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B Cell Development Is Arrested at the Immature B Cell Stage in Mice Carrying a Mutation in the Cytoplasmic Domain of Immunoglobulin β

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Abstract

The B cell receptor (BCR) regulates B cell development and function through immunoglobulin (Ig)α and Igβ, a pair of membrane-bound Ig superfamily proteins, each of which contains a single cytoplasmic immunoreceptor tyrosine activation motif (ITAM). To determine the function of Igβ, we produced mice that carry a deletion of the cytoplasmic domain of Igβ (IgβΔC mice) and compared them to mice that carry a similar mutation in Igα (MB1ΔC, herein referred to as IgαΔC mice). IgβΔC mice differ from IgαΔC mice in that they show little impairment in early B cell development and they produce immature B cells that respond normally to BCR cross-linking as determined by Ca^{2+} flux. However, IgβΔC B cells are arrested at the immature stage of B cell development in the bone marrow and die by apoptosis. We conclude that the cytoplasmic domain Igβ is required for B cell development beyond the immature B cell stage and that Igα and Igβ have distinct biologic activities in vivo.

Key words: B cell receptor • immunoglobulin α • immunoglobulin β • immunoreceptor tyrosine activation motif • apoptosis

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Introduction

Signals from the B cell receptor (BCR)[1] regulate many of the essential physiologic activities in the B cell pathway. These include several different transitions in B cell development, allelic exclusion, central and peripheral tolerance, as well as B cell survival and response to antigen (1). All of these functions appear to be induced by signals emanating from the Ig-associated heterodimer of Igα and Igβ (2–4). Signals initiated by ligand binding to membrane (m)IgM are communicated to the Igα–Igβ transducer through a noncovalent interaction that involves polar residues in the plane of the cell membrane (5–7). Mutations that disrupt these polar residues interfere with signal transduction and early B cell development (5–8).

The discovery that the BCR signal transducer is a heterodimer led to the proposal that the Igα and Igβ subunits might have distinct biological functions. Biochemical studies showing that the cytoplasmic tails of Igα and Igβ bind to different sets of cellular kinases (9) and transfection experiments showing differences in the signaling activities of Igα and Igβ cytoplasmic domains support this idea (6, 7, 10–14). However, experiments performed in mice have failed to show any differences in the biologic activities of Igα and Igβ. Similarly, there are no known qualitative differences in the activities of any of the immunoreceptor tyrosine activation motifs (ITAMs) in the CD3 chains of the TCR (15–21).

Three approaches have been used to determine the function of Igα and Igβ in vivo: transgenic expression of chimeric proteins (8, 22, 23), Igβ gene deletion (24), and Igα cytoplasmic tail mutation (25). Transgenic experiments showed that the cytoplasmic domain of either Igα or Igβ was sufficient to activate allelic exclusion and pre-B cell development and led to the conclusion that Igα and Igβ are redundant in early B cell development (8, 22, 23). Deletion of Igβ resulted in B cells that failed to assemble a BCR and

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[1]*Abbreviations used in this paper:* BCR, B cell receptor; CGG, chicken gamma globulin; ITAM, immunoreceptor tyrosine activation motif; RAG, recombination activating gene; SH2, src homology 2.

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were arrested at the pre-BI cell stage, suggesting that BCR assembly is essential for B cell development (24). Deletion of 40 of the 61 cytoplasmic amino acids of Igα, including both ITAM tyrosines (IgαΔC [25]), produced B cells that assembled a mutant BCR composed of mIgμ and an Igα–Igβ heterodimer with a truncated Igα tail. In agreement with the transgenic experiments, the single Igβ cytoplasmic domain in the IgαΔC BCR was enough to induce pre-B cell development and allelic exclusion (8, 25). However, the number of pre-B cells in IgαΔC mice was reduced by 50%, immature B cells were reduced by 80%, and the number of mature B cells in spleen was only 1% of control. Thus, a BCR with only an Igβ cytoplasmic domain was unable to support later stages of B cell development. Furthermore, increased tyrosine phosphorylation in IgαΔC B cells and increased calcium flux in response to receptor cross-linking suggested a unique negative regulatory role for the Igα cytoplasmic domain (26, 27).

To compare the biologic function of Igα and Igβ directly, we produced mice that carry a targeted deletion of the cytoplasmic domain of Igβ.

Materials and Methods

Mice. IgβΔC mice were created by gene targeting in 129/Sv embryonic stem cells (24). To shorten the cytoplasmic tail of Igβ by 45 amino acids and delete the ITAM, the stop codon TGA was introduced by PCR at amino acid 184 (4). A unique HindIII site was placed into the targeting vector between the exons as indicated (see Fig. 1 A). A lox-P–flanked neomycin resistance gene was inserted between two XbaI sites, and sequence coding for diphtheria toxin (DTA [28]) was added to the 3′ end of the targeted locus at the XhoI site (see Fig. 1 A). Homologous recombination was confirmed by Southern blotting after digestion with HindIII (see Fig. 1 B). The rate of homologous recombination was 1:80. The genomic fragment used as a probe for Southern blotting was amplified by PCR using the specific primers GGATTCGAATGGTGAATGTTGG and AGGCTCTAGCTCAGTGAAGGGAG. PCR conditions were: 94°C for 5 min, and 30 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 1 min, followed by extension at 72°C for 7 min. To delete the neomycin gene, mice carrying the targeted Igβ gene were bred to C57BL/6 Cre transgenic mice (29). Deletion of the neomycin gene was confirmed by PCR using neomycin-specific primers ATGATTGAACAAGATGGATTGCAC and TCGTCCAGATCATCCTGATCGAC. PCR conditions were: 94°C for 3 min, and 30 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min, followed by extension at 72°C for 7 min. Heterozygous IgβΔC mice were backcrossed to C57BL/6 mice for three generations before intercrossing to produce homozygous IgβΔC mice. All mice were bred and maintained under specific pathogen–free conditions.

IgαΔC mice (25) were crossed with IgβΔC mice to create IgαΔC/IgβΔC mixed heterozygous and homozygous mice (25). IgβΔC mice were also bred to C57BL/6 Ig^{HEL} Ig transgenic mice (30).

