

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

By Amy Reichlin,* Yun Hu,* Eric Meffre,* Hitoshi Nagaoka,* Shiao-ching Gong,* Manfred Kraus,† Klaus Rajewsky,† and Michel C. Nussenzweig*

From the *Laboratory of Molecular Immunology, Howard Hughes Medical Institute, The Rockefeller University, New York, New York 10021; and the †Institute for Genetics, University of Cologne, 50931 Cologne, Germany

Abstract

The B cell receptor (BCR) regulates B cell development and function through immunoglobulin (Ig) α and Ig β , 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 Ig β , we produced mice that carry a deletion of the cytoplasmic domain of Ig β (Ig β Δ C mice) and compared them to mice that carry a similar mutation in Ig α (MB1 Δ C, herein referred to as Ig α Δ C mice). Ig β Δ C mice differ from Ig α Δ C 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 Ca²⁺ flux. However, Ig β Δ C 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 of Ig β is required for B cell development beyond the immature B cell stage and that Ig α and Ig β have distinct biologic activities in vivo.

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

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 Ig α and Ig β (2–4). Signals initiated by ligand binding to membrane (m)IgM are communicated to the Ig α –Ig β 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 Ig α and Ig β subunits might have distinct biological functions. Biochemical studies showing that the cytoplasmic tails of Ig α and Ig β bind to different sets of cellular kinases (9) and transfection experiments showing differences in the signaling activities of Ig α and Ig β 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 Ig α and Ig β . 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 Ig α and Ig β in vivo: transgenic expression of chimeric proteins (8, 22, 23), Ig β gene deletion (24), and Ig α cytoplasmic tail mutation (25). Transgenic experiments showed that the cytoplasmic domain of either Ig α or Ig β was sufficient to activate allelic exclusion and pre-B cell development and led to the conclusion that Ig α and Ig β are redundant in early B cell development (8, 22, 23). Deletion of Ig β resulted in B cells that failed to assemble a BCR and

A. Reichlin and Y. Hu contributed equally to this work.

Address correspondence to M.C. Nussenzweig, Laboratory of Molecular Immunology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: 212-327-8067; Fax: 212-327-8370; E-mail: nussen@rockvax.rockefeller.edu

¹Abbreviations 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.

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 Ig α , including both ITAM tyrosines (Ig $\alpha\Delta$ C [25]), produced B cells that assembled a mutant BCR composed of mIg μ and an Ig α -Ig β heterodimer with a truncated Ig α tail. In agreement with the transgenic experiments, the single Ig β cytoplasmic domain in the Ig $\alpha\Delta$ C BCR was enough to induce pre-B cell development and allelic exclusion (8, 25). However, the number of pre-B cells in Ig $\alpha\Delta$ C 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 Ig β cytoplasmic domain was unable to support later stages of B cell development. Furthermore, increased tyrosine phosphorylation in Ig $\alpha\Delta$ C B cells and increased calcium flux in response to receptor cross-linking suggested a unique negative regulatory role for the Ig α cytoplasmic domain (26, 27).

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

Materials and Methods

Mice. Ig $\beta\Delta$ C mice were created by gene targeting in 129/Sv embryonic stem cells (24). To shorten the cytoplasmic tail of Ig β 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 Ig β gene were bred to C57BL/6 Cre transgenic mice (29). Deletion of the neomycin gene was confirmed by PCR using neomycin-specific primers ATGATGTAACAAGATGGATTGCAC 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 Ig $\beta\Delta$ C mice were backcrossed to C57BL/6 mice for three generations before intercrossing to produce homozygous Ig $\beta\Delta$ C mice. All mice were bred and maintained under specific pathogen-free conditions.

Ig $\alpha\Delta$ C mice (25) were crossed with Ig $\beta\Delta$ C mice to create Ig $\alpha\Delta$ C/Ig $\beta\Delta$ C mixed heterozygous and homozygous mice (25). Ig $\beta\Delta$ C mice were also bred to C57BL/6 Ig^{H β} 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-Ig β (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 Ig μ staining, cells were first surface stained with anti-B220-allophycocyanin, anti-CD25-PE, and/or anti-IgM-PE, and anti-CD43-biotin, permeabilized with Intra-prep 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).

Ca²⁺ Flux. Bone marrow cells were adjusted to 5 × 10⁶/ml in PBS plus 1% FCS plus 1 mM CaCl₂ plus 1 mM MgCl₂ (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 10⁶/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 Ig $\beta\Delta$ C 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 NP₁₆BSA (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 Ig $\beta\Delta$ C Mice. Gene targeting was used to introduce a stop codon at position 184 in Ig β (Fig. 1 A). The mutant gene directs the expression of a truncated Ig β protein that resembles mIg μ in having only three charged cytoplasmic anchor amino acids (DKD).

B cell development in Ig $\beta\Delta$ C mice was analyzed by multiparameter flow cytometry. When compared with wild-type controls, Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C mice showed an increase

