A B Cell Receptor with Two Igα Cytoplasmic Domains Supports Development of Mature But Anergic B Cells

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Abstract

B cell receptor (BCR) signaling is mediated through immunoglobulin (Ig)α and Igβ a membrane-bound heterodimer. Igα and Igβ are redundant in their ability to support early B cell development, but their roles in mature B cells have not been defined. To examine the function of Igα–Igβ in mature B cells in vivo we exchanged the cytoplasmic domain of Igα for the cytoplasmic domain of Igβ by gene targeting ($Ig\beta_c \rightarrow \alpha_c$ mice). $Ig\beta_c \rightarrow \alpha_c$ B cells had lower levels of surface IgM and higher levels of BCR internalization than wild-type B cells. The mutant B cells were able to complete all stages of development and were long lived, but failed to differentiate into B1a cells. In addition, $Ig\beta_c \rightarrow \alpha_c$ B cells showed decreased proliferative and Ca^{2+} responses to BCR stimulation in vitro, and were anergic to T-independent and -dependent antigens in vivo.

Key words: B cell receptor • immunoglobulin • signal transduction • anergy • B cell development

Introduction

Signaling by membrane Ig (mIg) regulates antigen-independent B cell development, antigen-dependent adaptive immune responses, and B cell survival (1, 2). Signals are transmitted from mIg to the cytoplasm through Igα and Igβ, which form a disulfide-linked membrane-bound heterodimer that is noncovalently associated with mIg through polar residues in the plane of the cell membrane (3–7). Igα and Igβ contain tyrosine residues that are imbedded in immunoreceptor tyrosine-based activation motifs (ITAMs) that are essential for B cell receptor (BCR) signaling (6–11). Upon BCR cross-linking these tyrosine residues recruit and serve as substrates for src and syk family kinases (12–21). Deletion of src kinases blk, fyn, and lyn results in a severe, but incomplete, block at the preB cell stage of development and the combination of syk and zap70 mutation abrogates B cell development (15, 22–24).

Although the cytoplasmic domains of both Igα and Igβ contain essential ITAMs they are otherwise nonhomologous and they display different signaling properties in transfected cell lines (9, 16). Only Igα has non-ITAM tyrosines that bind SLP-65, which in turn recruit Grb2, Nck, Vav, Btk, and PLC-γ2 (20, 25–27). These differences, and the observation that Igα and Igβ cytoplasmic domains bind to distinct sets of kinases in cytoplasmic extracts, led to the suggestion that the two signal transducers have different functions in vivo (6, 7, 9, 11, 16, 28–30). However, studies in transgenic and gene-targeted mice showed that Igα and Igβ have nearly equivalent function for all aspects of early B cell development (31–35). Either the Igα or the Igβ cytoplasmic domain is sufficient to induce early B cell development in transgenic mice by a mechanism that requires ITAM tyrosine phosphorylation (32, 33). In addition, selective deletion of Igα or Igβ cytoplasmic domains by gene targeting (IgαΔC and IgβΔC mice, respectively) results in a block in B cell development that is more severe in early B cells in IgαΔC than in IgβΔC mice (34, 35). Both IgαΔC and IgβΔC mice display a near absence of mature B cells in the periphery (34–36).

To determine whether the cytoplasmic domains of Igα and Igβ have distinct signaling activities in mature B cells

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Abbreviations used in this paper: BCR, B cell receptor; BrdU, bromodeoxyuridine; CFDA,SE, 5-carboxyfluorescein diacetate succinimidyl ester; CGG, chicken gamma globulin; IP, immunoprecipitation; ITAM, immunoreceptor tyrosine activation motif; MIg, membrane Ig; NP, nitrophenol.

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in vivo we replaced the cytoplasmic tail of Igβ with the cytoplasmic domain of the Igα and produced a BCR that contains a homodimer of Igα tails instead of the normal physiologic heterodimer of Igα and Igβ. Here we report that all B cell subsets, with the exception of B1 cells, developed normally in $Ig\beta_c \rightarrow \alpha_c$ mice, but mature B cells had reduced cell surface BCR and were anergic.

Materials and Methods

Mice. $Ig\beta_c \rightarrow \alpha_c$ mice carry an Igβ gene in which the cytoplasmic tail of Igβ from positions 183–228 was replaced with the cytoplasmic tail of Igα from positions 161–220 by gene targeting in 129/Sv embryonic stem cells. A cDNA containing the coding sequence for Igα from positions 161–220 followed by a stop codon, TGA, was inserted after amino acid position 182 in the Igβ gene. A unique HindIII site was placed into the targeting vector as indicated (see Fig. 1 A). The long arm of the targeting construct was 16 kb, the short arm was 6 kb, a LoxP-flanked neomycin-resistance gene was used for positive selection, and a diphtheria toxin gene for negative selection (37). Homologous integrants were confirmed by Southern blotting after digestion with HindIII. The genomic fragment used as a probe for Southern blotting was generated by PCR with the primers 5′ GGA TTC GAA TGG TGA ATG TTG G 3′ and 5′ AGG CTC TAGG CTC AGT GAA GGC AG 3′. Screening by PCR was performed using the primers, 5′ AGC AGG AAG ATG GGC ACA ATG ATG AAG 3′ and 5′ TGG TAT CTA CTT CTG CAA GCA GAA ATG 3′: a 250-bp product represented the mutant allele and a 186-bp product corresponded to the wild-type allele. The rate of homologous recombination was 1:35. Neomycin primers 5′ ATG ATT GAA CAA GAT GGA TTG CAC 3′ and 5′ TCG TCC AGA TCA TCC TGA TCG AC 3′ were used to confirm neomycin gene deletion. Heterozygous $Ig\beta_c \rightarrow \alpha_c$ mice were backcrossed to C57BL/6 for two generations after Cre deletion before intercrossing. All mice were maintained under specific pathogen-free conditions. All experiments shown were performed with homozygous mice.

B Cell Purification and Western Blotting. B cells were purified by negative selection from spleen using anti-CD43 magnetic microbeads (Miltenyi Biotec). For cross-linking experiments B cells were resuspended at 10^8/ml in RPMI with 1% FBS and were cross-linked with 20 μg/ml goat anti–mouse IgM, μ chain specific $F(ab')_2$ fragments (Jackson ImmunoResearch) for the indicated times at 37°C. Cells were lysed in TX-100 buffer (50 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and inhibitors or 50 mM Tris/HCl, pH 7.7, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 0.1% deoxycholic acid, 10% glycerol, 1 mM PMSF, 0.2 mM Na_3VO_4, and protease inhibitors) for Western blotting and in RIPA buffer (50 mM Tris/HCl, pH 7.7, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% deoxycholic acid, 1 mM PMSF, 0.2 mM Na_3VO_4, and protease inhibitors) for immunoprecipitation (IP). Lysates were incubated for 15 min on ice and cellular debris were sedimented at 14,000 rpm for 15 min at 4°C. Lysates were resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and immunoblotted on Immobilon polyvinylidene fluoride membranes (Millipore). For IP, cellular lysates were precleared with protein A–Sepharose (Amersham Biosciences), incubated for 2 h with primary antibodies, and captured with protein A–Sepharose. Visualization was with protein A horse radish peroxidase (Amersham Biosciences), donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories) or goat and mouse IgG (Jackson ImmunoResearch Laboratories) and Western Plus chemiluminescent reagent (NEN Life Science Products). Antibodies used for IP and Western blotting were anti-Igα and anti-Igβ (38), 4G10 (Upstate Biotechnology), anti-Syk (Santa Cruz Biotechnology, Inc.), and Phospho-Syk (Cell Signaling Technologies).

