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Comments
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Flexible ordering of antibody class switch and V(D)J joining during B-cell ontogeny

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V(D)J joining is mediated by RAG recombinase during early B-lymphocyte development in the bone marrow (BM). Activation-induced deaminase initiates isotype switching in mature B cells of secondary lymphoid structures. Previous studies questioned the strict ontological partitioning of these processes. We show that pro-B cells undergo robust switching to a subset of immunoglobulin H (IgH) isotypes. Chromatin studies reveal that in pro-B cells, the spatial organization of the IgH locus may restrict switching to this subset of isotypes. We demonstrate that in the BM, V(D)J joining and switching are interchangeably inducible, providing an explanation for the hyper-IgE phenotype of Omenn syndrome.

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Humoral immunity is dependent on antigen receptors that are assembled from immunoglobulin (Ig) heavy chain V, D, and J segments during early B-cell development. RAG recombinase mediates V(D)J gene segment assembly in the bone marrow (BM) (Zhang et al. 2010; Schatz and Ji 2011). In mature B cells located in secondary lymphoid structures, activation-induced deaminase (AID) initiates a switch from IgM expression to secondary Ig classes (IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) that have specialized effector functions (Stavnezer 1996, 2000). Class switch recombination (CSR) occurs via an intrachromosomal deletional process while maintaining the original V(D)J assembly (Kenter 2012). The failure to assemble V(D)J exons or signal through the B-cell receptor (BCR) creates a block in the developmental progression of pro-B cells (Jankovic et al. 2004; von Boehmer and Melchers 2010). However, the strict ontological separation of V(D)J joining and CSR may break down under some circumstances [Milili et al. 1991; Rolink et al. 1996; Weller et al. 2001; Dudley et al. 2002; Mao et al. 2004; Han et al. 2007; Ueda et al. 2007; Scheeren et al. 2008; Kuraoka et al. 2009; Wesemann et al. 2011]. For example, in μMT mice, membrane IgM is not expressed, and B-cell development is halted at the pro-B-cell stage; nevertheless, IgA is selectively expressed (Melamed et al. 2000; Macpherson et al. 2001), implying an alternative pathway for CSR that can circumvent the pro-B-cell block. However, it is unclear where and under what circumstances the switch to IgA occurs.

Omenn syndrome is a severe combined immunodeficiency disease associated with autoimmunity and atopy, most commonly arising from hypomorphic mutations in the Rag recombinase genes. The syndrome is characterized by the severe paucity of B and T lymphocytes, hypogammaglobulinemia, and, paradoxically, elevated IgE levels [Wong and Roth 2007; Ozcan et al. 2008]. To account for hyper-IgE in Omenn syndrome, we hypothesized that (1) CSR occurs in BM pro-B cells prior to V(D)J joining, (2) CSR is predisposed to IgE, and (3) V(D)J joining events can follow CSR. Here, we show that Rag-deficient or Mb1-deficient pro-B cells can be induced to undergo robust CSR, albeit to a restricted subset of Ig constant (C) region genes, including IgG2b and IgE, prior to or following V(D)J joining. Immunization of Rag1−/− mice with LPS promotes IgG2b switching in BM pro-B cells. We also demonstrate that V(D)J joining can follow CSR in Abelson transformed pro-B-cell and pre-B-cell lines. Studies of IgH locus chromatin structure revealed a unique stage-specific organization in pro-B cells that is correlated with preferential IgG2b and IgE switching. The flexible expression of the V(D)J recombination and CSR programs has implications for the genesis of Omenn syndrome hyper-IgE, autoimmune repertoire development, and leukemogenesis via the coexpression of the RAG and AID recombinases [Tsai et al. 2008].