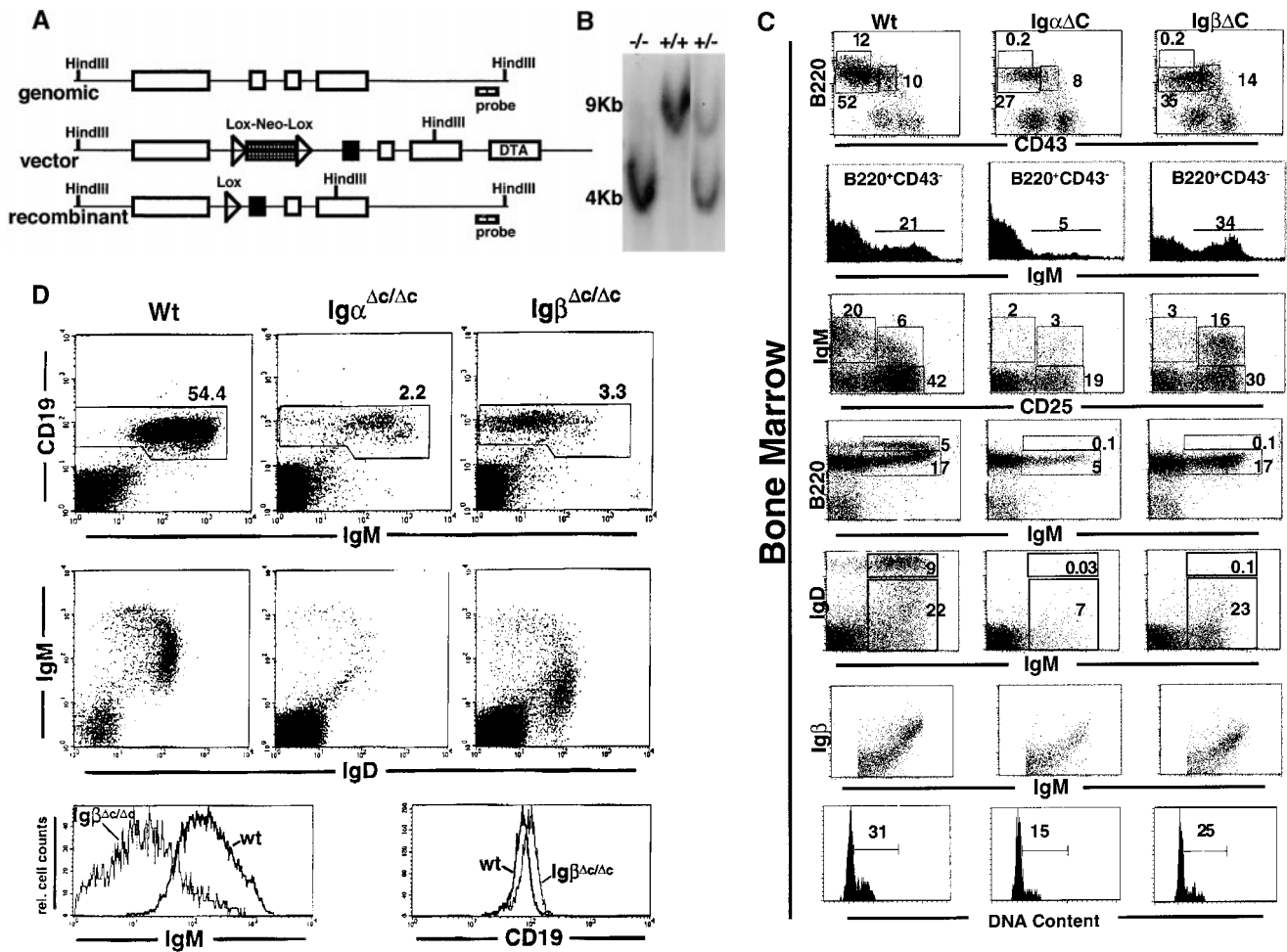


Figure 1. $Ig\beta\Delta C$ gene targeting. (A) Scheme for $Ig\beta\Delta C$ gene targeting showing the genomic $Ig\beta$ locus (top), targeting vector (middle), and recombinant locus after deletion of the neo gene (bottom). Exons are represented by open boxes, loxP sites by open triangles, and the exon 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), $Ig\alpha\Delta C$, and $Ig\beta\Delta C$ mice. In B220 vs. CD43 and B220 vs. IgM 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), $Ig\alpha\Delta C$, and $Ig\beta\Delta C$ mice. Spleen plots for $Ig\alpha\Delta C$ and $Ig\beta\Delta C$ 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 $Ig\beta\Delta C$ mice was less substantial than the 50% decrease found in $Ig\alpha\Delta C$ 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 $Ig\alpha$ cytoplasmic domain in the $Ig\beta\Delta C$ 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 $Ig\beta\Delta C$ mice was similar to that of control mice (Fig. 1 C and Table I). Thus, the single cytoplasmic tail of $Ig\alpha$ in the $Ig\beta\Delta C$

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 $Ig\alpha\Delta C$ 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, $Ig\beta\Delta C$ 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 $Ig\beta\Delta C$ B cells fail to progress further to the CD25⁻IgM⁺IgD⁺ transitional B cell stage (Fig. 1 C; IgM

