

Human IgE⁺ B cells are derived from T cell-dependent and T cell-independent pathways

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Background: The prevalence of IgE-mediated diseases has been increasing worldwide, yet IgE-expressing B cells are poorly characterized, mainly because of their scarcity and low membrane IgE levels.

Objective: We sought to study the immunobiology of human IgE-expressing B cells in healthy subjects and patients with allergic disease.

Methods: We used a stepwise approach for flow cytometric detection and purification of human IgE-expressing B cells in control subjects, CD40 ligand-deficient patients, and patients with atopic dermatitis. Molecular analysis of replication histories, somatic hypermutation (SHM), and immunoglobulin class-switching was performed.

Results: Using multicolor flow cytometry, we reliably detected IgE-expressing plasma cells and 2 IgE-expressing memory B-cell subsets. These IgE-expressing cells showed molecular and phenotypic signs of antigen responses. The replication history and SHM levels of IgE⁺ plasma cells and CD27⁺IgE⁺ memory B cells fitted with a germinal center (GC)-dependent pathway, often through an IgG intermediate, as evidenced from S_μ remnants in S_μ-Se switch regions. CD27⁺IgE⁺ cells showed limited proliferation and SHM and were present in CD40 ligand-deficient patients, indicating a GC-independent origin. Patients with atopic dermatitis had normal numbers of blood IgE⁺ plasma cells and CD27⁺IgE⁺ memory B cells but increased numbers of CD27⁺IgE⁺ memory B cells with high

SHM loads compared with those seen in healthy control subjects and patients with psoriasis.

Conclusions: We delineated GC-dependent and GC-independent IgE⁺ B-cell responses in healthy subjects and indicated involvement of the GC-independent pathway in a human IgE-mediated disease. These findings provide new insights into the pathogenesis of IgE-mediated diseases and might contribute to accurate monitoring of IgE⁺ B cells in patients with severe disease undergoing anti-IgE treatment. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: B cell, IgE, CD40 ligand, atopic dermatitis, plasma cell

Allergic disease incidence has increased over the past decades in most Western countries.¹ It has been estimated that approximately 8% of adults in the United States have asthma² and that 30% to 40% of the world population is affected by 1 or more allergies.³ Allergic responses are mediated by an expansion of T_H2 cells and isotype switching of B cells to generate allergen-specific IgE.

Allergic patients have IgE with specificities toward harmless food-borne or airborne particles (allergens) that underlie the disease.⁴ Despite the crucial role of IgE, little is known about the cellular aspects of IgE⁺ B-cell responses.⁵ The 3 main reasons for this are as follows: (1) the presumed scarcity of IgE-expressing B cells,⁶ (2) the poor expression of membrane IgE caused by suboptimal polyadenylation signals downstream of the exons encoding the cytoplasmic tail of IgE,⁷ and (3) the presence of the low-affinity receptor FcεRII (CD23) on B cells, which binds IgE and results in detection of IgE on non-IgE-producing B cells.⁸

Class-switching toward IgE is primarily thought to take place in lymphoid germinal centers (GCs), where it is mediated by IL-4, IL-13, IL-21, and CD40 ligand (CD40L) on activated T_H2 cells.⁹⁻¹² In addition, costimulatory signals can be provided by the interaction between CD23 and CD21 on B cells, as well as signaling through CD86.^{13,14} In addition, the respiratory mucosa and gastrointestinal tract also show molecular signs of IgE class-switching.¹⁵ This is reminiscent of mucosal IgA responses that can take place independently of cognate T-cell help, possibly against nonpeptide antigens.^{16,17}

In recent studies with mouse models, IgE-producing plasma cells in lymph nodes were found to be derived from GC reactions. These cells were mostly short-lived, and their participation in GCs seemed transient. Long-term memory was generated in the form of post-GC IgG₁⁺ cells that switched to IgE on subsequent antigen encounter.^{18,19} Direct IgM→IgE class-switching was found as well,^{20,21} but the generation of high-affinity IgE

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Abbreviations used

CD40L: CD40 ligand
 CDR: Complementarity determining region
 CSR: Class-switch recombination
 GC: Germinal center
 R/S: Replacement/silent mutation
 SHM: Somatic hypermutation
 TACI: Transmembrane activator and CAML interactor

antibodies depended on indirect $\text{IgM} \rightarrow \text{IgG}_1 \rightarrow \text{IgE}$ switching.²² IgE^+ memory B cells have only been observed by Talay et al,²³ which is potentially due to the manipulated IgE allele.²⁴

Because of continuous antigenic pressure, human lymph nodes have persistent GCs, and large numbers of memory B cells can be found in blood. These features limit direct extrapolation from (manipulated) mouse models to human disease. We have recently delineated the human memory B-cell compartment using direct *ex vivo* analysis of immunoglobulin genes in sorted B-cell subsets.²⁵ The immunoglobulin genes are targets of unique processes that leave permanent marks in genomic DNA: V(D)J recombination for immunoglobulin repertoire development, somatic hypermutation (SHM) for affinity maturation, and immunoglobulin class-switch recombination (CSR) for effector function. $\text{CD27}^- \text{IgA}^+$ B cells and $\text{CD27}^+ \text{IgM}^+ \text{IgD}^+$ natural effector B cells were derived, at least in part, from local and systemic GC-independent responses, respectively; $\text{CD27}^- \text{IgG}^+$ and $\text{CD27}^+ \text{IgM}^+ \text{IgD}^-$ “IgM-only” B cells were derived from primary GC responses, and $\text{CD27}^+ \text{IgG}^+$ and $\text{CD27}^+ \text{IgA}^+$ B cells (at least in part) were derived from consecutive GC responses.

We have now developed an 8-color flow cytometry-based strategy to reliably detect IgE^+ plasma cells and $\text{CD27}^- \text{IgE}^+$ and $\text{CD27}^+ \text{IgE}^+$ memory B-cell subsets after stepwise exclusion of IgM^+ , IgD^+ , IgG^+ , and IgA^+ B cells. These IgE^+ B cells lacked CD23 expression and carried IgE transcripts. We used molecular analysis of their replication history, SHM, and CSR profiles and studies in CD40L-deficient patients and patients with atopic dermatitis to reveal new insights into their maturation pathways and involvement in allergic disease.

METHODS**Patients**

Peripheral blood samples from patients and healthy donors and childhood tonsils were obtained with informed consent and according to the guidelines of the Medical Ethics Committee of Erasmus MC.

All CD40L-deficient patients lacked CD40L protein expression on activated T cells, as measured after a 5-hour stimulation with phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, Mo) and calcium ionophore (Sigma-Aldrich), and carried hemizygous mutations in their *CD40L* gene (see Table E1 in this article's Online Repository at www.jacionline.org).

Patients with atopic dermatitis and psoriasis vulgaris were selected from the outpatient clinic of Dermatology and Venereology at Erasmus MC in Rotterdam (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org). The diagnosis of psoriasis vulgaris was made by certified dermatologists based on results of clinical examination only. Patients with atopic dermatitis fulfilled the criteria of Hanifin and Rajka.²⁶ Disease severity was scored for both groups by the attending physician. Total serum IgE levels and levels of IgE against common inhalant allergens (Phadiatop) were measured with ImmunoCAP 250 (Phadia, Uppsala, Sweden), according to the manufacturer's manual.

Flow cytometric immunophenotyping and cell sorting

Absolute counts of blood CD3^+ T cells, $\text{CD16}^+/\text{56}^+$ natural killer cells, and CD19^+ B cells were obtained with a diagnostic lyse-no-wash protocol. Eight-color flow cytometry of IgE^+ B cells from peripheral blood and from tonsils was performed by using the mAbs listed in Table E4 in this article's Online Repository at www.jacionline.org. Intracellular staining was performed on fixation and permeabilization with the FIX&PERM Kit (An der Grub, Wien, Austria).

