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Immature B cells preferentially switch to IgE with increased direct S\(\mu\) to S\(\epsilon\) recombination

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Immunoglobulin heavy chain (IgH) class-switch recombination (CSR) replaces initially expressed C\(\mu\) (IgM) constant regions (C\(\mu\)) exons with downstream C\(\epsilon\) exons. Stimulation of B cells with anti-CD40 plus interleukin-4 induces CSR from C\(\mu\) to C\(\gamma\)1 (IgG1) and C\(\epsilon\) (IgE), the latter of which contributes to the pathogenesis of atopic diseases. Although C\(\epsilon\) CSR can occur directly from C\(\mu\), most mature peripheral B cells undergo CSR to C\(\epsilon\) indirectly, namely from C\(\mu\) to C\(\gamma\)1, and subsequently to C\(\epsilon\). Physiological mechanisms that influence CSR to C\(\gamma\)1 versus C\(\epsilon\) 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 C\(\mu\) to C\(\epsilon\). Our findings suggest that IgE dysregulation in certain immunodeficiencies may be related to impaired B cell maturation.

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 \(\mu\) constant region exons (C\(\mu\)) are deleted and replaced by one of several sets of downstream C\(\gamma\)1 exons (e.g., C\(\gamma\)1, C\(\gamma\)2, and C\(\gamma\)3), termed C\(\gamma\)\(\delta\) 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\(\gamma\)1 genes (Chaudhuri et al., 2007). During CSR, DNA double-strand breaks (DSBs) are specifically induced in a donor S region (S\(\delta\)) upstream of C\(\mu\) and a downstream region; SCID, severe combined immunodeficiency.
acceptor S region; these DSBs then are joined by classical non-homologous end-joining, or an alternative DNA end-joining pathway (Yan et al., 2007), replacing Cµ with a downstream CΗ 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 (Th2) T cells, leads to the activation of NF-κB and Stat6 transcription factors, respectively, which, together with other transcription regulators, induce germine (GL) transcription (GLT) from Ψμ1 and Ψε promoters and CSR to Ψγ1 or Ψε (Bacharier and Geha, 2000). Although αCD40 plus IL-4 treatment theoretically can lead to direct CSR from Cµ to either Ψγ1 or Ψε, direct CSR to Ψε occurs less frequently than to Ψγ1 (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 Ψγ1, followed by switching to IgE via a “second step” CSR from Ψγ1 to Ψε (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 Ψγ1 is abrogated through mutation of Ψγ1 or deletion of Ψγ1 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...
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.

**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 of pre–B versus more mature B cells might differ in fetal liver versus immature B cells. To test this, we compared the C57BL/6 and BALB/c mouse strains, which 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.

**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 another round of αCD40+IL-4 activation. 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 αCD43+ 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. 2A). 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 $\approx 50%$ were AA4.1/CD93$^+$, and AA4.1/CD93-depleted B cell fractions, in which nearly 95% were AA4.1/CD93$^-$ (Fig. 2B and C). After αCD40 plus IL-4 stimulation and assay of IgE switching via the hybridoma approach, we found that $\approx 51\%$ ± 2% of AA4.1/CD93-enriched splenic B cells versus only 34 ± 8% AA4.1/CD93-depleted splenic B cells switched to IgE (Fig. 2D; $P = 0.02$). These findings suggest that immature peripheral B cells, specifically transitional B cells, preferentially switch to IgE compared with mature B cells.

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...
than those of adult splenic B cells (Fig. 3, C and D). Strik-ingly, however, IBCs switched to IgE with significantly increased kinetics, reaching IgE expression levels \( \sim 1 \text{ d} \) 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 C\( \alpha \) 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 Labora-tory 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 compara-ble 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 ge-netic background.

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

Because CSR to IgE and IgG1 is de-pendent on Stat6 (Linehan et al., 1998; Bacharier and Geha, 2000), we compared Stat6 phosphorylation status of untreated and \( \alpha \)CD40 plus IL-4–activated splenic B cells and IBCs (Fig. 6 A). For this assay, B cells were activated for 45 min, 12 h,
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CD40/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 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 initiation 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 Cγ regions, 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 CD40/IL-4–activated mature B cells vs. IBCs, and then assayed for enrichment of γ1 and γ1 regions by quantitative PCR. Compared with adult splenic B cells, H3K4me3 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).

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 129Sv 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.

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.

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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 γ1 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

and 48 h, followed by staining with an antibody specific for tyrosine-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,
the levels of IgL transcripts compared with adult splenic B cells on day 2, and similar levels thereafter (Fig. 6 F). Thus, both γ1 H3K4me3 and γ1 IgL transcript levels appear to be consistently blunted in IBCs compared with adult splenic B cells, whereas more comparable levels of ε H3K4me3 and IgL transcript levels are shared between IBCs and adult splenic B cells.

