

Self-Restrained B Cells Arise following Membrane IgE Expression

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Brice Laffleur, Sophie Duchez, Karin Tarte, Nicolas Denis-Lagache, Sophie Péron, et al.. Self-Restrained B Cells Arise following Membrane IgE Expression. Cell Reports, 2015, 10 (6), pp.900-909. 10.1016/j.celrep.2015.01.023 . hal-01116949

HAL Id: hal-01116949

https://univ-rennes.hal.science/hal-01116949

Submitted on 16 Feb 2015

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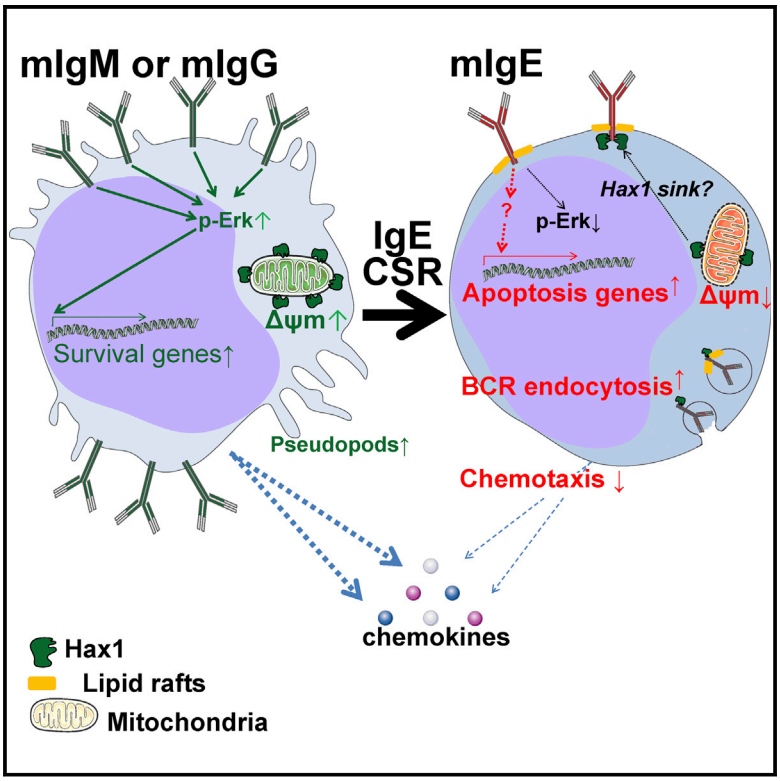
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Report

Cell Reports

Self-Restrained B Cells Arise following Membrane IgE Expression

Graphical Abstract



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In Brief

IgE responses may convey severe allergy and thus need tight control. Laffleur et al. demonstrate a self-restrained B cell stage where membrane IgE expression impacts the phenotype, shape, and mobility of B lymphocytes, promotes apoptosis, and severely shortens IgE$^+$ B cell fate.

Highlights

- Membrane IgE appears transiently and is then internalized
- The IgE B cell receptor (BCR) spontaneously co-localizes with lipid rafts
- IgE BCR expression restrains mobility responses to chemokines
- mIgE promotes apoptosis of IgE$^+$ cells, severely impacting their fate

Accession Numbers

GSE64130

Laffleur et al., 2015, Cell Reports *10*, 1–10
February 17, 2015 ©2015 The Authors
http://dx.doi.org/10.1016/j.celrep.2015.01.023

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Self-Restrained B Cells Arise following Membrane IgE Expression

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http://dx.doi.org/10.1016/j.celrep.2015.01.023
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SUMMARY

Among immunoglobulins (Igs), IgE can powerfully contribute to antimicrobial immunity and severe allergy despite its low abundance. IgE protein and gene structure resemble other Ig classes, making it unclear what constrains its production to thousand-fold lower levels. Whether class-switched B cell receptors (BCRs) differentially control B cell fate is debated, and study of the membrane (m)IgE class is hampered by its elusive in vivo expression. Here, we demonstrate a self-controlled mIgE$^+$ B cell stage. Primary or transfected mIgE$^+$ cells relocate the BCRs into spontaneously internalized lipid rafts, lose mobility to chemokines, and change morphology. We suggest that combined proapoptotic mechanisms possibly involving Hax1 prevent mIgE$^+$ memory lymphocyte accumulation. By uncoupling in vivo IgE switching from cytokine and antigen stimuli, we show that these features are independent from B cell stimulation and instead result from mIgE expression per se. Consequently, few cells survive IgE class switching, which might ensure minimal long-term IgE memory upon differentiation into plasma cells.

INTRODUCTION

In mammals, IgE contributes to immunity against pathogens and toxins (Marichal et al., 2013; Palm et al., 2013). It also yields severe allergies, making it crucial to restrain IgE production and control IgE immune memory. Since IgE$^+$ cells are scarce in vivo, it is unclear whether, as for other Igs, cells with an IgE B cell receptor (BCR) become memory lymphocytes. Globally, humoral memory relies on the dual ability of B cells to either differentiate into long-lived plasma cells (PCs) or survive as memory B lymphocytes. The BCR, providing both tonic and ligation-induced signals, includes membrane-anchored Igs (mIgs) differing from secreted Igs by inclusion of an extracellular membrane-proximal domain, a transmembrane segment, and a cytoplasmic tail varying between heavy chain (HC) classes (Venkitaraman et al., 1991). Human mIgE exists with either a long ("m_LIgE") or a short ("m_SIgE") membrane-proximal domain (Batista et al., 1995).

Mouse mIgE was studied in the abundant mIgE$^+$ B cells appearing in vitro after class switch recombination (CSR) in the presence of interleukin-4 (IL-4) (Anand et al., 1997; Coffman et al., 1986). Such mIgE$^+$ B cells are rare in vivo, except in T/B monoclonal or hyper-T_H2 LAT_{Y136F} mice (Aguado et al., 2002; Genton et al., 2006, Erazo et al., 2007). A single spontaneous mIgE$^+$ lymphoma cell line was reported (Sitia, 1985). In mIgE$^+$ cells, BCR expression is weak (Karnowski et al., 2006). That mIgE$^+$ B cells are mandatory for generating IgE PCs (similar to mIgG and mIgA) was proven by knocking out membrane exons (Achatz et al., 1997; Amin et al., 2012; Kaisho et al., 1997). To avoid Fcε receptor-mediated artifacts, IgE$^+$ B cells can be characterized by intracellular rather than surface staining (Wesemann et al., 2011). After tagging mIgE or knocking in an IRES-GFP cassette downstream from Cε, it was suggested that within germinal centers, caspase activation was higher in IgE$^+$ than in IgG1$^+$ cells (Talay et al., 2012; He et al., 2013). However, the IRES-GFP strategy monitors not only mIgE but also germline Cε transcription. It was also proposed that among antigen-activated cells, mIgE$^+$ cells vanished through accelerated differentiation into PCs and were more apoptotic and less mobile than IgG$^+$ cells (Yang et al., 2012). These observations did not provide an explanation for the increased ratio of PCs versus activated B lymphocytes in the IgE$^+$ compartment, and more importantly with regard to long-term immunity, they gave no clue about the status of memory B cells.