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 de-

velopment 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

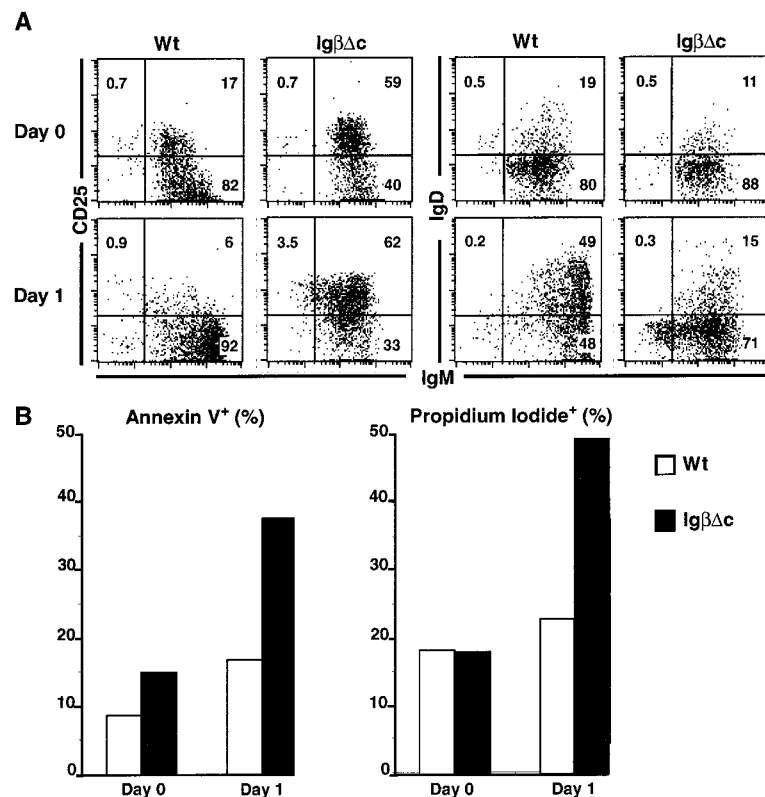


Figure 2. B cell development and death in bone marrow cultures. Bone marrow cells from IgβΔC and control mice were selected using CD19 MACS beads, sorted for B220⁺CD43⁻IgM^{low} immature B cells, and then cocultured with S17 stromal cells. (A) Dot plots show staining with anti-CD25 or anti-IgD and anti-IgM 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%.

in development (39). Freshly isolated immature $Ig\beta\Delta C$ 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 $Ig\beta\Delta C$ B cells did not progress beyond the $CD25^+IgM^{lo}IgD^-$ immature B cell stage. Instead, $Ig\beta\Delta C$ B cells became increasingly annexin V and propidium iodide positive (Fig. 2). Thus, $Ig\beta\Delta C$ 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 $Ig\alpha$ cytoplasmic domain in $Ig\beta\Delta C$ mice is sufficient for allelic exclusion, we bred $Ig\beta\Delta C$ mice to Ig^{HEL} transgenic mice which carry an allotype marked $Ig\mu^a$ H chain (30). Ig^{HEL} transgenic $Ig\beta\Delta C$ mice resemble non-transgenic $Ig\beta\Delta C$ 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 $Ig\beta\Delta C$ B cells. 96% of the immature B cells in the bone marrow of both Ig^{HEL} transgenic $Ig\beta\Delta C$ 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 $Ig\alpha$ cytoplasmic domain in $Ig\beta\Delta C$ BCRs is sufficient to maintain H chain allelic exclusion and that transgenic antibody ex-

pression is not sufficient to induce further B cell differentiation in $Ig\beta\Delta C$ mice.

Peripheral B Cells and Antibody Responses. Splenic B cell numbers were reduced to $\sim 2\%$ in $Ig\beta\Delta C$ 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 $Ig\alpha\Delta C$ mice (splenic B cell numbers are reduced to $\sim 1\%$ [$0.21 \pm 0.14 \times 10^6$, $n = 5$; reference 25]). The maturation status of splenic B lymphocytes was examined in $Ig\alpha\Delta C$ and $Ig\beta\Delta C$ 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 $Ig\alpha\Delta C$ and $Ig\beta\Delta C$ mice expressed higher levels of CD19 (Fig. 1 D). Splenic B cells in $Ig\alpha\Delta C$ mice expressed normal levels of cell surface IgM. In contrast, the splenic B cells found in $Ig\beta\Delta C$ 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 $Ig\alpha\Delta C$ mice produce specific antibody responses to T cell-dependent but not to T cell-independent antigens (25). To determine whether $Ig\beta\Delta C$ 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. $Ig\beta\Delta C$ B cells mount a hapten specific immune response to NP-CGG with class switching

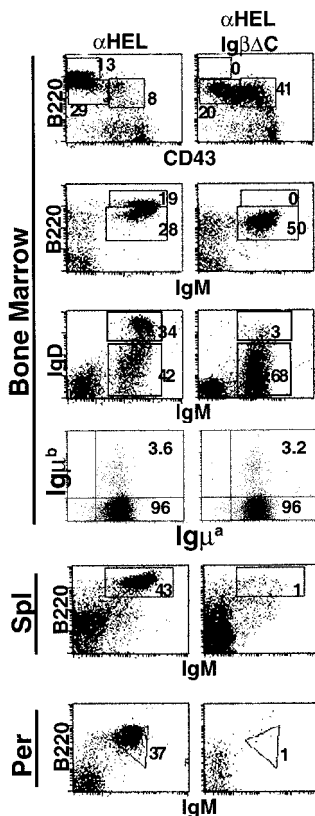


Figure 3. B cell development and allelic exclusion in $Ig\beta\Delta C$ Ig^{HEL} transgenic mice. In the B220 vs. CD43 and B220 vs. IgM plots, numbers indicate percentages of lymphocytes as determined by forward vs. side scatter parameters. In the IgD vs. IgM plots, numbers indicate percentages of B220⁺ 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.

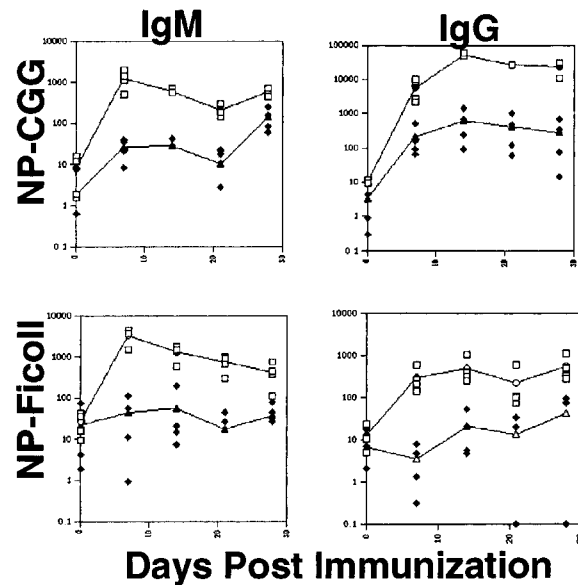


Figure 4. Antibody responses in $Ig\beta\Delta C$ mice. Plots show anti-NP IgM and IgG responses measured by ELISA on days 7, 14, 21, and 28 after immunization with NP-CGG or NP-Ficoll. The open squares represent individual wild-type controls and the filled diamonds represent individual $Ig\beta\Delta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