Flow Cytometry. Single cell suspensions from bone marrow, spleen, and peritoneal cavity were stained with FITC, PE, APC, and biotin-conjugated mAbs visualized with Strepavidin red 613 (GIBCO BRL) or streptavidin PerCp (Becton Dickinson). mAbs were anti-CD43, anti-IgM, anti-B220, anti-CD25, anti-IgD, anti-CD19, anti-CD5, anti-CD21, anti-HSA, and anti-CD23 (BD Biosciences). Data collection was performed on a FACSCalibur™ and analyzed using CellQuest™ software (Becton Dickinson).

In Vitro B Cell Cultures and Proliferation Assays. Purified spleen B cells were cultured in RPMI 1640 (GIBCO-BRL) supplemented with 10% FCS, 2 mM L-glutamine (GIBCO-BRL), 100 IU/ml penicillin/streptomycin (GIBCO-BRL), and 100 μM 2-mercaptoethanol (Sigma-Aldrich). Cells were stimulated with CD40L at a concentration of 1:100 (CD8–gp39 fusion protein derived from insect cells; a gift from Dr. Randolph Noelle, Dartmouth Medical School, Hanover, NH; reference 39), or 25 μg/ml LPS (Sigma-Aldrich), or 10 μg/ml goat anti–mouse $F(ab')_2$ IgM (Jackson ImmunoResearch), or 5 μg/ml RP105 (a gift from A. Tarahkovsky, The Rockefeller University), and 1:2,000 CpG (MWG Biotech).

For thymidine incorporation assays 3×10^5 cells were cultured in triplicate for 48 h in 96-well flat bottom plates and pulsed with 1 μCi/ml of [^{3}H]thymidine for 6 h. For labeling with 5-carboxyfluorescein diacetate succinimidyl ester (CFDA,SE; Molecular Probes) 10^7 cells were incubated at 37°C for 10 min in 1 ml RPMI 1640 supplemented with 5 μM CFDA,SE. Cultures were stained with PE-CD19 (BD Biosciences) and analyzed on a FACScalibur™ using CellQuest™ software.

BCR Internalization Assays. Single cell suspensions were equilibrated in ice water baths and stained with biotin-SP goat anti–mouse IgM Fab' fragment (μ specific; Jackson ImmunoResearch). An aliquot of cells was fixed with PBS containing 0.5% paraformaldehyde for the time 0 (T_0) cell surface expression value. The remaining cells were incubated at 37°C for the indicated times (T_n). Reactions were terminated with ice-cold PBS containing 0.5% paraformaldehyde and cells stained with PE-B220 (BD Biosciences) and Streptavidin red 670 (GIBCO BRL). Percent internalization was calculated by the formula % $sIgM(T_0) - \%\ sIgM(T_n)/\%\ IgM(T_0) \times 100$.

Bromodeoxyuridine (BrdU) Labeling. 8–12-wk-old $Ig\beta_c \rightarrow \alpha_c$ and age- and sex-matched control (B6/129F1) mice received BrdU (Sigma-Aldrich) in their drinking water at a concentration of 0.5 mg/ml. The number of $B220^+$ bone marrow and spleen cells was not significantly altered during the 63-d time course. Staining was with anti-BrdU mAb using the BrdU flow kit™ (BD Biosciences) and data was collected using a FACScalibur™ (Becton Dickinson). Fitted curves were drawn using Microsoft Excel software. Approximate half-life was equal to time point where 50% of cells stained positively for BrdU.

Ca^{2+} Flux. Spleen cell suspensions from C57BL/6 mice and $Ig\beta_c \rightarrow \alpha_c$ mice were adjusted to 5×10^6/ml in PBS containing 1% FCS, 1 mM $CaCl_2$, 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) to determine gating. Calcium flux was measured by fluorescence emission ratios of Indo-1-AM on a dual laser FACSVantage™ (Bec-

ton Dickinson) at 395/510 nm on $B220^+IgM^+$ cells. A baseline reading was collected for 60 s before cross-linking the BCR with 30 μg/ml goat anti–mouse IgM $F(ab')_2$ fragments (Southern Biotechnology Associates, Inc.).

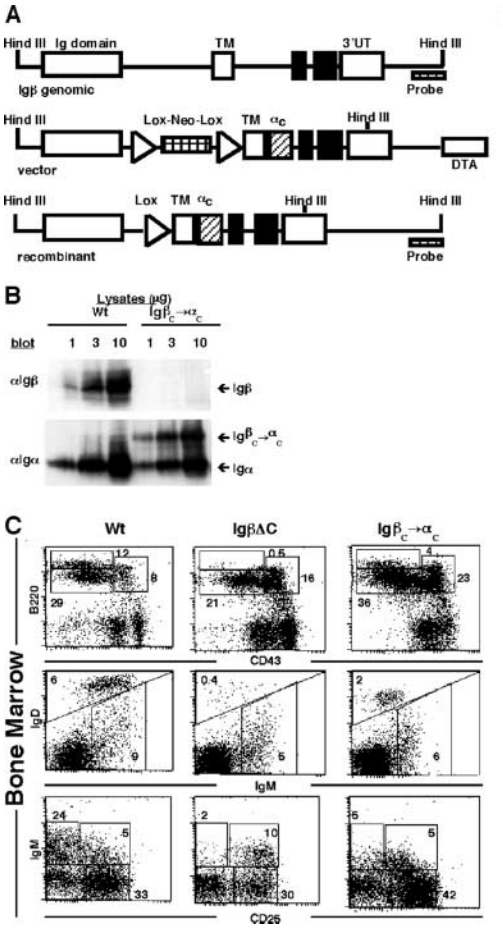
Immunization. 6–8-wk-old $Ig\beta_c \rightarrow \alpha_c$ and B6/129F1 controls were immunized intraperitoneally with either 50 μg alum precipitated 4-hydroxy-3-nitrophenylacetyl coupled to chicken gamma globulin (NP-CGG; Biosearch Technologies) or 50 μg NP-Ficoll in PBS. Blood was collected from the tail vein of each mouse before immunization and then again at days 7, 14, 21, and 31 postimmunization for the mice immunized with NP-CGG and at days 7, 14, 21, and 28 post immunization for the NP-Ficoll group. NP-specific IgM and IgG levels were measured by ELISA using $NP_{16}BSA$ as a capture and developed with goat anti-IgM or anti-IgG coupled to horse radish peroxidase (Southern Biotechnology Associates, Inc.). Immunoabsorbance was read at 415 nm and titers were calculated relative to control sera from un-immunized mice. Three mice were used in each group.