$\text{CD19}^+ \text{CD38}^{\text{dim}} \text{CD27}^+ \text{IgD}^- \text{IgM}^- \text{IgE}^+$ and $\text{CD19}^+ \text{CD38}^{\text{dim}} \text{CD27}^- \text{IgD}^- \text{IgM}^- \text{IgE}^+$ cells were purified (>95% purity) on a FACSria cell sorter (BD Biosciences, San Jose, Calif) after AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) enrichment of CD19^+ B cells from post-Ficoll mononuclear cells of healthy donors.^{25,27}

Molecular analysis of replication history and immunoglobulin gene rearrangements

DNA was isolated from each sorted B-cell subset with a mammalian DNA Miniprep Kit (Sigma-Aldrich) to assess replication history with the kappa-deleting recombination excision circle assay, and the frequency of mutated *IGK* alleles was determined with the *Igk* restriction enzyme hot-spot mutation assay.²⁷ RNA from $\text{CD19}^+ \text{CD38}^{\text{dim}} \text{CD27}^+ \text{IgD}^- \text{IgM}^-$ and $\text{CD19}^+ \text{CD38}^{\text{dim}} \text{CD27}^- \text{IgD}^- \text{IgM}^-$ B cells and total plasma cells was isolated by using a mammalian RNA Miniprep kit (Sigma-Aldrich) and reverse transcribed with random hexamers. Complete *IGH* gene rearrangements were amplified in a seminested approach with previously described L-VH forward primers²⁸ and 2 different *IGH*E primers in the first (5'-CATCACCG GCTCCGGAAGTAG-3') and second (5'-GTTTTCGACGAGCGGGT CAAG-3') reactions or in a 1-step PCR with either *IGH*M, *IGH*A, or *IGH*G consensus reverse primers.²⁵ PCR products were cloned into the pGEM T-easy vector (Promega, Madison, Wis) and sequenced on an ABI Prism 3130XL (Applied Biosystems, Foster City, Calif). Obtained sequences were analyzed with the IMGT database (www.imgt.org)²⁹ and the JoinSolver program.³⁰

Immunoglobulin switch region analysis

Hybrid μ -Se regions were amplified from genomic DNA in a nested long-range PCR by using 2 μ -Se primer sets for the first and second reactions.^{31,32} PCR products were sequenced on the ABI Prism 3130XL, and the results were aligned with the *IGH* reference sequence (NG_001019).

In vitro plasma cell differentiation

Naive mature, IgG^+ , and IgE^+ B-cell subsets were purified, and 10,000 to 25,000 cells of each subset were cultured in 100 μL of Iscove modified Dulbecco medium supplemented with 10% FCS, 10 $\mu\text{g}/\text{mL}$ anti-CD40 (Bioceros B.V.), and 50 ng/mL IL-21 (PeproTech, Rocky Hill, NJ).³³ Plasma cell maturation was assessed with flow cytometry after 6 days.

Statistical analyses

Statistical analyses were performed with the Mann-Whitney *U* test, paired *t* test, or χ^2 test, as indicated in detail in the table and figure legends. *P* values of less than .05 were considered statistically significant.

RESULTS **IgE^+ plasma cells**

The identification of IgE^+ B cells by using flow cytometry has been controversial because of the scarcity of these cells, the low IgE expression levels, and the presence of cells that have captured IgE with their $\text{Fc}\epsilon$ receptors.^{6,7} We acknowledged these difficulties by designing a novel 8-color flow cytometric approach to detect IgE^+ B cells in tonsils and in blood (Figs 1 and 2). We

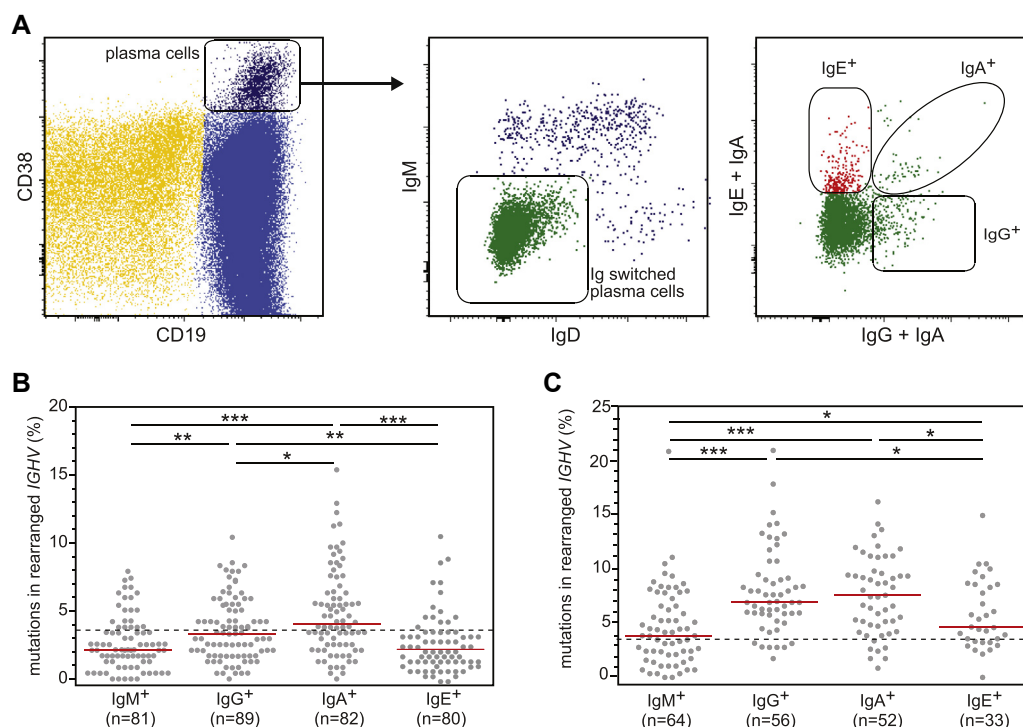


FIG 1. Detection of IgE⁺ plasma cells in tonsils and peripheral blood. **A**, Representative images from flow cytometric analysis of plasma cell subsets in childhood tonsils. IgM⁺IgD⁺ cells were gated within the total CD19⁺CD38^{high} plasma cell fraction and analyzed for surface IgG, IgA, and IgE expression. **B** and **C**, SHM frequencies in rearranged *IGHV* genes in *IGHM*, *IGHG*, *IGHA*, and *IGHE* transcripts from childhood tonsils (Fig 1, B) and adult blood (Fig 1, C). Each dot represents an individual sequence, red lines represent median values, and the dashed line indicates the median SHM frequency in centrocytes. Data were statistically analyzed with the Mann-Whitney test. **P* < .05, ***P* < .01, and ****P* < .001.

identified plasma cells based on the CD19⁺CD38^{high} phenotype. Subsequently, we selected IgM⁺IgD⁺ cells within the plasma cell gate and analyzed IgE, IgA, and IgG expression on these cells (Fig 1, A, and see Fig E1 in this article's Online Repository at www.jacionline.org). In childhood tonsils CD19⁺CD38^{high} plasma cells constituted approximately 4% of total CD19⁺ cells. These plasma cells were mostly CD138⁺, of which 66% coexpressed IgM and IgD. Approximately 30% of the total plasma cells were surface immunoglobulin negative but expressed cytoplasmic IgG (not shown). The remaining plasma cells either expressed IgG (2.6%), IgA (1.2%), or IgE (1.6%) on their membranes.

Sequencing of *IGHE* transcripts from plasma cells yielded diverse clones that nearly all contained SHMs in their *IGHV* regions (Fig 1, B and C). In both childhood tonsils and adult blood, the SHM levels were similar to those in IgM⁺ plasma cells and significantly lower than those in IgA⁺ and IgG⁺ plasma cells. Nonetheless, targeting of SHM to hypermutable motifs and selection against replacement mutations in complementarity determining regions (CDRs) were similar in all plasma cell subsets (data not shown).

Two IgE⁺ memory B-cell subsets in blood

Subsequently, we analyzed the blood of healthy adult donors, who carry large numbers of circulating memory B cells.²⁵ Within the CD19⁺CD38^{dim}IgM⁺IgD⁺ class-switched memory B cells, we detected 2 subsets of IgE⁺ B cells with differential

expression of CD27: CD27⁺IgE⁺ and CD27[−]IgE⁺ B cells (Fig 2, A).

In young children (<6 years old) CD27⁺IgE⁺ cells were scarce, with a median frequency of 0.03% of total CD19⁺ B cells (Fig 2, B). This frequency increased with age and reached 0.1% of total B cells in adults aged 31 to 40 years. In contrast, CD27[−]IgE⁺ cells were more abundant in children than in adults, with the highest frequency of 0.12% of total B cells in children aged 6 to 15 years. Thus 2 subsets of IgE⁺ B cells can be reproducibly detected in blood of healthy subjects and show distinct kinetics with age.

