Immature B cells preferentially switch to IgE with increased direct Sμ to Sε recombination

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Immunoglobulin heavy chain (IgH) class-switch recombination (CSR) replaces initially expressed Cμ (IgM) constant regions (C_H) exons with downstream C_H exons. Stimulation of B cells with anti-CD40 plus interleukin-4 induces CSR from Cμ to Cγ1 (IgG1) and Cε (IgE), the latter of which contributes to the pathogenesis of atopic diseases. Although Cε CSR can occur directly from Cμ, most mature peripheral B cells undergo CSR to Cε indirectly, namely from Cμ to Cγ1, and subsequently to Cε. Physiological mechanisms that influence CSR to Cγ1 versus Cε are incompletely understood. In this study, we report a role for B cell developmental maturity in IgE CSR. Based in part on a novel flow cytometric IgE CSR assay, we show that immature B cells preferentially switch to IgE versus IgG1 through a mechanism involving increased direct CSR from Cμ to Cε. Our findings suggest that IgE dysregulation in certain immunodeficiencies may be related to impaired B cell maturation.

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Abbreviations used: αCD40, anti-CD40; AID, activation-induced cytidine deaminase; C, constant region; ChIP, chromatin immunoprecipitation; CSR, class-switch recombination; DSB, double-strand break; GL, germline; GLT, GL transcript(ion); IBC, early lineage and immature B cell derived from fetal liver cultures; Rag, recombination-activating gene; S, switch region; SCID, severe combined immunodeficiency.

Ig and T cell receptor variable region exons are assembled from component V, D, and J gene segments via V(D)J recombination. V(D)J recombination is initiated in developing lymphocytes by the recombination-activating gene (RAG) endonuclease, which is comprised of the RAG1 and RAG2 proteins (Matthews and Oettinger, 2009). RAG endonuclease introduces DNA double strand breaks at the borders of V, D, or J segments, which are then joined by classical nonhomologous end-joining to form complete V(D)J exons (Jung and Alt, 2004; Rooney et al., 2004; Weterings and Chen, 2008). In developing B lineage cells, the Ig heavy (IgH) chain variable region exon is assembled first in progenitor (pro) B cells, followed by Ig light (IgL) chain V-to-J recombination in precursor (pre) B cells (Bassing et al., 2002). Productive assembly of both IgH and IgL variable region exons gives rise to a diverse repertoire of IgM-expressing early lineage and immature B cell derived from fetal liver cultures (IBCs). Deficiency of either the RAG1 or RAG2 protein leads to a complete severe combined immune deficiency (SCID) as a result of inability to initiate V(D)J recombination (Schwarz et al., 1996). Mutations in mice or humans that severely impair, but do not totally block RAG1 or RAG2 function, can lead to a "leaky" SCID phenotype in which there are low numbers of peripheral B or T lymphocytes (Villa et al., 2001).

Upon activation by antigen in peripheral lymphoid organs, mature B cells may undergo IgH class-switch recombination (CSR), a process in which the IgH μ constant region exons (Cμ) are deleted and replaced by one of several sets of downstream C_H exons (e.g., Cγ, Cε, and Cα), termed C_H genes. CSR is the basis for IgH switching from IgM to other Ig classes (e.g., IgG, IgE, or IgA). CSR occurs within switch regions (S), which are 1–10-kb sequences located 5′ to each set of C_H genes (Chaudhuri et al., 2007). During CSR, DNA double-strand breaks (DSBs) are specifically induced in a donor S region (Sμ) upstream of Cμ and a downstream

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acceptor S region; these DSBs then are joined by classical nonhomologous end-joining, or an alternative DNA end-joining pathway (Yan et al., 2007), replacing Cμ with a downstream C_H gene. The activation-induced cytidine deaminase (AID) enzyme initiates both CSR and the related process of somatic hypermutation of Ig variable region exons via cytidine deamination activity. During CSR, AID-induced mutations in S regions are converted into DSBs. AID is targeted to S regions during CSR by transcription. In this regard, each S region is preceded by a promoter and a noncoding exon termed an "I" exon (Chaudhuri and Alt, 2004). Different forms of activation and/or cytokines provided by helper T cells or other cells can direct AID and, as a result, CSR to a particular target S region by specifically stimulating transcription from upstream I region promoters (Chaudhuri and Alt, 2004; Chaudhuri et al., 2007).

Stimulation of cultured splenic IgM^+ B cells with an anti-CD40 antibody (αCD40) plus IL-4, which mimics in vivo activation by T helper type 2 (T_H2) T cells, leads to the activation of NF-κB and Stat6 transcription factors, respectively, which, together with other transcription regulators, induce germline (GL) transcription (GLT) from Iγ1 and Iε promoters and CSR to Cγ1 or Cε (Bacharier and Geha, 2000). Although αCD40 plus IL-4 treatment theoretically can lead to direct CSR from Cμ to either Cγ1 or Cε, direct CSR to Cε occurs less frequently than to Cγ1 (Snapper et al., 1988; Bottaro et al., 1994; Jung et al., 1994; Purkerson and Isakson, 1994). Various studies have shown that IgE switching largely occurs through a sequential CSR mechanism, in which activated B cells first switch from IgM to IgG1 via direct CSR from Cμ to Cγ1, followed by switching to IgE via a "second step" CSR from Cγ1 to Cε (Yoshida et al., 1990; Siebenkotten et al., 1992; Mandler et al., 1993; Hodgkin et al., 1994). Indeed, these two CSR steps leading up to IgE switching can be separated by cellular division, hypermutation, and selection within germinal centers (Erazo et al., 2007). In this regard, $IgG1^+$ B cell intermediates in sequential CSR have been proposed to be a required part of the development of high-affinity IgE responses in vivo (Erazo et al., 2007). Sequential CSR to IgE has also been shown to occur through IgG intermediates in human B cells (Jabara et al., 1993; Baskin et al., 1997; Zhang et al., 1994; Mills et al., 1995). However, mice in which CSR to Sγ1 is abrogated through mutation of Iγ1 or deletion of Sγ1 can still undergo substantial switching to IgE, showing that an IgG1 intermediate is not absolutely required for switching to IgE (Jung et al., 1994; Misaghi et al., 2010). Whether or not intrinsic B cell properties exist that would favor direct IgE CSR versus direct IgG switching is unknown.

IgE antibodies play a central role in atopic diseases and in host protection against parasites. When engaged with antigen, specific IgE may activate mast cells and basophils to release potent inflammatory mediators (Geha et al., 2003). Serum IgE is thus normally found at levels 3–4 orders of magnitude less that the other Ig isotypes and is produced by a correspondingly low number of IgE-secreting cells (Saxon et al., 1980; King et al., 1990). Multiple different primary immunodeficiency conditions are associated with markedly elevated

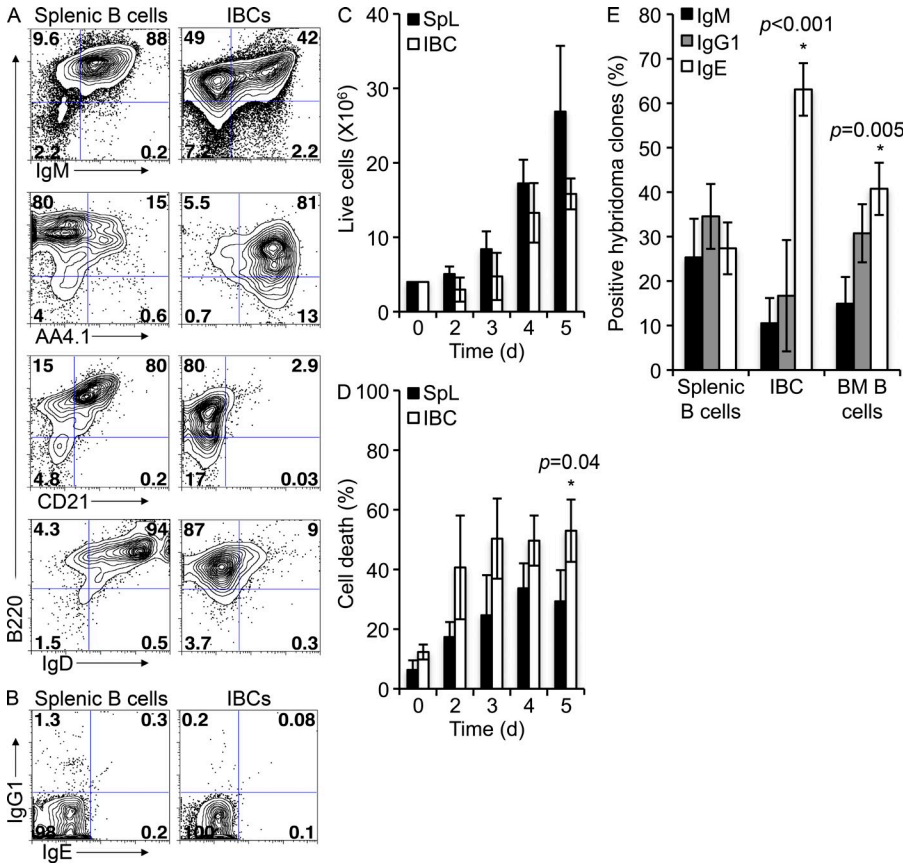

Figure 1. Fetal liver–derived IBCs display IgE CSR preference. (A) WT BALB/c fetal liver cells from embryonic day 15 were cultured in the presence of IL-7–producing T220 fibroblasts for 7 d to form IBCs. These cells were subjected to $B220^+$ magnetic separation and compared with adult splenic B cells purified in the same way. Immunophenotyping with the indicated surface markers is shown. (B) IgG1 and IgE stains of unstimulated adult splenic B cells and IBCs. (C and D) Graphs showing total number of live B cells in millions (C) and percent death rates by trypan blue inclusion (D) during activation with αCD40 plus IL-4. The experiments in A–D are representative of three independent experiments. (E) $B220^+$ purified adult splenic B cells, IBCs, and BM B cells were then stimulated with αCD40 plus IL-4 for 4 d, followed by fusion with the NS-1 myeloma cell line. Hybridoma clones were analyzed by ELISA for IgM, IgG1, and IgE secretion. Shown are the mean values ± SD for three independent experiments. Over 100 clones were analyzed per experiment. The p-values were calculated using the two-tailed Student's *t* test.