Results

$Ig\beta_c \rightarrow \alpha_c$ Mice. To determine whether Igα and Igβ have unique or redundant functions in mature B cells we replaced the cytoplasmic tail of Igβ with the cytoplasmic tail of Igα by gene targeting (Fig. 1 A, $Ig\beta_c \rightarrow \alpha_c$). $Ig\beta_c \rightarrow \alpha_c$ protein expression in B cells was confirmed by Western blotting of purified B cell lysates using antibodies specific for the cytoplasmic domains of Igα and Igβ (38, 40). Control lysates showed the presence of both Igα and Igβ (Fig. 1 B). In contrast, there was no wild-type Igβ in the lysates from $Ig\beta_c \rightarrow \alpha_c$ B cells. Instead we found a new species reactive with the anti-Igα antibody that had the predicted mobility of the $Ig\beta_c \rightarrow \alpha_c$ protein and no additional Igβ species (Fig. 1 B). We conclude that B cells from $Ig\beta_c \rightarrow \alpha_c$ mice do in fact express only the chimeric Igβ protein with the cytoplasmic tail of Igα.

B Cell Development in $Ig\beta_c \rightarrow \alpha_c$ Mice. To examine the effects of the Igα cytoplasmic tail on development of B lineage cells we compared B cells from $Ig\beta_c \rightarrow \alpha_c$ mice (BCR with two Igα tails) with B cells from IgβΔC mice (BCR with a single Igα tail; reference 35) and wild-type controls (Igα-Igβ).

Like IgβΔC, $Ig\beta_c \rightarrow \alpha_c$ mice showed an increase in $IgM^-B220^{low}CD43^+$ pro-B cells, normal levels of $IgM^-B220^{low}CD25^+$ preB cells and a decrease in the number of immature $IgM^+B220^{low}IgD^{-/low}$ B cells. In addition, immature $Ig\beta_c \rightarrow \alpha_c$ B cells displayed decreased surface IgM and IgD expression (Figs. 1 C and 2 A). Lower IgM levels were also found in IgβΔC B cells but these cells were severely impaired in B cell development (35). $Ig\beta_c \rightarrow \alpha_c$ differed from IgβΔC in that BCRs with two Igα tails sup-



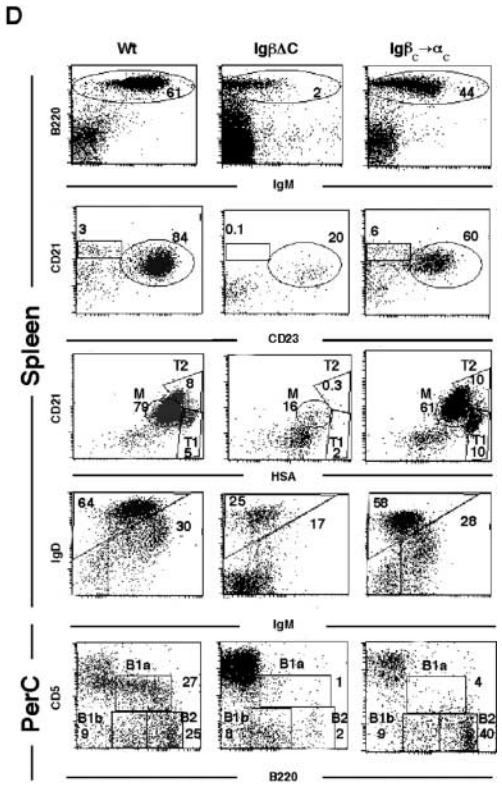


Figure 1. $Ig\beta_c \rightarrow \alpha_c$ mice. (A) Scheme for gene targeting depicts the genomic Igβ locus (top), targeting vector (middle), and recombinant allele after neomycin gene deletion (bottom). Exons are represented by boxes (Igβ cytoplasmic exons [black] and Igα cytoplasmic exons [cross-hatched]), lox P sites by open triangles, and the probe is shown by a striped box. (B) Western blot analysis. Wild-type (wt) and $Ig\beta_c \rightarrow \alpha_c$ B cell lysates were probed with antibodies specific for the cytoplasmic domains of Igα and Igβ. Numbers above the lanes represent micrograms of lysate per sample. Bands with the expected relative mobility of Igα, Igβ, and $Ig\beta_c \rightarrow \alpha_c$ proteins are indicated to the left of the figure. (C) B cell development in the bone marrow of wild-type, IgβΔC, and $Ig\beta_c \rightarrow \alpha_c$ mice. Numbers represent the percentages of lymphocytes except for the plots depicting CD25 versus IgM that were gated on $B220^+$ cells. (D) Spleen B cells in wild-type, IgβΔC, and $Ig\beta_c \rightarrow \alpha_c$ mice. Numbers represent the percentages of lymphocytes. Rows 2 through 4 were pregated on the $B220^+$ population. Transitional 1 cells (T1) were HSA^+CD21^-, transitional 2 cells (T2) were HSA^+CD21^+, and mature (M) B cells were $HSA^{lo}CD21^+$. Peritoneal B cells were gated on lymphocytes as per forward versus side scatter parameters and then fractionated into B1a ($B220^{lo}$ $CD5^+$), B1b ($B220^{lo}$ $CD5^-$), and B2 ($B220^{hi}CD5^-$).

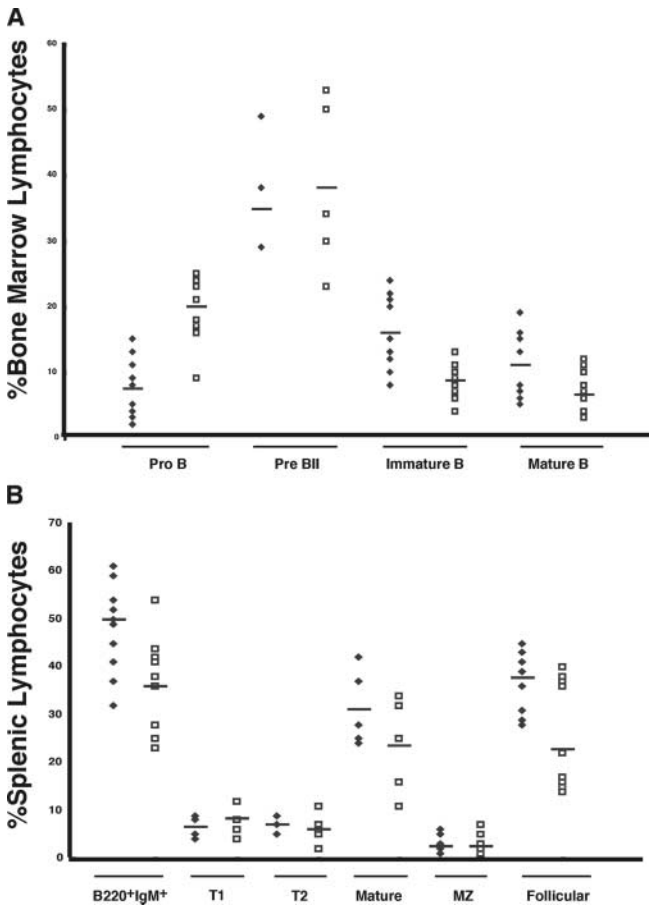


Figure 2. B cells in bone marrow and spleen in wild-type and Ig$\beta_c \rightarrow \alpha_c$ mice. Numbers show percentages of bone marrow or splenic lymphocytes. (A) Bone marrow. Pro B cells were $CD43^+B220^+$, pre BII cells were $CD25^+IgM^-$, immature B cells were IgM^+IgD^{lo}, and mature B cells were IgM^+IgD^{hi}. (B) Spleen. T1, T2, and mature B cells were defined as in Fig. 1. Marginal zone B cells were $CD21^+CD23^-$ and follicular B cells were $CD21^+CD23^+$. Filled diamonds represent wild-type and open squares represent Ig$\beta_c \rightarrow \alpha_c$ mice. Each symbol shows an individual mouse. Bars represent the means of all mice in a group.