IgE expression seemed dim on memory B cells, which could be due to suboptimal polyadenylation signals in transcripts encoding membrane IgE.⁷ To study whether B-cell receptor levels were indeed low, we determined expression levels of the B-cell antigen receptor complex member CD79b on all memory B-cell subsets. CD79b expression was high on IgG⁺ and natural effector B cells, intermediate on naive mature B cells, reduced on IgA⁺ cells, and clearly the lowest on IgE⁺ memory B cells (Fig 2, C and D). The differences in CD79b expression were not due to steric hindrance of the anti-IgA and anti-IgE isotypes (see Fig E1, D). The low IgE membrane levels might have prevented prior detection of IgE⁺ memory B cells.

Subsequently, we studied the immunophenotype of the IgE⁺ memory B cells and compared this with that of naive mature B cells and the 6 previously defined memory subsets.²⁵ All analyzed B-cell subsets had high expression of CD21 (Fig 2, E). A minor fraction of CD27[−]IgA⁺ and CD27[−]IgG⁺ cells lacked

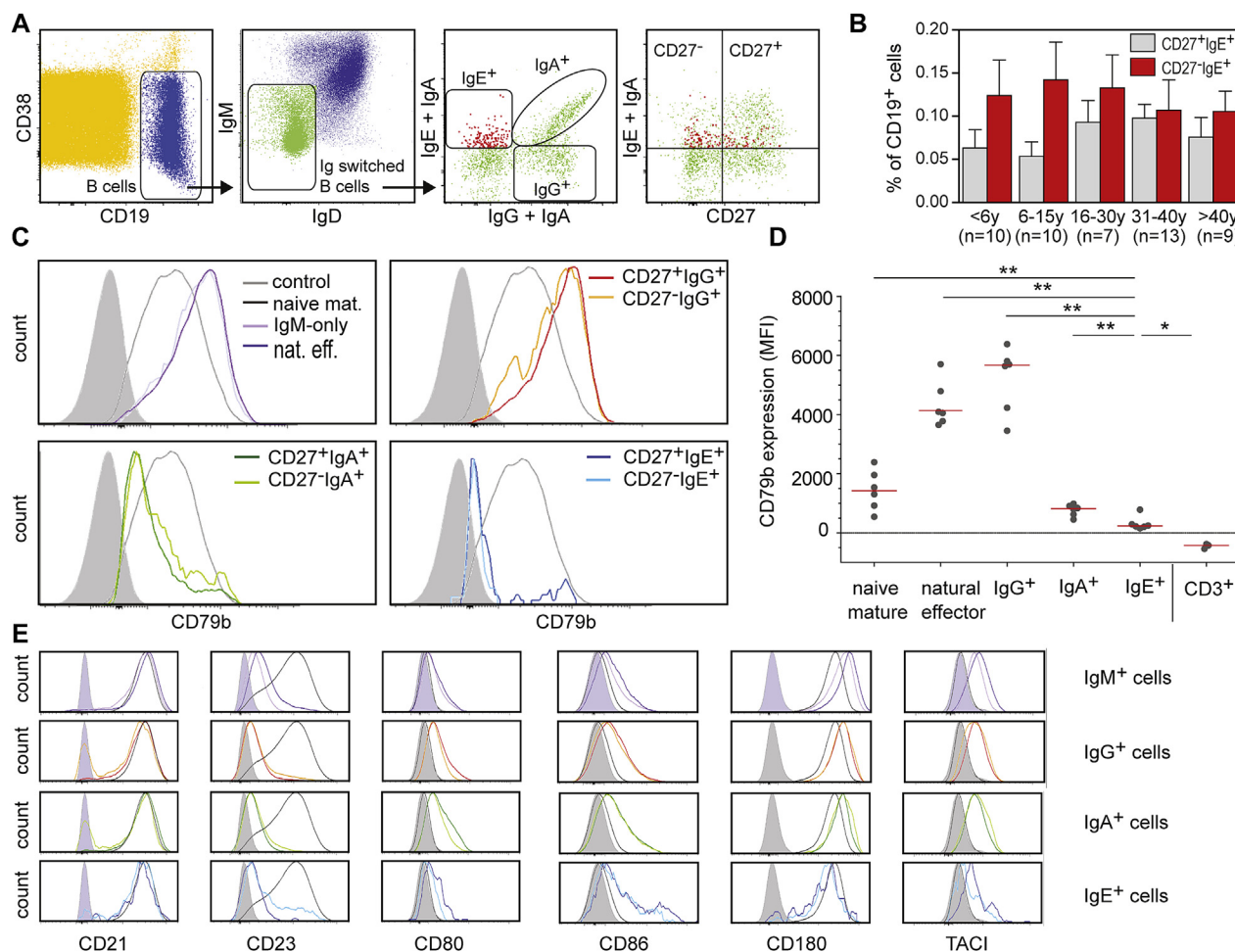


FIG 2. Two IgE⁺ memory B-cell subsets in blood. **A**, Stepwise gating strategy for identification of CD27⁺IgE⁺ and CD27⁻IgE⁺ subsets. **B**, CD27⁺IgE⁺ and CD27⁻IgE⁺ B-cell frequencies in healthy children and adults. Bars represent mean values with SEMs. **C**, Expression levels of CD79b on IgM⁺, IgG⁺, IgA⁺, and 2 IgE⁺ memory B-cell subsets. Each histogram is composed of merged data from 6 healthy donors and contains a light gray filled histogram representing CD3⁺ T cells and a dark gray histogram representing naive mature B cells. **D**, Median fluorescence intensity of CD79b on B-cell subsets and T cells from 6 individual donors. Data were statistically analyzed with the Mann-Whitney test. *P < .05 and **P < .01. **E**, Expression levels of selected markers on IgM⁺, IgG⁺, IgA⁺, and IgE⁺ memory B-cell subsets. Light gray filled histograms represent isotype controls, and dark gray histograms represent naive mature B cells.

CD21 expression, confirming previous observations that part of the CD21^{low} B-cell subset is negative for IgM and IgD.³⁴ CD23 was highly expressed on naive B cells but not on memory B cells.^{35,36} Therefore the detection of IgE on CD27⁺ and CD27⁻ memory B-cell subsets is not the result of FcεR-bound molecules. Furthermore, CD27⁺IgE⁺ and CD27⁻IgE⁺ B cells showed an immunophenotype that was characteristic of activated cells, with increased expression of the B7 family members CD80 and CD86 and the TNF receptor superfamily member transmembrane activator and CAML interactor (TACI) compared with naive mature B cells. In contrast to other memory B cells, IgE⁺ B cells did not show upregulation of Toll-like receptor-related CD180. Nonetheless, CD27⁺IgE⁺ and CD27⁻IgE⁺ B cells carry a typical memory B-cell immunophenotype with absence of CD23, presence of CD21, and upregulation of activation markers.^{25,37}

Replication history, SHMs, and class-switching patterns in IgE⁺ memory B cells

CD27⁺IgE⁺ and CD27⁻IgE⁺ memory B cells were purified from healthy subjects for molecular analysis of their *in vivo* replication history and immunoglobulin gene maturation. CD27⁺IgE⁺ memory B cells showed a replication history of approximately 9 cell divisions (Fig 3, A). This was similar to that seen in CD27⁺ IgM-only and CD27⁻IgG⁺ B cells and higher than that seen in centrocytes. In contrast, CD27⁻IgE⁺ B cells had a limited replication history of only approximately 4 cell divisions, which was similar to that in CD27⁻ IgA⁺ memory B cells. In agreement with the replication history, the frequency of mutated *IGKV3-20* alleles and SHM levels in rearranged *IGHV* genes of CD27⁺IgE⁺ cells were similar to those of memory B cells from primary immune responses (Fig 3, B and C). In contrast, CD27⁻IgE⁺ B cells showed few mutated *IGKV3-20* alleles and low SHM levels in *IGHV*, which are reminiscent of GC-independent responses.