In immature mouse B cells, transfected IgE expression resulted in BCR ligation-inducible growth inhibition as with IgM (Batista et al., 1996). In mature cells binding antigen, it is unknown whether class-switched and naive IgM$^+$ cells are differentially stimulated or eventually undergo activation-induced cell death (Guzman-Rojas et al., 2002; Figgett et al., 2013; Péron et al., 2012). Cross-linking of a transfected mIgE was reported to induce apoptosis (Poggianella et al., 2006). In vivo, the rarity of mIgE$^+$ cells could involve either a specific influence of cytokines and cell interactions promoting IgE CSR or specific signals from the mIgE BCR. Globally, mIgE$^+$ cells have only been observed transiently and in minute amounts in vivo after B cell activation, but never as resting memory B cells. These peculiar restrictions of IgE synthesis prompted us to look for a putative mIgE-BCR-dependent and B cell intrinsic self-control. To explore mIgE

specificities, we designed models uncoupling mIgE expression from immune activation, and we evaluated the potential changes of B cell fate resulting from mIgE expression per se. We checked whether mIgE can mimic mIgM and support B cell survival, as shown for other HCs (Duchez et al., 2010; Horikawa et al., 2007; Lutz et al., 1998). We show that, independent of stimulation, mIgE expression per se alters the B cell phenotype in multiple regards, with notably a short lifespan.

RESULTS

Premature Cε Expression Ablates Mature B Cells

While abundant in vitro upon stimulation in the presence of IL-4, mIgE$^+$ cells barely appear in vivo. We forced mIgE expression and set up the εKI mutation replacing Sμ with a human C_ε encoding both secreted IgE and mIgE (Figures S1A and S1B). Human IgE weakly binding mouse Fcε receptors was chosen to prevent nonspecific staining (Wesemann et al., 2011). Heterozygous wt/εKI mice were derived, but no B cell expressing IgE appeared (Figure S1C). This culminated in complete B cell lymphopenia in εKI/εKI animals, with bone marrow expansion of CD43$^+$/CD25$^-$ pro-B (16.5% to 94.7% of all B220$^+$/CD19$^+$ cells, $p < 0.0001$) but lack of CD25$^+$/B220$^+$ pre-B cells (43.4% to 0.05% of all B220$^+$/CD19$^+$ cells, $p < 0.0001$), while both follicular and marginal zone B cells were absent (Figure S1D). Neither murine Ig nor human IgE were detected in blood (Figure S1E). This did not involve defective association of human ε HC with mouse surrogate light chains since a transfected human ε HC efficiently reached the surface of mouse 18–81 pre-B cells (Figure S1F).

Apoptosis and Poor Survival of Primary mIgE$^+$ Cells

We set up a staining protocol that removed bound soluble IgE from the cell surface (Figure S1G) and confirmed that true mIgE$^+$ cells appear in vitro under anti-CD40/IL-4 stimulation of wt mouse B cells. They proved more prone to spontaneous or cytokine-deprivation induced apoptosis than mIgG1$^+$ cells (Figures 1A and 1B). Decreased ΔΨm potential suggested mitochondrial apoptosis, although use of bcl2 transgenic cells preserved a difference between mIgG1$^+$ and mIgE$^+$ cells (Figures 1A and 1B).

LAT_{Y136F} mutant mice overproducing T_H2 cytokines (Genton et al., 2006) generated [B220$^+$, IgE$^+$] cells in vivo, expressing CD138 much more frequently than IgM$^+$ or IgG$^+$ cells and thus likely engaged in PC differentiation (Figure 1C). Two days after bromodeoxyuridine (BrdU) injection, these cells stained as short-lived BrdU$^+$ cells (Figure 1D).

To compare survival according to Ig class, we transferred LAT_{Y136F} splenocytes into RAG2$^{-/-}$ γC$^{-/-}$ mice. Cell survival was estimated by monitoring levels of μ, γ1, and ε HC transcripts in spleen 48-hr post-transfer compared with their "input" amount in transferred cells. Differences appeared between persisting μ expression, falling γ1 levels, and still more strongly vanishing ε expression. The faster disappearance of ε versus γ1 and μ transcripts affected not only membrane but also secreted Ig transcripts, refuting the hypothesis that mIgE$^+$ cells disappeared by differentiating into PCs (Figure 1E).

Convergent observations were made with in vitro CD40L/IL4-stimulated human cells grafted to RAG2$^{-/-}$ γC$^{-/-}$ mice: transcripts evaluated after 48 hr indicated collapsed IgE production compared with IgM (Figure 1F).