Flow Cytometry. Single cell suspensions from bone marrow, spleen, and peritoneal cavity were stained with FITC, PE, allophycocyanin, and biotin-conjugated monoclonal antibodies visualized with streptavidin red 613 (GIBCO BRL). Monoclonal antibodies were anti-CD43, anti-IgM, anti-B220, anti-CD25, anti-IgD, anti-CD19, anti-IgM^a, anti-IgM^b (BD PharMingen), biotin anti-Igβ (a gift from H. Karasuyama, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), and anti-493 (a gift from A. Rolink, Basel Institute for Medical Science, Basel, Switzerland). For intracellular Igμ staining, cells were first surface stained with anti-B220–allophycocyanin, anti-CD25–PE, and/or anti-IgM–PE, and anti-CD43–biotin, permeabilized with Intraprep permeabilization kit (Immunotech), and incubated with Fab′ fragments of FITC-goat anti–mouse IgM. Data were collected on a FACSCalibur™ and analyzed using CELLQuest™ software (Becton Dickinson).

Cell Cycle Analysis. Bone marrow cells were incubated at 37°C for 40 min with Hoechst 33342 (Molecular Probes) diluted 1:1,000, then stained for cell surface markers with anti-B220, anti-IgM, anti-CD43, and anti-CD25. Data were collected on a FACS Vantage™ and analyzed with CELLQuest™ software (Becton Dickinson).

Ca^{2+} Flux. Bone marrow cells were adjusted to 5×10^6/ml in PBS plus 1% FCS plus 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (loading buffer), and incubated with 1.5 μM Indo-1-AM (Molecular Probes) for 30 min at 37°C. Cells were stained with PE–anti-B220 and Fab′ FITC-goat anti–mouse IgM (Jackson ImmunoResearch Laboratories). Calcium flux was measured by fluorescence emission ratios of Indo-1-AM on a dual laser FACS Vantage™ (Becton Dickinson) at 395/510 nm on $B220^{low}IgM^+$ cells. Data were acquired for 60 s before BCR cross-linking with $F(ab')_2$ goat anti–mouse IgM (Southern Biotechnology Associates, Inc.) at 10 or 20 μg/ml.

B Cell Cultures. Bone marrow B cells from mutant or wild-type mice were enriched by positive selection using MACS mouse CD19 microbeads (Miltenyi Biotech) and stained with Fab′ anti-IgM, and monoclonal anti-CD25, anti-CD43, and anti-B220. B cells were then sorted into $B220^+CD43^-IgM^{low}$ immature B cells and cocultured at 10^6/ml with irradiated S17 stromal cells in RPMI 1640 supplemented with 10% FCS and 10 ng/ml IL-7 (BD PharMingen [31]). B cell viability was assessed on days 0 and 1 by flow cytometry using PE–annexin V (BD PharMingen) and propidium iodide staining.

Immunization. 6–8-wk-old IgβΔC and C57BL/6 mice were immunized intraperitoneally with either 50 μg alum-precipitated 4-hydroxy-3-nitrophenylacetyl coupled to chicken gamma globulin (NP-CGG) or 50 μg NP-Ficoll in PBS. Blood was collected from the tail vein of each mouse before immunization and at days 7, 14, 21, and 28 after immunization. NP-specific IgM and IgG levels were measured by ELISA using plates coated with $NP_{16}BSA$ (5 μg/ml) and developed with anti-IgM coupled to horseradish peroxidase or anti-IgG coupled to horseradish peroxidase (Southern Biotechnology Associates, Inc. [32]). Immunoabsorbance was read at 415 nm and titers were calculated relative to control sera from unimmunized mice. Four mice were used in each group.

Results

B Cell Development in IgβΔC Mice. Gene targeting was used to introduce a stop codon at position 184 in Igβ (Fig. 1 A). The mutant gene directs the expression of a truncated Igβ protein that resembles mIgμ in having only three charged cytoplasmic anchor amino acids (DKD).

B cell development in IgβΔC mice was analyzed by multiparameter flow cytometry. When compared with wild-type controls, IgαΔC and IgβΔC mice showed an increase

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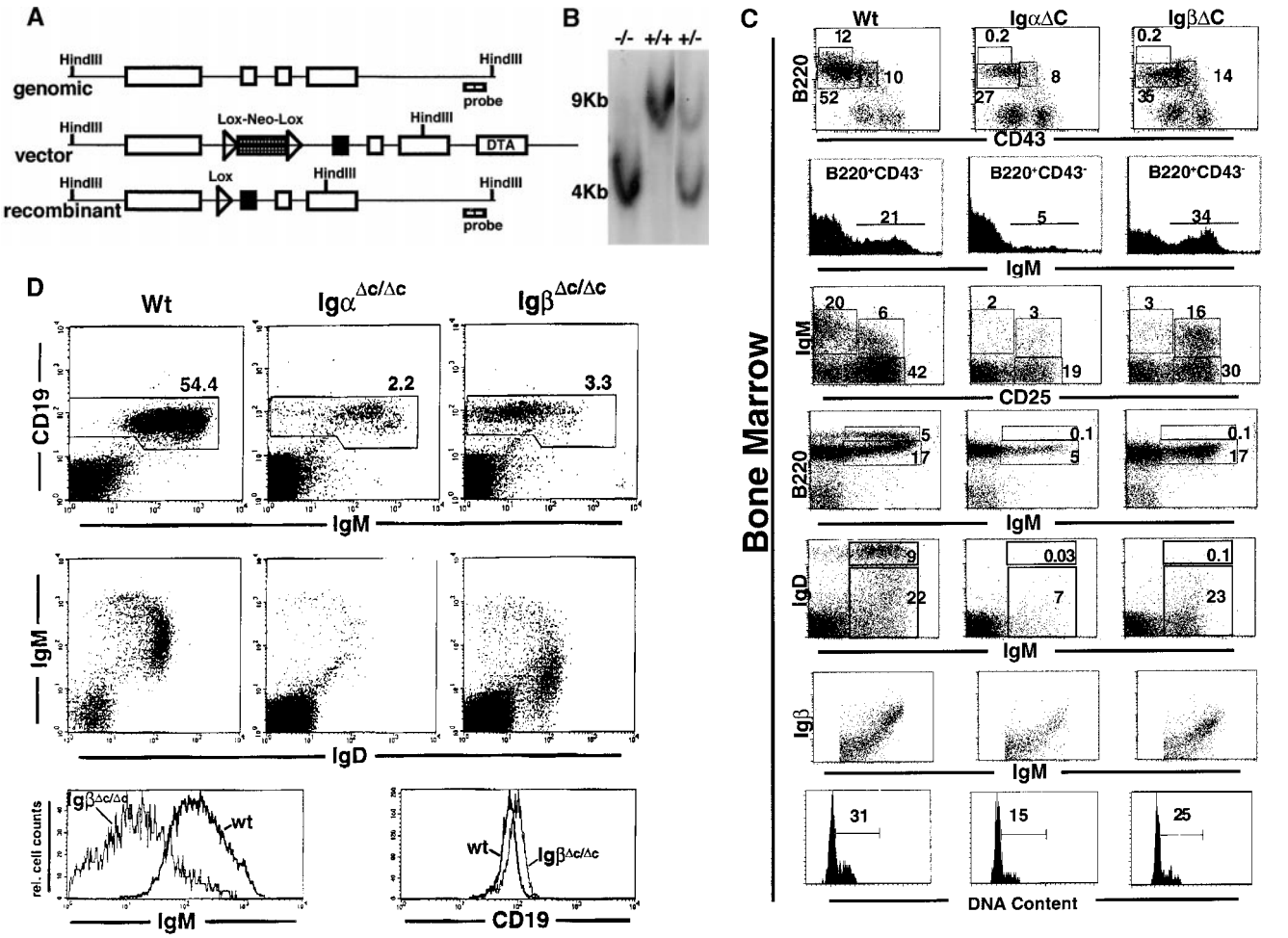