serum IgE levels (Ozcan et al., 2008). In this context, "leaky" human SCID patients (e.g., having very low peripheral B and T cell numbers) caused by debilitating, but not complete, inactivation of RAG proteins are associated with severe manifestations of immune dysregulation as exemplified by Omenn syndrome, which is characterized by immune deficiency, erythroderma, lymphadenopathy, tissue inflammation, and high IgE levels (Villa et al., 1998). Certain mouse models, such as mice harboring a homozygous point mutation in which serine 723 of RAG1 is converted to a cysteine (referred to here as $Rag1^{S723C}$ mutant mice) recapitulate features of Omenn syndrome. In particular, $Rag1^{S723C}$ mutant mice have a partial block of lymphocyte development at progenitor T and B cell stages caused by impaired ability to initiate V(D)J recombination; but despite severely reduced peripheral B cells numbers, they may paradoxically have abundant serum IgE (Giblin et al., 2009; Walter et al., 2010). Although the molecular underpinnings of the different primary immunodeficiency states associated with hyper-IgE are diverse, many lead to impaired lymphocyte development and function (Geha et al., 2003; Ozcan et al., 2008). In the current study, we test the hypothesis that B lineage cell developmental stage influences IgH CSR preference.

RESULTS

Fetal liver–derived IBCs display IgE CSR preference

Although CSR is usually considered in the context of mature B cells, previous studies have shown that immature B cells derived from fetal liver and BM cultures, as well as transformed pre–B cell lines have the capacity to undergo CSR (Akira et al., 1983; Rothman et al., 1990a,b; Rolink et al., 1996). Based on the finding that increased IgE is often associated with primary immune deficiencies that impair early B cell development, we hypothesized that CSR preference to Cγ1 versus Cε might differ in mature versus immature B cells. To test this, we compared the Cγ1/Cε preference of αCD40 plus IL-4–activated mature B cells from adult (age 2–4 mo) BALB/c spleens to that of immature B cells derived from BALB/c fetal liver cultures. Progenitor cells in the liver of WT day 15 mouse embryos develop into early B lineage cells, including pre–B and immature B cells, in the presence of IL-7–producing fibroblasts (Borzillo et al., 1992; Milne et al., 2004). We used this system to develop early B lineage and immature B cells that we will collectively call IBCs.

After 8 d in culture, embryonic day 15 liver cells develop into a population that expresses the pan–B lineage B220 and is uniformly positive for the early marker AA4.1, which stains the CD93 antigen. AA4.1/CD93 is found on all early stages of B lineage development, including the transitional B cell stage shortly after emigration from the BM to the spleen (Li et al., 1996; Allman and Pillai, 2008; Yamane et al., 2009). The IBCs are negative for markers found on more mature B cells such as IgD and CD21 (Fig. 1 A). About half of the IBCs are positive for surface IgM (Fig. 1 A) and all are negative for IgG1 and IgE (Fig. 1 B). When removed from the IL-7–containing culture conditions and stimulated for 4 d with αCD40 plus IL-4, IBCs formed blasts and proliferated similarly to mature splenic B cells (Fig. 1 C), with at most modestly increased death rates (Fig. 1 D). To measure IgH CSR, activated B cells from day 4 cultures were fused to the NS1 hybridoma fusion partner, and the resulting hybridomas were tested by ELISA for secretion of IgM, IgG1, or IgE. These analyses revealed that, under the same stimulation conditions, ∼63 ± 6% of IBC-derived hybridomas secreted IgE versus ∼27 ± 6% of splenic B cells (Fig. 1 E). Correspondingly, we observed a trend, although nonsignificant, toward decreased IgG1-secreting hybridomas from IBCs (17 ± 11%) versus those from splenic B cells (35 ± 7%; Fig. 1 E). We also found that BM B lineage cells stimulated with αCD40 plus IL-4 generated more (41 ± 6%) IgE-expressing hybridomas compared with splenic B cells (Fig. 1 E). Overall, these findings demonstrate that αCD40+IL-4–activated IBCs and BM B cells have a stronger tendency to undergo CSR to IgE compared with mature splenic B cells.

Splenic B cells from young mice and adult transitional B cells preferentially switch to IgE

Based on our finding that IBCs from fetal liver culture and BM B cells preferentially undergo class switching to IgE, we asked if immature B cells in the spleen also show such a preference.

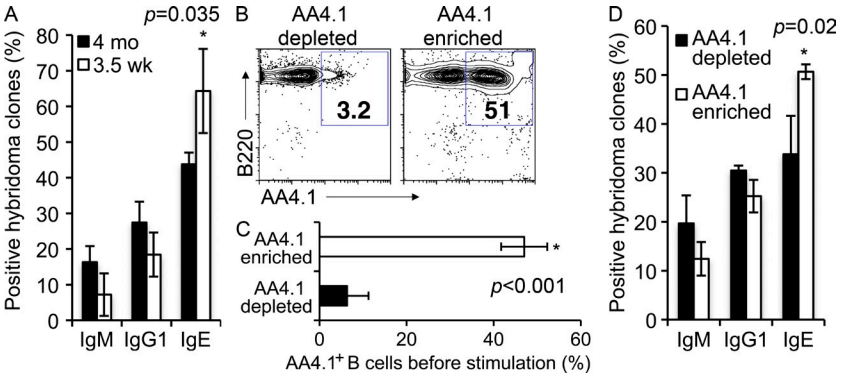

Figure 2. Splenic B cells from young mice and adult transitional B cells preferentially switch to IgE. (A) Splenocytes from BALB/c mice age 4 mo versus 3.5 wk were enriched by $B220^+$ selection before stimulation with αCD40 plus IL-4. Activated B cells were fused to NS-1 myeloma cells. Class switching was assessed by ELISA analysis of hybridoma clones. (B) Adult splenic B cells were enriched by $CD43^-$ selection before another round of $AA4.1^+$ separation. Shown is a representative example of a FACS plot of purified B cells that was performed independently three times. (C) Bar graph showing mean percentage ± SD of purified B cells expressing AA4.1 in AA4.1-enriched versus AA4.1-depleted fractions. (D) AA4.1-enriched versus -depleted fractions were stimulated, processed, and analyzed as in A. Shown are mean values ± SD. Each experiment shown was independently performed three times. Over 100 clones were analyzed per experiment. The p-values were calculated using the two-tailed Student's *t* test.

Splenic B cells from young mice are known to naturally harbor substantially more immature B cells than older mice (Melamed et al., 1998; Monroe et al., 1999). Therefore, we first assayed for IgE switching after αCD40 plus IL-4 activation of purified splenic B cells from young (3.5 wk) and older (4 mo) WT mice. Based on the aforementioned hybridoma/ELISA method, 68 ± 11% of activated B cells from young mice versus 44 ± 3% of those from older mice switch to IgE after such activation (Fig. 2 A). To assess whether the relative B cell maturity could explain the IgE CSR preference in younger versus older mice, we assayed switching in mature and immature splenic B cells isolated from spleens of adult mice (ages 2–4 mo). B cells that have recently migrated to the mouse spleen, termed transitional B cells, generally make up a small percentage of adult splenic B cells and are defined by surface expression of AA4.1/CD93 (Li et al., 1996; Allman and Pillai, 2008; Yamane et al., 2009). To test CSR preference of transitional B cells compared with more mature splenic B cells, we purified splenic B cells from other splenocytes (based on CD43-negative selection), and then enriched for transitional B cells based on presence or absence of surface AA4.1/CD93. This procedure generated AA4.1/CD93-enriched B cells, in which ∼50% were AA4.1/CD93$^+$, and AA4.1/CD93-depleted B cell fractions, in which nearly 95% were AA4.1/CD93$^-$ (Fig. 2, B and C). After αCD40 plus IL-4 stimulation and assay of IgE switching via the hybridoma approach, we found that ∼51 ± 2% of AA4.1/CD93-enriched splenic B cells versus only 34 ± 8% AA4.1/CD93-depleted splenic B cells switched to IgE (Fig. 2 D; P = 0.02). These findings suggest that immature peripheral B cells, specifically transitional B cells, preferentially switch to IgE compared with mature B cells.