mIgE-Dependent Changes in B Cells

The short lifespan of IgE-switched wt or LAT_{Y136F} cells might relate to either mIgE expression per se or a short-lived program promoted by T_H2 stimuli. We designed models for mIgE expression in B cells without prior stimulus by transiently transfecting human or murine cell lines. Expression vectors encoded either the short or long (m_S or mL) form of human mIgE, or mIgM as a control. In all cases, cytometry showed increased early apoptosis (with annexin V staining) specific to mIgE$^+$ cells. Decreased ΔΨm potential confirmed mitochondrial apoptosis (Figures 2A and 2B). Accordingly, apoptosis was inhibited by cyclosporin A or caspase inhibitor Q-VD-OPh (Figure 2B). Thus, mIgE expression promotes apoptosis independently of B cell stimulation in cell lines. The 28 amino acid long mIgE tail was reported to interact with Hax1, putatively through a conserved tyrosine-based DYANILQ motif in mouse IgE (found as DYTNVLQ in human IgE, but absent in tails of all other Ig classes) (Oberndorfer et al., 2006). It was recently shown that Hax1 is an apoptosis inhibitor binding mitochondria and that the vpr protein triggers apoptosis by relocalizing Hax1 (Simmen, 2011). We wondered whether mIgE expression could also relocate Hax1 and carried out flow imaging of BL41 transfectants. While transfection with control vectors preserved homogeneous Hax1 staining, mIgE expression associated with Hax1 relocalization and heterogeneous staining (quantified by flow-imaging bright detail evaluation) (Figures 2C and 2D). Mouse IgE$^+$ primary cells also featured increased heterogeneous Hax1 staining (Figures 2D and S2A). Co-localization experiments of Hax1 and mitochondria-targeted DsRed showed mitochondrial Hax1 depletion in mIgE$^+$ cells (Figure 2E). Finally, Hax1 staining globally increased in mIgE$^+$ cells (mean fluorescence intensity 36,824 for mIgG1$^+$ versus 68,864 for mIgE$^+$ mouse primary B cells and 145,667 for empty vector-transfected versus 332,353 for mIgE-transfected BL41 cells). This might result from lowered Hax1 catabolism upon binding IgE, since Hax1 normally undergoes rapid proteosomal degradation (Li et al., 2012).

We also inserted a VDJ segment upstream of Cε in the εKI construct, checking that it supported mIgE expression in transfected murine A20 cells. While a Cμ control construct readily yielded stable mIgM$^+$ transfectants, mIgE expression first appeared transient. Obtaining stable transfectants required repeated sorting of mIgE$^+$ cells. Both transiently transfected and sorted/stabilized mIgE$^+$ cells constantly showed higher apoptosis than untransfected cells or mIgM$^+$ transfectants (Figure S2B). We looked for transcriptional changes between untransfected, stable mIgM$^+$ and two independent stable mIgE$^+$ A20 transfectants. Unsupervised analysis clustered both εHC transfectants together, while the μHC transfectant clustered with untransfected A20. The strongest (over 3-fold) changes in mIgE$^+$ cells fell into four major categories (Figure 3A; Table S1):

- Apoptosis: several *pro-apoptotic genes* were strongly upregulated including Phlda3, Card12, and two inducers of mitochondrial apoptosis (Map3K9 and Bim), while some

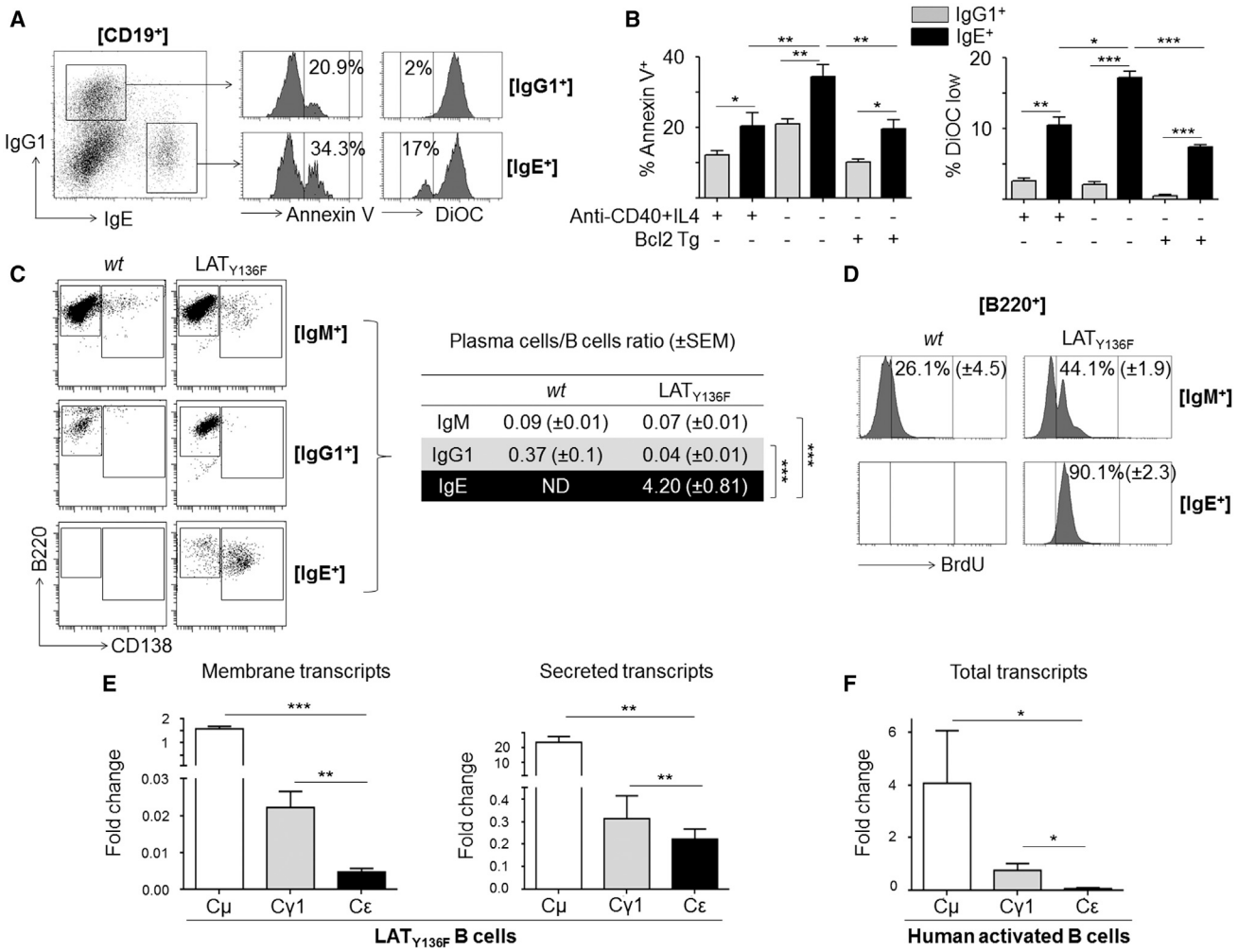


Figure 1. Primary Class-Switched IgE$^+$ B Cells Are Short Lived and Sensitive to Apoptosis

(A and B) After stimulation of splenocytes from wt (n = 8) or bcl2 transgenic mice (n = 6), apoptosis was determined after 24 hr cytokine deprivation (data are means ± SEM, three experiments, unpaired t test between mice and paired t test between isotypes).