ported development of mature recirculating IgM^+B220^{high} B cells whereas a single Igα tail in IgβΔC mice did not (Figs. 1 C and Fig. 2 A; reference 35), but the number of mature recirculating IgM^+B220^{high} B cells in the bone marrow in Ig$\beta_c \rightarrow \alpha_c$ was somewhat lower than wild-type controls (12% Wt vs. 4% Ig$\beta_c \rightarrow \alpha_c$; Figs. 1 C and Fig. 2 A). The total number of cells in spleens of Ig$\beta_c \rightarrow \alpha_c$ mice was similar to wild type (Table I). However, we found a 30% overall decrease in $B220^+IgM^+$ B cells with a small relative increase in marginal zone B cells (Figs. 1 D and Fig. 2 B). In the peritoneal cavity Ig$\beta_c \rightarrow \alpha_c$ mice showed a sevenfold relative decrease in the number of B1a cells but normal number of B1b and B2 cells (Fig. 1 D). Heterozygous Ig$\beta_c \rightarrow \alpha_c$ mice were no different than wild-type controls in B cell development or any of the signaling assays discussed below. We conclude that BCRs with two Igα tails support development of all B cell subtypes except B1a cells.

Decreased Levels of Cell Surface BCR. Mutation of the Igα ITAM ($Ig\alpha^{FF/FF}$mice) increased the level of cell surface BCR expression suggesting that phosphorylation of ITAM tyrosines in the cytoplasmic tail of Igα might regulate cell surface BCR levels (41). Consistent with this idea B cells with two cytoplasmic Igα chains showed decreased levels of cell surface BCR (Fig. 3 A). The decrease in surface BCR expression on Ig$\beta_c \rightarrow \alpha_c$ B cells was not due to a change in steady-state levels of Igμ mRNA or in Igμ protein synthesis as measured by RNase protection and [^{35}S]methionine metabolic labeling experiments (unpublished data). However, the decreased surface BCR level was associated with increased BCR internalization as measured with a biotinylated Fab' anti-IgM (Fig. 3 B). Ig$\beta_c \rightarrow \alpha_c$ B cells showed increased and more rapid receptor internalization than wild-type controls. We conclude that lower levels of cell surface BCR in Ig$\beta_c \rightarrow \alpha_c$ mice are associated with increased BCR internalization.

Table I. *Total Numbers of Nucleated Cells in Bone Marrow and Spleens of 8–12-wk-old Ig$\beta_c \rightarrow \alpha_c$ Mice and Wild-type Controls*

	Bone marrow (n = 9)	Spleen (n = 17)
Wild type	27.4 × 106 SD ± 6.80	71.5 × 106 SD ± 21.6
Ig$\beta_c \rightarrow \alpha_c$	24.2 × 106 SD ± 7.26	61.5 × 106 SD ± 19.9

n, number of mice tested.

To determine whether decreased cell surface BCR expression in Ig$\beta_c \rightarrow \alpha_c$ mice is the result of BCR signaling we bred Ig$\beta_c \rightarrow \alpha_c$ and $Ig\alpha^{FF/FF}$ mice (Ig$\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$ mice; reference 41). Ig$\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$ B cells differ from Ig$\beta_c \rightarrow \alpha_c$ B cells in that only one of the two cytoplasmic Igα tails in Ig$\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$ B cells carries a functional ITAM. We found that B cells with two functional Igα ITAMs (Ig$\beta_c \rightarrow \alpha_c$) had the lowest BCR levels, those with no functional Igα ITAMs ($Ig\alpha^{FF/FF}$) had the highest levels and B cells with one functional Igα ITAM (Ig$\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$) were intermediate (Fig. 3 A). We conclude that an active feedback signaling mechanism mediated by tyrosine phosphorylation of the Igα cytoplasmic domain is responsible for the low levels of BCR on Ig$\beta_c \rightarrow \alpha_c$ B cells.

Half-Life of Ig$\beta_c \rightarrow \alpha_c$ B Cells In Vivo. Ig$\beta_c \rightarrow \alpha_c$ mice displayed a small decrease in the numbers of mature B cells in spleen and a more significant decrease in immature B cells in the bone marrow (Fig. 2 B). B cell half-life is influenced by the rate of B cell influx and is increased in the absence of bone marrow B cell production (42). To determine the life span of Ig$\beta_c \rightarrow \alpha_c$ B cells we performed continuous labeling experiments using BrdU. In these experiments the half-life of cells in any compartment is the time taken to label 50% of the population. We found an increase in the half-life of Ig$\beta_c \rightarrow \alpha_c$ B cells in spleen (Fig. 4 A, 58.3 d vs. 23.4 d for the wild type). We conclude that Ig$\beta_c \rightarrow \alpha_c$ B cells have a significant defect in passing early B cell checkpoints in the bone marrow but once established in the periphery these cells have a longer life span than wild-type B cells.