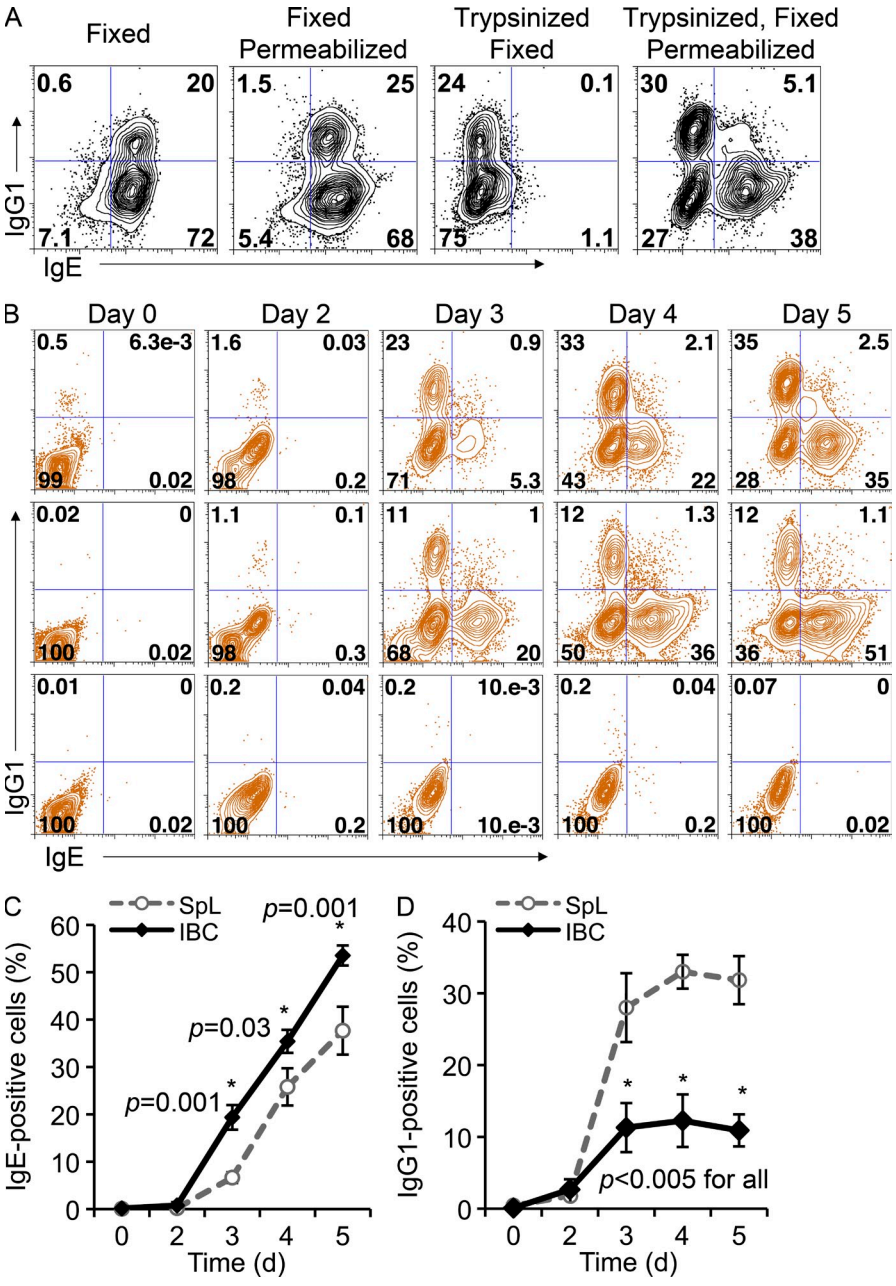

Figure 3. Different kinetics of CSR to IgE in IBCs versus mature B cells. (A) Development of an assay to assess IgE switching. Activated B cells were subjected to fixation alone, or fixation and permeabilization with or without pretreatment with trypsin before staining for IgE and IgG1. This assay allows for the detection of intracytoplasmic IgE in the absence of receptor-bound (cytophilic) IgE. (B) B cells from BALB/c spleen (top row), IBCs (middle row), or splenic B cells from AID-deficient mice (bottom row) were stimulated with αCD40 plus IL-4. At indicated time points, cells were analyzed for IgG1 and IgE switching using the method outlined in A. (C and D) The experiment in B was independently repeated a total of four times and the mean IgE (C) and IgG1 (D) expression ± SD are shown. The p-values were calculated using the two-tailed Student's *t* test.

Different kinetics of CSR to IgE in IBCs versus mature B cells

In activated mature B cells, IgE class switching is kinetically delayed relative to that of IgG1 potentially caused by the two-step sequential CSR mechanism operative in most of these cells (Yoshida et al., 1990; Siebenkotten et al., 1992; Mandler et al., 1993; Hodgkin et al., 1994). Kinetically, measurement of class switching is best done by staining for surface expression of the

various IgH isotypes on activated B cells. However, such an assay has not been available for IgE switching because activated B cells express CD23/FcεRII, which binds to secreted IgE present in activated B cell culture medium, thereby rendering all cells positive (Rolink et al., 1996). Therefore, we developed a method to remove both membrane and receptor-bound IgE from the surface of cultured B cells, allowing us to assay for intracytoplasmic IgE to provide a sensitive and reliable measure of IgE expression. For this purpose, we used trypsin treatment to effectively remove both membrane-bound and cytophilic IgE from activated B cell surfaces (Fig. 3 A). As a result, subsequent fixation and permeabilization of the cells allowed for specific staining of intracytoplasmic IgE-expression in the activated B cells (Fig. 3 A). Notably, membrane-bound IgG1 was not susceptible to trypsin-mediated removal from the cell surface because IgG1 (but not IgE) is still present after trypsinization/fixation and before permeabilization (Fig. 3 A, third panel from the left).

We used this novel intracytoplasmic IgE switching assay to measure the kinetics of IgG1 versus IgE expression in splenic B cells after 0, 2, 3, 4, and 5 d of in vitro stimulation with αCD40 plus IL-4. In adult splenic B cells, we observed the expected rapid induction of IgG1 versus IgE switching, with IgG1 switching reaching peak levels of ∼30% by days 3 and 4, whereas IgE switching was low at day 3 and continued to increase between days 4 and 5 (Fig. 3 B). Activated IBCs also reached peak IgG1 switching with similar kinetics to adult splenic B cells, although their maximal IgG1 levels were more than threefold less than those of adult splenic B cells (Fig. 3, C and D). Strikingly, however, IBCs switched to IgE with significantly increased kinetics, reaching IgE expression levels ∼1 d before adult splenic B cells (Fig. 3, C and D). Purified splenic B cells from 1-wk-old mice, consisting mostly of transitional B cells (Loder et al., 1999; Fig. 4 A), show a similar kinetic preference for IgE CSR (Fig. 4, A–C). CSR to IgG1 is also attenuated in transitional B cells compared with adult splenic B cells, although not to the degree of IgG1 CSR attenuation seen in IBCs (compare Fig. 3 D and Fig. 4 C). Thus, the IgE class switch preference of IBCs is associated with increased switching to this IgH isotype at earlier time points, suggesting that immature and transitional B cells may preferentially undergo direct CSR to Cε as compared with adult mature B cells.

Because genetic background has been shown to affect IgE responses (Drazen et al., 1996; Hogan et al., 1997; Mirotti et al., 2010), we tested whether the IgE CSR preference we observe in BALB/c mice applies to other genetic backgrounds. IBCs from pure 129/Sv mice purchased directly from The Jackson Laboratory have reduced total IgE CSR (Fig. 5 A), reaching only 10 ± 1.3% by day 5 in adult splenic B cells and 24 ± 2.1% in IBCs (Fig. 5, B and C); however, the IBC IgE CSR preference is maintained. In addition, IgE CSR appears earlier as it does in IBCs from BALB/c mice (compare Fig. 3 [C and D] to Fig. 5 [B and C]). Transitional B cells from 1-wk-old C57BL/6 mice achieve total IgE and IgG1 levels similar to transitional B cells from 1-wk-old BALB/c mice (Fig. 5 A), and exhibit a comparable IgE CSR preference as well (compare Fig. 4 [B and C] to Fig. 5 [E and F]). These data suggest that the IgE CSR preference is generalizable beyond the BALB/c genetic background.