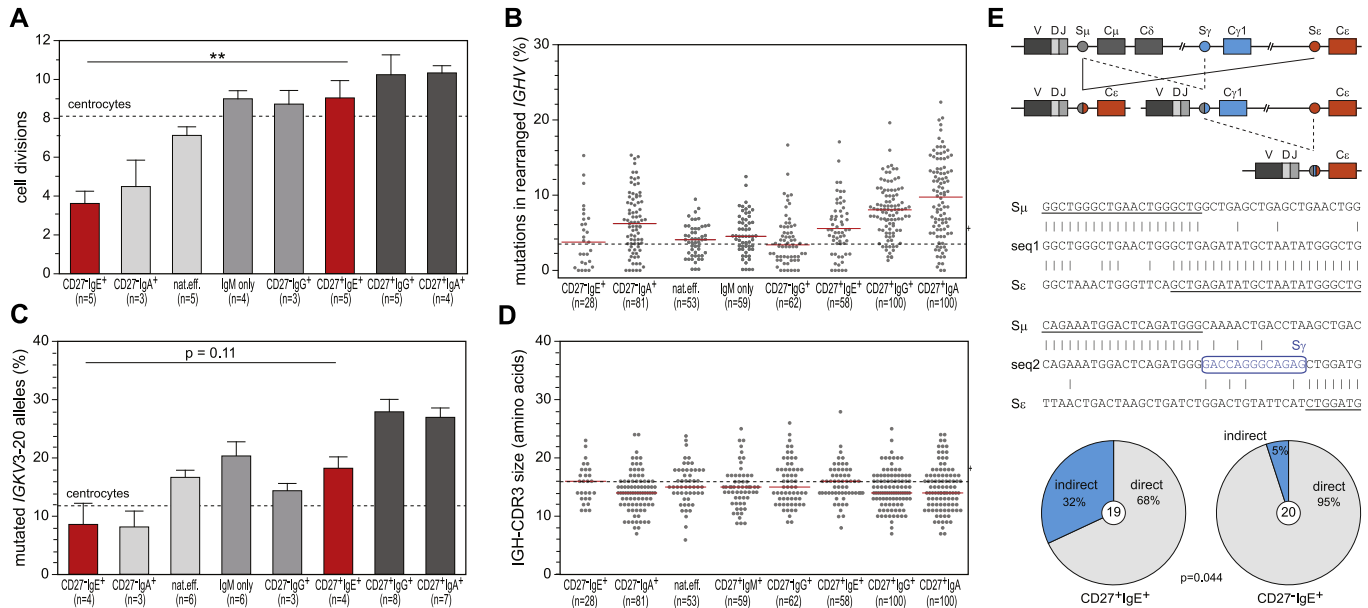


FIG 3. Distinct levels of molecular maturation in CD27⁺IgE⁺ and CD27⁻IgE⁺ memory B cells. **A**, Replication histories of memory B-cell subsets, as measured with the kappa-deleting recombination excision circle assay.^{25,27} Bars represent mean values with SEMs. Dashed lines represent values for centrocytes (n = 55).²⁷ **B**, Frequencies of mutated nucleotides in rearranged IGHV genes. Each gray dot represents a unique rearrangement, and red lines indicate median values. **C**, Frequencies of mutated IGKV3-20 genes as measured with the I κ restriction enzyme hot-spot mutation assay.²⁷ **D**, IGH-CDR3 size distributions in memory B-cell subsets. Differences between CD27⁺IgE⁺ and CD27⁻IgE⁺ cells were statistically analyzed with the Mann-Whitney test. ***P* < .01. **E**, Schematic representation of direct (solid lines) and indirect (dashed lines; through IgG₁) class-switching to IGHE. Examples of hybrid S_μ-S_ε switch regions generated from direct (upper sequence) and sequential (lower sequence) class-switching to IGHE. Frequencies of direct and indirect class-switching in CD27⁺IgE⁺ and CD27⁻IgE⁺ memory B cells. The numbers of analyzed sequences are indicated in the inner circles. Data were statistically analyzed with the χ^2 test.

Thus both the replication history and SHM levels of CD27⁺IgE⁺ memory B cells suggest that these are post-GC cells, whereas CD27⁻IgE⁺ and CD27⁻IgA⁺ B cells share an origin from local T cell-independent (non-GC) responses.

Further analysis of SHM revealed high replacement/silent mutation (R/S) ratios in IGHV-CDRs of CD27⁺IgE⁺ (2.5) and CD27⁻IgE⁺ (4.7) memory B cells (see Table E5 in this article's Online Repository at www.jacionline.org) and were indicative of antigenic selection.²⁵ Interestingly, detailed analysis of the SHM targeting showed less abundant targeting of RGYW motifs in IgE⁺ memory B cells than in most of the other memory B-cell subsets (see Table E5), suggesting lower activity of activation-induced cytidine deaminase in the formation of these cells.

IgM⁺, IgG⁺, and IgA⁺ memory B-cell subsets in human subjects have undergone selection against long IGH-CDR3 regions.²⁵ The median IGH-CDR3 sizes of both CD27⁺IgE⁺ and CD27⁻IgE⁺ memory B cells were 16 amino acids, which was similar to centrocytes but larger than the 14 to 15 amino acids found in other memory B-cell subsets (Fig 3, D). Thus selection mechanisms for IgE⁺ memory B cells seem different than for other memory B-cell subsets.

Class-switching to IgE can occur indirectly through an upstream IGHG or IGHA constant region (Fig 3, E). Theoretically, this would leave a S α or S γ remnant in approximately half of the S_μ-S_ε switch regions. Therefore we studied these molecular signs of indirect switching in hybrid S_μ-S_ε switch regions in genomic DNA of purified IgE⁺ memory B cells. CD27⁻IgE⁺ memory B cells hardly contained molecular signs of indirect switching (5%; Fig 3, E). However, a substantial fraction of

S_μ-S_ε switch regions in CD27⁺IgE⁺ memory B cells (32%) contained remnants of an S γ (mainly S γ 1) region. Thus CD27⁻IgE⁺ cells seem to be almost exclusively generated directly from IgM⁺ cells, whereas IgE class-switching through an IgG intermediate is a frequent event in CD27⁺IgE⁺ memory B cells.

T cell-independent generation of CD27⁻IgE⁺ memory B cells

To study whether CD27⁻IgE⁺ memory B cells can be formed independently of cognate T-cell help, we studied these cells in the blood of 5 CD40L-deficient patients (Fig 4, A, and see Table E1). These patients lack IgG⁺ memory B cells and carry strongly reduced IgM⁺ and IgA⁺ memory B-cell numbers.²⁵ Although the CD27⁺IgE⁺ events were on the borderline of detection, CD27⁻IgE⁺ B cells were reliably detected with approximately 30% reduced frequencies compared with those in healthy age-matched control subjects (Fig 4, B). From this, we concluded that IgE⁺ B cells can be formed in the absence of cognate T-cell help and that these cells are nearly all CD27⁻.

Efficient plasma cell differentiation of IgE⁺ memory B cells

Plasma cell differentiation and immunoglobulin class-switching can be mediated by the cytokines IL-4, IL-13, and IL-21.^{10,13,38} The receptor for IL-13 was not expressed on naive and memory B-cell subsets, IL-4 receptor was weakly expressed, and IL-21 receptor was clearly detectable (Fig 5, A). To study whether the IgE memory B cells could differentiate into plasma

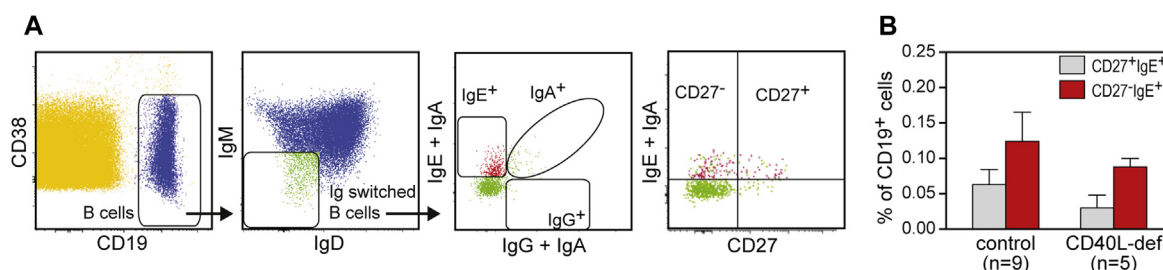


FIG 4. GC-dependent and GC-independent origin of IgE⁺ memory B-cell subsets in blood. **A**, Representative images of flow cytometric analysis of circulating memory B-cell subsets in CD40L-deficient patients. **B**, CD27⁺IgE⁺ and CD27⁻IgE⁺ B-cell frequencies in CD40L-deficient patients and healthy age-matched donors (0-3 y). Bars represent mean values with SEMs.