Results and Discussion

To test the proposition that robust CSR can occur in BM pro-B cells prior to V(D)J recombination, we evaluated Rag2, AID, and isotype-specific germline transcript (GLT) expression in an E2A−/− pre-pro-B cell line, in pro-B cells isolated from Rag1- or Mb1-deficient mice, and in a panel of Abelson transformed pro-B-cell lines [Rag-deficient R2K2 and 445.3] and pre-B-cell lines [PA112.1, PA112.2, PA48.1, A70.2, and ATM2A]. E2A and Rag deficiencies preclude V(D)J joining, whereas Mb1-deficient pro-B cells assemble V(D)J exons but cannot signal through the pre-BCR and are blocked at the pro-B-cell stage (Pelanda et al. 2002). Elevated Rag2 but not AID transcripts are detected in all strains of unstimulated pre-pro-B and pro-B cells but not in the Abelson transformed cell lines, as expected [Fig. 1A, top; Supplemental Fig. S1; Muljo and Schlissel 2003]. Upon activation with the CSR inducers LPS+CD40L

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or LPS+CD40L+IL4, Rag2 expression is reduced in E2A deficient pre-pro-B cells and Rag1-deficient or Mb1-deficient pro-B cells (Fig. 1A). In contrast, AID expression is markedly induced in Rag1-deficient and Mb1-deficient pro-B cells but not E2A-/- pre-pro-B cells [Fig. 1A, bottom]. Similarly, in R2K2 and PA112.2 as well as other Abelson transformed cell lines, AID expression substantially increases in response to CSR stimuli [Fig. 1A, bottom, Supplemental Fig. S1A,B]. E2A-/- pre-pro-B cells were not further considered because AID is not expressed. Previous studies of BM pro-B cells concluded that AID transcription was undetectable because AID is not expressed. Previous studies of BM pro-B cells from Rag1-/- mice injected with LPS or PBS. Wild-type or AID-/- splenic B cells activated with LPS or no template control [C] are representative of three independent experiments.

Activation of AID expression and germline transcription prompted us to investigate whether CSR occurs in pro-B or pre-B cells. We used semiquantitative digestion circularization PCR (DC-PCR) to detect CSR events, since pro-B cells do not express surface Ig [Fig. 1C, top]. The nonrearranging Acetylcholine receptor [nAChR] gene loading control. The nAChR gene was used as a loading control [Fig. 1C]. DC-PCR products representing μ → γ2b and μ → ε CSR events are detected in the Rag1-deficient and Mb1-deficient pro-B cells and Abelson transformed cell lines, whereas μ → γ3 or μ → γ1 events are not detected in either case [Fig. 1C, bottom, Supplemental Fig. S2]. In contrast, in mature splenic B cells activated with LPS or LPS+IL4, a full spectrum of μ → γ3/γ2b and μ → γ1/ε CSR events is detected, respectively [Fig. 1C]. This restricted pattern of CSR in the pro-B versus mature B cells parallels their GLT expression profiles. Strikingly, the levels of DC-PCR products in primary pro-B cells and activated splenic B cells were similar, indicating robust CSR in early B cells [Fig. 1C, Supplemental Fig. S2].

High-frequency μ → γ2b/ε CSR in pro-B cells was confirmed by several independent methods, including analysis of post-switch transcripts [PSTs], circle transcript PCR [CT-PCR] assays, and S/S junction analyses. Following CSR, the ιμ exon in is close proximity with a new downstream Cμ region, thereby permitting PST expression that was detected using a forward primer in ιμ together with a reverse primer in the relevant Cμ exon. PST γ2b and ε are induced in Rag1-deficient and Mb1-deficient pro-B cells and Abelson transformed cell lines.
upon activation with CSR stimuli (Supplemental Fig. S3). CT-PCR assays indicate that \( \mu \rightarrow \gamma 2b \) and \( \mu \rightarrow e \) CSR occurs dynamically in Rag1\(^{-/-}\) pro-B cells and Abelson transformed cell lines (Supplemental Fig. S4). We sequenced S/S recombination junctions from appropriately activated Rag1\(^{-/-}\) pro-B cells or R2K2 cells and found that S4 joined to a variety of sites in Sy2b or S6 (Supplemental Figs. S5–S8). Switch junctions were blunt or contained short junctional microhomologies or occasional insertions in normal proportions (Stavnezer et al. 2010) and confirmed that the mechanism of CSR in pro-B cells is operationally indistinguishable from that observed in mature B cells.