Figure 1. IgβΔC gene targeting. (A) Scheme for IgβΔC gene targeting showing the genomic Igβ locus (top), targeting vector (middle), and recombinant locus after deletion of the neo gene (bottom). Exons are represented by open boxes, loxP sites by open triangles, and the exon containing the mutant stop codon at position 184 by a filled box. The probe used to identify homologous recombinants is shown. (B) Southern blot analysis of wild-type, homozygous, and heterozygous mice. HindIII digestion of genomic DNA yields restriction fragments of 4 or 9 kb corresponding to targeted or wild-type alleles, respectively. (C) Analysis of bone marrow B cells in wild-type (Wt), IgαΔC, and IgβΔC mice. In B220 vs. CD43 and B220 vs. IgM plots, numbers indicate the percentages of lymphocytes determined by forward versus side scatter parameters. In IgD vs. IgM and IgM vs. CD25 plots, numbers indicate the percentages of $B220^+$ cells in the boxed region. In the IgM histograms, numbers indicate the percentage of $B220^+CD43^-$ that are IgM positive (immature B cells). IgD vs. IgM, IgM vs. CD25, and Igβ vs. IgM dot plots show bone marrow cells that were pregated on $B220^+$ cells. DNA content is shown through Hoechst 33342 staining of large $B220^+CD25^+$ pre-B cells, and the percentage of cells in S and G2/M phase is indicated. (D) Analysis of spleen B cells in wild-type (Wt), IgαΔC, and IgβΔC mice. Spleen plots for IgαΔC and IgβΔC mice show a fivefold greater number of events than the wild-type. Antibodies used for staining are indicated.

in the number of $IgM^-B220^+CD43^+CD25^-$ pro-B cells (25; Table I and Fig. 1 C). Both strains also showed smaller numbers of $IgM^-B220^+CD43^-CD25^+$ pre-BII cells (fraction C′/D) than wild-type, although the 25% decrease found in IgβΔC mice was less substantial than the 50% decrease found in IgαΔC mice (25; Table I and Fig. 1 C).

After H chain expression, pre-BI cells become large dividing pre-BII cells (fraction C′ [33–36]). To determine whether the single Igα cytoplasmic domain in the IgβΔC BCR is sufficient to trigger normal pre-BII cell division, we measured the DNA content of these cells. We found that the cell cycle distribution of large pre-BII cells in IgβΔC mice was similar to that of control mice (Fig. 1 C and Table I). Thus, the single cytoplasmic tail of Igα in the IgβΔC BCR is sufficient to trigger pre-BII cell (fraction C′) proliferation.

After mIgμ triggered proliferative expansion, B cells rearrange Ig L chain genes, express surface IgM, lose CD25 expression, and then express IgD (37). Few B cells in IgαΔC mice progress to the $B220^+CD43^-IgM^+IgD^-$ "immature" B cell stage (fraction E) (25; Fig. 1 C, second row; $B220^+CD43^-$ gated IgM histograms; IgM versus CD25, B220 versus IgM, and IgM versus IgD dot plots). In contrast, IgβΔC B cells proceed to this immature B cell stage in normal numbers, although the level of IgM expressed on the cell surface is lower than control (Fig. 1 C). Immature IgβΔC B cells fail to progress further to the $CD25^-IgM^+IgD^+$ transitional B cell stage (Fig. 1 C; IgM

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Table I. *B Cell Development and Cell Cycle Analysis*

	Percentage of B220$^+$ bone marrow cells				Percentage of B220$^+$ cells in S/G2/M	
	Pre-BI*	Pre-BII‡	Immature§	Recirculating‖	Pre-BII (small)¶	Pre-BII (large)**
C57BL/6	27 ± 2.2	37 ± 5.3	23 ± 12.6	7.0 ± 3.6	0.4 ± 0.3	33 ± 6.0
IgβΔC	55 ± 4.0	27 ± 2.8	28 ± 11.9	0.1 ± 0.1	0.7 ± 0.8	20 ± 6.5

Data represent the mean from four mice ± SD.
*CD43$^+$IgM$^-$CD25$^-$.
‡CD43$^-$IgM$^-$CD25$^+$.
§B220^{lo}CD43$^-$IgM$^+$.
‖B220^{hi}CD43$^-$IgM$^+$.
¶IgM$^-$CD25$^+$.
**IgM$^-$CD25$^+$.

versus CD25, B220 versus IgM, and IgM versus IgD). Failure to progress to the CD25$^-$IgM$^+$IgD$^+$ transitional B cell stage is reflected in the near absence of recirculating B cells in the bone marrow and mature B cells in spleen (Fig. 1, C and D). To determine whether this failure to mature is due to low levels of surface Igα–Igβ expression, we stained developing B cells with anti-Igβ monoclonal antibody (38). We found that for any given level of surface IgM expression, the level of cell surface Igβ on B220$^+$IgM$^+$ immature B cells was similar in IgβΔC B cells and controls (Fig. 1 C). Thus, IgαΔC mice suffer a continuous loss of B cell precursors beginning at the pre-B cell stage, whereas B cell development is terminated abruptly at the immature B cell stage in IgβΔC mice (25; Fig. 1 C and Table I).

