

Counterselection against D μ Is Mediated through Immunoglobulin (Ig) α -Ig β

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Summary

The pre-B cell receptor is a key checkpoint regulator in developing B cells. Early events that are controlled by the pre-B cell receptor include positive selection for cells express membrane immunoglobulin heavy chains and negative selection against cells expressing truncated immunoglobulins that lack a complete variable region (D μ). Positive selection is known to be mediated by membrane immunoglobulin heavy chains through Ig α -Ig β , whereas the mechanism for counterselection against D μ has not been determined. We have examined the role of the Ig α -Ig β signal transducers in counterselection against D μ using mice that lack Ig β . We found that D μ expression is not selected against in developing B cells in Ig β mutant mice. Thus, the molecular mechanism for counterselection against D μ in pre-B cells resembles positive selection in that it requires interaction between mD μ and Ig α -Ig β .

The object of B lymphocyte development is to produce cells with a diverse group of clonally restricted antigen receptors that are not self reactive (1). Antigen receptor diversification is achieved through regulated genomic rearrangements that result in the random assembly of Ig gene segments into productive transcription units (2, 3). These gene rearrangements are in large part regulated by the pre-B cell receptor (BCR)¹.

B cells undergoing Ig heavy chain gene rearrangements (pre-B) can express at least two types of BCRs. One form of the receptor is composed of membrane immunoglobulin heavy chain (mIg μ), λ 5, V-pre-B, and Ig α -Ig β , and is referred to as the pre-BCR (4–6). A second form of the pre-B cell receptor, known as the D μ pre-BCR (7), is found only in pre-B1 cells (8) and contains truncated mIg μ chains lacking a V_H domain (mD μ). mD μ is produced by Ig genes that have rearranged DJ_H gene segments in reading frame (RF) 2 producing an in-frame start codon and a truncated transcription unit (7). Like authentic mIg μ , mD μ is a membrane protein that forms a complex with λ 5, V-pre-B, and Ig α -Ig β , and in tissue culture cell lines the D μ pre-BCR can activate cellular signaling responses (9–14). But despite its ability to activate nonreceptor tyrosine kinases, D μ pre-BCR producing pre-B cells are selected against by a process that is mediated through the transmembrane domain of the mD μ protein (15). In contrast, pre-B cells that express intact mIg μ containing pre-BCRs are positively selected.

Counterselection is reflected in the relative lack of mature B cells that express mIg μ in RF2 (15–17). The mechanism by which mD μ activates counterselection has not been defined, but is known to require expression of syk (18). Here we report on experiments showing that Ig β is essential for counterselection against mD μ in vivo.

Materials and Methods

Mice. Ig β ^{-/-}, mIg μ , and Bcl-2 transgenic strains have been previously described and were maintained by backcrossing with BALB/c mice under specific pathogen-free conditions (19–21). All experiments were performed with 4–8-wk-old female mice.

Fluorescence Analysis and Cell Sorting. Single cell suspensions prepared from bone marrow or spleen were stained with PE-labeled anti-B220 and FITC-labeled anti-CD43 (PharMingen, San Diego, CA) or FITC-labeled anti-IgM, and analyzed on a FACScan[®]. For cell sorting, bone marrow cells from four to six mice were stained with the same reagents and separated on a FACSvantage[®]. CD43⁺B220⁻ and CD43⁺B220⁺ cells were collected based on gating with RAG-1^{-/-} controls.

DNA and PCR. Total bone marrow DNA was prepared for PCR as previously described (22). DNA from sorted cells was prepared for PCR in agarose plugs (23). Primers for V_H-DJ_H and D_H-J_H rearrangement were as in reference 22; these primers are mouse specific and do not detect the human Ig μ transgene. All experiments were performed a minimum of three times with two independently derived DNA samples. Nonrearranging Ig gene intervening sequences were amplified in parallel with other reactions and used as a loading control (22). Amplified DNA was visualized after transfer to nylon membranes by hybridization with a 6-kb EcoR1 fragment that spans the mouse J_H region.

Isolation and Sequencing of V_H-DJ_H and D_H-J_H Joints. A J_H4 primer was combined with either a D_H primer or a VHJ558L primer to

¹Abbreviations used in this paper: BCR, B cell receptor; mIg μ , membrane immunoglobulin heavy chain; RF, reading frame.