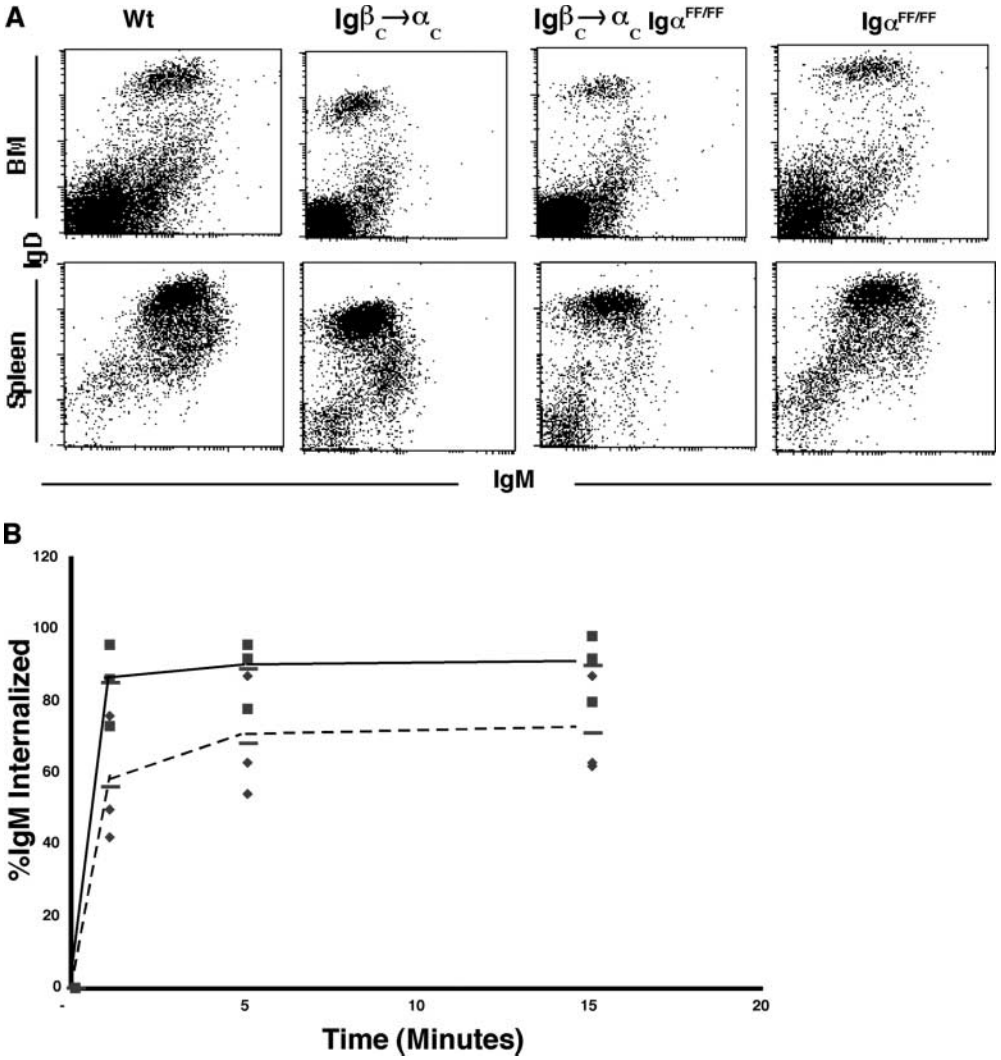


Figure 3. Cell surface BCR expression. (A) IgM and IgD surface expression on B cells from bone marrow and spleen of wild-type, $Ig\beta_c \rightarrow \alpha_c$, $Ig\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$, and $Ig\alpha^{FF/FF}$ mice. Splenic B cells were purified by negative selection before staining. (B) IgM internalization assessed by flow cytometry. Percent internalization was calculated by the formula % $sIgM(T_0)$ − % $sIgM(T_n)$/% $IgM(T_0)$ × 100. The graph shows three separate experiments. Wild type (filled diamonds) and $Ig\beta_c \rightarrow \alpha_c$ (filled squares).

$Ig\beta_c \rightarrow \alpha_c$ B Cells Are Relatively Unresponsive to T Cell–dependent and –independent Antigens In Vivo. To determine whether $Ig\beta_c \rightarrow \alpha_c$ B cells respond to antigen in vivo we immunized mice with nitrophenol-28 coupled to NP-CGG, a T cell–dependent antigen or nitrophenol-59 coupled to ficoll (NP-Ficoll) a T cell–independent antigen. $Ig\beta_c \rightarrow \alpha_c$ mice showed a three- to fivefold reduction in specific IgM and IgG responses to T-dependent antigens when compared with wild-type controls and they were unable to respond to NP-Ficoll (Fig. 5). We conclude that despite their longevity $Ig\beta_c \rightarrow \alpha_c$ B cells are relatively anergic to stimulation with antigen in vivo.

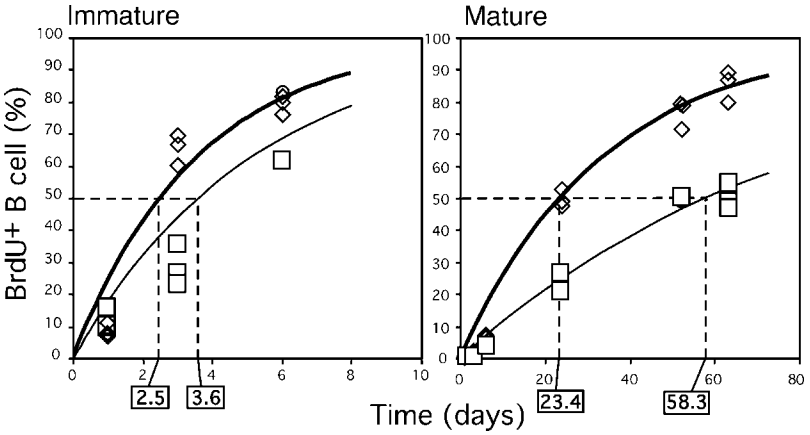


Figure 4. (A) B cell lifespan in bone marrow and spleen in wild-type and $Ig\beta_c \rightarrow \alpha_c$ mice measured by BrdU. Diamonds show wild-type B cells and circles show the $Ig\beta_c \rightarrow \alpha_c$ B cells. Immature B cells (bone marrow) and mature B cells (spleen) were defined as in previous figures. Half-life was the time when 50% of the cells were BrdU labeled. Immature B cells were as in previous figures. Spleen immature B cells were IgM^+IgD^{lo}, mature B cells were $IgM^{lo}IgD^{hi}$, and T cells were $CD3^+$.

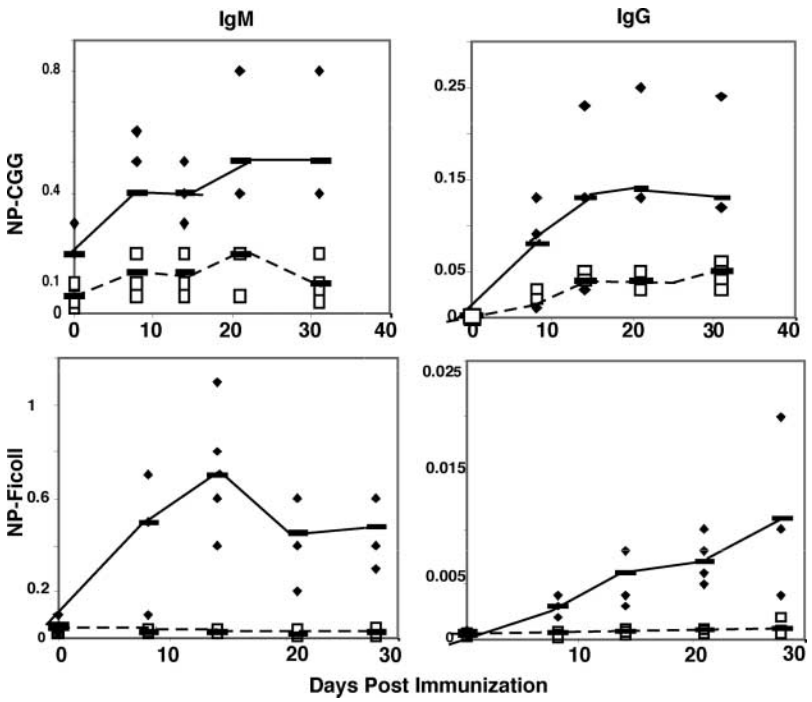


Figure 5. Antibody responses in $Ig\beta_c \rightarrow \alpha_c$ mice. Graphs show anti-NP IgM and IgG responses measured by ELISA on days 7, 14, 21, and 28 (for NP-CGG) and days 7, 14, 21, and 31 for (NP-Ficoll) after immunization with T-dependent (NP-CGG) or -independent (NP-Ficoll) antigens. The filled diamonds and the open squares represent serum titers in individual wild-type and $Ig\beta_c \rightarrow \alpha_c$ mice, respectively. The ordinate shows OD 415 nm relative to nonimmunized controls. The line shows the means for each group at a given time point. There were three mice in each group.