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

Ca²⁺ Flux. Ca²⁺ flux responses are enhanced in immature B cells from Ig^{HEL} transgenic, $Ig\alpha\Delta C$ mice (27). This increase in the Ca²⁺ response could be due to a unique negative regulatory role for $Ig\alpha$ in developing B cells or, alternatively, to a difference in Ca²⁺ responses induced by Ig^{HEL} transgene expression in the $Ig\alpha\Delta C$ background (27). To determine whether altered responses to BCR cross-linking were $Ig\alpha\Delta C$ specific, we measured Ca²⁺ flux in response to BCR cross-linking in immature $Ig\beta\Delta C$ 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 Ca²⁺ responses to anti-BCR cross-linking between immature $Ig\beta\Delta C$ B cells and control immature B cells (Fig. 5). In contrast, Ig^{HEL} transgenic $Ig\beta\Delta C$ B cells produced a higher magnitude Ca²⁺

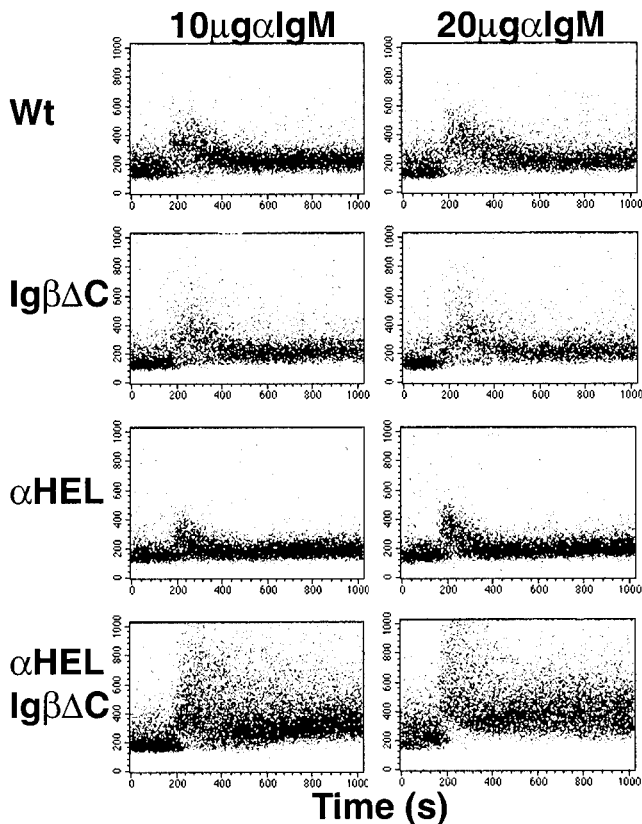


Figure 5. Ca²⁺ flux response to BCR cross-linking in immature B cells in $Ig\beta\Delta C$ and Ig^{HEL} transgenic $Ig\beta\Delta C$ mice. Dot plots represent Ca²⁺ flux of immature B cells measured by the fluorescence 395/510 nm ratio of Indo-1-AM emission accumulated over 512 s. Immature B cells were 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.

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 $Ig\beta\Delta C$ B cells induces normal Ca²⁺ flux responses, whereas B cells in $Ig\beta\Delta C$ mice carrying the Ig^{HEL} transgene have hyperactive receptors.

B Cell Development in the Absence of $Ig\alpha$ and $Ig\beta$ Tails. To determine whether the cytoplasmic domain of either $Ig\alpha$ and $Ig\beta$ is essential for pre-B cell development, we produced double mutant $Ig\alpha\Delta C/Ig\beta\Delta C$ mice by crossing $Ig\alpha\Delta C$ and $Ig\beta\Delta C$ mice. $Ig\alpha\Delta C/Ig\beta\Delta C$ 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 $Ig\alpha$ and $Ig\beta$.

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 $Ig\beta$, 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 $Ig\alpha$ and $Ig\beta$ are required for Ig H chain recombination and expression, we stained for intracellular Ig μ . Developing B cells in $Ig\alpha\Delta C/Ig\beta\Delta C$, $Ig\beta^{-/-}$, μ MT, 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 Ig μ to measure H chain expression. Consistent with previous reports, intracellular Ig μ 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). $Ig\alpha\Delta C/Ig\beta\Delta C$ and μ MT mice resembled $Ig\beta^{-/-}$ mice in that their B220⁺CD43⁺ cells also showed lower levels of intracellular Ig μ expression than controls. However, intracellular Ig μ levels in pre-BI cells in $Ig\alpha\Delta C/Ig\beta\Delta C$ mice were similar to $Ig\beta^{-/-}$, μ MT, and wild-type controls (B220⁺CD43⁺IgM⁻CD25⁻ cells; Fig. 6 B). Therefore, the decreased Ig μ 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 $Ig\alpha$ and $Ig\beta$ are essential for B cell development past the pre-BI stage, and that $Ig\alpha\Delta C/Ig\beta\Delta C$, $Ig\beta^{-/-}$, and μ MT are all arrested at a similar stage in development.