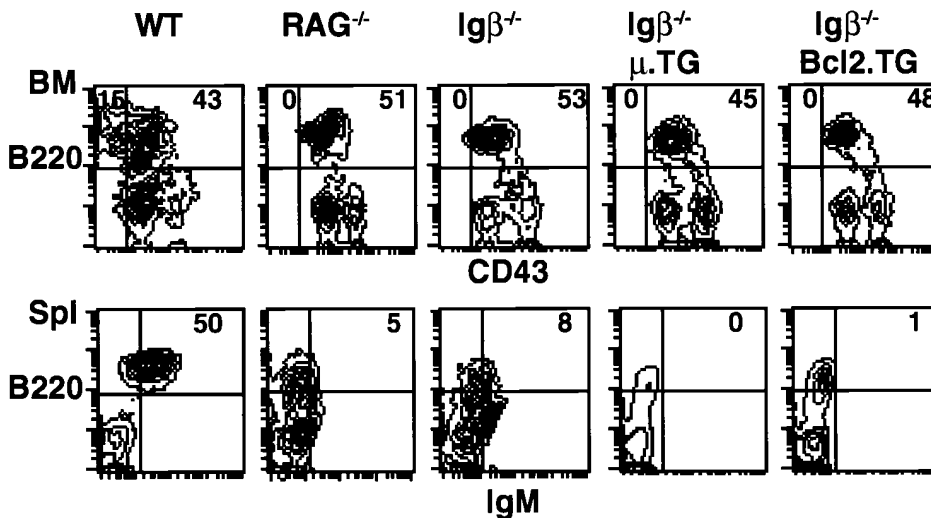


Figure 1. Flow cytometric analysis of spleen (*Spl*) and bone marrow cells (*BM*) from $Ig\beta^{-/-}$, transgenic, and wild-type mice. Single-cell suspensions from lymphoid organs of 6-wk-old mice were prepared and analyzed on FACScan[®]. Bone marrow cells were stained with PE-anti-B220 and FITC-anti-CD43. Spleen cells were stained with FITC-anti-IgM and PE-anti-B220. The lymphocyte population was gated according to standard forward- and side-scatter values. The numbers in each quadrant represent the percentages of gated lymphocytes. *WT*, wild type; *RAG*^{-/-}, RAG-1 mutant; *Igβ*^{-/-}, *Igβ* mutant; *Igβ*^{-/-} μ .TG, *Igβ* mutant, *mIgμ*, transgenic.

amplify DJ_H and VDJ_H rearrangements, respectively. The primers were: (a) J_H4, ACGGATCCGGTGACTGAGGTTCT; (b) D_H, ACAAGCTTCAAAGCACAATGCCTGGCT; and (c) VHJ558L, GCGAAGCTTA(A,G)GCCTGGG(A,G)CTTCAGTGAAG. PCR amplification for DJ_H joints was for 35 cycles of 0.5 min at 94°C, and 2 min at 72°C; for VDJ_H joints, it was for 0.5 min at 94°C, 1 min at 68°C, and 1.5 min at 72°C. PCR products were purified by agarose gel electrophoresis, subcloned into pBluescript, sequenced using an Applied Biosystems (Foster City, CA) DNA sequencing kit, and analyzed on a genetic analyzer (ABI-310; Applied Biosystems).

Results

mIgM Cannot Induce the Pre-B Cell Transition or Allelic Exclusion in the Absence of Igβ. Expression of *Igβ* is required for B cells to efficiently complete *Ig V_H* to DJ_H gene rearrangements (19). B cells in $Ig\beta^{-/-}$ mice fail to express normal levels of *mIgμ*, and B cell development is arrested at the CD43⁺B220⁺ pre-B1 stage (19). A similar celltype specific developmental arrest is also found in mice that carry a mutation in the transmembrane domain of *mIgμ* (24), and mice that fail to complete *Ig V(D)J* recombination (25–29). In view of the abnormally low levels of *mIgμ* in $Ig\beta^{-/-}$ mice, failed pre-B cell development might simply be due to lack of *Ig* expression.