Decreased Stat6 phosphorylation and increased Iε GLTs in activated IBCs versus mature B cells

Because CSR to IgE and IgG1 is dependent on Stat6 (Linehan et al., 1998; Bacharier and Geha, 2000), we compared Stat6 phosphorylation status of untreated and αCD40 plus IL-4–activated splenic B cells and IBCs (Fig. 6 A). For this assay, B cells were activated for 45 min, 12 h,

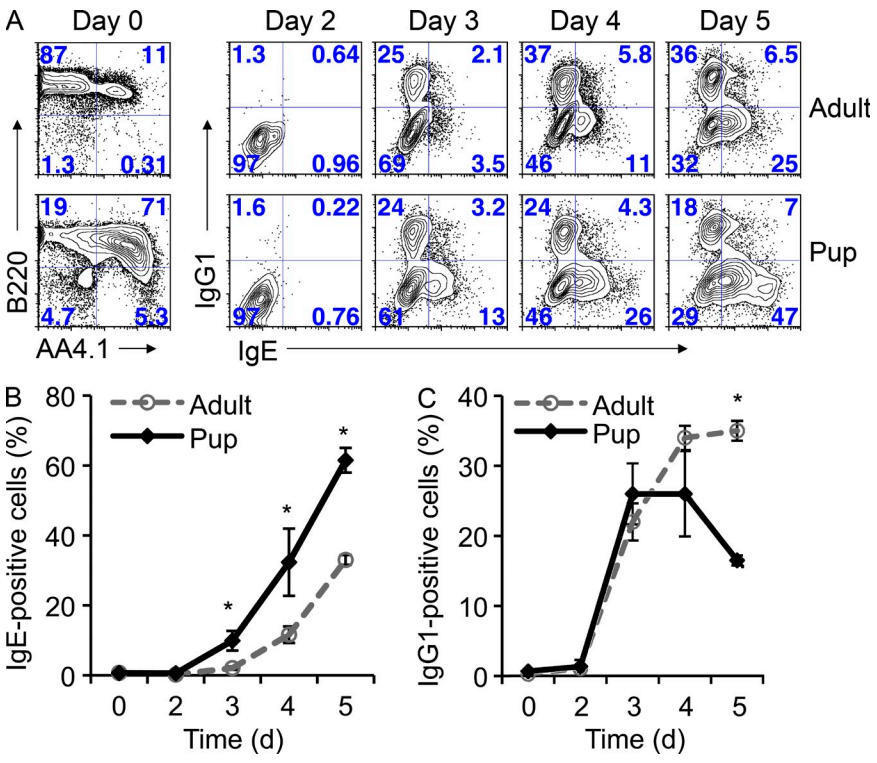

Figure 4. IgE CSR preference in transitional B cells from 1-wk-old mice. (A) B cells from BALB/c adult spleen (top row) or splenic B cells from 1-wk-old BALB/c pups (bottom row) were stained with B220 and AA4.1 (left) and stimulated with αCD40 plus IL-4 for the indicated time points (right). Cells were analyzed for IgG1 and IgE switching using the method outlined in Fig. 3 A. (B and C) The experiment in A was independently repeated a total of three times and the mean IgE (B) and IgG1 (C) expression ± SD are shown. The p-values were calculated using the two-tailed Student's *t* test.

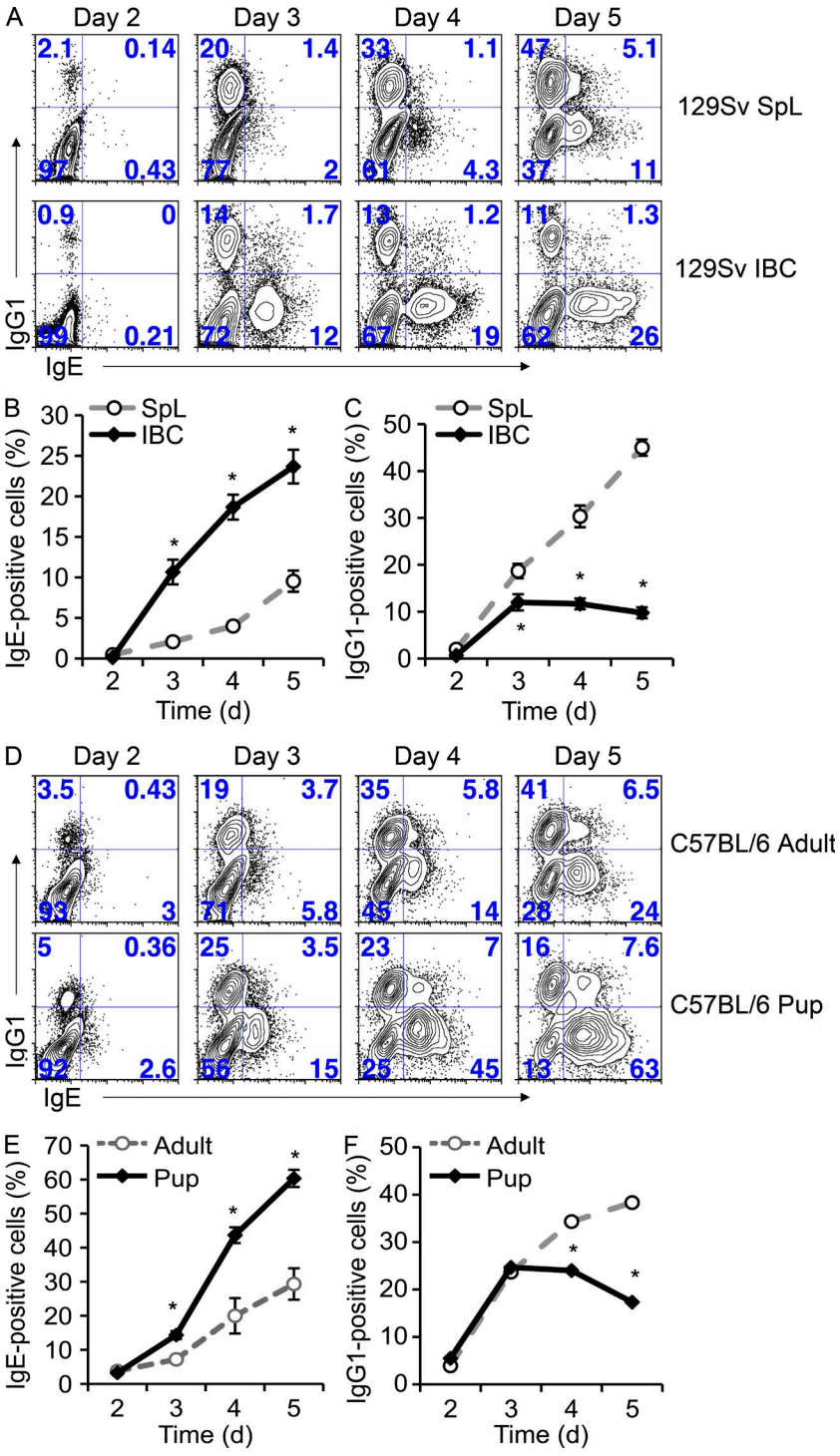

Figure 5. IgE CSR preference in immature and transitional B cells from other genetic backgrounds. (A) Adult spleen (top row), or IBCs (bottom row) from 129/Sv mice were stimulated with αCD40 plus IL-4. Cells were analyzed for IgG1 and IgE switching using the method outlined in Fig. 3A. (B and C) The experiment in A was repeated in parallel for three separate mice, and fetal liver preparations and the mean IgE (B) and IgG1 (C) expression ± SD are shown. (D) Splenic B cells from adults (top row) or 1-wk-old pups (bottom row) of C57BL/6 mice were stimulated with αCD40 plus IL-4. Cells were analyzed for IgG1 and IgE switching using the method outlined in Fig. 3 A. (E and F) The experiment in D was performed for three separate mice per group, and the mean IgE (E) and IgG1 (F). Shown are averages ± SD. The p-values were calculated using the two-tailed Student's *t* test.

and 48 h, followed by staining with an antibody specific for tyrosine^{641}-phosphorylated Stat6. Phosphorylated Stat6 levels were then measured by flow cytometry. As expected, αCD40 plus IL-4 treatment induced robust Stat6 phosphorylation in splenic B cells (Linehan et al., 1998); however, relative phospho-Stat6 levels were substantially reduced in activated IBCs at all time points tested (Fig. 4 A). In addition, αCD40/IL-4–mediated induction of phospho-Stat6 is blunted in transitional B cells from 1-wk-old pups compared with adult splenic B cells, although the difference is more modest compared with IBCs (Fig. 6 B). Thus, the propensity of IBCs and transitional B cells to preferentially switch to IgE as compared with mature B cells does not appear to result from an increase in total phospho-Stat6 levels.