Proliferation Responses In Vitro. To determine whether the inability to respond to antigen reflects a B cell autonomous defect we stimulated $Ig\beta_c \rightarrow \alpha_c$ B cells with mitogens in vitro. Purified B cells were labeled with CFDA,SE and stimulated with anti-IgM, CD40L, RP105, CpG, or LPS (Fig. 6, A and E). $Ig\beta_c \rightarrow \alpha_c$ B cells responded to anti-IgM stimulation by up-regulating CD69 and CD86 expression (Fig. 6 B). However, $Ig\beta_c \rightarrow \alpha_c$ B cells failed to proliferate in response to BCR cross-linking. Only a small number of the B cells stimulated with anti-IgM diluted CFSE; thymidine incorporation was also severely decreased compared with the wild-type control (Fig. 6, A and C). To determine whether the lack of responsiveness to BCR stimulation was due to a signaling defect as opposed to a problem with receptor assembly we measured responses to anti-IgM by $Ig\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$ B cells, which carry ITAM tyrosine mutations in one of the two Igα tails. We found that mutating the ITAM tyrosines in Igα partially rescued the unresponsive phenotype in $Ig\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$ B cells (Fig. 6 A). Whereas $Ig\beta_c \rightarrow \alpha_c$ B cells fail to proliferate in response to anti-IgM treatment, $Ig\beta_c \rightarrow \alpha_c$/$Ig\alpha^{FF/FF}$ B cells divided, although the average number of divisions was somewhat lower than wild-type or $Ig\alpha^{FF/FF}$ B cells (Fig. 6 A). Thus, the lack of responsiveness found in $Ig\beta_c \rightarrow \alpha_c$ B cells is at least in part due to a process that requires Igα ITAM tyrosine phosphorylation.

$Ig\beta_c \rightarrow \alpha_c$ showed normal levels of cell surface CD40L and RP105, but diminished responses to CD40L, RP105, CpG, and LPS stimulation (Fig. 6, C–E). There was less cell division, as measured by CFSE dye dilution, in response to CD40L and RP105, and fewer cells were induced to divide by LPS or CpG (Fig. 6 E). These results were confirmed by measuring [^{3}H]thymidine uptake in response to the same stimuli (Fig. 6 C). These differences were not due to a change in the number of cells undergoing apoptosis (Fig. 6; and unpublished data). We conclude that $Ig\beta_c \rightarrow \alpha_c$ B cells show modestly decreased responses to a broad range of mitogenic stimuli in vitro.

To determine whether proximal receptor signaling is decreased in $Ig\beta_c \rightarrow \alpha_c$ B cells we measured Ca^{2+} flux responses to BCR cross-linking. We found that $Ig\beta_c \rightarrow \alpha_c$ B cells showed impaired Ca^{2+} responses compared with wild-type controls (Fig. 6 F). To further characterize the proximal BCR signaling in $Ig\beta_c \rightarrow \alpha_c$ mice we examined total phosphorylation, as well as Igα and Syk phosphorylation after BCR cross-linking (12–18). We found an overall decrease in total phosphorylation consistent with the lower levels of Ca^{2+} signaling in $Ig\beta_c \rightarrow \alpha_c$ B cells (Fig. 7 A). In addition, $Ig\beta_c \rightarrow \alpha_c$ B cells showed little Igα phosphorylation in response to BCR cross-linking (Fig. 7 B). Finally, phosphorylation of Syk was substantially reduced in $Ig\beta_c \rightarrow \alpha_c$ mice when compared with wild-type controls (Fig. 7 C). We conclude that decreased surface BCR levels on $Ig\beta_c \rightarrow \alpha_c$ B cells are associated with decreased intensity of proximal signaling.

Discussion

Our experiments extend previous work by examining the function of Igα and Igβ in mature B cells in vivo. We show that a BCR containing a signaling module composed of a homodimer of Igα cytoplasmic tails can support the development of all currently defined subgroups of B cells with the exception of B1a cells and therefore Igα and Igβ are redundant for all stages of B cell development. In contrast, Igα and Igβ have distinct functions in regulating BCR surface expression and setting thresholds for mature B cell activation.