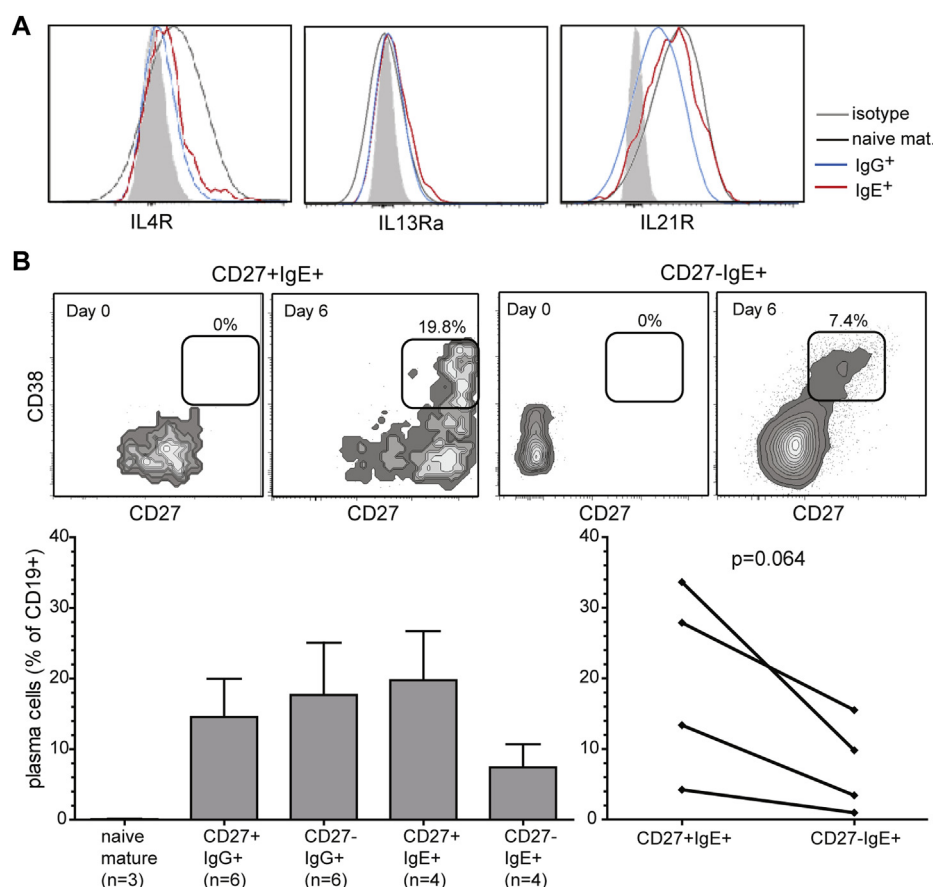


FIG 5. **A**, Expression levels of IL-4 receptor (*IL4R*), IL-13 receptor alpha (*IL13Ra*), and IL-21 receptor (*IL21R*) on naive mature and IgG⁺ and IgE⁺ memory B cells. **B**, Generation of plasma cells defined as CD38^{hi}CD27⁺ after 6-day cultures. Average values for independent experiments with SEMs are shown, as well as paired analysis of CD27⁺IgE⁺ and CD27⁻IgE⁺ memory B-cell subsets (paired *t* test).

cells, we cultured fluorescence-activated cell sorting-purified cells for 6 days in the presence of anti-CD40 and IL-21. Both IgG and IgE memory B cells generated CD38^{hi}CD27⁺ plasma cells (Fig 5, B), although the CD27⁺ subsets seemed more efficient than the CD27⁻ cells.

Increased numbers of circulating CD27⁻IgE⁺ memory B cells in patients with atopic dermatitis

We exploited the reliable detection of IgE⁺ memory B-cell subsets in blood to study 23 patients with atopic dermatitis, an

allergic response often accompanied by increased serum IgE levels (see Table E2).³⁹ IgE⁺ B-cell frequencies were compared with those of 16 age-matched healthy control subjects and with 14 patients with T_H1/T_H17-mediated psoriasis (see Table E3). Frequencies of IgE⁺ plasma cells and CD27⁺IgE⁺ memory B cells were not significantly different between the groups (Fig 6, A). However, the median frequency of CD27⁻IgE⁺ cells in atopic patients (0.12%) was significantly higher than in healthy donors (0.8%, *P* < .05) and patients with psoriasis (0.5%, *P* < .05).

Patients with a high disease severity according to the criteria by Hanifin and Rajka²⁶ had more CD27⁺IgE⁺ and CD27⁻IgE⁺ cells

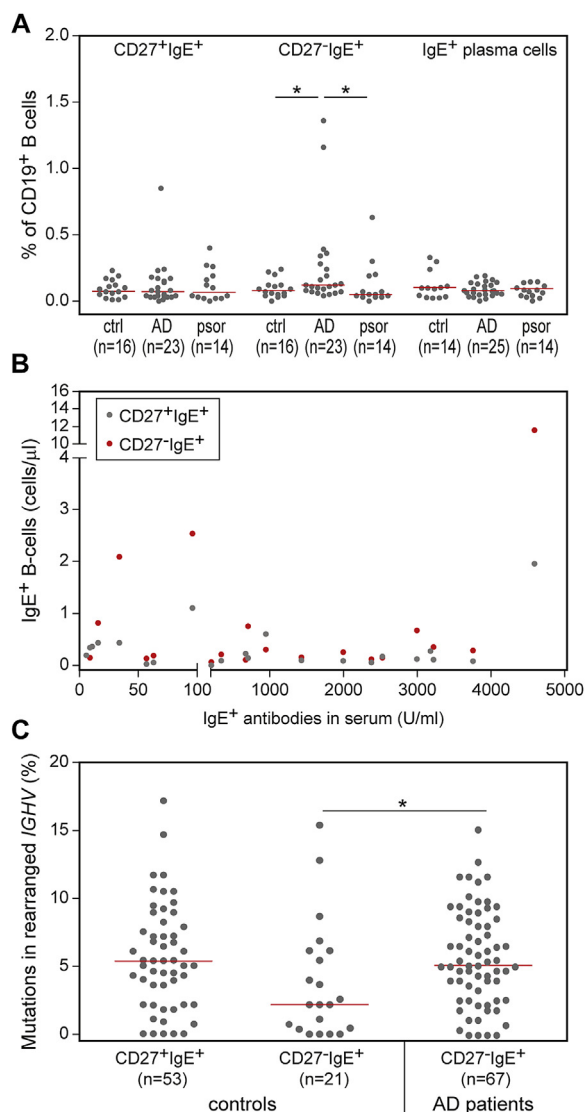


FIG 6. Increased frequencies of CD27⁻ IgE⁺ memory B cells in patients with atopic dermatitis. **A**, Frequencies of CD27⁺ IgE⁺ and CD27⁻ IgE⁺ memory B cells and IgE⁺ plasma cells in patients with atopic dermatitis (AD), patients with psoriasis (psor), and healthy age-matched donors (ctrl). Individual data points are shown as gray dots, with red lines indicating median values. **B**, Correlation between CD27⁺ IgE⁺ and CD27⁻ IgE⁺ memory B-cell numbers and IgE serum levels in 23 patients with atopic dermatitis. **C**, Frequencies of mutated nucleotides in rearranged IGHV genes. Data were statistically analyzed with the Mann-Whitney test: * $P < .05$.

than patients with mild or moderate manifestations of the disease (data not shown).²⁶ Because of the low numbers of patients per group, these differences did not reach statistical significance. The frequencies of IgE⁺ memory B cells did not correlate with total or allergen-specific IgE serum levels (Fig 6, B).

To study the effects of chronic disease on IgE responses, we analyzed SHMs in variable regions of *IGHV* transcripts amplified from 5 patients with atopic dermatitis with a selective increase in CD27⁻ IgE⁺ memory B cells (≥ 6 -fold more CD27⁻ IgE⁺ than CD27⁺ IgE⁺ B cells). The frequency of SHMs in *IGHV* genes of CD27⁻ IgE⁺ in patients with atopic dermatitis was significantly higher than in control CD27⁻ IgE⁺ memory B cells and similar to that seen in control CD27⁺ IgE⁺ B cells (Fig 6, C).

Patients' transcripts showed molecular signs of affinity maturation, with a high IGHV-CDR R/S ratio (3.0). Thus patients with atopic dermatitis have increased levels of circulating CD27⁻ IgE⁺ memory B cells that show molecular signs of extensive antibody maturation.

