of atopic donors, and we directly demonstrate that allergen-specific IgE can be produced by these cells. BMPCs from two donors with different allergic profiles produce IgE that can induce mast cell degranulation in FcεRI humanized mice in response to their respective allergens, a direct indication that IgE⁺ BMPCs contribute to IgE serological memory in atopic patients. Our ability to induce passive anaphylaxis in mice with IgE generated from either human BMPCs or from chronically HDM-exposed mice is indicative of the potential pathogenicity of these cells in both species.

Our findings highlight a population of IgE-producing cells that is highly relevant in the pathophysiology of allergic disorders and which, once generated, appears largely resistant to currently available targeted therapies for allergic disorders. Efforts to further characterize these cells will be necessary to devise strategies to target them in ways that could improve on currently available therapies.

MATERIALS AND METHODS

Study design

To study the IgE response in vivo, we used an HDM-driven lung inflammation model in WT and membrane IgE^{venus}/Blimp-1^{mCherry} single or dual reporter mice. Cellular responses were tracked by harvesting lymphoid tissues from the mice at the end of each experiment and analyzing them by flow cytometry. Molecular readouts were assessed using prepared RNA from harvested cells. Serum readouts, such as IgE or IgG1 levels, were assessed using ELISA. Pathogenicity of serum IgE derived from short-term and chronic HDM-exposed mice was determined by PCA and PSA assays. The human IgE response was also examined ex vivo using BM samples from allergic and nonallergic donors. Human IgE-producing BMPCs were quantified

using intracellular IgE staining, secretion of IgE was determined by ELISA from cultured BM supernatants, and the capacity of the IgE to induce anaphylaxis was determined by PCA. Statistical significance was calculated using GraphPad Prism.

Animals

All procedures were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee of Regeneron Pharmaceuticals. All mice used in this study were generated in a hybrid 129S6/C57BL/6 background. Previously described strategies were used to generate IgE^{Venus} (9) and Blimp-1^{mCherry} (28) reporter mice. Briefly, the coding sequence of Venus (yellow fluorescent reporter) was inserted downstream of the last membrane IgE exon (M2), linked by the ribosomal skipping porcine teschovirus-1 (P2A) (52) sequence to allow simultaneous expression of Venus with membrane IgE. Both endogenous membrane IgE polyadenylation sites were left intact. The coding sequence of mCherry was inserted at the 3' end of exon 7 in *Prdm-1* (Blimp-1) gene linked by P2A. Self-deleting technology was used to remove the hygromycin or neomycin cassettes before phenotypic analysis of both reporters. VelociGene and VelociMouse methods (53–56) were used to generate heterozygous (57) reporter mice. Blimp1^{mCherry} F0 mice (50% B6/50% 129) were crossed to C57BL/6 mice to obtain F1 heterozygotes (75% B6/25% 129); IgE^{Venus} F0 mice (50% B6/50% 129) were crossed to C57BL/6 mice to obtain F1 heterozygotes (75% B6/25% 129). Double reporter mice were generated by crossing F1 generations of IgE^{Venus} heterozygous and Blimp-1^{mCherry} heterozygous mice. All experiments were performed using IgE^{Venus} homozygous and Blimp-1^{mCherry} heterozygous mice unless otherwise indicated. For FcεRIα humanization, the mouse *Fcer1a* locus, located on mouse chromosome 1, was humanized by construction of unique targeting vectors from human and mouse bacterial artificial chromosomes DNA using VelociGene technology (53–56).

In some experiments (Figs. 1, A and B, and 3A and figs. S1 and S3C), mice with IL-33 humanized locus (*Il33*^{hu/hu}) were used. The IgE response in this background is comparable with WT (hybrid 129S6/C57BL/6) mice (23), and experiments performed in this background have been repeated in WT or in IgE^{Venus}/Blimp-1^{mCherry} reporter mice for consistency.

Four- and 15-week HDM-induced allergic inflammation model

Mice were exposed to 50 µg of HDM extract (Greer) diluted in 20 µl of saline solution intranasally three times per week for either 4 or 15 weeks. Saline (20 µl) was administered intranasally in control mice. For rest experiments, the dose of HDM was lowered to 25 µg. At the end of the experiment, blood was collected for determination of serum concentrations of total IgE and HDM-specific IgG1, and spleen and BM were collected for flow cytometry.