To determine whether arrest at the CD25$^+$IgM$^+$IgD$^-$ immature B cell stage is associated with increased cell death, we established in vitro bone marrow cultures (31). Immature B cells were purified by cell sorting using a Fab′ anti-IgM to avoid receptor cross-linking. Cell death was measured by propidium iodide exclusion and annexin V staining (Fig. 2). Annexin V staining varies between different stages of B cell development and is therefore unreliable when comparing B cells in different stages (39). However, annexin is a reliable marker for apoptosis when comparing cells at similar stages

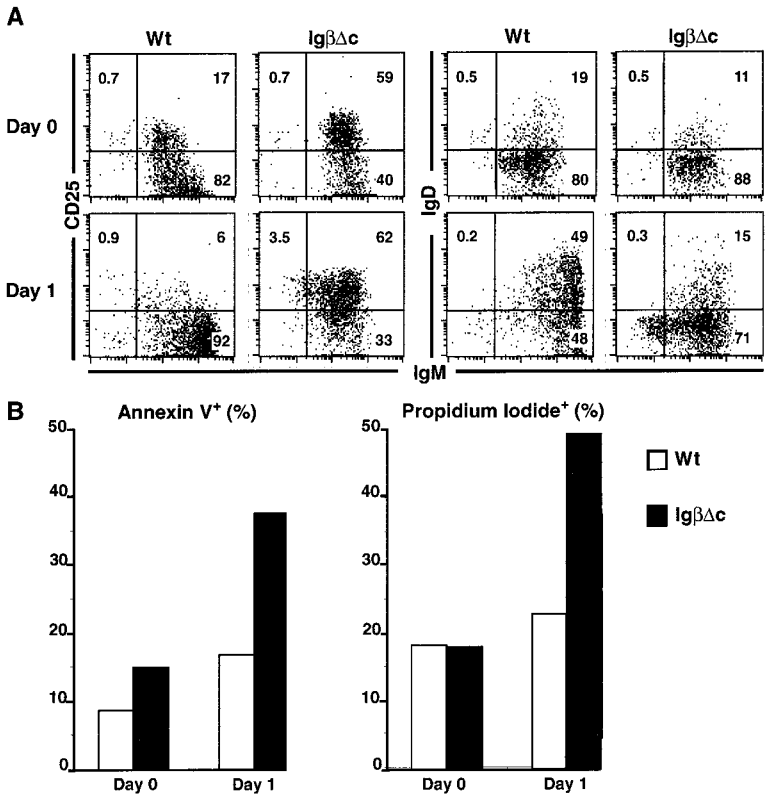

Figure 2. B cell development and death in bone marrow cultures. Bone marrow cells from IgβΔC and control mice were selected using CD19 MACS beads, sorted for B220$^+$CD43$^-$IgM^{low} immature B cells, and then cocultured with S17 stromal cells. (A) Dot plots show staining with anti-CD25 or anti-IgD and anti-IgM at the initiation of culture (left) and after 24 h (right). Numbers indicate percentages of B220$^+$ cells in each quadrant. Wt, wild-type. (B) Bar graphs show the percentage of B220$^+$ cells that were annexin V or propidium iodide. Results are the average of duplicate cultures performed on two independent mice. The variation between samples and mice was <5%.

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in development (39). Freshly isolated immature IgβΔC and control B cells were equally viable as measured by exclusion of propidium iodide. In culture, the control immature B cells developed into $CD25^{-}IgM^{hi}IgD^{+}$ transitional B cells, whereas the IgβΔC B cells did not progress beyond the $CD25^{+}IgM^{lo}IgD^{-}$ immature B cell stage. Instead, IgβΔC B cells became increasingly annexin V and propidium iodide positive (Fig. 2). Thus, IgβΔC B cells that reach the $CD25^{+}IgM^{+}IgD^{-}$ immature B cell stage fail to progress and die by apoptosis.

Allelic Exclusion. In addition to supporting pre-B cell development, cell surface expression of the BCR induces Ig H chain allelic exclusion (40, 41). To determine whether the single Igα cytoplasmic domain in IgβΔC mice is sufficient for allelic exclusion, we bred IgβΔC mice to Ig^{HEL} transgenic mice which carry an allotype marked $Ig\mu^{a}$ H chain (30). Ig^{HEL} transgenic IgβΔC mice resemble nontransgenic IgβΔC mice in that their B cells fail to progress beyond the immature stage of B cell development, they express lower levels of surface IgM than controls, and there are few detectable B cells in the spleen and the peritoneal cavity (Fig. 3). Nevertheless, allelic exclusion is established normally in Ig^{HEL} transgenic IgβΔC B cells. 96% of the immature B cells in the bone marrow of both Ig^{HEL} transgenic IgβΔC mice and control mice expressed the Ig^{HEL} $Ig\mu^{a}$ H chain, whereas only 3–4% coexpressed the endogenous $Ig\mu^{b}$ H chains (Fig. 3). We conclude that the single Igα cytoplasmic domain in IgβΔC BCRs is sufficient to maintain H chain allelic exclusion and that transgenic antibody expression is not sufficient to induce further B cell differentiation in IgβΔC mice.