Discussion

Ig α or $Ig\beta$ Signaling Is Essential for Pre-B Cell Development. We have shown that the cytoplasmic domain of either $Ig\alpha$ or $Ig\beta$ 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 μ MT (33) B cells all fail to progress beyond the pre-BI stage. Although it has been assumed that this early block in development is

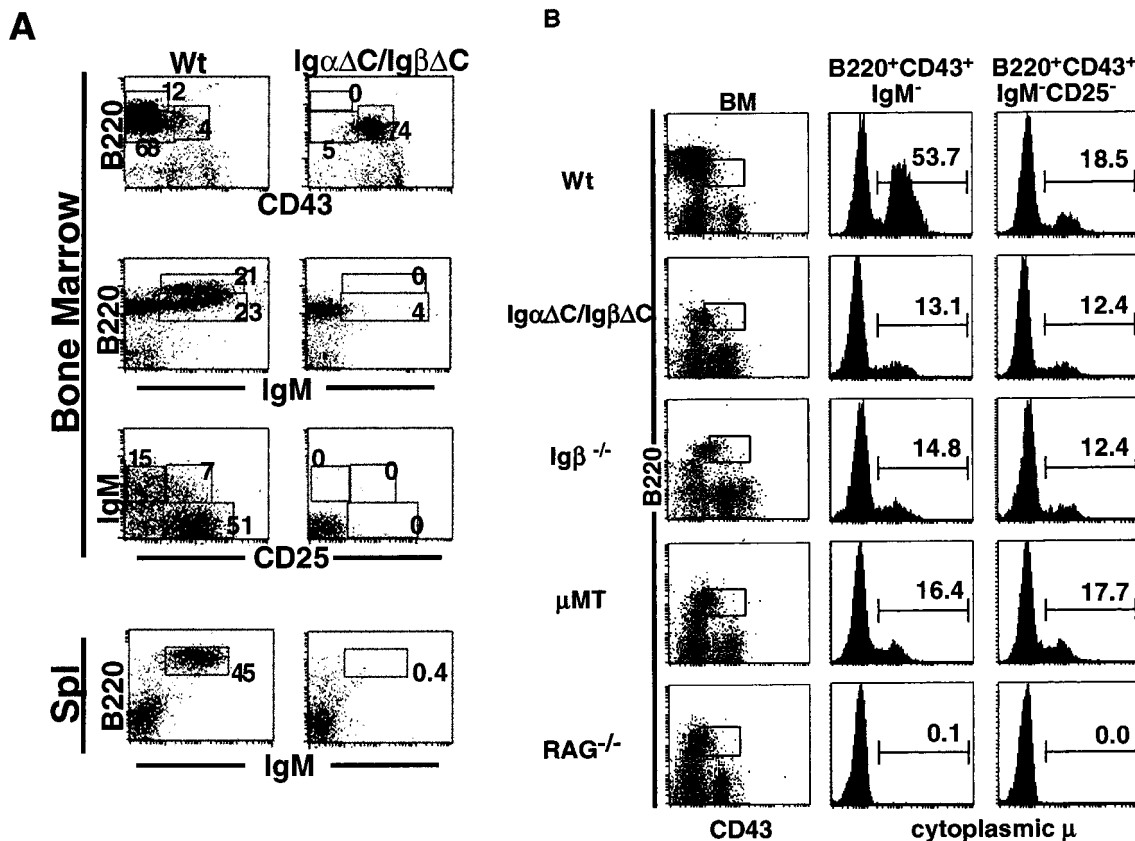


Figure 6. Cytoplasmic domains of Ig α and Ig β are essential for B cell development. (A) Dot plots show staining with combinations of anti-B220, anti-CD43, anti-IgM, and anti-CD25 antibodies in bone marrow and spleen. In the B220 vs. CD43 and B220 vs. IgM plots, numbers indicate percentages of lymphocytes 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 Ig μ expression in Ig α Δ C/Ig β Δ C, Ig β ^{-/-}, μ MT, 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 Ig μ 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 Ig μ ⁺ 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 Ig μ and Ig α in the absence of Ig β in Ig β ^{-/-} 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 Ig α -Ig β are expressed on the cell surface in the absence of mIg μ in RAG^{-/-} B cells (45). In contrast, the combination of Ig α Δ C and Ig β Δ C mutations produces surface BCRs that are simply unable to signal. Therefore, the finding that Ig α Δ C/Ig β Δ C B cells arrest at the pre-BI stage shows that the cytoplasmic domain of either Ig α Δ C or Ig β Δ C 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 Ig α Δ C and Ig β Δ C Mice. Many aspects of B cell development are similar in Ig α Δ C and Ig β Δ C 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 Ig α Δ C mice, whereas significant B cell loss is not apparent in

Ig β Δ C mice until the late stages of B cell maturation. Ig β Δ C B cell development is arrested before high level surface IgM expression and acquisition of surface IgD. Low surface IgM expression is not characteristic of Ig α Δ C mice and appears to be specific for Ig β Δ C, suggesting that the cytoplasmic domain of Ig β plays an important role in regulating surface BCR expression. Alternatively, the single Ig α molecule may interfere with receptor assembly or enhance receptor degradation in Ig β Δ C 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 Ig β Δ C 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 Ig β Δ C mice. We would like to speculate that decreased surface BCR expression is a consequence of altered BCR signaling in Ig β Δ C B cells.

The differences in B cell development between Ig α Δ C and Ig β Δ C mice are reminiscent of the differences in signaling between Ig α and Ig β chimeras in transfected cell lines. B and T cell lines transfected with Ig α chimeras

showed higher levels of signaling than those transfected with Ig β chimeras (6, 7, 10–14). Furthermore, in some cell lines, chimeric receptors required both Ig α and Ig β cytoplasmic domains to trigger cell death (13). However, the differences in the Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C mice were unexpected because transgenic mice that carry Ig μ –Ig α or Ig μ –Ig β chimeric receptors showed equivalent function in early (8, 22) and late stages of development (23). Furthermore, in both Ig μ –Ig α or Ig μ –Ig β transgenics, B cells developed fully and left the bone marrow whereas Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C mice show few mature B cells in the spleen (23). Several differences between the chimeric antibody transgenics and Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C mice could account for these apparent discrepancies. First, the transgenic mice carried artificial receptors in which the tails of Ig α or Ig β 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 Ig α and Ig β . Finally, the transgenic receptors carried dimers of Ig α or Ig β tails instead of the normal monomers and therefore had twice as many signaling ITAMs as the BCRs in Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C 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 Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C mice cannot simply be due to a difference in the number of ITAMs (46).