To determine whether *mIgμ* could induce the pre-B cell transition in the absence of *Igβ*, we introduced a productively rearranged immunoglobulin gene (20) into the $Ig\beta^{-/-}$ background (TG. μ $Ig\beta^{-/-}$). We then measured B cell development by staining bone marrow cells with anti-CD43 and anti-B220 monoclonal antibodies (30). We found that expression of a pre-rearranged *Ig* transgene was not sufficient to activate the pre-B cell transition in the absence of *Igβ* (Fig. 1). TG. μ $Ig\beta^{-/-}$ B cells did not develop past the CD43⁺B220⁺ pre-B cell stage (Fig. 1). In control experiments, the same *mIgμ* transgene did induce the appearance of more mature CD43⁻B220⁺ pre-B cells in a RAG^{-/-} mutant background where B cell development was similarly arrested at the CD43⁺B220⁺ stage (20,

25, 26; data not shown). We conclude that in the absence of *Igβ*, a productively rearranged *mIgμ* is unable to activate the pre-B cell transition.

Allelic exclusion is established as early as the CD43⁺B220⁺ stage of B cell development (31–33). This early stage of development is found in the bone marrow of $Ig\beta^{-/-}$ mice (19). However, we were initially unable to measure allelic exclusion in *Igβ* mutant mice due to the low efficiency of complete *Ig V_H* to DJ_H gene rearrangements and absence of surface *Igμ* expression (19). To determine whether expression of *mIgμ* could activate allelic exclusion in TG. μ $Ig\beta^{-/-}$ mice, we measured inhibition of *V_H* to DJ_H gene rearrangements by PCR (34). In controls, the *mIgμ* transgene inhibited *V_H* to DJ_H gene rearrangement (22), but the same transgene had no effect in the $Ig\beta^{-/-}$ background (Fig. 2). We had previously shown that the cytoplasmic domains of *Igα* and *Igβ* are sufficient to activate allelic exclusion (20, 35). The finding that *mIgμ* is unable to induce allelic exclusion in the absence of *Igβ* suggests that *Igβ* is essential for allelic exclusion.

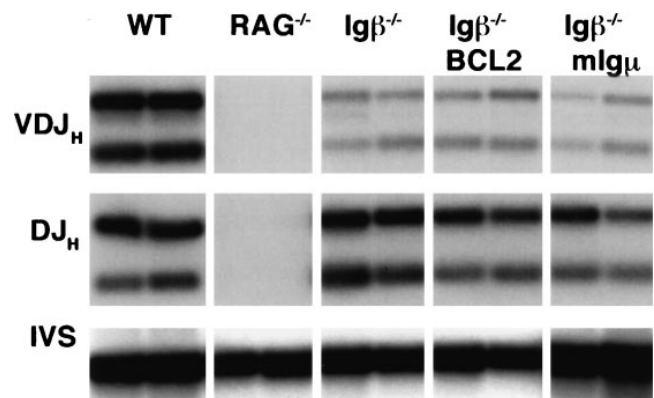


Figure 2. *Ig* gene rearrangements in $Ig\beta^{-/-}$, transgenic, and wild-type mice. Bone marrow DNA from 6-wk-old mice was amplified with VH558L, VH7183 (not shown), or D_H and J_H2 primers. Control primers were from the J-CH1 intervening sequence (*IVS*) (22).