Peripheral B Cells and Antibody Responses. Splenic B cell numbers were reduced to ~2% in IgβΔC mice in comparison with wild-type controls ($0.64 \pm 0.66 \times 10^{6}$ B cells, $n = 5$ versus $27.51 \pm 5.86 \times 10^{6}$ B cells, $n = 8$). A similar block in the development and maintenance of mature B lymphocytes was present in IgαΔC mice (splenic B cell numbers are reduced to ~1% [$0.21 \pm 0.14 \times 10^{6}$, $n = 5$; reference 25). The maturation status of splenic B lymphocytes was examined in IgαΔC and IgβΔC mice. More than 80% of B lymphocytes did not stain for the immature B cell marker 493 (42) and displayed a mature phenotype (data not shown). We also examined splenic B cells for surface expression of CD23 and MHC class II and found no effect of the cytoplasmic truncations (data not shown). However, peripheral B lymphocytes in IgαΔC and IgβΔC mice expressed higher levels of CD19 (Fig. 1 D). Splenic B cells in IgαΔC mice expressed normal levels of cell surface IgM. In contrast, the splenic B cells found in IgβΔC mice resembled their bone marrow precursors and continued to express 10 times lower levels of surface IgM and 0.5 times lower levels of IgD than controls (Fig. 1 D).

The scarce peripheral B cells in IgαΔC mice produce specific antibody responses to T cell–dependent but not to T cell–independent antigens (25). To determine whether IgβΔC B cells can respond to antigens, we immunized mice with T cell–dependent (NP-CGG) and T cell–independent (NP-Ficoll) antigens and measured specific antibody responses by ELISA. IgβΔC B cells mount a hapten specific immune response to NP-CGG with class switching

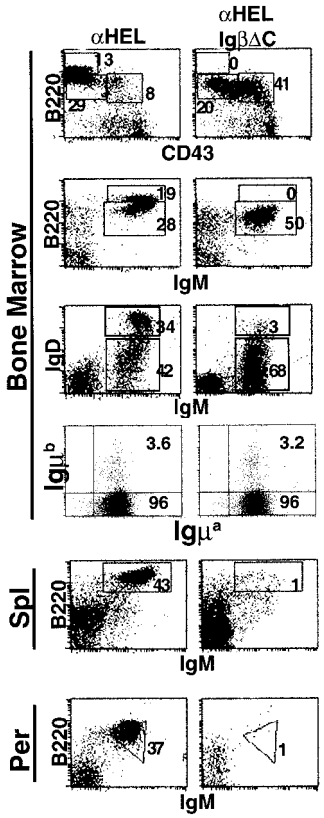

Figure 3. B cell development and allelic exclusion in IgβΔC Ig^{HEL} transgenic mice. In the B220 vs. CD43 and B220 vs. IgM plots, numbers indicate percentages of lymphocytes as determined by forward vs. side scatter parameters. In the IgD vs. IgM plots, numbers indicate percentages of $B220^{+}$ cells in the boxed region. For H chain allelic exclusion, dot plots show B cells gated on $B220^{+}IgM^{+}$ cells stained with IgM^{a} and IgM^{b}, and the numbers indicate the percentage of $B220^{+}IgM^{+}$ cells in each quadrant. B220 vs. IgM staining in the spleen (Spl) and peritoneal cavity (Per) is shown.

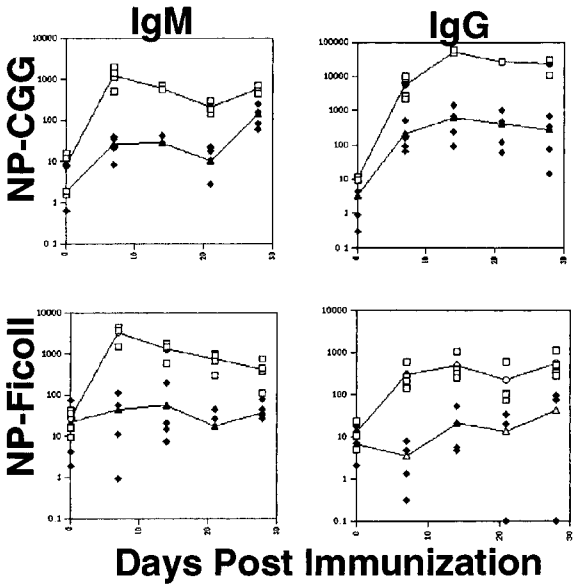

Figure 4. Antibody responses in IgβΔC mice. Plots show anti-NP IgM and IgG responses measured by ELISA on days 7, 14, 21, and 28 after immunization with NP-CGG or NP-Ficoll. The open squares represent individual wild-type controls and the filled diamonds represent individual IgβΔC mice. The y axis indicates OD415 relative to unimmunized controls. The lines represent the means.

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to IgG, but do not appear to respond to NP-Ficoll. Consistent with the small number of peripheral B cells in the IgβΔC mice, anti-NP antibody titers were two orders of magnitude lower than controls (Fig. 4). We conclude that like IgαΔC B cells, IgβΔC B cells respond to T cell–dependent but not T cell–independent antigens.

Ca^{2+} Flux. Ca^{2+} flux responses are enhanced in immature B cells from Ig^{HEL} transgenic, IgαΔC mice (27). This increase in the Ca^{2+} response could be due to a unique negative regulatory role for Igα in developing B cells or, alternatively, to a difference in Ca^{2+} responses induced by Ig^{HEL} transgene expression in the IgαΔC background (27). To determine whether altered responses to BCR cross-linking were IgαΔC specific, we measured Ca^{2+} flux in response to BCR cross-linking in immature IgβΔC bone marrow cells. B cells expressing similar levels of surface IgM were compared by electronically gating on surface IgM expression after staining with an Fab′ anti-IgM. We found no measurable differences in Ca^{2+} responses to anti-BCR cross-linking between immature IgβΔC B cells and control immature B cells (Fig. 5). In contrast, Ig^{HEL} transgenic IgβΔC B cells produced a higher magnitude Ca^{2+} response than either wild-type controls or Ig^{HEL} transgenic B cells despite lower surface IgM expression (Fig. 5). We conclude that cross-linking the BCR in immature IgβΔC B cells induces normal Ca^{2+} flux responses, whereas B cells in IgβΔC mice carrying the Ig^{HEL} transgene have hyperactive receptors.