These differences in signaling between Ig α and Ig β may be attributed to the two additional non-ITAM tyrosines in the cytoplasmic domain of Ig α (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 Ig α may recruit a distinct set of SH2 domain-containing signaling proteins, or simply enhance signaling through Ig α by increasing the number of SH2 docking sites on Ig α . Other differences between Ig α and Ig β that could account for the differences in signaling include higher levels of serine and threonine phosphorylation on Ig β (9) and nonconserved residues between the tyrosines in the ITAMs of Ig α and Ig β that appear to modulate src kinase binding (49).

An additional distinction between Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C mice is that the Ig α tail truncation created by Torres et al. (25) shortened the cytoplasmic tail of Ig α by 40 amino acids leaving 21 amino acids, including one non-ITAM ty-

rosine intact. Our strategy shortened the Ig β cytoplasmic tail by 45 amino acids, leaving a 3 amino acid anchor, DKD. The considerably longer remaining cytoplasmic sequence in the Ig α 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 Ig α and Ig β tail truncation.

Hyperresponsive BCRs in Ig $\beta\Delta$ C Ig^{HEL} Transgenic B Cells. The hyperresponsive phenotype found in Ig $\beta\Delta$ C 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 Ig $\alpha\Delta$ C and Ig $\beta\Delta$ C 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 Ig $\beta\Delta$ C Ig^{HEL} transgenic B cells, nontransgenic Ig $\beta\Delta$ C B cells are indistinguishable from controls in Ca²⁺ flux experiments. Thus, the hyperactive phenotype appears to be Ig transgene specific. The discrepancy between Ig $\beta\Delta$ C Ig^{HEL} transgenic B cells and nontransgenic Ig $\beta\Delta$ C B cells could be due to partial compensation for abnormal B cell development in Ig $\beta\Delta$ C 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 Ig α was suggested by experiments with Ig $\alpha\Delta$ C mice (26, 27). However, Ig $\beta\Delta$ C Ig^{HEL} transgenic B cells resemble Ig $\alpha\Delta$ C Ig^{HEL} transgenic B cells in that they too were hyperresponsive compared with Ig^{HEL} controls in Ca²⁺ flux experiments. Thus, the absence of either Ig α or Ig β produces a hyperreactive Ig^{HEL} transgenic B cell and this negative regulatory effect is not specific for Ig α or Ig β .

Arrested B Cell Development in Ig $\beta\Delta$ C Mice. Several mutations in signaling molecules and B cell coactivators have phenotypes similar to Ig $\beta\Delta$ C. 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 Ig $\beta\Delta$ C mice.

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

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

We thank H. Karasuyama for the Ig β monoclonal antibody, Dr. A. Rolink for anti-493 monoclonal antibody, members of the Nussenzweig lab for helpful discussions, and F. Isdell and M. Genova for flow cytometry.

A. Reichlin was supported by a National Institutes of Health KO8 training grant. This work was funded in part by a grant to K. Rajewsky from Deutsche Forschungsgemeinschaft through SFB 243, The Max Planck Research Award to K. Rajewsky, and grants from the National Institutes of Health to M.C. Nussenzweig. M.C. Nussenzweig is an Investigator in the Howard Hughes Medical Institute.