Cell preparation and flow cytometry

Spleens were collected and mashed on 12-well, 70-µm filter plates (Corning Costar) in RPMI 1640 media to generate single cell suspensions. For BM extraction, femurs were cut at both ends, placed in a PCR plate with holes punched at the bottom, and spun down for 3 min at 500g. RBC lysis was performed on single cell suspensions from spleen and BM, and samples were labeled with LIVE/DEAD solution (Thermo Fisher Scientific) for 10 min in the dark at room temperature (RT). Cells were then blocked using Fc Block (Tongo Biosciences) for 15 to 30 min at 4°C, followed by incubation with a primary (surface) antibody mix for 30 min at 4°C in Brilliant Stain Buffer (BD Biosciences). Samples were washed,

fixed (BD Cytotfix; 1:4 diluted), and run on autoMACS running buffer (Miltenyi Biotech). For intracellular staining, samples were fixed and permeabilized (BD Cytotfix/Cytoperm and BD Perm/Wash buffer) and resuspended in intracellular mix for 30 min at 4°C in the dark.

RNA isolation, sequencing, and analysis

Single cell suspensions from BM were prepared from two to five mice in each group, and samples were pooled to generate enough cells for sequencing. Cells were stained with LIVE/DEAD dye, blocked with Fc Block, and stained with Ly6G, TCRβ, CD11b, CD49b, and CD117 (see antibody table) for dump gating and CD138 for PC gating. In Blimp-1^{mCherry} reporter mice, PCs were sorted on the basis of Blimp-1^{mCherry} expression on MoFlo Astrios (Beckman Coulter). All protocols for RNA extraction and sequencing library preparation were similar to those described previously (58). IgE and other Ig transcripts were mapped to mouse reference genome B38, with GENCODE V19.

Measurement of serum IgE

Whole blood was collected into Microtainer SST serum tubes and pelleted by centrifuging at 15,000g for 10 min at 4°C. For mice, serum samples were used to determine total IgE concentrations by IgE sandwich ELISA OptEIA kit (BD Biosciences) according to the manufacturer's instructions. For human IgE ELISA, serum and BM supernatant were used to measure total IgE concentration using Human IgE ELISA^{PRO} kit (Mabtech). Data analysis was performed using GraphPad Prism (GraphPad Software).

IgE ELISpot

IgE ELISpots on human BM cells were performed following the manufacturer's instructions (Mabtech) with few modifications. Briefly, ELISpot plates (Millipore) were coated with capture antibody (15 µg/ml) in phosphate-buffered saline overnight. Plates were washed and blocked with media containing 10% fetal bovine serum. BM samples were added to each well (500 K per well), and plates were incubated at 37°C overnight. Plates were washed, and IgE detection antibody (1 µg/ml) was added for 2 hours at RT. Plates were washed and incubated with Streptavidin-alkaline phosphatase (ALP) for 1 hour. Spots were developed by adding 100 µl of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) plus substrate (Mabtech).

Human BM and serum tests

Human cat-allergic and nonallergic donor BM mononuclear cells and matching donor sera were obtained from HemaCare (Los Angeles, CA). Allergy status of each donor sera was checked by ImmunoCAP rapid test (Phadia, Thermo Fisher Scientific) following the manufacturer's instructions. Serum and BM supernatant samples were also sent to Phadia Immunology Reference Laboratory (Portage, MI) for ImmunoCAP analysis to determine the concentration of cat-specific IgE levels.

For culturing human BM mononuclear cells in vitro, frozen samples were thawed at 37°C for 3 min and washed in MarrowMAX BM media (Thermo Fisher Scientific). Cells were incubated in media that contains deoxyribonuclease I (0.1 mg/ml) (Roche) for 15 min at RT and washed twice. Samples were resuspended in MarrowMAX media and plated in six-well plates for 8 days.

Passive cutaneous anaphylaxis

PCA was performed as described previously (59). For sensitization, ears of naïve mice received 10-µl intradermal injection of sera derived

from either HDM-exposed mice or saline-exposed controls. All sera were normalized to 25 ng of total IgE/10 μ l intradermal sensitization. After 24 hours, the mice were challenged by intravenous injection of 1 μ g of Der p 1 (Indoor Biotechnologies) diluted in 0.5% Evans blue dye (Sigma-Aldrich). One hour after allergen challenge, mice were euthanized, and Evans blue dye was extracted from ear tissue and spectrophotometrically quantitated using a standard curve (59).

For validation of Fc ϵ R1 α humanized mice, groups of naïve Fc ϵ R1 $\alpha^{hu/hu}$ mice were sensitized with a cocktail of two allergen-specific human IgE antibodies or an irrelevant IgG antibody (negative control) into the right and left ears, respectively. For PCA using human sera and BM supernatants, 10 μ l of neat sera or concentrated BM supernatants were used to sensitize ears of naïve Fc ϵ R1 $\alpha^{hu/hu}$ mice.