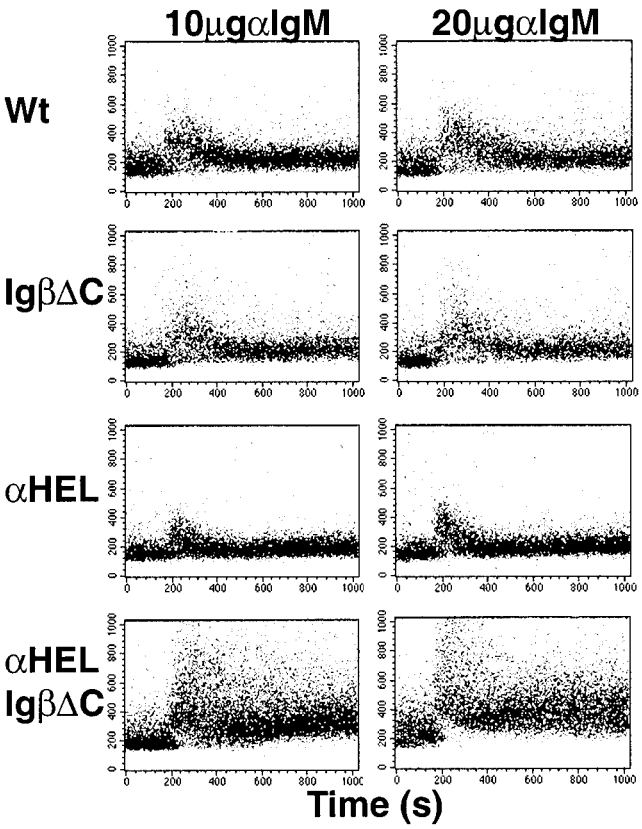

Figure 5. Ca^{2+} flux response to BCR cross-linking in immature B cells in IgβΔC and Ig^{HEL} transgenic IgβΔC mice. Dot plots represent Ca^{2+} flux of immature B cells measured by the fluorescence 395/510 nm ratio of Indo-1-AM emission accumulated over 512 s. Immature B cells were gated by staining with anti-B220 and Fab′ anti-IgM fragment. Baseline fluorescence was acquired for 60 s before the agonist, $F(ab')_2$ goat anti–mouse IgM at a final concentration of either 10 or 20 μg/ml, was added. Wt, wild-type.

B Cell Development in the Absence of Igα and Igβ Tails. To determine whether the cytoplasmic domain of either Igα and Igβ is essential for pre-B cell development, we produced double mutant IgαΔC/IgβΔC mice by crossing IgαΔC and IgβΔC mice. IgαΔC/IgβΔC mice resembled $Ig\beta^{-/-}$ mice in that B cell development was arrested at the $B220^+CD43^+CD25^-$ pre-BI stage (24; Fig. 6 A). We conclude that B cell development cannot proceed beyond the pre-BI stage in the absence of the cytoplasmic domains of both Igα and Igβ.

Mixtures of $B220^+CD43^+$ pro- and pre-B cells purified from $Ig\beta^{-/-}$ mice have fewer complete VDJ_H genes than wild-type $B220^+CD43^+IgM^-$ pro- and pre-B cells (24). This effect could be due to inefficient V to DJ_H recombination in pre-BI cells lacking Igβ, or to lack of positive selection and amplification of pre-BII cells with in-frame Ig H chains (24). To determine whether the cytoplasmic domains of Igα and Igβ are required for Ig H chain recombination and expression, we stained for intracellular Igμ. Developing B cells in IgαΔC/IgβΔC, $Ig\beta^{-/-}$, μMT, recombination activating gene $(RAG)^{-/-}$, and wild-type mice were compared after cell surface staining with anti-B220, anti-CD43, anti-CD25, and anti-IgM to separate pre-BI and pre-BII cell subpopulations, and intracellular staining for Igμ to measure H chain expression. Consistent with previous reports, intracellular Igμ levels in $B220^+CD43^+IgM^-$ mixtures of pre-BI and pre-BII cells were decreased in $Ig\beta^{-/-}$ mice compared with wild-type controls (Fig. 6 B). IgαΔC/IgβΔC and μMT mice resembled $Ig\beta^{-/-}$ mice in that their $B220^+CD43^+$ cells also showed lower levels of intracellular Igμ expression than controls. However, intracellular Igμ levels in pre-BI cells in IgαΔC/IgβΔC mice were similar to $Ig\beta^{-/-}$, μMT, and wild-type controls ($B220^+CD43^+IgM^-CD25^-$ cells; Fig. 6 B). Therefore, the decreased Igμ expression in the developing B cells in these mutant strains is due to arrest at the pre-BI stage and lack of positive selection for B cells with an in-frame Ig H chain during pre-BII cell expansion. We conclude that the cytoplasmic domains of Igα and Igβ are essential for B cell development past the pre-BI stage, and that IgαΔC/IgβΔC, $Ig\beta^{-/-}$, and μMT are all arrested at a similar stage in development.

Discussion

Igα or Igβ Signaling Is Essential for Pre-B Cell Development. We have shown that the cytoplasmic domain of either Igα or Igβ is essential for B cells to develop beyond the pre-BI (fraction B/C) stage. In the absence of BCR assembly, $RAG^{-/-}$ (43, 44), $Ig\beta^{-/-}$ (24), and μMT (33) B cells all fail to progress beyond the pre-BI stage. Although it has been assumed that this early block in development is