Submitted: 11 September 2000

Revised: 1 November 2000

Accepted: 3 November 2000

References

- Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature*. 381:751–758.
- Sakaguchi, N., S. Kashiwamura, M. Kimoto, P. Thalmann, and F. Melchers. 1988. B lymphocyte lineage-restricted expression of mb-1, a gene with CD3-like structural properties. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3457–3464.
- Hombach, J., L. Leclercq, A. Radbruch, K. Rajewsky, and M. Reth. 1988. A novel 34-kd protein co-isolated with the IgM molecule in surface IgM-expressing cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3451–3456.
- Hermanson, G.G., D. Eisenberg, P.W. Kincade, and R. Wall. 1988. B29: a member of the immunoglobulin gene superfamily exclusively expressed on B-lineage cells. *Proc. Natl. Acad. Sci. USA*. 85:6890–6894.
- Shaw, A.C., R.N. Mitchell, Y.K. Weaver, T.J. Campos, A.K. Abbas, and P. Leder. 1990. Mutations of immunoglobulin transmembrane and cytoplasmic domains: effects on intracellular signaling and antigen presentation. *Cell*. 63:381–392.
- Sanchez, M., Z. Misulovin, A.L. Burkhardt, S. Mahajan, T. Costa, R. Franke, J.B. Bolen, and M. Nussenzweig. 1993. Signal transduction by immunoglobulin is mediated through Ig α and Ig β . *J. Exp. Med.* 178:1049–1056.
- Williams, G.T., C.J.G. Peaker, K.J. Patel, and M.S. Neuberger. 1994. The α/β sheath and its cytoplasmic tyrosines are required for signaling by the B-cell antigen receptor but not for capping of for serine/threonine-kinase recruitment. *Proc. Natl. Acad. Sci. USA*. 91:471–478.
- Papavasiliou, F., Z. Misulovin, H. Suh, and M.C. Nussenzweig. 1995. The role of Ig β in precursor B cell transition and allelic exclusion. *Science*. 268:408–411.
- Clark, M.R., K.S. Campbell, A. Kazlauska, S.A. Johnson, M. Hertz, T.A. Potter, C. Pleiman, and J.C. Cambier. 1992. The B cell antigen receptor complex: association of Ig-alpha and Ig-beta with distinct cytoplasmic effectors. *Science*. 258:123–126.
- Kim, K.M., G. Alber, P. Weiser, and M. Reth. 1993. Differential signaling through the Ig-alpha and Ig-beta components of the B cell antigen receptor. *Eur. J. Immunol.* 23:911–916.
- Taddie, J., T.R. Hurley, B.S. Hardwick, and B.M. Sefton. 1994. Activation of B- and T-cells by the cytoplasmic domains of the B-cell antigen receptor proteins Ig- α and Ig β . *J. Biol. Chem.* 269:13529–13535.
- Luisiri, P., Y.J. Lee, B.J. Eisfelder, and M.R. Clark. 1996. Cooperativity and segregation of function within the Ig-alpha/beta heterodimer of the B cell antigen receptor complex. *J. Biol. Chem.* 271:5158–5163.
- Tseng, J., B.J. Eisfelder, and M.R. Clark. 1997. B-cell antigen receptor-induced apoptosis requires both Ig alpha and Ig beta. *Blood* 89:1513–1520.
- Pao, L., S.J. Famiglietti, and J.C. Cambier. 1998. Asymmetrical phosphorylation and function of the immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. *J. Immunol.* 160:3305–3314.
- Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for the induction of cytolysis by the T cell antigen/Fc receptor ζ chain. *Cell*. 68:889–897.
- Shores, E.W., K. Huang, T. Tran, E. Lee, A. Grinberg, and P.E. Love. 1994. Role of TCR zeta chain in T cell development and selection. *Science*. 266:1047–1050.
- Shinkai, Y., A. Ma, H.L. Cheng, and F.W. Alt. 1995. CD3 epsilon and CD3 zeta cytoplasmic domains can independently generate signals for T cell development and function. *Immunity*. 2:401–411.
- Malissen, M., A. Gillet, L. Ardouin, G. Bouvier, J. Trucy, P. Ferrier, E. Vivier, and B. Malissen. 1995. Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:4641–4653.
- Dave, V.P., Z. Cao, C. Browne, B. Alarcon, G. Fernandez-Miguel, J. Lafaille, A. de la Hera, S. Tonegawa, and D.J. Kappes. 1997. CD3 delta deficiency arrests development of the alpha beta but not the gamma delta T cell lineage. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:1360–1370.
- Haks, M.C., P. Krimpenfort, J. Borst, and A.M. Kruisbeek. 1998. The CD3gamma chain is essential for development of both the TCRalpha beta and TCRgamma delta lineages. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:1871–1882.
- Malissen, B., L. Ardouin, S.Y. Lin, A. Gillet, and M. Malissen. 1999. Function of the CD3 subunits of the pre-TCR and TCR complexes during T cell development. *Adv. Immunol.* 72:103–148.
- Papavasiliou, F., M. Jankovic, H. Suh, and M.C. Nussenzweig. 1995. The cytoplasmic domains of Ig α and Ig β can independently induce the pre-B cell transition and allelic exclusion. *J. Exp. Med.* 182:1389–1394.
- Teh, Y., and M.S. Neuberger. 1997. The immunoglobulin Ig α and Ig β cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice. *J. Exp. Med.* 185:1753–1768.
- Gong, S., and M.C. Nussenzweig. 1996. Regulation of an early developmental checkpoint in the B cell pathway by Ig β . *Science*. 272:411–414.
- Torres, R.M., H. Flaswinkel, M. Reth, and K. Rajewsky. 1996. Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science*. 272:1802–1804.
- Torres, R.M., and K. Hafen. 1999. A negative regulatory role for Ig-alpha during B cell development. *Immunity*. 11:527–536.
- Kraus, M., K. Saijo, R.M. Torres, and K. Rajewsky. 1999. Ig-alpha cytoplasmic truncation renders immature B cells more sensitive to antigen contact. *Immunity*. 11:537–545.