(C) Splenocytes split by flow cytometry into B lymphocytes (B220$^+$/CD138$^-$) and PCs (CD138$^+$). Ratios were quantified for each Ig class.

(D) Amounts of recently divided (BrdU$^+$) and long lived (BrdU$^-$) cells from wt or LAT$_{Y136F}$ mice (data are means ± SEM, n = 7, three experiments, paired t test).

(E) Lymphocytes from LAT$_{Y136F}$ mice were transferred into RAG2$^{-/-}$ γC$^{-/-}$ mice. Cμ, Cγ1, and Cε membrane and secreted transcripts were quantified by qPCR, estimating survival of the corresponding B cells (data are means ± SEM, n = 6, three experiments, paired t test).

(F) Human lymphocytes were stimulated and transferred into RAG2$^{-/-}$ γC$^{-/-}$ mice. Cμ, Cγ1, and Cε total transcripts were quantified by qPCR (data are means ± SEM, n = 4, four experiments, Mann-Whitney test).

See also Figure S1.

anti-apoptotic genes were underexpressed, including Bcl3 and Cdkn1a.

- Metabolism: upregulation of DecR1, a fatty acid catabolism enzyme inhibiting cell proliferation; Slc16a3, which extracts lactate; and Bhd1, a ketone pathway regulator.
- Signaling and mobility: two transporters extracting cytosolic Ca^{2+}, Slc24a3, and Npc1l1 were upregulated. The Rgs13 inhibitor of small GTP protein-coupled receptors (GPCRs) known to reduce intra-GC B cell mobility (Hwang et al., 2013) was overexpressed, which might alter cytoskeleton reorganization and responses to chemokines. Finally, downregulation affected the γc chain of IL-2-4-7-21 receptors (major receptors for B cell activation), the semaphorin receptor plexin D1 that normally promotes B cell mobility (Holl et al., 2011), S1pR1, promoting lymphoid egress from GCs, and the CXCL13 receptor CXCR5.
- Other surface receptors: these included underexpressed integrins and receptors involved in adhesion to laminins or glycosaminoglycans (Itga6, Vsig1, CD97…) and in B-T cell interactions or cytokine responses (CD70, CD69, IL2Rg) and increased expression of the IgG/IgE-receptor FcγR4.

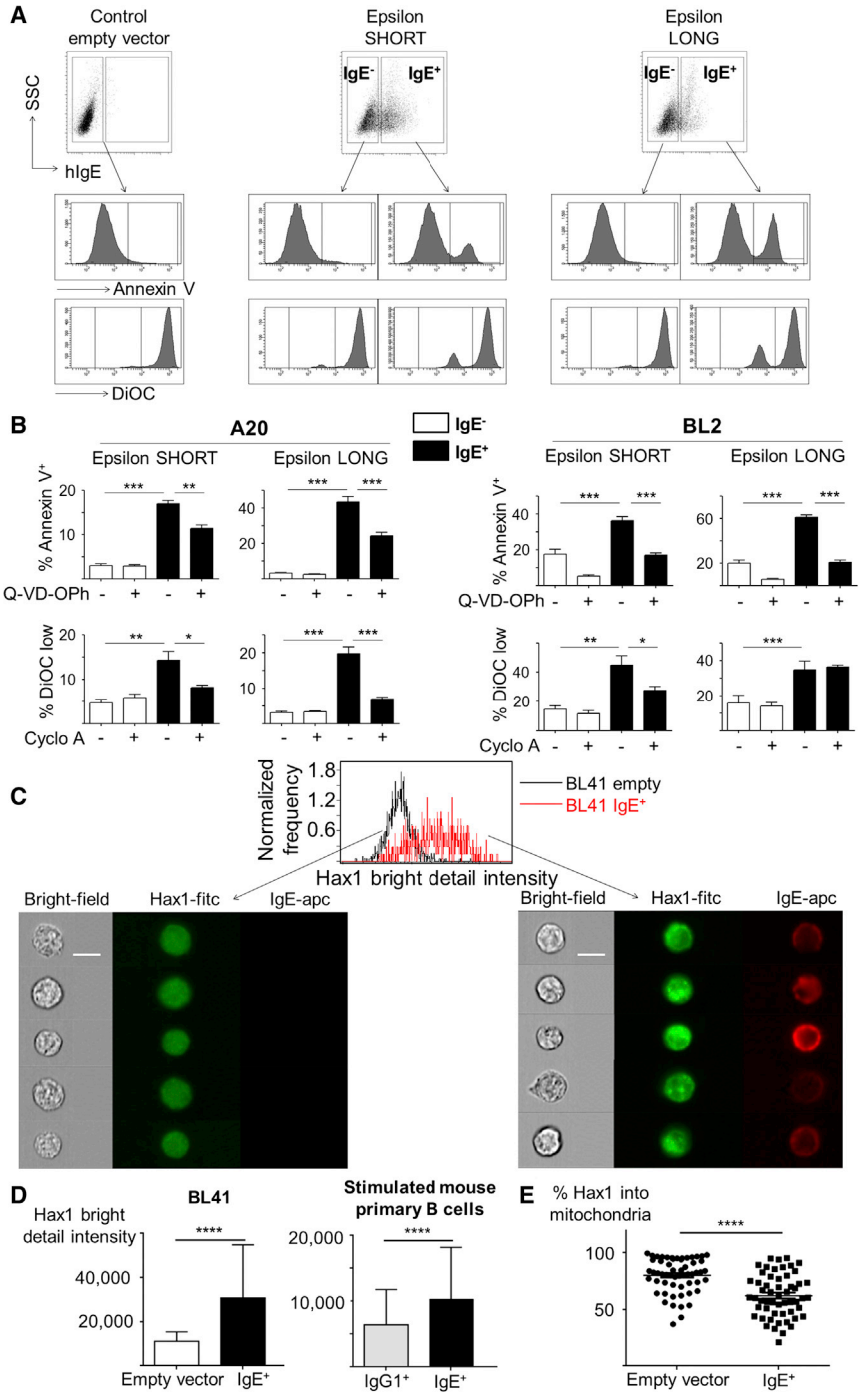


Figure 2. Membrane IgE Induces Apoptosis and Hax1 Relocalization in B Cells

(A and B) Transient transfection of A20 and BL2 B cell lines with expression vectors for SHORT or LONG mIgE. Annexin V and $DiOC_6$ staining were compared between $mIgE^+$ and $mIgE^-$ cells in the absence (–) or presence (+) of Q-VD-OPh or cyclosporine A (Cyclo A) (data are means ± SEM, n = 6, three experiments, paired t test).