Model of IgE⁺ plasma cell and memory B-cell formation in human subjects

To recapitulate our findings, we propose a model of IgE⁺ plasma cell and IgE⁺ memory B-cell formation in human subjects (Fig 7). On the basis of the distinct molecular features and similarity to previously characterized memory B-cell subsets, we distinguish CD27⁺ IgE⁺ memory B cells derived from a primary GC-dependent pathway and CD27⁻ IgE⁺ memory B cells from local (mucosal) tissue. IgE⁺ plasma cells are likely to be derived from both the GC and non-GC maturation pathways.

DISCUSSION

Using a novel 8-color flow cytometry strategy, we reliably detected IgE⁺ plasma cells and 2 IgE⁺ memory B-cell subsets. Purification and molecular analysis of these subsets allowed us to dissect their origins from GC responses (IgE⁺ plasma cells and CD27⁺ IgE⁺ memory B cells) and GC-independent responses (CD27⁻ IgE⁺). The GC-dependent responses generated more SHMs and frequently involved immunoglobulin CSR through IgG₁, contrasting non-GC responses generating CD27⁻ IgE⁺ memory B cells. Numbers of these CD27⁻ IgE⁺ cells were increased and showed extensive SHM in patients with atopic dermatitis.

Before flow cytometric selection of IgE⁺ B cells, we performed a stepwise exclusion of IgM⁺, IgD⁺, IgG⁺, and IgA⁺ cells to increase the specificity of detection of rare IgE⁺ B cells. IgE⁺ B cells carried low surface immunoglobulin levels compared with other B cells, possibly because of the suboptimal polyadenylation signals in membrane IgE transcripts.⁷ Moreover, with this strategy, we excluded CD23⁺ naive B cells that stained positively for IgE because of CD23-mediated capture from serum.³⁵ Thus far, the detection of IgE⁺ B cells has been problematic and often depended on cold acid washing to disrupt CD23-IgE binding,⁴⁰ which can be very damaging to the cells. Our direct flow cytometric approach on otherwise untreated cells allowed detailed immunophenotyping and molecular studies.

The majority of plasma cells in childhood tonsils had an immature phenotype, with surface immunoglobulin and low/absent CD138 expression, which is commonly seen in plasma cells in lymph nodes, but not in blood and bone marrow,^{41,42} and likely reflects recent generation. The IgE transcripts showed low mutation frequencies similar to IgM. Furthermore, blood IgE⁺ plasma cells had intermediate SHM levels compared with the 2 IgE⁺ memory B-cell subsets. Therefore IgE⁺ plasma cells are likely a mixture of cells from primary GC-dependent and GC-independent responses.^{25,27}

In blood we identified circulating IgE⁺ B cells that could be separated into CD27⁺ and CD27⁻ subsets. Both subsets showed immunophenotypic and molecular signs of B-cell memory: (1) high expression of activation and costimulatory molecules; (2) antigen-driven proliferation; and (3) SHM levels with high R/S ratios in IGHV-CDRs. The existence of IgE⁺ B-cell memory

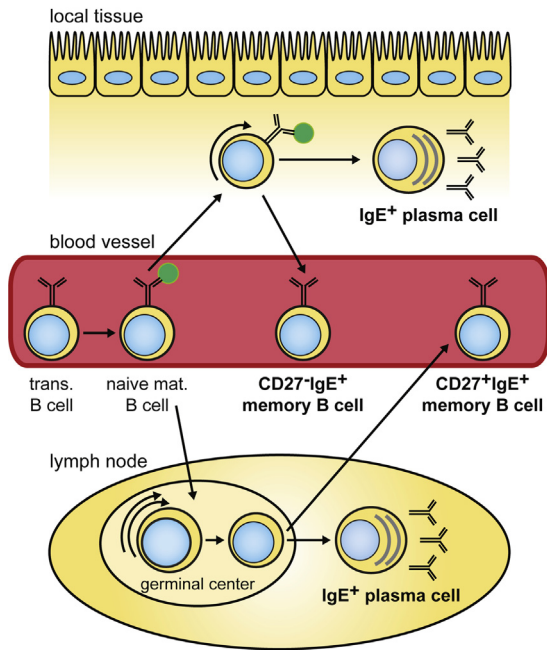


FIG 7. Model for GC-dependent and GC-independent maturation of IgE⁺ B cells. Naive mature B cells scavenge the peripheral lymphoid system for antigens. Responses to peptide allergens with T-cell help result in extensive proliferation and affinity maturation in GCs, generating IgE⁺ plasma cells and CD27⁺IgE⁺ memory B cells, potentially through an IgG intermediate. Antigen responses independently from T-cell help in local tissue (eg, mucosa and skin) induce limited proliferation, SHM, and direct IgE switching, generating plasma cells or CD27⁺IgE⁺ memory B cells.

has been disputed, although some indirect evidence exists from the kinetics of allergic responses.^{43,44} Moreover, several studies report detection of low numbers of circulating IgE⁺ B cells displaying a plasma cell-like phenotype.^{45,46} The IgE⁺ B cells we identified showed a memory B-cell phenotype with low CD38 expression and upregulation of CD80, CD86, and TACI. In addition, fluorescence-activated cell sorting-purified cells expressed IgE-switched transcripts with SHM. Thus IgE⁺ memory B cells can be formed in healthy subjects and circulate in peripheral blood.

The 2 circulating IgE⁺ memory B-cell subsets did not merely differ in their CD27 expression: the replication history, SHM levels, and frequency of indirect class-switching were significantly higher in CD27⁺IgE⁺ than in CD27⁺IgE⁺ memory B cells. In CD27⁺IgE⁺ memory B cells these levels were slightly higher than in centrocytes but lower than in CD27⁺IgG⁺ and CD27⁺IgA⁺ memory B cells, indicating that the majority of these cells in healthy adults are potentially derived from primary GC responses. This GC-dependent IgE response is most likely the source of high-affinity IgE in human subjects. In mice switching to IgE takes place in post-GC IgG⁺ B cells.²² Because CD27⁺IgE⁺ memory B cells show high frequencies of indirect switching through an IgG intermediate, it is possible that IgE switching does not take place in the GC but is a post-GC process.

CD27⁺IgE⁺ memory B cells had a limited replication history (3–4 cell divisions) and low SHM levels and were present in CD40L-deficient patients, supporting their origin from a T cell-independent pathway. Until recently, CD27 was considered a

pan-memory B-cell marker,⁴⁷ but there is growing evidence that also CD27⁺ immunoglobulin class-switched cells display memory B-cell characteristics.^{25,48} Importantly, the replication history and SHM levels of CD27⁺IgE⁺ memory B cells were similar to those of CD27⁺IgA⁺ memory B cells. Furthermore, both subsets showed predominantly direct immunoglobulin class-switching, and the frequently used *IGHA2* region in CD27⁺IgA⁺ memory B cells is genomically close to *IGHF*.²⁵ These comparable features support a similar origin of CD27⁺IgA⁺ and CD27⁺IgE⁺ memory B cells from local mucosal responses.²⁵ This contrasts with CD27⁺IgG⁺ cells, which are fully dependent on T-cell help and share clonal relationships with CD27⁺IgG⁺ B cells.^{25,49} Nonetheless, it cannot be excluded that such clonal relationships are also present between CD27⁺IgE⁺ and CD27⁺IgE⁺ subsets. Multiple studies demonstrated local IgE production in the respiratory tracts of patients with atopic rhinitis and asthma^{50,51} and in the gastrointestinal tracts of patients with food allergy and showed increased local IL-4 and IL-13 production in these pathologic conditions.^{52,53} Of these, IL-4 is crucial for CD40-independent class-switching and B cell-activating factor of the TNF family— or a proliferation-inducing ligand—induced switching to IgE.^{54,55} CD27⁺IgE⁺ memory B cells were the predominant IgE⁺ subset in children. Because GC-dependent responses remain underdeveloped in young children, involvement of CD27⁺IgE⁺ cells in local responses could possibly explain the early onset of some allergic diseases.