Both Iγ1 and Iε promoter regions contain Stat6-responsive elements, and transcription initiated at I regions is required for CSR (Chaudhuri and Alt, 2004). We therefore hypothesized that GLT through Sγ1 and Sε might be regulated differently in mature versus IBCs. Trimethylation of lysine 4 on histone 3 (H3K4me3) within IgH C_H promoters, I regions, and S regions has previously been shown to correlate with induction of CSR (Wang et al., 2009) and has recently been shown to be an initiating event in GLT (Daniel et al., 2010). We therefore performed H3K4me3 chromatin immunoprecipitation (ChIP) on resting and αCD40/IL-4–activated mature B cells vs. IBCs, and then assayed for enrichment of ε and γ1 regions by quantitative PCR. Compared with adult splenic B cells, H3K4me4 ChIP revealed a modest nonsignificant reduction of ε enrichment in IBCs (P = 0.17; Fig. 6 C) and a more striking reduction of γ1 enrichment (P = 0.004; Fig. 6 D). Indeed, day 2 IBCs reach levels of γ1 H3K4me3 similar to day 0 in mature B cells (Fig. 6 D). Using quantitative PCR to test for steady-state GL transcript levels, we observed decreased Iγ1 GL transcripts in IBCs relative to mature B cells (Fig. 6 E), correlating with diminished Stat6 phosphorylation in activated IBCs. Yet, activated IBCs revealed a modest increase in

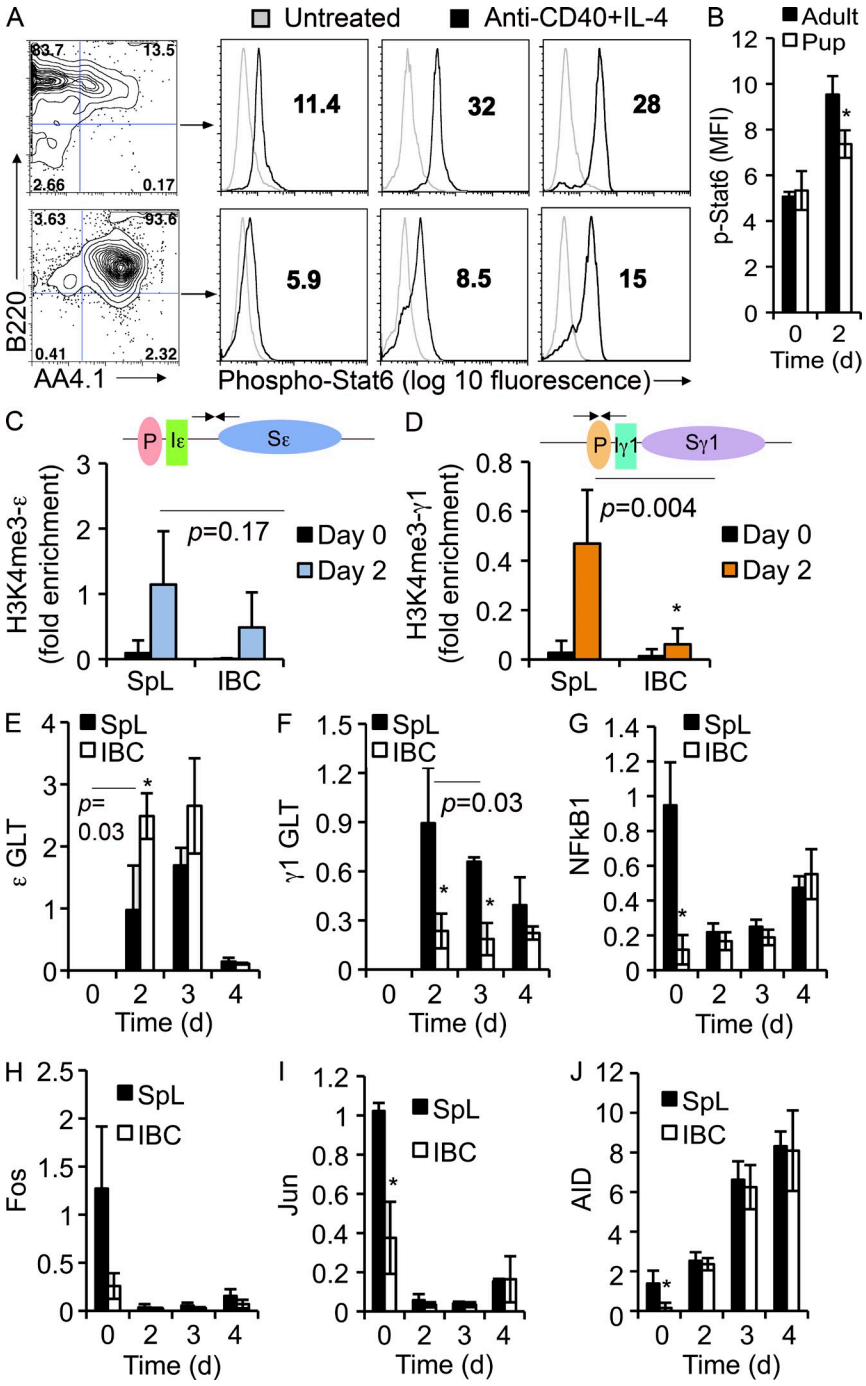

Figure 6. Decreased Stat6 phosphorylation and Iγ1 GLTs in activated IBCs versus mature B cells. (A) B220-enriched cells were separated from fresh WT mouse spleens (SpL) or from day 8 WT fetal liver cultures (IBC) and either left unstimulated or stimulated with αCD40 and IL-4 for the indicated times. Cells were then fixed and stained for intracellular tyrosine-phosphorylated Stat6 and analyzed via FACS. The numerical value displayed is the mean fluorescence intensity (MFI) of the treated sample. This experiment was repeated with nearly identical results. (B) The MFI of anti–phospho-Stat6–stained resting and αCD40+IL-4–activated adult splenic B cells versus transitional B cells from 1-wk-old pups. This experiment was performed in parallel for three independent mice from each group. (C and D) ChIP of resting and αCD40+IL-4–activated mature B cells and IBCs to test for enrichment of ε (C) and γ1 (D) sequences in anti-H3K4me3 precipitates using quantitative PCR. Signals were normalized to input after subtracting out the value of control IgG precipitates. Schematics of the ε (C) γ1 (D) loci show the location of the primers relative to promoter (P), I, and S regions. The ChIP experiments were performed on five independent samples per group. (E–J) Quantitative PCR (qPCR) for the indicated transcripts at the indicated time points after αCD40 plus IL-4 stimulation. The values presented in E–J represent relative levels of expression of the indicated transcripts normalized to GAPDH expression. Shown are the averages of three independent experiments. The error bars indicate ± SD. The p-values were calculated using the two-tailed Student's *t* test. Asterisks indicate statistical significance ($P < 0.05$).

the levels of Iε GL transcripts compared with adult splenic B cells on day 2, and similar levels thereafter (Fig. 6 F). Thus, both γ1 H3K4me3 and Iγ1 GL transcript levels appear to be consistently blunted in IBCs compared with adult splenic B cells, whereas more comparable levels of ε H3K4me3 and Iε GL transcript levels are shared between IBCs and adult splenic B cells.

NF-κB and AP-1 are important regulators of Iγ1 and Iε GLT (Iciek et al., 1997; Lin et al., 1998b; Shen and Stavnezer, 1998; Shen and Stavnezer, 2001). We therefore used quantitative PCR to assay for differences in NF-κB and AP-1 transcription factor components, namely, NF-κB1/p105 (NFkB1), which is a component of NF-κB, as well as jun and fos, which are components of the AP-1 transcription factor complex. We found that transcript levels of each of these factors is reduced in IBCs compared with adult splenic B cells before stimulation, but that these differences disappear during activation with αCD40 and IL-4 (Fig. 6, G–I). Additionally, differences in AID induction do not appear to explain differences in CSR preference between IBCs and splenic B cells because levels of AID are similar between the two groups (Fig. 6 J).

Factors previously shown to inhibit GLT of the Iε promoter region include Bcl6 and ID2. Bcl6 inhibits Iε GLT by antagonizing Stat6-binding activity (Harris et al., 1999, 2005), and ID2 down-regulates Iε GLT by inhibiting E2A proteins from binding to the Iε promoter (Sugai et al., 2003). In this context, both Bcl6-deficient (Harris et al., 2005) and ID2-deficient (Sugai et al., 2003) splenic B cells exhibit increased propensity to switch to IgE. Therefore, we investigated the

possibility that increased CSR to IgE in activated IBCs versus activated mature B cells might result from decreased Bcl6 and/or ID2 levels in IBCs. Indeed, measurement of steady-state Bcl6 and ID2 transcript levels by quantitative PCR revealed that before activation, IBCs expressed approximately sevenfold lower levels ($P < 0.001$) of Bcl6 transcripts (Fig. 7 A) and 50% lower ($P = 0.074$) levels of ID2 transcripts (Fig. 7 B) compared with mature B cells. To directly test such a role for Bcl6, we used IBCs that overexpress Bcl6 from a Bcl6 transgene under the control of the Iμ promoter (Cattoretti et al., 2005). The Iμ-Bcl6 IBCs express Bcl-6 at levels 5–30-fold higher than in WT IBCs (Fig. 7 C). Notably, these were within the range of adult splenic B cell Bcl6 expression levels (Fig. 7 A). After stimulating both Iμ-Bcl6 and WT IBCs with αCD40 plus IL-4, we found no difference in IgE or IgG1 switch preference (Fig. 7, D–F). Therefore, overexpression of Bcl6 alone does not change the IgE CSR preference within IBCs.