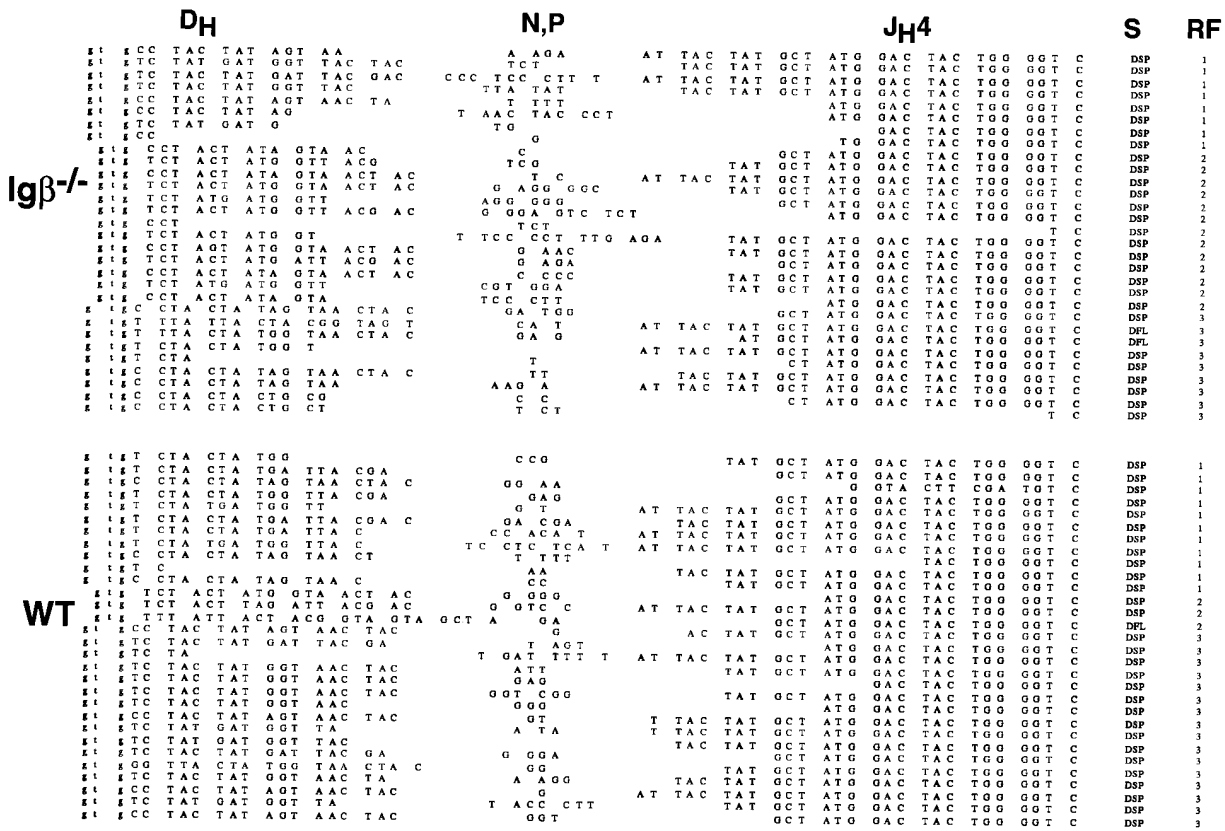


Figure 3. Nucleotide sequences of D_H - J_H joints from $Ig\beta^{-/-}$ and wild-type sorted bone marrow B cells. D_H segment, N or P nucleotides, and J_H4 segment sequences are shown. The number of DJ_H4 joints in D_H RF 1, 2, or 3 are noted. Stop codons in D_H segments are underlined.

Igβ Is Required for RF2 Counterselection. Igs with D_H joined to J_H in RF2 are rarely found in mature B cells (15–17). Genetic experiments in mice have shown that counterselection against RF2 requires the transmembrane domain of mIgμ and the syk tyrosine kinase (15, 18). To determine whether counterselection is mediated through $Ig\beta$, we sequenced DJ_H joints amplified from sorted $CD43^+B220^+$ pre-B cells from $Ig\beta^{-/-}$ mice and controls. In control samples, only 10% of the DJ_H joints were in RF2 (Fig. 3), which is in agreement with similar measurements performed in other laboratories (15–17, 31–33). In contrast, there was no counterselection in the bone marrow cells of $Ig\beta^{-/-}$ mice; 13 out of 30 DJ_H joints were in RF2 with the remainder being distributed in RF1 and 3 (Fig. 3). Thus, in the absence of $Ig\beta$, there was no RF2 counterselection at the level of DJ_H rearrangements in $CD43^+B220^+$ cells in the bone marrow.

V_H to DJ_H joining and counterselection are normally completed in $CD43^+B220^+$ pre-B cells (31–33), but in $Ig\beta^{-/-}$ mice, V_H to DJ_H joining is inefficient (19). To determine whether RF2 was counterselected in the few $Ig\beta$ mutant B cells that completed V_H to DJ_H rearrangements, we amplified and sequenced $VHJ558L$ - DJ_H4 joints from unfractionated bone marrow cells (Fig. 4). As with the DJ_H joints, we found no evidence for counterselection against RF2 in VDJ_H joints in $Ig\beta^{-/-}$ B cells. 10/33 $VHJ558L$ -

DJ_H4 joints sequenced from $Ig\beta^{-/-}$ mice were in RF2. By contrast, RF2 was only found in 1 of 11 mature Igs in the controls. The VDJ_H and DJ_H $Ig\beta^{-/-}$ joints otherwise resembled the wild type in the number of N and P nucleotides as well as in the extent of nucleotide deletion (Figs. 3 and 4). We conclude that there was no selection against RF2 in the absence of $Ig\beta$, and that the absence of $Ig\beta$ has no significant impact on the mechanics of recombination as measured by the variability of the joints.

