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Citation

Published Version
doi:10.1084/jem.20111155

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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 (Cµh) exons with downstream Cµl exons. Stimulation of B cells with anti-CD40 plus interleukin-4 induces CSR from Cµl to Cy1 (IgG1) and Ce (IgE), the latter of which contributes to the pathogenesis of atopic diseases. Although Ce CSR can occur directly from Cµl, most mature peripheral B cells undergo CSR to Ce indirectly, namely from Cµl to Cy1, and subsequently to Ce. Physiological mechanisms that influence CSR to Cy1 versus Ce 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 Cµl to Ce. Our findings suggest that IgE dysregulation in certain immunodeficiencies may be related to impaired B cell maturation.

Abbreviations used: αCD40, anti-CD40; AID, activation-induced cytidine deaminase; C, constant region; ChBP, chromatin immunoprecipitation; CSR, class-switch recombination; DSB, double-strand break; GL, germline; GLT, GL transcription(jon); 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 (Cµh) are deleted and replaced by one of several sets of downstream Cµl exons (e.g., Cy, Ce, and Cα), termed Cµl 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 Cµl genes (Chaudhuri et al., 2007). During CSR, DNA double-strand breaks (DSBs) are specifically induced in a donor S region (Sµ) upstream of Cµl and a downstream
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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 Cy1, followed by switching to IgE via a “second step” CSR from Cy1 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 Sy1 is abrogated through mutation of Iγ1 or deletion of Sy1 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 than 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...
serum IgE levels (Ozcán 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<sup>S723C</sup> mutant mice) recapitulate features of Omenn syndrome. In particular, Rag1<sup>S723C</sup> 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; Ozcán 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 Cy1 versus Ce might differ in mature versus immature B cells. To test this, we compared the Cy1/Ce 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.

**Splenial 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<sup>+</sup> 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<sup>+</sup> selection before another round of AA4.1<sup>+</sup> 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.

**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 IgE GLTs in activated B cells 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, 24 h, and 48 h with αCD40 plus IL-4 and subsequently stained for phospho-Stat6, IgE, and IgG1. As expected, Stat6 phosphorylation was increased in IBCs compared with splenic B cells at all time points (Fig. 6 A). However, the increase in Stat6 phosphorylation was more pronounced in IBCs than in splenic B cells at 48 h, suggesting that Stat6 phosphorylation is a marker of CSR failure in IBCs.

Data are presented as mean ± SD of three independent experiments. *p < 0.05. **p < 0.01. ***p < 0.001. 

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.
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 phosphorylation 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 Jy1 and Jε 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 Sy1 and Se 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 αCD40/IL-4–activated mature B cells vs. IBCs, and then assayed for enrichment of Sy1 and γ1 regions by quantitative PCR. Compared with adult splenic B cells, H3K4me3 ChIP revealed a modest nonsignificant reduction of γ1 enrichment (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 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

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NF-κB and AP-1 are important regulators of Iγ1 and Ie 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), 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 Ie promoter region include Bcl6 and ID2. Bcl6 inhibits Ie GLT by antagonizing Stat6-binding activity (Harris et al., 1999, 2005), and ID2 down-regulates Ie GLT by inhibiting E2A proteins from binding to the Ie 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
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 (Fig. 8A). 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 Iμ 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 Iμ-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 Iμ-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. 8B, middle). Levels of direct Iμ-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 et al., 2005).

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. 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.
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 Sp<sub>m</sub> to Se recombination. General location of PCR primers in I<sub>ɛ</sub> and C<sub>m</sub> are shown as half arrows. (B and C) PCR assay showing excision circle transcript signals unique to transcripts only from direct <sup>m</sup> to <sup>ɛ</sup> (top lanes) or direct <sup>m</sup> to <sup>γ1</sup> (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 <sup>m</sup> to <sup>ɛ</sup> 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 CD40/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 H3K4me3 and reduced GL transcripts in CD40 plus IL-4–activated IBCs compared with mature B cells suggests that GL is more poised for CD40+IL-4–mediated induction of GLT in mature cells, whereas H3K4me3 and 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 CD40+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 CD40+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 GL promoter. This change in promoter inducibility results in relatively more CD40+IL-4–mediated induction of GLT in mature cells, whereas H3K4me3 and 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 CD40+IL-4–induced phospho-Stat6 that we observed in the IBCs versus mature B cells.

Figure 9. Increased IgE, IgM double-positive cells in CD40+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 Rag1<sup>S723C</sup> 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<sup>+</sup> or CD19<sup>+</sup> selection before being stimulated for 4 d with CD40 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).
and Stavnezer, 2001), may potentially usurp Stat6 binding at the expense of 1y1, thus favoring 1e 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 1e GLT and Sε accessibility is reduced in mature B cells compared with IBCs, given the modest decrease in day 2 1e GL transcripts in mature B cells. In this regard, two important candidate 1e 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 1e 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<sup>S723C</sup> 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 1y1 and 1e 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<sup>+</sup> 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<sup>S723C</sup> knock-in mutation at the endogenous locus were previously described (Giblin et al., 2009). Heterozygous breeding pairs produced experimental homozygous mice with WT littersmates that served as controls. T. Honjo (Kyoto University, Kyoto, Japan) provided AID<sup>−/−</sup> mice. The 1y1-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<sup>+</sup> selection via magnetic column (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<sup>−</sup> separation followed by AA4.1<sup>+</sup> magnetic separation.

**Intracytoplasmic IgE staining.** Intracytoplasmic staining and flow cytometry were performed on days 0, 2, 3, and 5 with anti-IgE-FITC, anti-IgG1-PE, and anti-B220-PE-Cy5 after incubating cells in 0.05% trypan blue 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-aminopterinthymidine 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 50% CD7–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 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 proteinase K. Immunoprecipitation was reversed, and proteins were degraded with proteinase K. Immunoprecipitation of chromosomal V(D)J recombination. 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). IgE and Ig1 GLTs were then quantified using TaqMan qPCR (Applied Biosystems). Primers and probes were as follows: for IgE GLT, Eps GLT probe 5′-AGGGTTTCCTGATAGAGCT- GAGGT-3′, IgE forward primer 5′-GAGATTCCAAGGCTCCTG-3′, and Cε reverse primer 5′-CTTTACTAGGGCTTCAAGGG-3′; for Ig1 GLT, GLTY1 probe 5′-ACAGGTTGAGAGAACCAAGGAAGCTG-3′, and Cε reverse primer 5′-ATAGACAGATGGGGGTGTCG-3′. TaqMan assays for NfkB1 switching events were also sequenced to confirm product specificity.

REFERENCES


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