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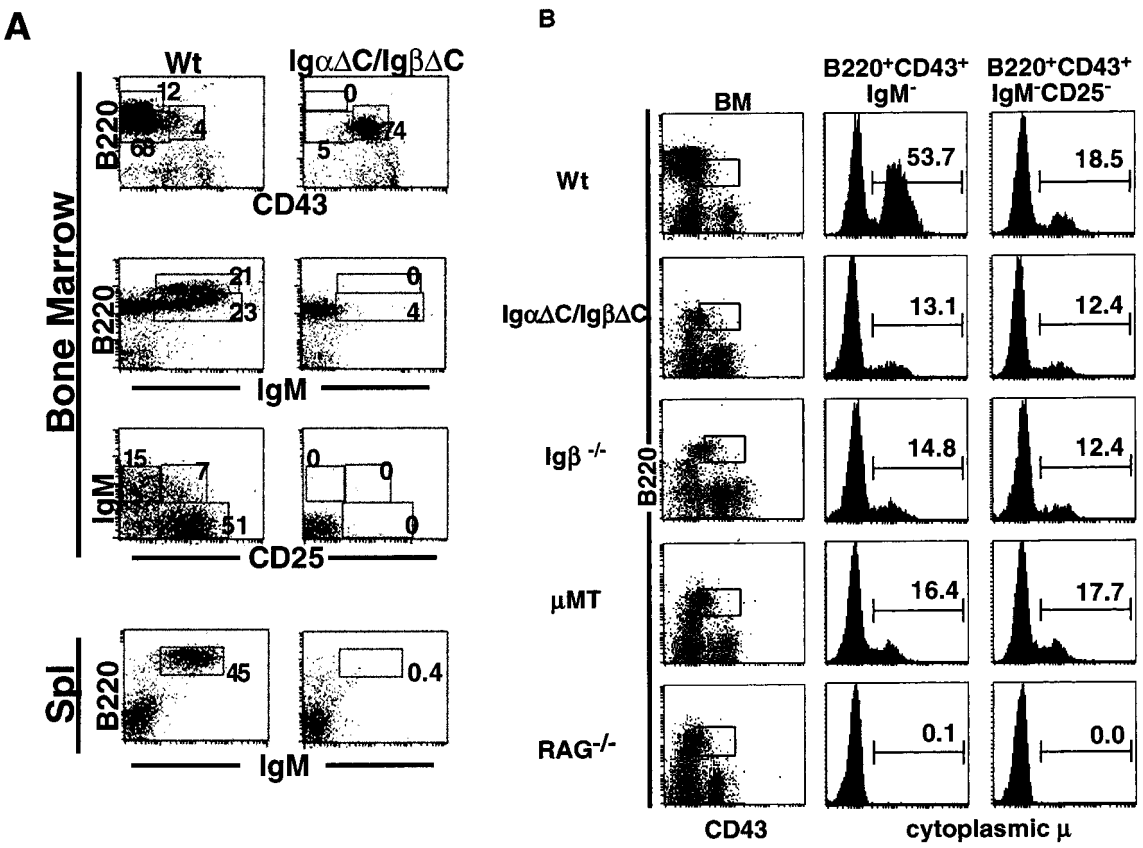

Figure 6. Cytoplasmic domains of Igα and Igβ are essential for B cell development. (A) Dot plots show staining with combinations of anti-B220, anti-CD43, anti-IgM, and anti-CD25 antibodies in bone marrow and spleen. In the B220 vs. CD43 and B220 vs. IgM plots, numbers indicate percentages of lymphocytes as determined by forward vs. side scatter parameters. In the IgM vs. CD25 plots, numbers indicate percentages of $B220^+$ cells in the boxed region. Wt, wild-type. (B) Intracytoplasmic Igμ expression in IgαΔC/IgβΔC, $Igβ^{-/-}$, μMT, $RAG^{-/-}$, and wild-type mice. Dot plots show staining with anti-B220 and anti-CD43, and the boxed region was used in further gating as indicated. Histograms show intracytoplasmic Igμ staining in surface $B220^+CD43^+IgM^-$ pro- and pre-B cells and $B220^+CD43^+IgM^-CD25^-$ pre-BI cells. Numbers indicate the percentages of intracellular $Igμ^+$ surface $B220^+CD43^+IgM^-$ pro- and pre-B cells and $B220^+CD43^+IgM^-CD25^-$ pre-BI cells.

due to failure to activate a BCR-dependent checkpoint, it might also be due to aberrant expression of BCR components. For example, expression of Igμ and Igα in the absence of Igβ in $Igβ^{-/-}$ mice produces an incomplete receptor that is not transported to the cell surface and might be toxic for developing pre-B cells. Similarly, only very low levels of Igα–Igβ are expressed on the cell surface in the absence of mIgμ in $RAG^{-/-}$ B cells (45). In contrast, the combination of IgαΔC and IgβΔC mutations produces surface BCRs that are simply unable to signal. Therefore, the finding that IgαΔC/IgβΔC B cells arrest at the pre-BI stage shows that the cytoplasmic domain of either IgαΔC or IgβΔC is essential for early B cell development, and that BCR signaling as opposed to assembly is required for later stages of B cell development.

B Cell Development Differs in IgαΔC and IgβΔC Mice. Many aspects of B cell development are similar in IgαΔC and IgβΔC mice. For example, pre-B cell development and allelic exclusion are activated in both strains, and there are few peripheral B cells in either. However, the two strains differ in that B cells are lost throughout development in IgαΔC mice, whereas significant B cell loss is not apparent in IgβΔC mice until the late stages of B cell maturation. IgβΔC B cell development is arrested before high level surface IgM expression and acquisition of surface IgD. Low surface IgM expression is not characteristic of IgαΔC mice and appears to be specific for IgβΔC, suggesting that the cytoplasmic domain of Igβ plays an important role in regulating surface BCR expression. Alternatively, the single Igα molecule may interfere with receptor assembly or enhance receptor degradation in IgβΔC mice. Failure to acquire high levels of surface IgM is not due to an intrinsic defect in BCR expression, as there is a broad spectrum of IgM expression in the selected B cells found in the spleen of IgβΔC mice including B cells that express high levels of surface IgM. Indeed, the heterogeneity of BCR surface expression suggests that antibody specificity contributes to setting the level of BCR expression in IgβΔC mice. We would like to speculate that decreased surface BCR expression is a consequence of altered BCR signaling in IgβΔC B cells.