(C and D) Hax1 staining patterns compared by flow imaging between BL41 $mIgE^-$ and $mIgE^+$. Increased width of "bright detail intensity" peak indicates increased heterogeneity of staining and was quantified in transfected BL41 (n = 3, two experiments each evaluating ~1,000 IgE^+ cells) or stimulated mouse splenocytes (n = 4, two experiments each evaluating ~1,800 IgE^+ cells) (scale bar represents ~20 μm; data are means ± SD).

(E) Hax1 colocalization to mitochondria in BL41 cells (three experiments evaluating ~60 IgE^+ cells, unpaired t test).

See also Figure S2.

Variations of apoptosis-related genes fitted with the observed ongoing apoptosis of $mIgE^+$ cells. Other changes likely to impact cell fate related to signaling, cell adhesion, mobility, and thus optimal interactions with the microenvironment, notably due to underexpressed plexin D1 and/or increased RGS13 and GPCR inhibition thereof. A20 $mIgE^+$ transfectants indeed showed actin cytoskeleton reorganization and loss of pseudopods by confocal microscopy (after phalloidin labeling) and increased circularity by flow imaging (Figure 3B). Increased circularity also marked primary cells induced in vitro to ε compared with γ1 CSR (Figure S3A).

Basal intracellular tyrosine phosphorylation and phospho-ERK levels were decreased in $mIgE^+$ A20 transfectants (Figure 3C). In addition, $mIgE^+$ cells showed spontaneous BCR co-localization with lipid rafts (Figure 3D) and increased abundance of rafts both in transfected A20 and $mIgE^+$ activated primary B cells compared with $mIgM^+$ or $mIgG1^+$ cells from the same culture (Figure S3B).

In $mIgE^+$ transfectants, the aforementioned increased FcγR4 transcription correlated with increased surface expression and binding of fluorescently labeled IgE (Figure S3C). Since genes connected to mobility showed variations, we assayed responses to chemokines of primary and transfected $mIgE^+$ cells. In all cases, $mIgE^+$ cells proved less reactive to either CXCL12, CXCL13, or mixed chemokines produced by lymphoid stromal cells (Figures 3E and S3D).

$mIgE^+$ *cre*-Switched B Cells Internalize mIgE BCR, Lose Mobility, and Are Short Lived

Since εKI mice were B-less, we generated μεKI animals whose B cells initially express Cμ and can later be *cre*-deleted toward Cε expression. The Cε gene encoded both secreted IgE and mIgE

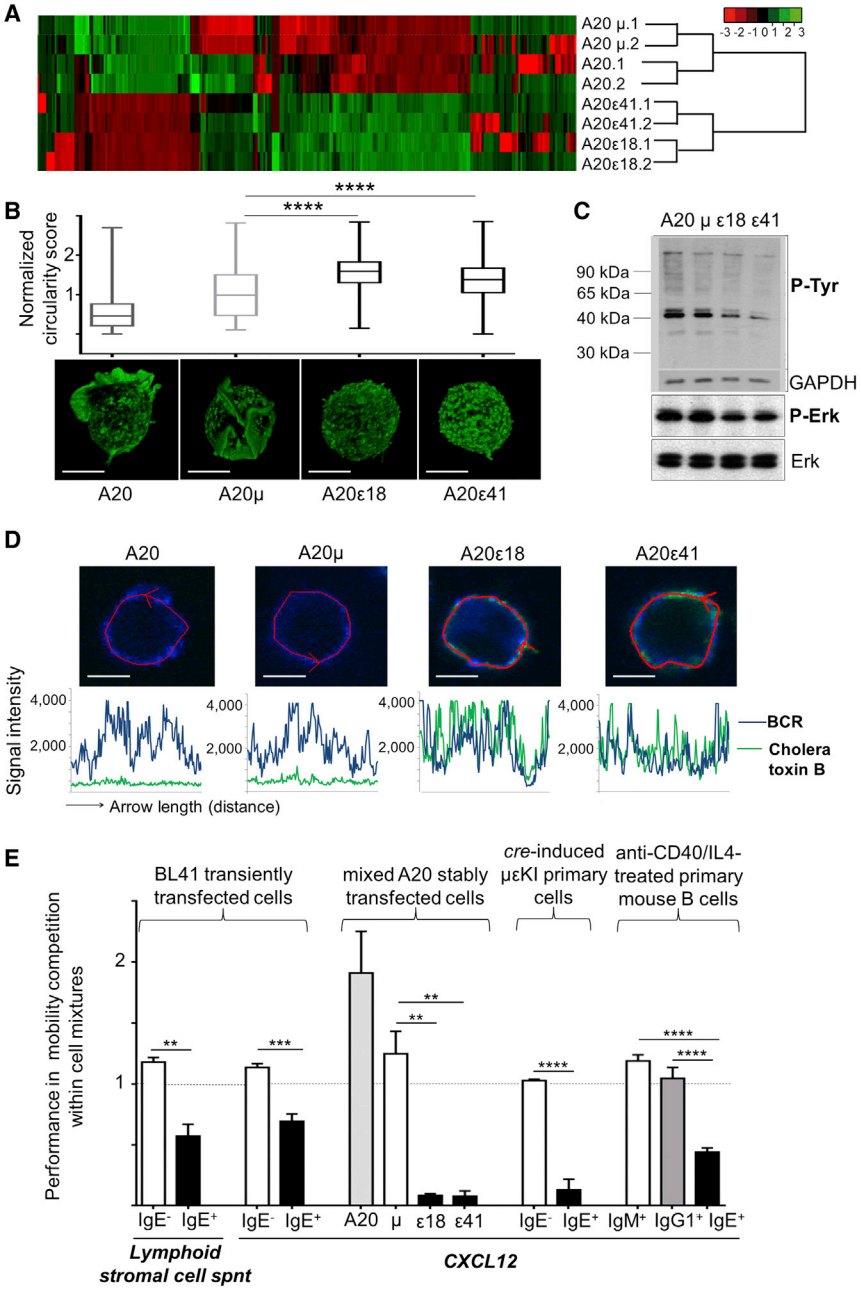


Figure 3. Phenotypic Changes Induced by Membrane IgE

(A) Unsupervised clustering split mIgE$^+$ transfectants and untransfected or mIgM$^+$ A20.