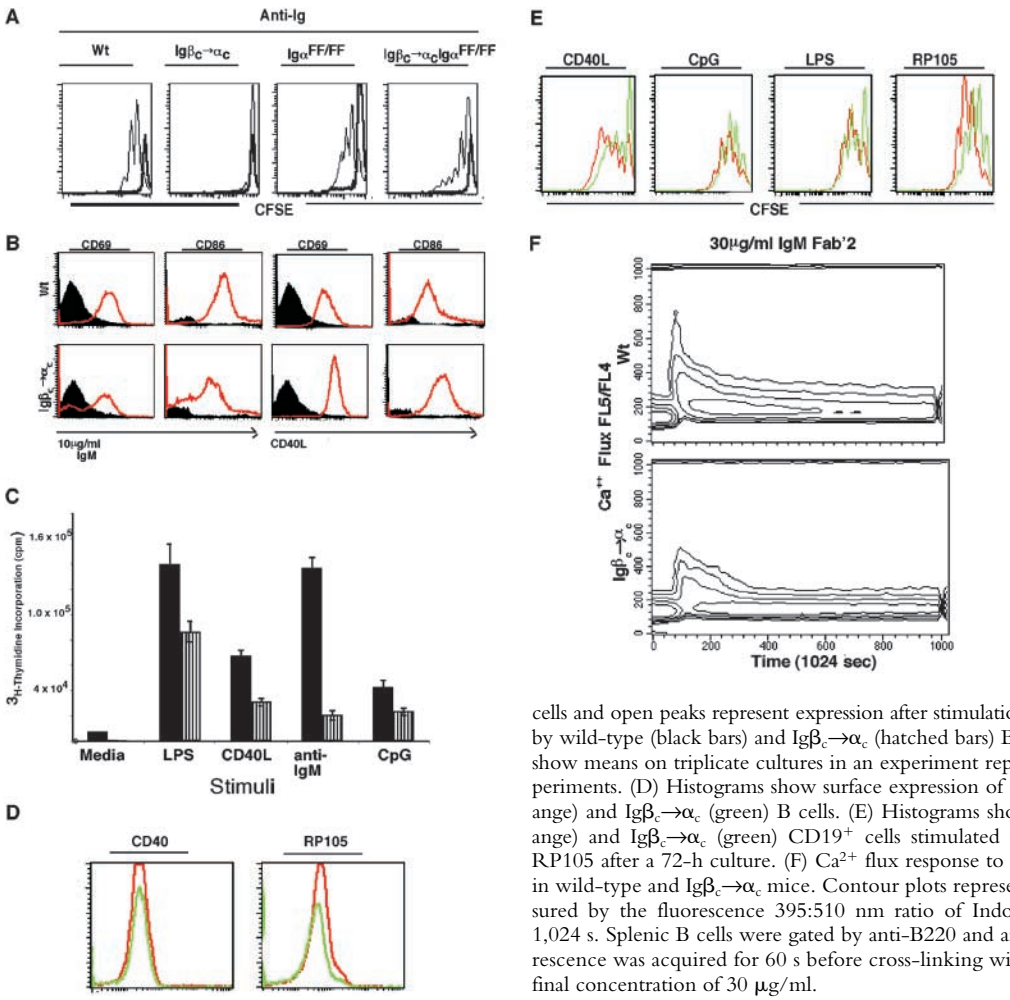


Figure 6. In vitro responses to mitogens. (A) Histograms show CFSE staining on wild-type, $Ig\beta_c \rightarrow \alpha_c$, $Ig\alpha^{FF/FF}$, and $Ig\beta_c \rightarrow \alpha_c / Ig\alpha^{FF/FF}$ $CD19^+$ cells cultured with Fab′2 anti-IgM. The dark line represents cells at time 0 with no stimulation, the light line represents number of cell divisions after 72 h in culture. (B) Up-regulation of activation markers in wild-type and $Ig\beta_c \rightarrow \alpha_c$ B cells. Cell surface expression of CD69 and CD86 after 24 h incubation with 10 mg/ml goat anti–mouse $(Fab')_2$ fragments or CD40L. Solid peaks show unstimulated cells and open peaks represent expression after stimulation. (C) [^{3}H]Thymidine incorporation by wild-type (black bars) and $Ig\beta_c \rightarrow \alpha_c$ (hatched bars) B cells after a 54-h culture. Error bars show means on triplicate cultures in an experiment representative of three independent experiments. (D) Histograms show surface expression of CD40 and RP105 by wild-type (orange) and $Ig\beta_c \rightarrow \alpha_c$ (green) B cells. (E) Histograms show CFSE dilution by wild-type (orange) and $Ig\beta_c \rightarrow \alpha_c$ (green) $CD19^+$ cells stimulated with CD40L, or CpG, or LPS, or RP105 after a 72-h culture. (F) Ca^{2+} flux response to BCR cross-linking in splenic B cells in wild-type and $Ig\beta_c \rightarrow \alpha_c$ mice. Contour plots represent Ca^{2+} flux of splenic B cells measured by the fluorescence 395:510 nm ratio of Indo-1-AM emission accumulated over 1,024 s. Splenic B cells were gated by anti-B220 and anti-Fab′ IgM staining. Baseline fluorescence was acquired for 60 s before cross-linking with goat anti–mouse IgM $(Fab')_2$ at a final concentration of 30 μg/ml.

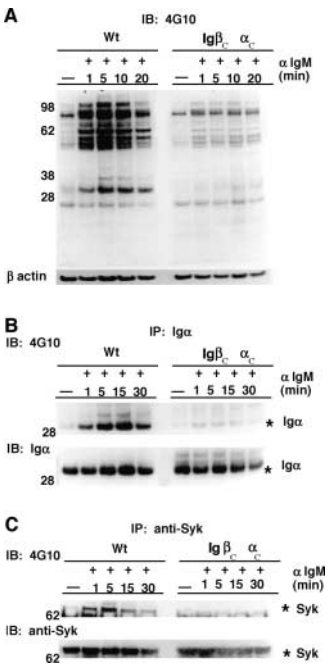


Figure 7. Protein tyrosine phosphorylation. Purified B cells were stimulated with 10 μg/ml goat anti–mouse $F(ab)'_2$ for the indicated times. (A) Tyrosine phosphorylation of total cellular proteins (5×10^6 cells/lane) detected by antiphosphotyrosine immunoblotting with the mAb 4G10. (B) Tyrosine phosphorylation in Igα immunoprecipitates. Igα immunoprecipitates were immunoblotted with mAb 4G10 to detect tyrosine phosphorylation. The lower panel shows the same blot stripped and reprobed with Igα antibody. (C) Syk tyrosine phosphorylation. Anti-Syk immunoprecipitates were immunoblotted with mAb 4G10 to detect tyrosine phosphorylation. The lower panel shows the same blot stripped and reprobed with anti-Syk antibody.

Igα and Igβ and B Cell Development. Early B cell development requires preBCR signaling and either Igα or Igβ cytoplasmic domains are sufficient to satisfy this requirement (32, 33). Signaling through BCR ITAM tyrosines was required for early B cell development but the BCR ITAMs were redundant and a single functional Igβ ITAM was sufficient to reconstitute development (32, 33, 41, 43). Transgenic Fc chimeras fused to the cytoplasmic tails of Igα or Igβ differed from Igμ chimeras in that they appeared to support mature B cell development, but the distribution of different B cell subpopulations was not examined and the Fc chimeras were not antigen receptors so B cell function could not be studied (32, 33).

Gene targeting confirmed the transgenic experiments and showed that BCRs lacking either the cytoplasmic domain of Igα or Igβ (IgαΔC and IgβΔC) were sufficient for B cells to progress to the immature stage of development but not the mature B cell stage (34, 35). IgαΔC and IgβΔC differed in that IgβΔC progressed to the immature stage of development nearly normally whereas IgαΔC B cells showed a partial block in the transition between preB and immature stage of development (34, 35). These findings

were reminiscent of the observation that thymocyte development is dependent on the number of T cell receptor ζ ITAMs (44–46). Single ITAM-containing BCRs in IgαΔC and IgβΔC mice were simply not sufficient for complete B cell maturation (34, 35).

In vitro experiments with isolated Igα and Igβ cytoplasmic domains suggested that the two transducers bind to different but overlapping sets of kinases (16). However, transfection experiments with chimeric receptors and transgenic experiments failed to show qualitative differences in early B cell development or signaling stimulated by Igα or Igβ (6, 7, 9, 11, 28–35). Mature B cells are therefore unique in their requirement for both Igα and Igβ for normal levels of BCR expression and physiologic responses to BCR cross-linking.

As both Igα and Igβ have a single ITAM, the physiologic differences in mature B cell signaling could be due to the non-ITAM residues. Among the non-ITAM residues in Igα implicated in signaling are two non-ITAM tyrosines, 176 and 204, that bind the adaptor molecule BLNK (20, 21, 26, 47, 48), which links the BCR with phospholipase Cγ, Vav, Grb2, Syk, Btk, and HPK1 (20, 25–27). Mice that are BLNK deficient show a block in early B cell development, decreased numbers of peripheral B cells, and defective B cell activation (20, 21, 25, 27, 49–53). BLNK-deficient mice also show an accumulation of mature B cells whose phenotype is $IgM^{high}IgD^{low}$ and increased expression of surface preBCR (54, 55). These features would lend credence to the idea that BCRs with two Igα cytoplasmic domains in $Ig\beta_c \rightarrow \alpha_c$ B cells may simply recruit abnormally high levels of BLNK and its signaling partners, however, we find lower levels of BLNK phosphorylation in $Ig\beta_c \rightarrow \alpha_c$ B cells (unpublished data) and therefore the two non-ITAM tyrosines in Igα that recruit BLNK are not likely to be responsible for the difference in signaling by Igα and Igβ in mature B cells in vivo.