To determine whether CSR can be induced in vivo, Rag1\(^{-/-}\) mice were injected with LPS or PBS, and pro-B cells were isolated directly from the BM and tested for CSR by DC-PCR. Wild-type or AID-deficient splenic B cells that were activated with LPS in ex vivo culture served as positive and negative controls for CSR, respectively. nAChR amplification is shown as a loading control, and the absence of PCR products in reactions devoid of template demonstrate PCR specificity. Under these activation conditions, \( \mu \rightarrow e \) CSR is negligible. Switching \( \mu \rightarrow \gamma 2b \) but not \( \mu \rightarrow \gamma 3 \) was observed in 67% (six out of nine) of LPS immunized mice \( \left(P = 0.007; \chi^2 \right) \) but not \( \chi^2 \) (zero out of six) for PBS-injected controls from three independent experiments (Fig. 1D). Targeting of CSR to the \( \gamma 2b \) but not the \( \gamma 3 \) locus reflects the established GLT expression pattern that we observed for pro-B cells activated in ex vivo culture (Fig. 1B,C). We conclude that CSR can occur in BM Rag1\(^{-/-}\) pro-B cells prior to V(D)J recombination.

To address the question of whether a B cell that experienced CSR can subsequently undergo V(D)J joining, switched clones from the R2K2 pro-B-cell line were isolated by limiting dilution. CSR was confirmed in clones B5 (\( \mu \rightarrow \gamma 2b \) and A2 (\( \mu \rightarrow e \) by DC-PCR, and clonality was demonstrated by virtue of unique intra-Sp region rearrangements (Fig. 2A; Supplemental Fig. S9). Treatment of these cell lines with STI571, a pharmacologic inhibitor of Abl kinase activity, resulted in activation of V(D)J joining and differentiation to the late pre-B-cell state (Muljo and Schlissel 2003). STI571 stimulation of B5 and A2 clones led to induction of the V(D)J joining gene program (Fig. 2B; Muljo and Schlissel 2003). The Rag2\(^{+/} \) but not the empty expression construct induced diverse D\(_{\text{DFL}16.1-J1} \) coding joins (CJs), while PST expression persisted (Fig. 2C; Supplemental Figs. S10, S11). Thus, pro-B-cell clones that experienced CSR retain the ability to undergo V(D)J recombination. Importantly, evidence indicates that BM pro-B cells expressing IgG1 (Waisman et al. 2007; Dougan et al. 2012) or transgenic Ig\( \gamma 2b \) chains (Storb et al. 1994) are capable of mature B-cell development that is indistinguishable from wild type.

To determine whether class-switched pre-B cells could complete the developmental program of recombination, we assayed V-J joining at the light chain Igk locus. PA112.2 cells (strain 129; Lk, germline) were exposed to CSR inducers for 48 h, and switching was activated as indicated by increased expression of AID and PSTs \( \gamma 2b \) or \( e \), which persisted for 8 h following withdrawal of the CSR inducers (Fig. 2D,E). In cells exposed to STI571, AID diminished, and Rag2 concomitantly increased, accompanied by active V\(_{6.23-J1} \) CJ formation (Fig. 2D,F) with appropriate junctional diversity (Supplemental Fig. S12). CSR induction had no effect on subsequent generation of V\(_{6.23-J1} \) CJs (Supplemental Fig. S13). Active PST expression following STI571 treatment indicates that populations of switched cells are fully capable of V(D)J joining (Fig. 2D,E). We conclude that B cells expressing BCR with secondary isotypes may emerge directly from the BM. Intriguingly, the CSR or V(D)J gene programs can be alternately expressed in response to specific inducers during early B-cell ontogeny.

The pattern of CSR in pro-B cells is skewed toward IgG2b and IgE and is atypical of mature B cells. Germline transcription in activated mature B cells is regulated by the spatial organization of the Igk locus through long-range chromatin contacts between the E\(_{\text{mu}} \) and \( 3' \) \( \text{Ea} \) enhancers and GLT promoters (Supplemental Fig. S14A; Wuerfel et al. 2007; Sellars et al. 2009). Could \( \gamma 3-\gamma 1 \) contacts in a developmental stage-specific chromatin structure restrict CSR in pro-B cells (Supplemental Fig. S14B)? Using the chromosome conformation capture (3C) carbon copy (5C) method for unbiased detection of chromatin contacts (Dostie et al. 2006), we simultaneously examined a matrix of 12,656 possible looping interactions in parallel across the entire Igk locus and focused here on the 220-kb C\(_{\text{H}} \) domain spanning E\(_{\text{mu}} \) and
3′Eα (Supplemental Material, Supplemental Fig. S15; Supplemental Tables S2, S3). We detected abundant γ3-γ1 chromatin interactions (Fig. 3, orange circle), indicating a compact conformation in Rag2−/− pro-B cells that is absent in resting splenic B cells (Fig. 3). We examined the log2 ratio pro-B/resting B 5C interactions [represented by red [pro-B] and blue [resting B] in Fig. 3] and confirmed that γ3-γ1 interactions are elevated in pro-B cells [Fig. 3]. We found that Eμ:γ2b-ε and Eμ:γ3-γ1 contacts are associated with pro-B and resting B cells, respectively, in accord with the pattern of potential GLT expression and CSR. In contrast, few long-range interactions were detected for a gene desert region on chromosome 5 (Supplemental Fig. S16).