Patients with atopic dermatitis had increased frequencies of CD27⁺IgE⁺ memory B cells with high SHM levels. Although this increase was not found in patients with psoriasis, the nature of these cells in patients with atopic dermatitis remains unclear. The CD27⁺IgE⁺ phenotype could reflect the mainly local manifestation of this allergic disease. These cells would then be abnormally expanded and gained increased SHM levels. Alternatively, the CD27⁺IgE⁺ memory B cells in these patients are derived from a GC response with normal SHM levels but display an abnormal phenotype. Nonetheless, these findings imply that analysis of CD27⁺IgE⁺ memory B-cell numbers can contribute to diagnosis and therapy monitoring of these patients, especially in cases with low IgE serum levels. Furthermore, IgE⁺ memory B-cell measurements could prove valuable in monitoring patients undergoing immunotherapy or anti-IgE antibody therapy (eg, with omalizumab). A recent study indicated that the beneficial effect of omalizumab is the result of reduced IgE production.⁵⁶ Therefore the kinetics of reduction in IgE⁺ memory B-cell levels could be an early marker to predict therapy outcome for patients with severe allergies.

Thus by implementing a new strategy to reliably detect IgE-expressing B cells, we provided new insights into the biology of human IgE⁺ plasma cells and dissected the molecular differences of the 2 memory B-cell subsets potentially derived from T cell-dependent and T cell-independent pathways. These insights open new possibilities to study the pathophysiologic mechanisms underlying IgE-mediated diseases, which can be directly translated into patient care.

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Key messages

- **IgE⁺ plasma cells and memory B cells can be detected in lymphoid tissue and blood of healthy subjects.**
- **GC responses generate CD27⁺IgE⁺ memory B cells, whereas CD27⁻IgE⁺ memory B cells do not depend on T-cell help.**
- **CD27⁻IgE⁺ memory B-cell numbers are increased in the blood of patients with atopic dermatitis.**

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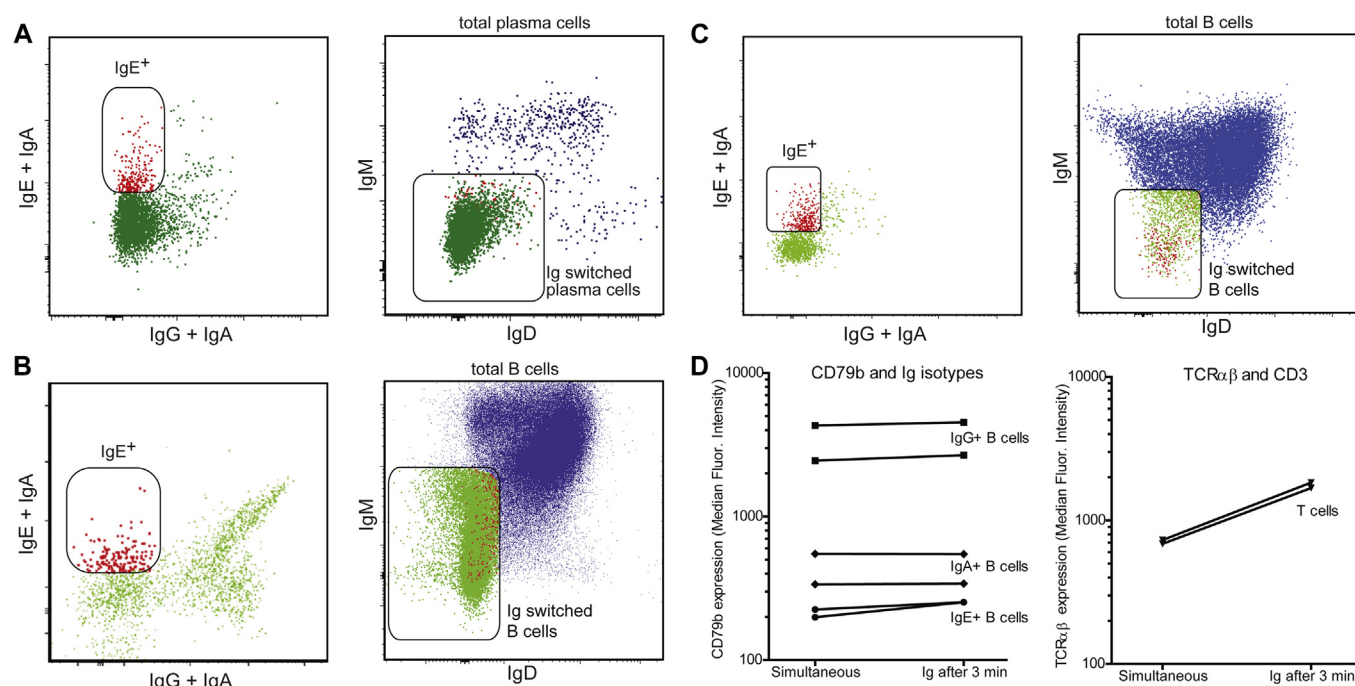


FIG E1. Immunophenotyping details of IgE⁺ memory B cells and plasma cells. **A-C**, Back gating of IgE⁺ plasma cells from tonsils (Fig E1, A) and memory B cells from blood of a healthy control subject (Fig E1, B) and a CD40L-deficient patient (Fig E1, C) within the IgM⁺IgD⁺ gate. **D**, Evaluation of steric hindrance of CD79b (left) and T-cell receptor αβ expression. Cells from 2 healthy donors were incubated simultaneously with anti-CD79b and anti-immunoglobulin isotypes or 3 minutes with CD79b before addition of anti-immunoglobulin isotypes. Preincubation with anti-CD79b before addition of the anti-immunoglobulin isotype antibodies did not lead to higher expression levels. In contrast, preincubation of T cells with the anti-T-cell receptor αβ clone IP26 before addition of the anti-CD3 clone OKT3 did lead to increased T-cell receptor αβ expression levels.

TABLE E1. General characteristics and laboratory findings in 5 CD40L-deficient patients

Patient	Age at analysis (y)		Sex	Mutation, cDNA	Mutation, protein	CD40L protein expression	CD3 ⁺ T cells (cells/μL)	CD19 ⁺ B cells (cells/μL)	CD16 ⁺ /CD56 ⁺		
									NK cells (cells/μL)	Serum IgG (g/L)	Serum IgA (g/L)
XHIM01-3	3	M	c.761C>T	p.Thr254Met	Absent	8720*	378	390*	0.8*	<0.01*	<0.3*
XHIM10	3	M	c.435 delC	p.Tyr146ThrfsX3	Absent	3910	1370	490	1.34	0.06	0.71
XHIM11	1	M	c.154 A>T	p.Lys52X	Absent	6013	796	367	1.52	0.06	1.78
XHIM14	1	M	c.302 delA	p.Asn101ThrfsX27	Absent	2720	1800	350	1.43	0.06	4.14
XHIM16	1	M	c.135 T>A	p.Tyr45Stp	Absent	5850	1600	360	9.7†	0.05	1.51

Values of less than the 5th percentile of age-matched control subjects are shown in boldface, and those greater than the 95th percentile are shown in italics.

M, Male; NK, natural killer.

*Measured at the age of 1 year.

[†]After IVIG treatment.

TABLE E2. General characteristics and laboratory findings in 23 patients with atopic dermatitis

Patient	Severity score*	Age at analysis (y)	Sex	CD3 ⁺ T cells (cells/ μ L)	CD19 ⁺ B cells (cells/ μ L)	CD16 ⁺ /CD56 ⁺ NK cells (cells/ μ L)	Total serum IgE (kU/mL)	Specific IgE (kU/mL) [†]	IgE reactivity [‡]
AD-1	3	54	F	730	180	140	34	8.5	Mite
AD-2	3	16	M	1150	850	110	2377	17.2	Cat
AD-3	1	25	F	990	360	120	2527	41.5	Birch
AD-4	3	33	M	1020	260	90	3219	100.0	ND
AD-5	3	58	M	4230	650	60	946	0.29	—
AD-6	3	38	F	1740	220	130	706	ND	—
AD-7	1	30	M	1170	240	230	107	18.7	Mite
AD-8	2	23	F	1060	150	260	342	77.3	Mite
AD-9	1	42	M	420	50	30	4589	3.2	Mite
AD-10	1	72	M	850	130	140	11	0.0	—
AD-11	3	39	M	650	170	70	3755	100.0	Mite, cat, dog
AD-12	1	45	M	1130	240	140	96	0.0	—
AD-13	1	24	M	1200	270	200	57	2.1	Grass
AD-14	3	60	M	2120	430	240	6	0.8	Mite
AD-15	1	32	F	1670	240	410	674	78.6	Grass
AD-16	3	35	M	2410	680	130	208	4.9	Mite
AD-17	1	27	F	1710	470	220	63	2.9	Mite
AD-18	3	53	F	920	160	130	16	0.0	—
AD-19	3	22	M	1040	310	130	3178	100.0	Mite, birch
AD-20	3	70	M	1270	40	70	1430	1.2	Mite
AD-21	1	79	F	700	110	100	9	0.0	—
AD-22	3	39	F	800	240	110	2995	65.0	Mite, grass, birch
AD-23	2	30	F	1440	210	90	1996	100.0	Mite

Increased total IgE serum levels (>100 kU/mL) and increased inhalant allergen-specific IgE serum levels (>0.35 kU/mL) are indicated in boldface.