(B) (Top) Circularity was quantified for A20 cells and IgM or IgE transfectants by flow imaging (data are represented as boxplot with whiskers from min to max, ~5,000 cells, two experiments, unpaired t test) and parallel confocal microscopy (bottom, representative cells).

(C) (Top) Phosphorylation of proteins from A20 and transfectants; GAPDH is a loading control (two experiments). (Bottom) Erk-phosphorylation (two experiments).

(D) BCR (in blue) and lipid rafts stained by cholera toxin (green) show increased co-localization (i.e., superimposition of curves) in IgE+ cells (representative cells, three experiments) (quantification followed red arrows along cell contours; scale bar represents ~5 μm).

(E) Cell mobility experiments in response to gradients of (left) a mix of chemokines produced by human stromal cells, and (right) recombinant CXCL12 (data are means ± SEM, n = 3 to 8, five experiments, paired t test).

See also Figure S3.

(Karnowski et al., 2006) (Figure 4A). In homozygous mice, the μεKI mutation and associated Sμ deletion homogenously imposed human IgM expression instead of any mouse Ig (Figure 4B). Mating with creERT2 mice yielded tamoxifen-inducible IgE expression. In vivo, *cre* deletion was efficient at the DNA level in spleen (Figure S4A). It also massively occurred in vitro in immortalized IgM$^+$ hybridomas from μεKI/creERT2 mice (Figure S4B). While inducible in primary B cells in vivo and in vitro, IgE detection required intracellular staining, reminiscent of requirements for staining mIgE$^+$ primary cells (Wesemann et al., 2011). Contrasting with massive *cre* deletion at the DNA level in spleen, most primary B cells retained IgM expression in vivo, with IgE expression remaining below 4% and vanishing within 4 weeks after *cre* induction (Figure 4C). Cμ deletion and subsequent mIgE "*cre*-CSR" were thus counterselected in B cells and did not yield long-lived mIgE$^+$ cells. Also, similar to normal primary mIgE$^+$ cells (and to transfected cells), *cre*-switched cells showed reduced chemokine-induced mobility (Figure 3E). Feeding mice with BrdU for 10 days, 20 days after tamoxifen induction, distinguished nonproliferating "long-lived" cells from recently divided "short-lived" cells: IgE$^+$ cells overwhelmingly scored as BrdU$^+$, i.e., short lived (Figure 4D), in agreement with transient mIgE expression in blood or spleen lymphocytes. In serum, human IgE peaked at the same time as IgE$^+$ cells, but persisted at levels around 1,000 KU/l after IgE$^+$ cells completely vanished from blood and spleen, over 5 months after tamoxifen induction. Memory of *cre* switching was thus only attested to by a few remaining IgE secreting cells (Figure 4C).

Direct mIgE staining has been documented as weak or absent on primary B cells (Karnowski et al., 2006; Wesemann et al., 2011). Since it was also virtually absent in μεKI B cells, we wondered whether this could reflect spontaneous mIgE internalization. To preserve membrane receptor trafficking, B cells were stained at 37°C instead of 4°C, with fluorescently labeled anti-IgE antibodies. This clearly and specifically stained tamoxifen-induced IgE$^+$ μεKI cells, showing that mIgE BCRs do reach the