Increased direct μ to ε CSR in IBCs

Based on the increased kinetics of CSR to IgE in IBCs relative to mature B cells, we hypothesized that IBCs might be relatively more prone to undergo direct CSR to the Sε region than mature B cells. To assess direct μ to ε CSR in IBCs versus adult splenic B cells, we used a semi-quantitative PCR assay to detect transcripts from the circular DNA that is excised in the context of direct Sμ to Sε CSR. In this assay, a forward primer in Iε together with a reverse primer within Cμ detects hybrid Iε-Cμ transcripts that arise from excised circular DNA generated via direct Sμ to Sε CSR (Fig. 8 A). A single band is observed in this assay because the intronic region (containing the hybrid Sμ-Sε junction) is spliced out of the excision circle transcript (Fagarasan et al., 2001), leaving the processed RNA containing Iε and Cμ (Fig. 8, A and B). Because the largest burst of IgE expression in IBCs occurs between day 2 and 3 of stimulation, we assayed Iε-Cμ hybrid transcripts at day 2 of stimulation. Densitometry values were used to evaluate fold change in circular transcript levels between IBCs and adult splenic B cell levels, which revealed a 2.6-fold increase ($P = 0.025$) in direct Iε-Cμ CSR in the IBCs compared with the adult splenic B cells (Fig. 8, B and D) on day 2. There was also a mild, although nonsignificant decrease (33%; $P = 0.12$) in the levels of Iγ1-Cμ hybrid transcripts (Fig. 8 B, middle). Levels of direct Iε-Cμ circle transcripts between splenic B cells and IBCs were similar on day 4 (Fig. 8, C and E). We also used our intracytoplasmic IgE-switching flow cytometry assay to measure the proportion of cells double-positive for both IgM plus IgE and both IgG1 plus IgE, which may represent intermediate states for cells that undergo direct CSR versus indirect switching to IgE (Kitayama

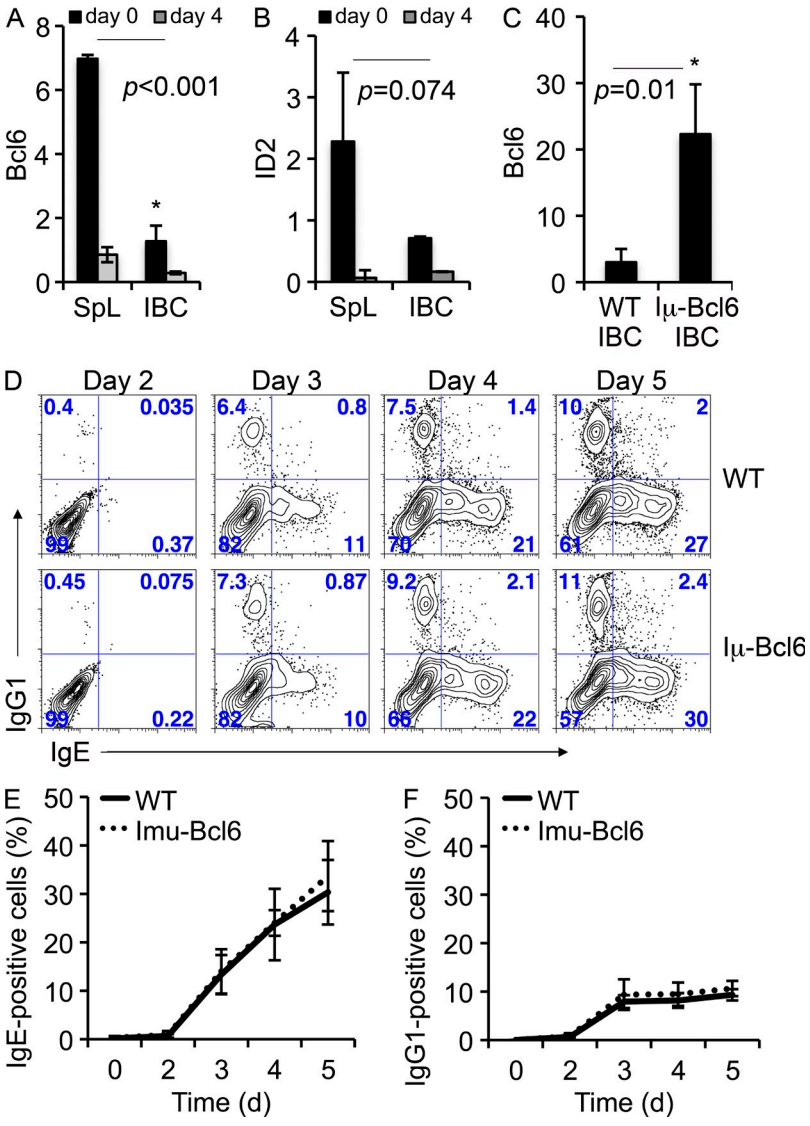

Figure 7. Assessment of Bcl6 and ID2 transcript levels in IBCs compared with splenic B cells. Quantitative PCR for Bcl6 (A) and ID2 (B) expression was performed on samples from unstimulated and αCD40+IL-4–activated splenic B cells and IBCs. (C) Quantitative PCR of Bcl6 expression in WT IBCs versus IBCs from mice heterozygous for the Iμ-Bcl6 transgene. Values were normalized to GAPDH. Shown are mean values ± SD of three independent experiments. The p-values were calculated using the two-tailed Student's *t* test. (D) IBCs from WT mice (top row), and IBCs from Iμ-Bcl6 transgenic mice (bottom row) were stimulated with αCD40+IL-4. Cells were analyzed for IgG1 and IgE switching using the intracytoplasmic staining method outlined in Fig. 3 A. The experiment in D was repeated a total of three times from three independent samples per group and the mean IgE (E) and IgG1 (F) expression ± SD for are shown.

et al., 2008; Zhang et al., 2008). Notably, we found significantly increased IgM/IgE double-positive cells in activated IBCs versus activated mature B cells at day 3 of stimulation (Fig. 8, F and G). There were also significantly fewer IgG1/IgE double-positive cells at day 4 and 5 in activated IBCs relative to activated mature B cells (Fig. 3 B and Fig. 8 H). We also find that transitional B cells from 1-wk-old pups harbor more IgM/IgE double-positive intermediates on day 3 (Fig. 9, A and B). Moreover, there is a trend, though not significant ($P = 0.1$), of decreased IgG1/IgE double-positive cells in the transitional B cells compared with adult splenic B cells (Fig. 9 C). Together, these data are consistent with IBCs being more prone to undergo direct CSR to IgE.

Peripheral Rag1^{S723C} B lineage cells exhibit CSR preference to IgE

Despite a blockade in lymphocyte development and decreased peripheral B cell numbers, some Rag1^{S723C} mice have elevated IgE levels (Giblin et al., 2009). Because we found that IBCs preferentially undergo CSR to IgE versus IgG1, we hypothesized that increased peripheral B cell immaturity could contribute to increased IgE CSR in the RAG1^{S723C} primary immune deficiency mouse model. Most B lineage splenic cells in these mutant mice are progenitor/precursor or IBCs based on AA4.1 staining (Fig. 10 A). To determine whether splenic B cells from Rag1^{S723C} mutant mice display IgE CSR preference, B lineage cells from spleens of 7–12 wk old mutant and WT littermates were enriched using B220^{+} selection