$Ig\beta_c \rightarrow \alpha_c$ mice show a small relative enlargement of the MZ compartment but a near absence of B1 cells. Several signaling molecules have been shown to be essential for normal MZ B cell development including CD19, *btk*, *pyk2*, NF-κB, aiolos, DOCK2, and Lsc (56–61). In addition, Kraus et al. showed that impaired Igα signaling in a BCR with point mutations of the Igα ITAM tyrosines results in decreased MZ B cells production (41). Together our data suggests that the size of the MZ compartment is directly related to the number of Igα ITAM tyrosines.

Similarly B1 development is BCR dependent and requires positive selection by low affinity interaction with self-antigen (62–64) The reduced cell surface BCR and decreased signaling by $Ig\beta_c \rightarrow \alpha_c$ B cells may simply be insufficient for normal B1 selection. Alternatively, there may be a specific requirement for Igβ signaling in the development of the B1 population but this seems unlikely as Igβ is not sufficient to produce B1 cells in $Ig\alpha^{FF/FF}$ mice.

Decreased Surface BCR Expression in $Ig\beta_c \rightarrow \alpha_c$ Mice. Cell surface expression of mIg requires coexpression of either Igα–Igβ or Igβ (6, 38, 65). This requirement is linked to the presence of polar residues in the transmembrane domain of mIg that interact with Igα–Igβ (6). Once on the cell surface the BCR is constitutively recycled from the plasma membrane to endosomes where captured antigens are processed for presentation to cognate T cells. Signaling by the cytoplasmic domains of Igα and Igβ is essential for BCR internalization and targeting to MHC II–containing endosomes (47, 66). The idea that tyrosines in the cytoplasmic domains of Igα and Igβ may be critical for constitutive BCR internalization derived from studies of endocytosis of the transferrin and LDL receptors (67, 68). Bulky hydrophobic amino acids such as phenylalanine or tyrosine in the cytoplasmic domains of those receptors were required for constitutive endocytosis. Consistent with the role of such residues in endocytosis Igα tyrosines were found to be essential for both signal transduction and antigen presentation in cells lines transfected with Fc–Igα chimeras (69). In contrast Igβ tyrosine residues were dispensable for internalization (70). In addition, experiments with PDGFR chimeras that contained both Igα and Igβ showed that both cytoplasmic domains were required to regulate ligand-induced internalization (47). The implication of these in vitro studies was that Igα and Igβ had distinct but complementary roles in mediating BCR internalization.

A role for Igα in regulating BCR surface expression in mature B cells in vivo was confirmed by gene targeting (41). B cells with Igα ITAM tyrosines mutated to phenylalanine displayed increased surface BCR expression in vivo (41). Although the mechanism for increased receptor expression in $Ig\alpha^{FF/FF}$ mice was not determined three alternatives were suggested. First, B cells with increased levels of surface BCR might be selected during development to compensate for the decreased signaling activity of the mutant BCR (41). Second, Igα signaling might normally inhibit Igβ function (36, 71). In this scheme uninhibited Igβ signaling would account for increased BCR surface expression in $Ig\alpha^{FF/FF}$ B cells and increased BCR signaling in IgαΔC B cells (36, 71). However, IgβΔC mutant B cells that lack the Igβ cytoplasmic tail showed the same hyper-reactive phenotype as IgαΔC B cells (35). Thus, the negative regulatory effect is not specific for either Igα or Igβ and is more likely due to inability of the single chain BCRs in IgαΔC and IgβΔC B cells to recruit negative regulators of BCR function such as Src homology 2 domain–containing phophatase 1 (35). Finally, Igα signaling might directly control BCR surface expression or internalization (41). According to this hypothesis signals emanating from the Igα ITAM would be required to set cell surface BCR levels and the absence of such signals in $Ig\alpha^{FF/FF}$ B cells would lead to increased expression and a proportional increase in BCR signaling. Our data is most consistent with the idea that Igα functions as such a regulator as we find lower surface BCR levels when the BCR contains an additional Igα cytoplasmic domain. This decrease in BCR expression is directly correlated with, and may be responsible for, the decreased signaling by BCRs with two Igα ITAMs. Furthermore, phosphorylation of ITAM tyrosines is critical for regulating surface BCR levels because $Ig\beta_c \rightarrow \alpha_c/Ig\alpha^{FF/FF}$ BCRs with a lower total number of active Igα ITAMs have increased BCR surface expression. Active feedback

regulation of cell surface receptor levels is a common mechanism for regulating cellular sensitivity to persistent stimuli. We speculate that Igα performs this feedback function for regulating BCR surface expression in vivo.

$Ig\beta_c \rightarrow \alpha_c$ Mature B Cells Are Anergic. Subthreshold signaling leads to anergy in both T cells and B cells, possibly by partial stimulation of Ca^{2+}-dependent pathways (72, 73). B cells stimulated with submitogenic doses of anti-BCR antibodies in vitro showed decreased surface BCR expression and became relatively anergic to further stimulation (74, 75). In the same way, anti-hen egg lysozyme–specific B cells became anergic when exposed to cognate antigen in vivo, and this was accompanied by decreased BCR surface expression (76). Finally, similar effects including decreased surface BCR expression and inability to secrete antibody were found in anti-double– (77) and anti-single–stranded DNA Ig transgenic mice (78). In Ig-transgenic models, subthreshold BCR signaling was associated with increased baseline Ca^{2+} levels (73,79), PKCδ-dependent changes in Ca^{2+} responses, and nuclear signaling pathways including NFκB and JNK (79–82). In contrast, we found no differences in resting Ca^{2+} levels in $Ig\beta_c \rightarrow \alpha_c$ B cells. Anergy in this case may be due to an overall decrease in signaling as a result of low levels of cell surface receptor expression.

$Ig\beta_c \rightarrow \alpha_c$ B cells spontaneously display many of the features of anergic B cells, including decreased surface BCR expression and diminished responses to BCR cross-linking. In addition $Ig\beta_c \rightarrow \alpha_c$ B cells showed impaired polyclonal responses to T cell–independent and –dependent antigens in vivo. These changes in sensitivity to BCR cross-linking required signal transduction and Igα tyrosine phosphorylation because they were partially reversed in $Ig\beta_c \rightarrow \alpha_c/Ig\alpha^{FF/FF}$ B cells in which one of the two BCR ITAMs is inactivated. The major difference between $Ig\beta_c \rightarrow \alpha_c$ B cells and other anergic B cells is that they are long lived when compared with wild-type B cells (77, 83). Little is known about how B cell longevity is regulated but our experiments suggest that Igα may play an important role in this process. We conclude that Igα and Igβ signaling have distinct roles in regulating mature B cell physiology in vivo.

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