F, Female; M, male; ND, not determined; NK, natural killer.

*The following denotations are used: 1, mild; 2, moderate; and 3, severe.

[†]Concentration of IgE specific for common inhalant allergens, as measured with the ImmunoCAP Phadiatop.

[‡]The most common IgE-reactive allergens were tested: dust mite, rye grass, birch, mugwort, cat, and dog.

TABLE E3. General characteristics and laboratory findings in 14 patients with psoriasis

Patient	Severity score*	Age at analysis (y)	Sex	CD3 ⁺ T cells (cells/ μ L)	CD19 ⁺ B cells (cells/ μ L)	CD16 ⁺ /CD56 ⁺ NK cells (cells/ μ L)	Total serum IgE (kU/mL)	Specific IgE (kU/mL) [†]	IgE reactivity [‡]
AP-1	3	61	F	1310	490	250	76	0.0	—
AP-2	3	36	M	1310	460	210	215	29.3	Birch
AP-3	ND	45	F	290	20	130	4	0.1	—
AP-4	ND	58	M	970	240	220	65	0.0	—
AP-5	1	64	F	1340	390	60	10	0.0	—
AP-6	3	45	M	1260	360	220	34	0.0	—
AP-7	2	35	M	1040	220	200	58	0.0	—
AP-8	2	37	M	2280	590	130	253	0.1	—
AP-9	2	43	M	1630	780	130	114	3.2	Mite
AP-10	0	35	F	1040	180	100	101	0.2	—
AP-11	1	25	M	960	120	320	26	0.6	Grass
AP-12	2	50	M	620	150	220	43	5.8	Grass
AP-13	0	64	M	290	70	50	13	0.0	—
AP-14	1	58	F	1640	110	70	27	0.1	—

Increased total IgE serum levels (>100 kU/mL) and increased inhalant allergen-specific IgE serum levels (>0.35 kU/mL) are indicated in boldface.

F, Female; M, male; ND, not determined; NK, natural killer.

*The following denotations are used: 1, mild; 2, moderate; and 3, severe.

[†]Concentration of IgE specific for common inhalant allergens, as measured with the ImmunoCAP Phadiatop.

[‡]The most IgE-reactive allergens from the following list were tested: dust mite, rye grass, birch, mugwort, cat fur, and dog fur.

TABLE E4. Antibodies used for flow cytometric immunophenotyping

Antibody	Clone	Conjugate	Supplier
CD21	M-T271	BV421	BD PharMingen, San Jose, Calif
IgD	IA6-2	Biotin	BD PharMingen
IgM	G20-127	HorV450	BD PharMingen
CD3	UCHT1	PE-CF594	BD Biosciences, Mississauga, Ontario, Canada
CD19	SJ25C1	PE-Cy7	BD Biosciences
CD20	L27	PE-Cy7	BD Biosciences
CD21	CR2	APC	BD Biosciences
CD23	EBVCS-5	APC	BD Biosciences
CD27	L128	PerCP-Cy5.5	BD Biosciences
CD27	L128	APC	BD Biosciences
CD38	HB7	PE	BD Biosciences
CD38	HB7	APC-H7	BD Biosciences
CD79b	SN8	PerCP-Cy5.5	BD Biosciences
CD124	hIL4-M57	biotin	BD Biosciences
CD138	MI15	APC	BD Biosciences
IgD	IA6-2	PE-CF594	BD Biosciences
IgG	G18-145	FITC	BD Biosciences
IgG	G18-145	PE	BD Biosciences
CD3	OKT3	BV711	BioLegend, San Diego, Calif
CD360	2G1-K12	APC	BioLegend
IgM	MHM-88	PerCP-Cy5.5	BioLegend
IgM	MHM-88	BV510	BioLegend
IgD	Goat polyclonal	FITC	SouthernBiotech, Birmingham, Ala
IgA	IS11-8E10	FITC	Miltenyi Biotec, Bergisch Gladbach, Germany
IgA	IS11-8E10	PE	Miltenyi Biotec
IgE	Fc Sp	FITC	Invitrogen, Carlsbad, Calif
Streptavidin	—	Pacific Orange	Invitrogen
CD80	MAB104	FITC	Beckman Coulter, Fullerton, Calif
CD180	MHR73-11	unlabeled	eBioscience, San Diego, Calif
TCR $\alpha\beta$	IP26	APC	eBioscience
Streptavidin	—	PE-Cy7	eBioscience
TACI	G112	biotin	PeptoTech, Rocky Hill, NJ
IL-13Ra1	419718	APC	R&D Systems, Minneapolis, Minn

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TCR $\alpha\beta$, T-cell receptor $\alpha\beta$.

TABLE E5. Targeting and selection of individual mutations in rearranged *IGHV* in IgE⁺ memory B-cell subsets

	Natural effector cells (n = 53)	IgM-only cells (n = 59)	CD27 ⁺ IgA ⁺ cells (n = 100)	CD27 ⁺ IgA ⁺ cells (n = 81)	CD27 ⁺ IgG ⁺ cells (n = 100)	CD27 ⁺ IgG ⁺ cells (n = 62)	CD27 ⁺ IgE ⁺ cells (n = 57)	CD27 ⁺ IgE ⁺ cells (n = 24)
RGYW (%)	142.3/500 (28.5)*†	184.4/650 (28.4)*†	591.1/2104 (27.1)*†	321.5/1176 (27.3)	501.3/1760 (28.5)*†	159.8/618 (25.8)	206.4/858 (24.1)	74.5/308 (24.2)
WRCY (%)	77.8/500 (15.6)	111.4/650 (17.1)	318.9/2104 (15.2)	178.5/1176 (15.2)	273.0/1760 (15.5)	89.8/618 (14.5)	125.4/858 (14.6)	42.3/308 (13.7)
WA (%)	72.1/500 (14.4)	89.8/650 (13.8)	299.0/2104 (14.2)	161.6/1176 (13.7)	261.8/1760 (14.9)	84.6/618 (13.7)	115.1/858 (13.4)	51.2/308 (16.6)
TW (%)	25.9/500 (5.2)*	36.4/650 (5.6)*	142.0/2104 (6.7)	81.4/1176 (6.9)	131.9/1760 (7.5)	47.9/618 (7.8)	73.1/858 (8.5)	15.9/308 (5.2)
Transitions (%)	273/500 (54.6)	359/650 (55.2)	1100/2104 (52.3)	610/1176 (51.9)	890/1760 (50.6)	336/618 (54.4)	444/858 (51.7)	155/308 (50.3)
Transversions (%)	227/500 (45.4)	291/650 (44.8)	1004/2104 (47.7)	566/1176 (48.1)	870/1760 (49.4)	282/618 (45.6)	414/858 (48.3)	153/308 (49.7)
FR (R/S)	202/112 (1.8)	239/156 (1.5)	770/568 (1.4)*†	463/286 (1.6)	654/402 (1.6)	242/149 (1.6)	344/216 (1.6)	142/80 (1.8)
CDR (R/S)	143/40 (3.6)	181/50 (3.6)	588/159 (3.7)*	341/86 (4.0)*	504/155 (3.3)	151/65 (2.3)	214/85 (2.5)	71/15 (4.7)

The numbers of analyzed sequences are indicated in parentheses next to the population names. All analyses were performed with the JoinSolver program, and the differences between IgE⁺ memory B-cell subsets and all other memory B-cell subsets were analyzed with the χ^2 test. Significant differences ($P < .05$) are depicted in boldface. RGYW, WRCY, TW and WA are DNA sequence motifs, where R denotes A/G; Y, C/T; W, A/T.

FR, Framework region.

*Statistical significance between the indicated subsets and CD27⁺IgE⁺ memory B cells.

†Statistical significance between the indicated subset and CD27⁺IgE⁺ memory B cells; only the difference in CDR R/S ratios was statistically significant between CD27⁺IgE⁺ and CD27⁺IgE⁺ memory B cells.