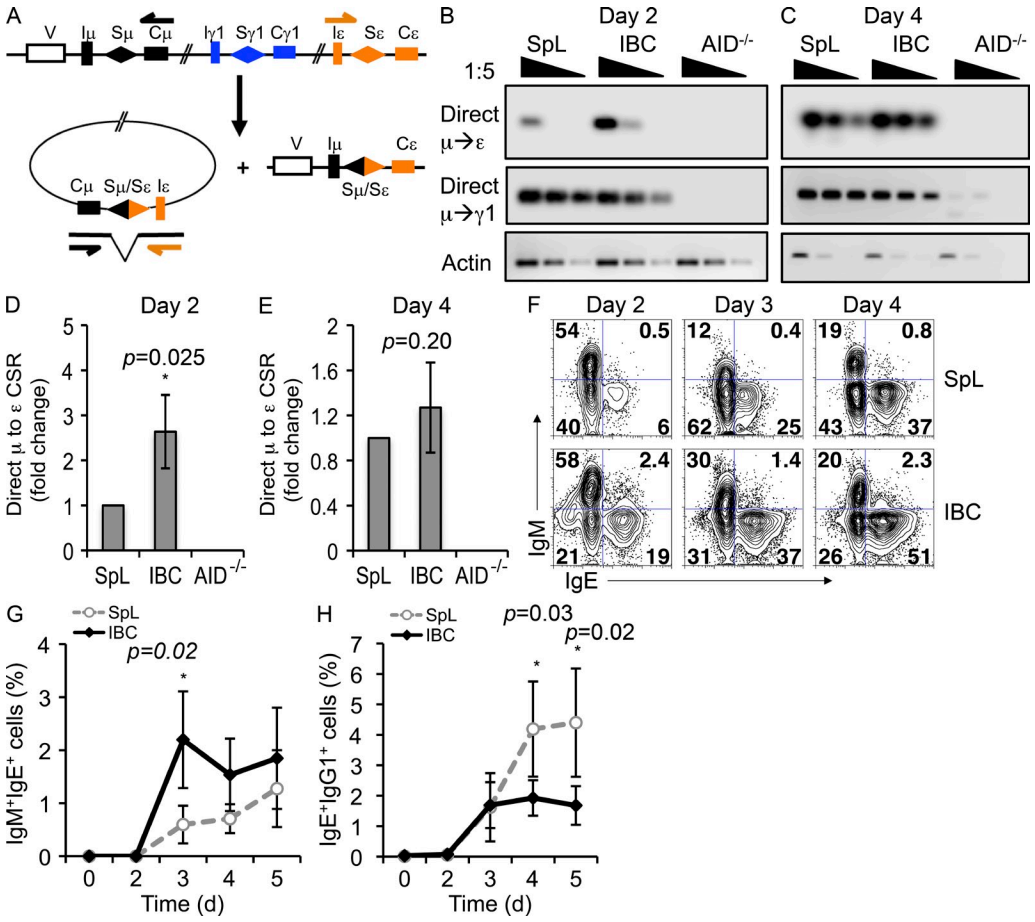

Figure 8. Increased direct IgM to IgE CSR in IBCs. (A) Simplified schematic of the IgH locus showing class switch recombination products resulting from direct Sμ to Sε recombination. General location of PCR primers in Iε and Cμ are shown as half arrows. (B and C) PCR assay showing excision circle transcript signals unique to transcripts only from direct μ to ε (top lanes) or direct μ to γ1 (middle lanes) CSR from adult splenic B cells versus IBCs after 2 (B) or 4 d (C) of culture. Actin was used as a loading control (bottom lanes). Results are representative of three independent experiments. (D and E) Quantification of densitometry of direct μ to ε CSR. Fold induction was calculated by measuring fold change compared with adult splenic B cells on day 2 (D) and 4 (E). Shown are mean values ± SD for three independent experiments. The p-values were calculated using the two-tailed Student's *t* test. (F–H) The intracytoplasmic staining technique described in Fig. 3 A was used to simultaneously detect expression of IgE and IgM (F), as well as simultaneous IgE and IgG1 expression (shown in Fig. 3 B) on αCD40 plus IL-4–activated B cells for the indicated times. (G and H) Line graphs show percent IgE, IgM double-positive B cells (G) and percent IgG1, IgE double-positive B cells (H) in activated adult splenic B cells compared with IBCs. Shown are mean values ± SD of three independent experiments. The p-values were calculated using the two-tailed Student's *t* test. Asterisks indicate statistical significance ($P < 0.05$).

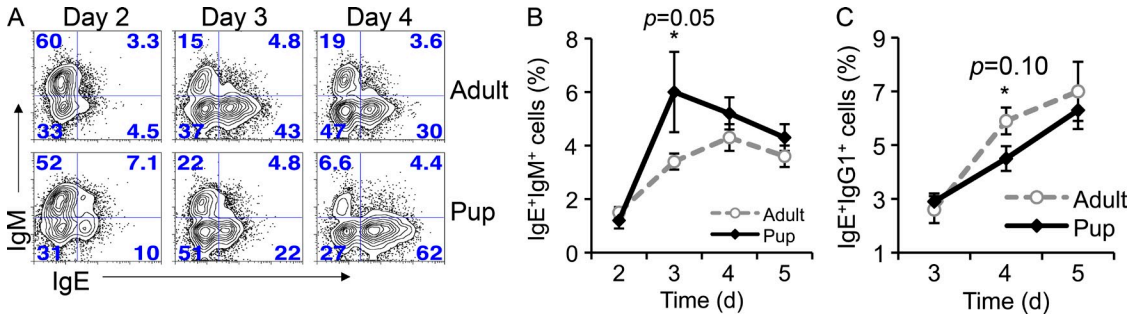

Figure 9. Increased IgE, IgM double-positive cells in αCD40+IL-4–activated transitional B cells. (A) The intracytoplasmic staining technique described in Fig. 3 A was used to simultaneously detect expression of IgE and IgM (A), as well as simultaneous IgE and IgG1 expression (shown in Fig. 4 A) in adult splenic B cells versus transitional B cells from 1-wk-old mouse pups. (B and C) Line graphs show percent IgE, IgM double-positive B cells (B), and percent IgG1+IgE double-positive B cells (C) in activated adult splenic B cells compared with transitional B cells. Shown are mean values ± SD of three independent experiments. The p-values were calculated using the two-tailed Student's *t* test. Asterisks indicate statistical significance ($P < 0.05$).

before stimulation with αCD40 plus IL-4 for 4 d to induce IgE and IgG1 CSR. IgE CSR occurred in 55 ± 9% of the Rag1^{S723C} B cells compared with 18 ± 3% in WT B cells from littermate mice (Fig. 10 B). Southern blotting of hybridoma DNA of IgE$^+$ clones confirmed IgE CSR at the DNA level (unpublished data). Together, these results suggest that Rag1^{S732C} splenic B lineage cells are skewed toward an early developmental phenotype and preferentially switch to IgE compared with IgG1 when stimulated with αCD40 and IL-4.

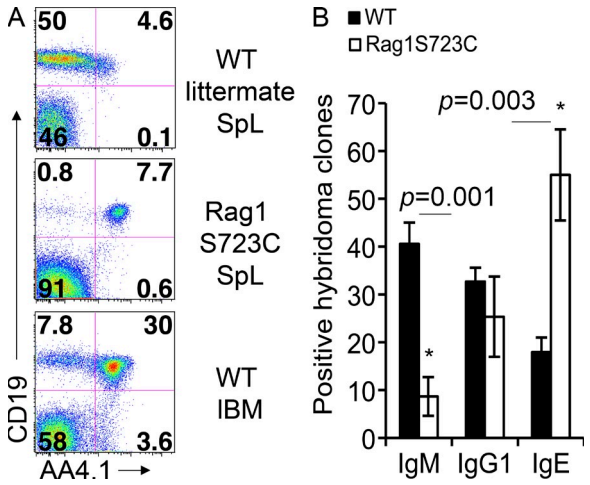

Figure 10. Peripheral Rag1^{S723C} B lineage cells exhibit CSR preference to IgE. (A) Rag1 S723C splenic (SpL) cells and cells from WT littermate control spleen and BM were stained for CD19 (to define B lineage cells) and AA4.1. The plots shown are representative of four experiments. (B) WT and Rag1 S723C B cells were purified based on B220$^+$ or CD19$^+$ selection before being stimulated for 4 d with αCD40 plus IL-4. They were then fused with the NS-1 myeloma fusion partner. Individual hybridoma clones were then assayed for IgG1, IgE, and IgM secretion by ELISA. Shown are mean values ± SD for four independent experiments. The p-values were calculated using two-tailed Student's *t* test. Asterisks indicate statistical significance ($P < 0.05$).

DISCUSSION

In this study, we report that αCD40/IL-4–activated immature and transitional B cells preferentially undergo CSR to IgE versus IgG1 compared with mature splenic B cells. We also show that IBCs switch to IgE though a mechanism involving increased direct CSR from IgM to IgE. The observation of decreased Sγ1 H3K4me3 and reduced Iγ1 GL transcripts in αCD40 plus IL-4–activated IBCs compared with mature B cells suggests that Iγ1 is more poised for αCD40+IL-4–mediated induction of GLT in mature cells, whereas ε H3K4me3 and Iε GL transcripts are closer to mature B cell levels. In addition, we find that these differences occur in the context of a lower amount of total αC40+IL-4–induced phospho-Stat6 levels in the IBCs. Our studies therefore identify B cell maturity as an intrinsic B cell property that affects CSR choice of IgE versus IgG1.

Our proposed mechanistic model to explain the differential preference of αCD40+IL-4-activated immature versus mature B cells to undergo CSR to IgG1 versus IgE is that, upon developmental transition from the immature stage to the mature B cell stage, B cells reduce suppressive activity at the Iγ1 promoter. This change in promoter inducibility results in relatively more αCD40+IL-4–mediated Iγ1 GLT and AID targeting of Sγ1 in mature B cells, leading them to switch first to IgG1. Correspondingly, the weaker induction of γ1 H3K4me3 and Iγ1 GLT compared with ε H3Keme4 and Iε GLT induction makes Sε a relatively favorable target for direct CSR to Sμ within αCD40+IL-4-activated immature and transitional B cells. Indeed, increased IgE CSR is observed in mature B cells that cannot switch to IgG1 because of deletion of Sγ1 (Misaghi et al., 2010). Because Stat6 is involved in the regulation of both Iγ1 and Iε GLT (Linehan et al., 1998), one may speculate that the decreased levels of total αCD40+IL-4–induced phospho-Stat6 that we observed in the IBCs versus mature B cells may endow the Iε promoter with a competitive advantage over the Iγ1 promoter. In this regard, it is conceivable that the Iε promoter, which binds Stat6 with 10-fold higher affinity compared with the Iγ1 promoter (Mao

and Stavnezer, 2001), may potentially usurp Stat6 binding at the expense of Iγ1, thus favoring Iε GL transcription within immature and transitional B cells. However, when we manufactured a modest reduction phospho-Stat6 levels in mature B cells by using decreased amounts of IL-4 (while maintaining αCD40 levels at a constant), we observed a reduction in both IgG1 and IgE CSR (unpublished data). In this regard, IgE CSR preference in IBCs cannot be explained by weaker IL-4 signals alone.

Although not apparent by the H3K4me3 ChIP experiments, one may also consider the possibility that Iε GLT and Sε accessibility is reduced in mature B cells compared with IBCs, given the modest decrease in day 2 Iε GL transcripts in mature B cells. In this regard, two important candidate Iε transcriptional inhibitors are Bcl6 and ID2 (Ozcan et al., 2008). Indeed, we find that Bcl6 expression is significantly lower in IBCs versus mature splenic B cells, which may play a role in the increased Iε GLT observed in αCD40+IL-4-activated IBCs compared with mature B cells. However, our Bcl6 overexpression experiments suggest that decreased Bcl6 in IBCs is not solely responsible for their IgE switch preference.