PSA and histamine ELISA

For PSA sensitization, mice received an intravenous injection of sera derived from either HDM-exposed mice or saline-exposed controls (IgE concentration/dilution for each experiment indicated in figure captions). Fc ϵ R1 $\alpha^{hu/hu}$ mice were sensitized with a cocktail of two allergen-specific human IgE antibodies (0.5 μ g total) or an irrelevant IgG antibody (5 μ g total). After 24 hours, basal core temperature measurements were taken for all mice, followed by intravenous injection of 1 μ g of Fel d 1. Core temperature measurements were taken at the indicated time points after the allergen challenge and graphed as changes in core temperature at each time point relative to basal temperature. Histamine ELISA was performed on plasma of mice following the manufacturer's protocol (Immuno-Biological Laboratories)

Switch region junction cloning

Single cell suspensions from BM or spleen were prepared from five to seven mice, and samples were pooled to analyze IgE switch junction sequences. S μ -S ϵ junction sequences were amplified as described previously (34). Briefly, DNA from each sample was prepared using DNeasy kit (QIAGEN) following the manufacturer's protocol. S μ -S ϵ PCR was set up using Advantage 2 PCR (Takara) with the following primers: S μ forward primer, ACTCAGTCAGTCAGTGGCGT-GAAGGGCT; S ϵ reverse primer, CATCAGGCTTTGCTCACTCA. Amplification was performed at 95°C for 1 min, 35 cycles of 95°C for 30 s, 68°C for 4 min, and a final cycle of 68°C for 4 min. PCR products were checked on a 1% agarose gel, purified, and cloned into pGEM-T cloning vector (Promega) following the manufacturer's instructions. The ligation products were transformed into TOP10F' competent cells (Invitrogen) for blue/white selection. Ninety-five white colonies were selected for each group for sequencing with T7 forward and M13 reverse primers. Sequenced inserts that contained both S μ -S ϵ sequences were analyzed, and the selected clone sequences were aligned to the 49-bp Sy1 switch region repeat using EMBOSS pairwise sequence alignment. Clones with alignment score above 50 were marked positive for Sy1 remnant.

Statistical analysis

Statistical and graphical analyses were performed using GraphPad Prism software (version 7.0). Normality was determined by Shapiro-Wilk normality test. One-way analysis of variance (ANOVA) or unpaired Student's *t* test was used on normally distributed samples, and Mann-Whitney or Kruskal-Wallis tests were performed on samples that did not pass the normality test. Two-way ANOVA was used on experiments that had two independent variables. Results were considered statistically significant at *P* < 0.05.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/5/43/eaav8402/DC1

Fig. S1. Chronic HDM exposure induces PC accumulation in the BM.

Fig. S2. Generation and characterization of IgE^{Venus}/Blimp-1^{mCherry} reporter mice.

Fig. S3. IgE persists in HDM-exposed mice during rest.

Fig. S4. IgE⁺ PCs are detected in the lung but not the spleen of mice exposed to HDM for 15 weeks and rested for 8 months.

Fig. S5. IgE generated after chronic HDM exposure can drive systemic anaphylaxis.

Fig. S6. Characterization of allergic and nonallergic donors.

Fig. S7. Validation of Fc ϵ R1 α humanized mice.

Fig. S8. Summary model.

Table S1. Sy1 switch region sequence alignment of IgE⁺ clones from spleen of 4-week HDM-exposed mice and BM of 15-week HDM-exposed mice.

Table S2. Mouse antibodies used in the study.

Table S3. Human antibodies used in the study.

Data file S1. Raw data in Excel spreadsheet.

[View/request a protocol for this paper from Bio-protocol.](#)

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Gene Expression Omnibus under accession number GSE140435. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The reporter and humanized mouse strains described in this manuscript are available from Regeneron through a material transfer agreement; requests to obtain these mice may be submitted through Regeneron's web portal: https://regeneron.envisionpharma.com/vt_regeneron/.

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Chronic allergen exposure drives accumulation of long-lived IgE plasma cells in the bone marrow, giving rise to serological memory

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Locating the reservoir for IgE memory

Allergic diseases persist when the immune system chronically churns out allergen-specific IgE antibodies. Identifying the tissue location of IgE⁺ memory plasma cells is complicated by their very low frequency. Asrat *et al.* tracked IgE⁺ memory plasma cell development after intranasal exposure of mice to house dust mite allergen using fluorescent reporter transgenes to mark IgE⁺ plasma cells. In mice repeatedly exposed to allergen for 15 weeks, long-lived IgE⁺ memory plasma cells emerged in the bone marrow. IgE⁺ memory plasma cells in the bone marrow of both mice and allergic human patients yielded pathogenic IgE antibodies capable of eliciting anaphylaxis after transfer. Identification of long-lived plasma cells as the chief source of IgE memory may assist in developing new therapeutic approaches for chronic allergic diseases.

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