Discussion

The transmembrane domain of mIgμ is required to produce the signals that mediate several antigen-independent events in developing B cells, including allelic exclusion and the pre-B cell transition (24, 36–39). However, mIgμ itself is insufficient for signal transduction (40), and it requires the $Ig\alpha$ and $Ig\beta$ signaling proteins to activate B cell responses *in vitro* and *in vivo*.

The earliest developmental checkpoint regulated by $Ig\alpha$ - $Ig\beta$ appears to involve either activation of cellular competence to complete V_H to DJ_H rearrangements, or positive selection for cells that express mIgμ (19). In the next phase of the B cell pathway, the same transducers are necessary (Fig. 2) and sufficient to produce the signals that activate allelic exclusion and the pre-B cell transition (19, 20, 35, 41).

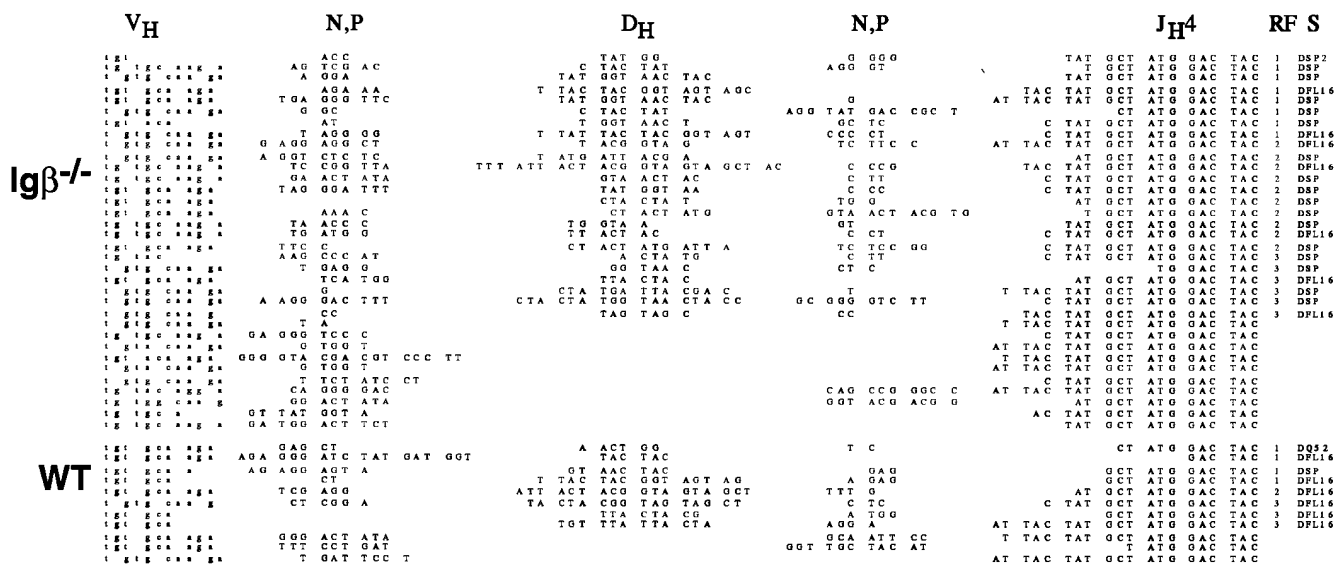


Figure 4. Nucleotide sequences of V(D)J (VH558L to DJH4) joints from $Ig\beta^{-/-}$ and wild-type bone marrow B cells. D_H segment, N or P nucleotides, and J_H4 segment sequences are shown. The number of DJ_H4 joints in D_H RF 1, 2, or 3 are noted. Stop codons in D_H segments are underlined, and the D_H segments used (S) are indicated.

In the present report, we show that in addition to these functions, $Ig\alpha$ - $Ig\beta$ transducers are also necessary for negative selection against $D\mu$.