3C studies confirmed abundant γ3-γ1a (1.7-fold, \(P = 0.053\)) and γ3:γ1b (twofold, \(P = 0.01\)) chromatin contacts in Rag2−/− pro-B cells as compared with resting B cells or ConA-activated splenic T cells, where γ1a and γ1b are opposite ends of Hind III fragment C [Fig. 4A,B]. 

Rag2−/− pro-B cells and the 445.3 pro-B-cell line display a similar profile of γ3:γ1 interactions, indicating that in early B cells, a compacted higher-order chromatin structure involving γ3:γ1 may limit that accessibility to the CSR machinery [Supplemental Fig. S17]. In contrast, γ3:γ2b contacts observed in both Rag2−/− pro-B cells and wild-type resting B cells are similar to each other and may be related to a spatial organization that is absent in T cells [Fig. 4B].

Because interaction of distal Igh enhancers with the targeted Cμ locus is essential to bring participating S regions into close proximity (Wuerffel et al. 2007), we examined Rag2−/− pro-B and 445.3 cells for these chromatin contacts in 3C assays. 3′Eα:Eμ [H-A] interactions in Rag2−/− pro-B cells and resting splenic B cells are similarly abundant and significantly higher than in ConA-stimulated splenic T cells [Fig. 4C, left panel]. When CSR is induced in LPS+IL4-activated B cells, 3′Eα:Eμ [H-A] interactions become elevated relative to resting B cells [Fig. 4C, left panel]. Notably, when the 445.3 pro-B-cell line is activated with LPS+CD40L to initiate CSR, 3′Eα:Eμ [H-A] \(P < 0.006\) and hs3b,4-γ2b \(P < 0.002\) interactions significantly increase, whereas γ3:3′Eα interactions remain unchanged [Fig. 4C, right panel]. Thus, our results demonstrate a unique three-dimensional chromatin structure in pro-B cells that supports isotype-specific CSR and appears to preclude γ3 and γ1 GLT expression and CSR in a developmental stage-specific fashion [Supplemental Fig. S14B].

Our findings establish that pro-B cells in the BM can undergo robust CSR, biased toward IgG2b and IgE, in response to specific stimuli followed by V(D)J recombination. Thus, pro-B cells that are delayed in the BM by genetic predisposition [as in Omenn syndrome] or environmental cues have the capacity to undergo CSR in response to bacterial infections and to eventually populate the periphery following V(D)J joining. Two additional conclusions flow from our findings. The origin of DNA damage reminiscent of both RAG and AID activity in some human leukemias (Tsai et al. 2008) has been difficult to explain. This footprint of DNA damage in leukemia may be related to coexpression of AID and RAG1/2 that we observed in pro-B cells induced to switch. Most notably, alternating expression of CSR versus V(D)J joining in early B cells reveals an unanticipated flexibility of these gene programs, with implications for our understanding of developmental and lineage commitment.

Materials and methods

In brief, mice were handled according to institutional and National Institutes of Health (NIH) guidelines. Standard protocols were used for cell culture, quantitative PCR, RT-PCR, DC-PCR, CT-PCR, retroviral transductions, statistical analyses, and switch, Vκ-Jκ, and Dκ-Jκ junction.
cloning. Abelson-MuLV transformed pro-B-cell or pre-B-cell lines were kindly provided by Dr. B. Sleckman (Washington University, St. Louis). 3C assays were carried out (Wuerffel et al. 2007) in combination with 5FAM/3BHQ1-modified probes. 5C primers were designed using online tools (http://my5C.umassmed.edu). 5C library construction was performed as described (Dostie et al. 2006). Additional methods are available in the Supplemental Material.

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