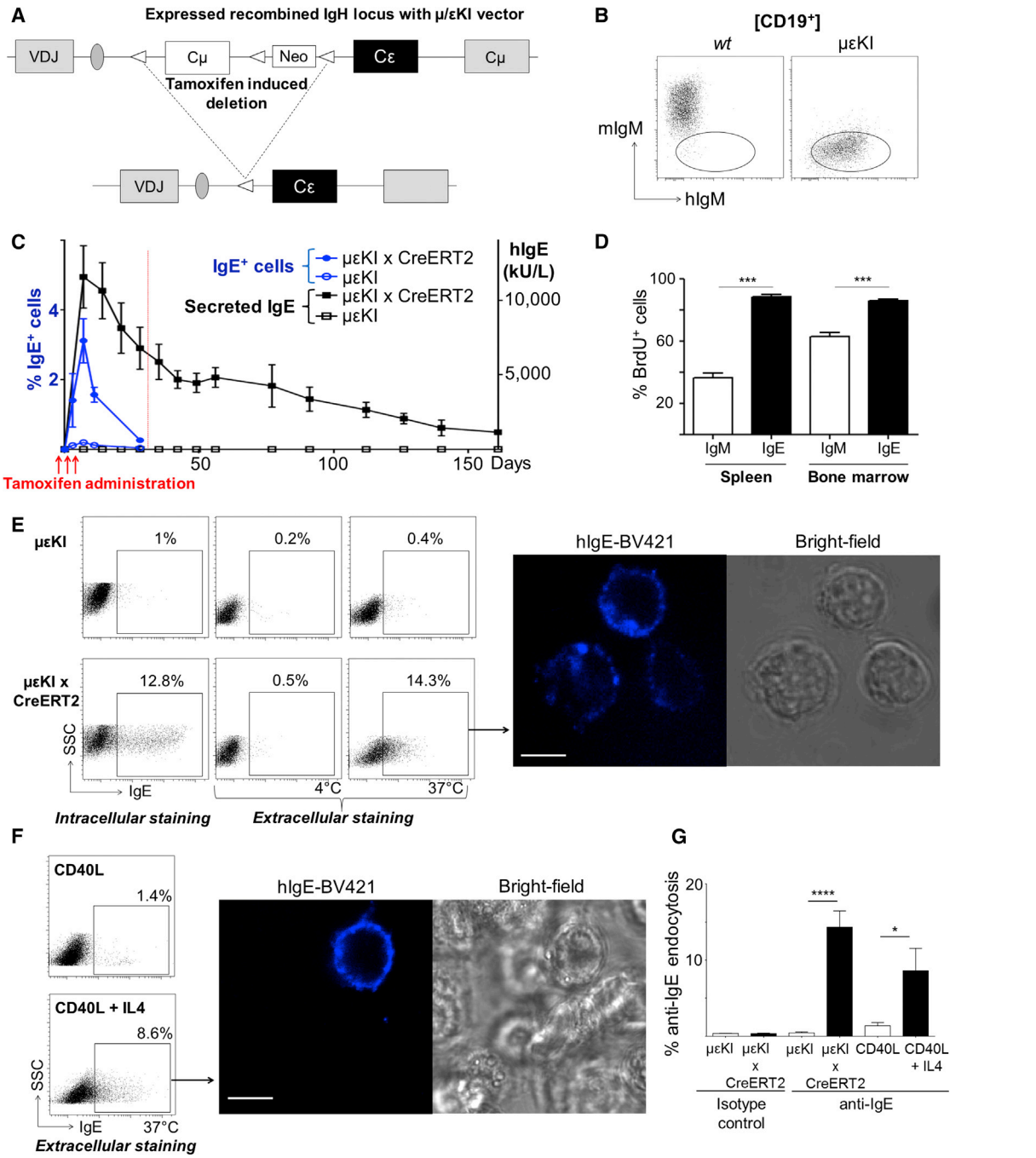


Figure 4. μεKI Mutant Mice

(A) (Top) Targeted IgH locus (not to scale) with floxed human Cμ gene, neo^r cassette, and Cε gene. (Bottom) The μεKI locus normally undergoes V(D)J rearrangement, expressed with human Cμ; after breeding with CreERT2 mice, Cε is expressed upon *cre* deletion of Cμ.

(B) B cells from μεKI mice express human IgM BCR (n = 20, five experiments).

(C) μεKI x CreERT2 mice were sacrificed 1 day to 5 months after tamoxifen administration. IgE B cells among splenocytes were quantified by cell cytometry after intracellular staining (mean ± SEM, n = 3 to 7 mice per group, four experiments); human IgE was evaluated in sera.

(D) BrdU was injected 10 days before sacrifice, 20 days after tamoxifen treatment, and incorporation was evaluated (mean ± SEM, n = 5, two experiments, paired t test).

(E) Cell cytometry (left) of IgE expression among [CD19$^+$] cells in μεKI × CreERT2 mice 24 hr after *cre* induction, readily detects IgE after permeabilization but not by surface labeling at 4°C. In contrast, 6-hr incubation at 37°C with fluorescent anti-IgE antibodies stains cells specifically from mice expressing *cre* (n = 9, four experiments, paired t test). Confocal imaging of internalized fluorescent antibodies (right ; scale bar represents ~10 μm).

(legend continued on next page)

membrane but are then internalized, eventually together with a labeling antibody (Figures 4E and 4G).

Similarly, in CD40L-stimulated human primary B cells, IgE expression appeared faint after staining at 4°C but strongly increased by staining at 37°C and was restricted to IL-4-exposed cells (Figures 4F and 4G).

DISCUSSION

Class-specific variations of BCR functions are incompletely understood (Laffleur et al., 2014). Compared with IgM, cross-linking of class-switched BCRs more strongly boosts PC differentiation (Horikawa et al., 2007; Pogue and Goodnow, 2000; Sato et al., 2007; Waisman et al., 2007; Wakabayashi et al., 2002). Specific mIgG or mIgE interactions were reported with Grb2 (Engels et al., 2009). The mIgE tail also interacts with Hax1, which binds the Syk target Hs1 (Batista et al., 1996; Oberndorfer et al., 2006). However, transfected cells reacted similarly to mIgE or mIgM cross-linking (Poggianella et al., 2006).

Independent of cross-linking, it is unknown whether constitutive signals from the various HC classes differentially support B cell survival. Rare primary class-switched mIgE$^+$ lymphocytes are mandatory precursors of IgE PCs (Achatz et al., 1997; Brightbill et al., 2010). The elusive nature of the mIgE$^+$ stage in vivo has been attributed to accelerated PC differentiation, increased apoptosis, or poor Sε efficacy during CSR, three non-mutually exclusive explanations (He et al., 2013; Misaghi et al., 2013; Wu and Zarrin, 2014; Yang et al., 2012). The T_H2 context yielding mIgE$^+$ cells might also shorten their survival, similar to some T cells in which Socs-1 induction by IL-4, IL-13, and Stat6 promotes activation-induced cell death (Alexander, 2002; Hebenstreit et al., 2003; Oh et al., 2012). Whatever the mechanisms involved, altogether they limit IgE production in vivo to levels 10,000- to 100,000-fold lower than other classes.

Since mIgE$^+$ cells abundantly arise in vitro from B cell stimulation, we suspected that one basis for their in vivo rarity might be that mIgE expression per se modulates B cell fate. To assay such a potential mIgE effect independently of any antigen-dependent cross-linking and T cell help, we first forced mIgE expression in mice by replacing Sμ with a Cε Ig gene. Early in vivo ε expression instead of μ HC in homozygous εKI mice abrogated B cell lymphopoiesis. Tonic signals successively provided by the pre-BCR and BCRs support B cell development (Mårtensson et al., 2007; Rossi et al., 2006). Albeit with variable efficiencies, tonic signals provided by δ, γ, or α HCs can replace μ during lymphopoiesis (Duchez et al., 2010; Horikawa et al., 2007; Lutz et al., 1998). The εKI lymphopenia shows that ε differs from other HCs in this regard.

We explored mIgE expression in [VDJ-Cε]-transfected cells and found it unstable in prolonged cultures. Additionally and reminiscent of the εKI B cell defect, mIgE$^+$ transfected cells showed multiple functional and morphologic changes, with defective chemokine-triggered mobility, decreased intracellular tyrosine phosphorylation, increased circularity, BCR clustering into lipid rafts, and finally increased apoptosis. Associated transcriptional changes had the potential to inhibit responses to chemokines (with modified RGS13, integrins, and plexin D1 expression), modulate receptors involved in T-B interactions, affect metabolism, and promote apoptosis. Multiple phenotypic alterations were common to various mIgE$^+$ cells (transfected cells, primary cells, and knock-in models). Interestingly, mIgE expression induced relocalization of the Hax1 anti-apoptotic protein, suggesting that the mIgE intracellular tail could be a sink for Hax1.

Endogenous mIgE expressing B cells obtained in vitro (after stimulation of human or wt mouse B cells) or ex vivo showed mIgE to be transiently expressed and internalized. These mIgE$^+$ cells, recently divided, survived less than other B cells upon transfer into immunodeficient mice.

To characterize mIgE function in primary B cells independently of immune stimulation, we studied μεKI B cells expressing mIgE upon *cre*ERT2 induction. It is known from previous studies that creERT2 has no major toxicity in B cells (Dogan et al., 2009). In μεKI mice, the *cre* deletion was counter-selected in B cells, and rare mIgE$^+$ B cells were again short lived both in vivo and in vitro. Similar to transfectants or classical switched B cells, they expressed low levels of a rapidly internalized IgE BCR. They also poorly responded to CXCL12 and easily underwent apoptosis. In contrast, IgE secretion lasted for months in μεKI mice after induction, indicating long-term survival of IgE PCs.