The observation that certain primary immune deficiencies with seemingly unrelated genetic underpinnings are associated with very elevated levels of IgE has been the subject of great interest since these primary immune deficiencies may provide clues into the regulation of IgE CSR and production (Geha et al., 2003; Ozcan et al., 2008). Proposed mechanisms for increased IgE in primary immune deficiency include defective and/or imbalanced cytokine production and regulatory T cell malfunction, but a unifying mechanism remains elusive (Ozcan et al., 2008). The effect of B cell developmental differences on class switch preference has not been addressed until now. Indeed, these studies highlight a need to examine B cell developmental effects in targeted knockout or transgenic mice that modulate CSR because it is not difficult to foresee situations where deletion of genes involved in immature/mature B cell ratios could indirectly affect CSR. In this regard, we find that the murine $Rag1^{S723C}$ immune deficiency model contains increased proportions of peripheral immature B cells that preferentially switch to IgE in ex vivo cultures when stimulated with αCD40 plus IL-4, suggesting a model whereby positioning of developmentally immature B cells (in places where they may be exposed to activating cytokines and T cell help) may permit a higher frequency of CSR to IgE. Consistent with the idea that intrinsic differences within B cells may play a role in regulating probability of CSR to IgE, perturbations in B cell development have been reported in a specific primary immune deficiency (Wiskott-Aldrich syndrome) associated with elevated IgE levels (Park et al., 2005). In addition, allogeneic BM transplantation, which temporarily places developing B lineage cells in the periphery, is associated with a transient, but sharp increase in IgE as early as 14 d after transplant (Geha et al., 1980). Our studies also provide potential insights for why children have higher total IgE levels compared with adults (Grundbacher, 1976).

Our findings reveal a functional link between B cell developmental maturity and inducibility of the Iγ1 and Iε promoter regions. Accordingly, our data reveal B cell maturity as an intrinsic regulatory factor that can affect downstream antibody functionality by impacting IgH isotype preference. In addition, the B cell developmental state may have an impact on IgE antibody specificity by regulating direct versus sequential CSR to IgE. In this regard, an $IgG1^+$ B cell intermediate has been proposed to be required for development of somatically hypermutated and affinity matured antigen-specific IgE molecules in vivo (Erazo et al., 2007) because IgE-expressing B cells, but not those expressing IgG1, are excluded from germinal center reactions (Erazo et al., 2007). Our finding of earlier progression to IgE in IBCs via increased direct IgM to IgE CSR may also imply that antibodies that mature this way may bypass the process of hypermutation and selection within germinal centers. Whether increased direct CSR to IgE plays a role in primary immune deficiencies with high IgE, and whether this has an effect on IgE affinity/specificity in these conditions, is the subject of ongoing study.

MATERIALS AND METHODS

Mice. Mice harboring the RAG1-S723C knock-in mutation at the endogenous locus were previously described (Giblin et al., 2009). Heterozygous breeding pairs produced experimental homozygous mice with WT littermates that served as controls. T. Honjo (Kyoto University, Kyoto, Japan) provided $AID^{-/-}$ mice. The Iμ-Bcl6 mice were a gift from R. Dalla-Favera (Columbia University, New York, NY) and were maintained on a C57BL/6 background. WT BALB/c, 129/Sv, and C57BL/6 mice were purchased from The Jackson Laboratory. All experiments with mice followed the protocols approved by the Boston Animal Care Facility of the Children's Hospital, Boston, MA 02115.

Splenic B cell purification and culture. Splenic and BM B lineage cells were isolated by $B220^+$ selection via magnetic columns (Miltenyi Biotec) before stimulation with αCD40 plus IL-4 to induce CSR to IgG1 and IgE, as previously described (Yan et al., 2007). Cytokine sources were αCD40 (eBioscience) and IL-4 (eBioscience). For AA4.1 enrichment, splenic B cells were first enriched by $CD43^-$ separation followed by $AA4.1^+$ magnetic separation.

Intracytoplasmic IgE staining. Intracytoplasmic staining and flow cytometry were performed on days 0, 2, 3, 4, and 5 with anti–IgE-FITC, anti–IgG1-PE, and anti–B220-PE-Cy5 after incubating cells in 0.05% trypsin for 2 min in PBS at room temperature, followed by fixation in 3% buffered paraformaldehyde for 10 min at 37°C. Cells were then permeabilized with 90% cold methanol for 30 min on ice, washed twice with PBS, and stained for flow cytometry.

Hybridoma analysis for CSR. 5–10 million αCD40/IL-4–stimulated B cells from each mouse were fused with NS-1 fusion partner myeloma cells on day 4 and recovered after 7 d selection with 1× hypoxanthine-aminopterin-thymidine medium. Single clones were isolated and grown before ELISA was done on their supernatants to measure secreted IgM, IgG1, and IgE. Over 100 clones were analyzed for each individual experiment, and at least 3 experiments were performed per condition. Only clones that were single positive for one of the three IgH isotypes tested were counted. Clones that were negative for all IgH isotypes or positive for more than one IgH isotype were found at very low levels (<5% of total clones) in all of the samples.

Early B lineage cell culture. Early B lineage cells were derived from fetal liver mononuclear cells from 15-d-old embryos (10^6/ml) as previously described (Lin et al., 1998a). Cells were suspended in IL-7–conditioned medium, consisting of 20% supernatant from IL-7–producing T220 fibroblast (Borzillo et al., 1992) and 80% fresh RPMI-1640 medium with 5% FCS. Cells were then cultured on a T220 cell monolayer grown on 10-cm plates. Media was changed after 4 d of culture. After 8 d of culture, cells were separated from the T220 fibroblasts and purified by magnetic separation using B220 magnetic beads (Miltenyi Biotech). Viability and purity was verified by flow cytometry. Cells were then stimulated with αCD40 plus IL-4, and assays for switching by the hybridoma/ELISA and FACS staining were performed described in the previous section.

ChIP. ChiP was performed essentially as previously described (Yoon and Boss, 2010). In brief, resting and day 2–activated adult splenic B cells and IBCs were cross-linked in 1% formaldehyde for 10 min at room temperature. The reaction was stopped by adding glycine to a final concentration of 0.125 M. Chromatin was isolated and sonicated to a mean size of 300–500 bp. Precleared chromatin was divided into tubes and used for input, IP with anti-H3K4me3 and IP with IgG control antibody (both from Abcam). Cross-linking was reversed, and proteins were degraded with proteinase K. Immunoprecipitates and input samples were analyzed by SYBER-Green real-time quantitative PCR using the primers, γ 1 region forward, 5′-ACCCTCAC-CCACACATTCAC-3′, and reverse, 5′-CCTTCATTCTGGGGGTTTCT-3′. Epsilon region forward, 5′-CTTGACCACCGAATGTCCTT-3′, reverse, 5′-GATTCCTCTCCAGCCTCTCC-3′. To ensure primer specificity, dissociation curves were analyzed and PCR products were run on agarose gels for each primer set. Mean pPCR values for duplicate and triplicate samples were calculated. For each primer set and time point, the value of the control IgG immunoprecipitation was subtracted from the values of the H3K4me3 immunoprecipitation. Background-subtracted values were then normalized to the qPCR value obtained from running 1% of the input sample to calculate fold enrichment.

Quantitative and semiquantitative PCR. Total RNA was extracted using the TRIzol method (Invitrogen) and reverse transcribed into cDNA using qScript (Quanta Biosciences). Iε and Iγ1 GLTs were then quantified using TaqMan qPCR (Applied Biosystems). Primers and probes were as follows: for Iε GLT, Eps GLT probe 5′-AGGGTTCCTGATAGAGGCT-GAGGT-3′, Iε forward primer 5′-GAGATTCACAACGCCTGG-3′, and Cε reverse primer 5′-CTTTACAGGGCTTCAAGGG-3′; for Iγ1 GLT, GLTγ1 probe 5-′ACAGGTTGAGAGAACCAAGGAAGCTG-3′, Iγ1 forward primer 5′-TCGAGAAGCCTGAGGAATGT-3′, and Cγ1 reverse primer 5′-ATAGACAGATGGGGGTGTCG-3′. TaqMan assays for NfkB1 (Mm00476361_m1), Jun (Mm00495062_s1), Fos (Mm00487425_m1), and AID (Mm00507774_m1) were purchased from ABI. Detection of excision circular transcripts from direct μ to ε switching events was performed using a forward primer in Iε (5′-CTGGCCAGCCACTCACTTAT-3′) together with a reverse primer in Cμ (5′-AGGTGAAGGAAATGGTGCTC-3′). Detection of excision circular transcripts from direct μ to γ1 switching events was done using a forward primer in Iγ1 (5′-TCGAGAAGCCT-GAGGAATGT-3′) together with the reverse Cμ primer. Reaction products were subjected to Southern blotting and probed with an internal oligo (5′-AAATGTCTTCCCCCTCGTCT-3′). Reaction products for direct μ to ε switching events were also sequenced to confirm product specificity.

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