28. Yagi, Y., Y. Ikawa, K. Yoshida, Y. Shigetani, N. Takeda, I. Mabuchi, T. Yamamoto, and S. Aizawa. 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. *Proc. Natl. Acad. Sci. USA.* 87:9918–9922.
29. Lakso, M., J.G. Pichel, J.R. Gorman, B. Sauer, Y. Okamoto, E. Lee, F.W. Alt, and H. Westphal. 1996. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci. USA.* 93:5860–5865.
30. Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherpoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature.* 334:676–682.
31. Cumano, A., K. Dorshkind, S. Gillis, and C.J. Paige. 1990. The influence of S17 stromal cells and interleukin 7 on B cell development. *Eur. J. Immunol.* 20:2183–2189.
32. Qin, X.F., A. Reichlin, Y. Luo, R.G. Roeder, and M.C. Nussenzweig. 1998. OCA-B integrates B cell antigen receptor-, CD40L-, and IL4-mediated signals for the germinal center pathway of B cell development. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:5066–5075.
33. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell deficient mouse by targeted disruption of the membrane exons of the immunoglobulin μ chain gene. *Nature.* 350:423–426.
34. Hardy, H., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213–1225.
35. Rolink, A., U. Grawunder, T.H. Winkler, H. Karasuyama, and F. Melcher. 1994. IL-2 receptor alpha chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. *Int. Immunol.* 6:1257–1264.
36. Spanopoulou, E., C.A.J. Roman, L.M. Corcoran, M.S. Schlisel, D.P. Silver, D. Nemazee, M.C. Nussenzweig, S.A. Shinton, R.R. Hardy, and D. Baltimore. 1994. Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. *Genes Dev.* 8:1030–1042.
37. Rolink, A.G., E. ten Boekel, T. Yamagami, R. Ceredig, J. Andersson, and F. Melchers. 1999. B cell development in the mouse from early progenitors to mature B cells. *Immunol. Lett.* 68:89–93.
38. Koyama, M., K. Ishihara, H. Karasuyama, J.L. Cordell, A. Iwamoto, and T. Nakamura. 1997. CD79 alpha/CD79 beta heterodimers are expressed on pro-B cell surfaces without associated mu heavy chain. *Int. Immunol.* 9:1767–1772.
39. Dillon, S., M. Mancini, A. Rosen, and M.S. Schlissel. 2000. Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation. *J. Immunol.* 164:1322–1332.
40. Nussenzweig, M.C., A.C. Shaw, E. Sinn, D.B. Danner, K.L. Holmes, H.C. Morse, III, and P. Leder. 1987. Allelic exclusion in transgenic mice that express the membrane form of immunoglobulin μ . *Science.* 236:816–819.
41. Kitamura, D., and K. Rajewsky. 1992. Targeted disruption of μ chain membrane exon causes loss of heavy-chain allelic exclusion. *Nature.* 356:154–156.
42. Rolink, A.G., J. Andersson, and F. Melchers. 1998. Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. *Eur. J. Immunol.* 28:3738–3748.
43. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1 deficient mice have no mature B and T lymphocytes. *Cell.* 68:869–877.
44. Shinkai, Y., G. Rathbun, K.-P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell.* 68:855–867.
45. Nagata, K., T. Nakamura, F. Kitamura, S. Kuramochi, S. Taki, K.S. Campbell, and H. Karasuyama. 1997. The Ig alpha/Ig beta heterodimer on mu-negative proB cells is competent for transducing signals to induce early B cell differentiation. *Immunity.* 7:559–570.
46. Reth, M. 1989. Antigen receptor tail clue. *Nature.* 338:383–384.
47. Songyang, Z., S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, et al. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell.* 72:767–778.
48. Wang, Q., J.B. Smith, M.L. Harrison, and R.L. Geahlen. 1991. Identification of tyrosine 67 in bovine brain myelin basic protein as a specific phosphorylation site for thymus p56lck. *Biochem. Biophys. Res. Commun.* 178:1393–1399.
49. Clark, M., S.A. Johnson, and J.C. Cambier. 1994. Analysis of Iga-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-stimulation of Fyn activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1911–1919.
50. Cyster, J.G., and C.C. Goodnow. 1995. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in lymphocytes and determines thresholds for negative selection. *Immunity.* 2:13–24.
51. Cornall, R., J.G. Cyster, M.L. Hibbs, A.R. Dunn, K.L. Otipoby, E.A. Clark, and C.C. Goodnow. 1998. Polygenic autoimmune traits: Lyn, CD22, and SHP-1, are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity.* 8:497–508.
52. Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarstrom, C. Kinnon, R. Levinsky, M. Bobrow, et al. 1993. The gene involved in X-linked agammaglobulinemia is a member of the src family of protein-tyrosine kinases. *Nature.* 364:226–233.
53. Conley, M., O. Parolini, J. Rohrer, and D. Campana. 1994. X-linked agammaglobulinemia: new approaches to old questions based on the identification of the defective gene. *Immunol. Rev.* 138:5–21.
54. de Weers, M., G.S. Brouns, S. Hinshelwood, C. Kinnon, R.K. Schuurman, R.W. Hendriks, and J. Borst. 1994. B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. *J. Biol. Chem.* 269:23857–23860.
55. Hardy, R.R., K. Hayakawa, D.R. Parks, and L.A. Herzenberg. 1983. Demonstration of B cell maturation in X-linked immunodeficient mice by simultaneous three-color immunofluorescence. *Nature.* 306:270–272.
56. Khan, V., F.W. Alt, R.M. Gerstein, B.A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A.B. Kantor, and L.A. Hersenber. 1995. Defective B cell development and function in Btk deficient mice. *Immunity.* 3:283–299.
57. Oka, Y., A.G. Rolink, J. Andersson, M. Kananka, Y. Uchida, T. Yasui, T. Kishimoto, H. Kikutani, and F. Melchers. 1996. Profound reduction of mature B cell numbers, reactivities and serum immunoglobulin levels in mice which simultaneously carry the XID and CD40-deficiency

- genes. *Int. Immunol.* 8:1675–1685.
58. Rolink, A., T. Brocker, H. Bluethmann, M.H. Kosco-Vilbois, J. Andersson, and F. Melchers. 1999. Mutations affecting either generation or survival of cells influence the pool size of mature B cells. *Immunity.* 10:619–628.
 59. Fruman, D., S.B. Snapper, C.M. Yballe, L. Davidson, J.Y. Yu, F.W. Alt, and L.C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85a. *Science.* 283:393–397.
 60. Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science.* 283:390–392.
 61. O'Keefe, T., G.T. Williams, S.L. Davies, and M.S. Neuberger. 1996. Hyperresponsive B cells in CD22-deficient mice. *Science.* 274:798–801.
 62. Sato, S., A.S. Miller, M. Inaoki, C.B. Bock, P.J. Jansen, M.L.K. Tang, and T.F. Tedder. 1996. CD22 is both a positive and a negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity.* 5:551–562.
 63. Engel, P., L.J. Zhou, D.C. Ord, S. Sato, B. Koller, and T.F. Tedder. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity.* 3:39–50.
 64. Rickert, R.C., K. Rajewsky, and J. Roes. 1995. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature.* 376:352–355.
 65. Hibbs, M., D.M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglitto, S.A. Stacker, and A.R. Dunn. 1995. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell.* 83:301–311.
 66. Kishihara, K., J. Penninger, V.A. Wallace, T.M. Kundig, K. Kawai, A. Wakeham, E. Timms, K. Pfeffer, P.S. Ohashi, and M.L. Thomas. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell.* 74:143–156.
 67. Norvell, A., L. Mandik, and J.G. Monroe. 1995. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J. Immunol.* 154:4404–4413.
 68. Sandel, P.C., and J.G. Monroe. 1999. Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. *Immunity.* 10:289–299.