The differences in B cell development between IgαΔC and IgβΔC mice are reminiscent of the differences in signaling between Igα and Igβ chimeras in transfected cell lines. B and T cell lines transfected with Igα chimeras

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showed higher levels of signaling than those transfected with Igβ chimeras (6, 7, 10–14). Furthermore, in some cell lines, chimeric receptors required both Igα and Igβ cytoplasmic domains to trigger cell death (13). However, the differences in the IgαΔC and IgβΔC mice were unexpected because transgenic mice that carry Igμ–Igα or Igμ–Igβ chimeric receptors showed equivalent function in early (8, 22) and late stages of development (23). Furthermore, in both Igμ–Igα or Igμ–Igβ transgenics, B cells developed fully and left the bone marrow whereas IgαΔC and IgβΔC mice show few mature B cells in the spleen (23). Several differences between the chimeric antibody transgenics and IgαΔC and IgβΔC mice could account for these apparent discrepancies. First, the transgenic mice carried artificial receptors in which the tails of Igα or Igβ were grafted onto heterologous transmembrane and external domains (8, 22, 23). Second, the genes coding for the transgenic receptors were controlled by Ig regulatory elements in multicopy randomly integrated loci and therefore the regulation of expression was not that of endogenous Igα and Igβ. Finally, the transgenic receptors carried dimers of Igα or Igβ tails instead of the normal monomers and therefore had twice as many signaling ITAMs as the BCRs in IgαΔC and IgβΔC mice.

Experiments performed on TCR CD3 proteins suggest that the ITAM-containing cytoplasmic domains of γ, ϵ, δ, and ζ proteins are functionally equivalent and that multiple ITAMs merely amplify signal strength (15–21). However, the TCR is a complex with 4 signaling proteins containing 10 ITAMs, and the role of individual ITAMs in T cell function has not been fully explored. In contrast to the TCR, the BCR has only two transducers, each with a single ITAM, and therefore differences between IgαΔC and IgβΔC mice cannot simply be due to a difference in the number of ITAMs (46).

These differences in signaling between Igα and Igβ may be attributed to the two additional non-ITAM tyrosines in the cytoplasmic domain of Igα (nos. 204 and 176; references 2 and 46). Neither of these tyrosine residues is known to be phosphorylated upon BCR cross-linking. Nevertheless, the sequence around tyrosine 204, YDQV, conforms to a consensus src homology 2 (SH2) docking site (47), and the acidic residues surrounding tyrosine 176 resemble those found in the cytoplasmic domain of erythrocyte band 3 protein, a target of ptk72 (48). Therefore, tyrosine 204 and 176 in Igα may recruit a distinct set of SH2 domain–containing signaling proteins, or simply enhance signaling through Igα by increasing the number of SH2 docking sites on Igα. Other differences between Igα and Igβ that could account for the differences in signaling include higher levels of serine and threonine phosphorylation on Igβ (9) and nonconserved residues between the tyrosines in the ITAMs of Igα and Igβ that appear to modulate src kinase binding (49).

An additional distinction between IgαΔC and IgβΔC mice is that the Igα tail truncation created by Torres et al. (25) shortened the cytoplasmic tail of Igα by 40 amino acids leaving 21 amino acids, including one non-ITAM tyrosine intact. Our strategy shortened the Igβ cytoplasmic tail by 45 amino acids, leaving a 3 amino acid anchor, DKD. The considerably longer remaining cytoplasmic sequence in the Igα tail truncation may have some signaling function beyond that attributable to the ITAM sequence. Thus, there may be an even greater difference between a complete Igα and Igβ tail truncation.

Hyperresponsive BCRs in IgβΔC Ig^{HEL} Transgenic B Cells. The hyperresponsive phenotype found in IgβΔC Ig^{HEL} transgenic mice resembles the effects found in Ig^{HEL} transgenic Src homology 2 domain–containing phosphatase 1 (SHP1) and lyn-deficient mice (50, 51). In the absence of these negative regulators, B cells are hyperresponsive to BCR cross-linking. Therefore, one explanation for the hyperreactive phenotype in IgαΔC and IgβΔC Ig^{HEL} transgenic B cells might be that their BCRs are unable to recruit negative regulators of signal transduction such as SHP1 and lyn.

In contrast to IgβΔC Ig^{HEL} transgenic B cells, nontransgenic IgβΔC B cells are indistinguishable from controls in Ca^{2+} flux experiments. Thus, the hyperactive phenotype appears to be Ig transgene specific. The discrepancy between IgβΔC Ig^{HEL} transgenic B cells and nontransgenic IgβΔC B cells could be due to partial compensation for abnormal B cell development in IgβΔC mice by the Ig^{HEL} transgene. Alternatively, the difference between transgenic and nontransgenic B cells could be due to artificially accelerated and altered B cell development in the transgenic mice.

A unique negative regulatory role for Igα was suggested by experiments with IgαΔC mice (26, 27). However, IgβΔC Ig^{HEL} transgenic B cells resemble IgαΔC Ig^{HEL} transgenic B cells in that they too were hyperresponsive compared with Ig^{HEL} controls in Ca^{2+} flux experiments. Thus, the absence of either Igα or Igβ produces a hyperreactive Ig^{HEL} transgenic B cell and this negative regulatory effect is not specific for Igα or Igβ.

Arrested B Cell Development in IgβΔC Mice. Several mutations in signaling molecules and B cell coactivators have phenotypes similar to IgβΔC. In humans, Btk mutation interferes with B cell development at several stages, beginning at the pre-B cell stage resulting in a near absence of peripheral B cells (X-linked agammaglobulinemia [52–54]). In mice, Btk mutation results in a four- to fivefold decrease in the number of recent bone marrow emigrants. Although the number of mature B cells is near normal, T cell–independent responses are severely diminished in these mice (55–58). Phosphoinositide 3-kinase deficiency in mice resembles Btk mutation in that there are decreased numbers of mature peripheral B cells and decreased levels of serum Ig (59, 60). Mouse mutations in B cell coreceptors CD22 (61, 62), CD19 (63, 64), the lyn kinase (65), and the CD45 phosphatase (66) all interfere with B cell development at the immature to mature B cell transition, but these effects are more subtle and less specific than the block in B cell development seen in IgβΔC mice.

Immature B cells are highly susceptible to deletion induced by BCR cross-linking, a feature which is likely to

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contribute to B cell tolerance by removing cells with self-reactive receptors (67, 68). Our work shows that this checkpoint is regulated by Igα–Igβ and that Igβ plays a particularly important role in setting the threshold for B cell development beyond the immature B cell stage.

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