NF-κB and AP-1 are important regulators of IgL and IgGLT (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 (NFκB1), 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 IgL promoter region include Bcl6 and ID2. Bcl6 inhibits IgL GLT by antagonizing Stat6-binding activity (Harris et al., 1999, 2005), and ID2 down-regulates IgL GLT by inhibiting E2A proteins from binding to the IgL 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...
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. 7A) and 50% lower (P = 0.074) levels of ID2 transcripts (Fig. 7B) 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 Ilμ promoter (Cattoretti et al., 2005). The Ilμ-Bcl6 IBCs express Bcl-6 at levels 5–30-fold higher than in WT IBCs (Fig. 7C). Notably, these were within the range of adult splenic B cell Bcl6 expression levels (Fig. 7A). After stimulating both Ilμ-Bcl6 and WT IBCs with αCD40 plus IL-4, we found no difference in IgE or IgG1 switch preference (Fig. 7D–F). Therefore, overexpression of Bcl6 alone does not change the IgE CSR preference within IBCs.

Increased direct μ to e 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 Ilε together with a reverse primer within Cμ detects hybrid Ilμ-Cμ transcripts that arise from excised circular DNA generated via direct Sμ to Sε CSR (Fig. 8A). A single band is observed in this assay because the intronic region (containing the hybrid Ilμ-Sε junction) is spliced out of the excision circle transcript (Fagarasan et al., 2001), leaving the processed RNA containing Ilε and Cμ (Fig. 8A and B). Because the largest burst of IgE expression in IBCs occurs between day 2 and 3 of stimulation, we assayed Ilμ-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 Ilμ-Cμ CSR in the IBCs compared with the adult splenic B cells (Fig. 8B and D) on day 2. There was also a mild, although nonsignificant decrease (33%; P = 0.12) in the levels of Ilγ1-Cμ hybrid transcripts (Fig. 8B, middle). Levels of direct Ilμ-Cμ circle transcripts between splenic B cells and IBCs were similar on day 4 (Fig. 8C 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 et al., 2003).

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 Ilμ-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 Ilμ-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. 3A. 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.

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Peripheral Rag1<sup>S723C</sup> B lineage cells exhibit CSR preference to IgE

Despite a blockade in lymphocyte development and decreased peripheral B cell numbers, some Rag1<sup>S723C</sup> 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<sup>S723C</sup> 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<sup>S723C</sup> mutant mice display IgE CSR preference, B lineage cells from spleens of 7–12 wk old mutant and WT littermates were enriched using B220<sup>+</sup> 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 S<sub>μ</sub> to S<sub>ε</sub> recombination. General location of PCR primers in I<sub>ε</sub> and C<sub>μ</sub> are shown as half arrows. (B and C) PCR assay showing excision circle transcript signals unique to transcripts only from direct S<sub>μ</sub> to S<sub>ε</sub> (top lanes) or direct S<sub>μ</sub> 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 S<sub>μ</sub> to S<sub>ε</sub> 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).
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 αCD40+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 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 αCD40+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.

Before stimulation with αCD40 plus IL-4 for 4 d to induce IgE and IgG1 CSR, IgE CSR occurred in 55 ± 9% of the Rag1S723C 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 Rag1S723C 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 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).

**Figure 10.** Peripheral Rag1S723C B lineage cells exhibit CSR preference to IgE. (A) Rag1 S723C splenic (SpL) cells and cells from WT littermate control spleens 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).
Our findings reveal a functional link between B cell developmental maturity and inducibility of the IgY1 and IgE promoters. 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.
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 6-well 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 Biotec). Viability and purity was verified by flow cytometry. Cells were then stimulated with oCD40 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 glycin 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 proteasome K. Immunoprecipitates and input samples were analyzed by SYBER–Green real-time quantitative PCR using the primers, γ1 region forward, 5′-ACCTCTCCACCCACATTCCAC-3′, and reverse, 5′-CTCTTTACAGGGCTTCAAGGG-3′ (Mm00495062_s1), Fos (Mm00487425_m1), and Jun (Mm00476361_m1). Reaction products for direct switching events were done using a forward primer in IGL1 GLTs (5′-TCGAGAAGCCTGAGGAATGT-3′) and a reverse primer in Cε (5′-CCACACATTCAC-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′-AGGGTTCCTGATAGAAGGCTTGAGGT-3′, Iγ1 forward primer 5′-GAGCTTACACCGCTCCGTT-3′, and reverse primer 5′-CTTTACAGGGCTTCAAGGG-3′ for Iγ1 GLT, GLT1 γ1 probe 5′-ACAGGTTGAGAGAACCAAGGAAGCTG-3′, Iγ1 reverse primer 5′-ATAGACAGATGGGGGTGTCG-3′, and Cε reverse primer 5′-GAGATTCACAACGCCTGG-3′. Switching events was performed using the ABI PRISM 7900 HT Sequence Detector (Applied Biosystems). Reaction products for direct switching events were also sequenced to confirm product specificity.

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