Altogether, our data suggest an immobile, reprogrammed, and rapidly apoptotic phenotype of mIgE$^+$ cells notably involving Hax1 sequestration by mIgE and inhibition of responses to chemokines. This phenotype directly imposed by mIgE expression prior to any antigen encounter may explain the short lifespan and in vivo rarity of mIgE$^+$ cells. It is thus questionable whether, among IgE$^+$ cells, the high ratio of PCs represents boosted PC differentiation or accelerated death of non-PC cells. Since the mIgE$^+$ stage is mandatory for PC differentiation (Achatz et al., 1997), it appears as a bottleneck limiting IgE memory (prohibiting survival of mIgE$^+$ memory B lymphocytes, but allowing a few to escape deletion, reach the mIgE stage, and survive as PCs). Existence of long-lived PCs is consistent with the common observation of long-term IgE atopy. Noticeably, PCs deriving from cells with a mIgE BCR poorly respond to CXCL12 (Achatz-Straussberger et al., 2008), possibly accounting for their absence in bone marrow (He et al., 2013). This suggests that part of the mIgE$^+$ phenotype is durably imprinted into PC differentiation afterward.

Altogether, a reasonable picture of IgE responses in light of our data and previous studies is that multiple mechanisms concur to restrict IgE production to minute amounts. IgE CSR is less efficient than IgG CSR; it yields mIgE$^+$ cells only appearing

(F) Cell cytometry (left) of IgE expression among stimulated human B cells, incubated 6 hr at 37°C with fluorescent anti-IgE. Confocal imaging of internalized fluorescent antibodies (right; scale bar represents ~10 μm) (n = 6, three experiments, Mann-Whitney test).

(G) Quantification of endocytosis from data of (E) and (F). BCR endocytosis was quantified in mouse primary cells with the 37°C IgE staining procedure, using either μεKI or μεKI × creERT2 cells treated with tamoxifen, and in human B cells stimulated for 4 days with either CD40L or CD40L + IL4. Data are means ± SEM. See also Figures S1A and S4.

transiently in both humans and mice, poorly effecting migrations associated with maturation within lymphoid organs and thus quickly eliminated by spontaneous and activation-induced apoptosis without survival as mIgE$^+$ memory cells. Only few IgE PCs might eventually survive this pathway, become long-lived, and solely ensure true long-term IgE memory. Given the short half-life of IgE, such rare long-lived IgE PCs are likely responsible for immediate IgE allergy, while mIgM$^+$ or mIgG$^+$ memory cells can eventually generate new IgE cells after CSR. Direct or sequential CSR was indeed documented as providing new IgE$^+$ cells from either Ag-experienced mIgM$^+$ or mIgG1$^+$ cells (Xiong et al., 2012).

This multifaceted restriction of IgE responses most likely attests to the beneficial accumulation throughout evolution of multiple means to both keep on producing the most powerful magic bullet of adaptive immunity while maintaining its hazardous production under tight control. Each of these specific constraints on the IgE B cell compartment will clearly deserve in-depth molecular analysis in the future.

EXPERIMENTAL PROCEDURES

Mice

RAG2$^{-/-}$ γC$^{-/-}$ (Colucci et al., 1999), AID$^{-/-}$ (Muramatsu et al., 2000), and LAT$_{Y136F}$ (Aguado et al., 2002; Genton et al., 2006) mice were used. *εKI* mice carried an inserted human Cε gene replacing Sμ, *μεKI* mice carried a floxed human Cμ followed by Cε (expressed upon *cre* deletion of Cμ). Detailed methods are described in Supplemental Experimental Procedures.

B Cell Transfectants

Expression of rearranged Ig chains was in A20, BL2, and BL41 cell lines. Detailed methods are described in the Supplemental Experimental Procedures.

Transcription Analysis

RNA was analyzed by qPCR for expression of μ, γ, and ε Ig HC secreted and membrane forms. Microarray analyses were with Agilent chips. Detailed methods are described in the Supplemental Experimental Procedures.

Cell Cultures

Sorted B cells were stimulated for IgE CSR in the presence of IL-4. In transfer experiments, cells were injected intravenously into RAG2$^{-/-}$ γC$^{-/--}$ mice. Detailed methods are described in Supplemental Experimental Procedures.

Cytometry, Confocal Microscopy, and Flow Imaging

Proliferation was monitored by BrdU incorporation. Apoptosis was monitored by Annexin V and DiOC$_6$ staining. Detailed methods are described in Supplemental Experimental Procedures.

Cell Mobility

Evaluation was done using transwells and recombinant or natural chemokines. The detailed method is described in Supplemental Experimental Procedures.

Statistical Analyses

Student's t test or Mann-Whitney tests were used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

ACCESSION NUMBERS

Microarray data have been deposited to the GEO database under accession number GSE64130.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.023.

AUTHOR CONTRIBUTIONS

M.C. designed experiments and wrote the manuscript. B.L. and M.C. performed the experiments, analyzed data, and prepared the figures. S.D., N.D.-L., S.P., C.C., and Y.D. participated in the experiments. K.T. discussed the data and contributed useful reagents. M.C. obtained financial grants and agreements to perform the study.

ACKNOWLEDGMENTS

We thank the staff of our transgenic facility and cell imaging facilities; Mylène Brousse and Sandrine Lecardeur for technical assistance; Tasuku Honjo, Bernard Malissen, Marie-Christine Birling, and James Di Santo for respectively providing AID$^{-/-}$, LAT$_{Y136F}$, ubiquitous creERT2, and RAG2$^{-/-}$ γC$^{-/-}$ mice; Patrick Smith for anti-FcγRIV antibody; and Jeanne Cook-Moreau for critical reading of the manuscript. This work was supported by Ligue Nationale Contre le Cancer, Association pour la Recherche sur le Cancer (grant SL220100601332), Institut Universitaire de France, and Conseil Régional du Limousin. B.L. and S.D. PhD fellowships were from CNRS, Région Limousin and Association pour la Recherche sur le Cancer.

Received: September 8, 2014
Revised: October 21, 2014
Accepted: January 8, 2015
Published: February 12, 2015

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