Two models have been proposed to explain counterselection against $mD\mu$. The first model states that $mD\mu$ is toxic, and that cells expressing this protein are deleted by a mechanism that involves inhibition of proliferation (31). A second theory postulates that $D\mu$ proteins produce the signal for heavy chain allelic exclusion and block the completion of productive heavy chain gene rearrangements (15). According to this second model, cells expressing $mD\mu$ are then unable to continue along the B cell pathway. Support for the active signaling model comes from three sets of observations: (a) that there is no counterselection in the absence of a $Ig\mu$ transmembrane exon (15); (b) that there is no RF counterselection in the absence of syk (18); and (c) that there is no counterselection in early $CD43^+B220^+$ B cell precursors in the absence of $\lambda 5$ (33). These experiments partially define the receptor structure for counterselection as composed of $mD\mu$ associated with $\lambda 5$. Our observation that negative selection against $D\mu$ does not occur in the absence of $Ig\beta$ supports the signaling model, and identifies $Ig\alpha$ - $Ig\beta$ as the transducers that activate counterselection possibly by linking $mD\mu$ to nonreceptor tyrosine kinases.

Why does the expression of the $D\mu$ pre-BCR lead to arrested development, whereas mature $mIg\mu$ in the same complex activates positive selection in early B cells? Both signals are produced in $CD43^+B220^+$ pre-B cells, both require $\lambda 5$ (33, 39, 42), and the $Ig\alpha$ - $Ig\beta$ coreceptors (19, 41), and both are transmitted through a cascade that induces syk (18, 43). One way to explain the difference between the cellular response to $mD\mu$ pre-BCR and $mIg\mu$ pre-BCR expression might be an inability of $D\mu$ to pair with conventional κ or

λ Ig light chains (14). According to this model, cells expressing $mD\mu$ should be trapped in the $CD43^+B220^+$ pre-B cell compartment since B cell development can progress to the $CD43^+B220^+$ stage in the absence of conventional light chains (44, 45). However, elegant single cell sorting experiments have shown that $mD\mu$ -producing cells are selected against before this stage in $CD43^+B220^+$ pre-B cells (33, 42). Thus, the idea that abnormal pairing of $mD\mu$ with light chains is responsible for counterselection fails to take into account the observation that counterselection normally occurs independently of light chain gene rearrangements.

Two alternative explanations for the disparate cellular responses to the $D\mu$ pre-BCR and the $mIg\mu$ pre-BCR are: (a) that there are qualitative differences between signals generated by a $mD\mu$ and a $mIg\mu$ receptor complex, and (b) pre-B-I cells that contain DJ_H rearrangements are in a different stage of differentiation than pre-B-II cells that have completed VDJ_H and express $mIg\mu$ (8). An example of two qualitatively distinct signals resulting in alternative biologic responses has been found in the highly homologous TCR receptor (46, 47). TCR interaction with ligand can produce either anergy or activation, depending on the affinity of the TCR for the peptide-MHC complex (48). High affinity ligands that produce T cell responses fully activate CD3 tyrosine phosphorylation, whereas peptides that induce anergy bind with low affinity and induce a reduced level of CD3 phosphorylation. The low level CD3 phosphorylation induced by the anergizing peptides is associated with less than optimal ZAP-70 kinase activation (46, 47).

Less is known about the physiologic responses activated by $Ig\alpha$ - $Ig\beta$ in developing B cells, but experiments in transgenic mice have shown that early B cell development requires tyrosine phosphorylation of $Ig\beta$ (20), and by

inference, receptor cross-linking. Although the cytoplasmic domains Ig α and Ig β appear to have redundant functions in allelic exclusion and the pre-B cell transition (20, 35), neither Ig α , (41) nor Ig β (Papavasiliou, N., and M.C. Nussenzweig, manuscript in preparation) alone are able to fully restore B cell development in the bone marrow, suggesting that there are specific functions for Ig α and Ig β , or the Ig α -Ig β heterodimer. Biochemical support for the idea that individual coreceptors could have unique biologic functions also comes from transfection experiments in B cell lines (49–51) and from the observation that the cytoplasmic do-

mains of Ig α and Ig β bind to different sets of nonreceptor tyrosine kinases (52).

We would like to propose that positive and negative selection in developing B cells, like activation and anergy in T cells, may be mediated by differential phosphorylation of Ig α and Ig β in the pre-BCR. Given the requirement for cross-linking in pre-BCR activation, the mechanism that produces the proposed differential phosphorylation of the mD μ and mIg μ pre-BCRs may be a function of their affinities for the cross-linker.

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