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

Amy Reichlin,1 Anna Gazumyan,1 Hitoshi Nagaoka,3 Kathrin H. Kirsch,4 Manfred Kraus,5 Klaus Rajewsky,3 and Michel C. Nussenzweig1,2

1Laboratory of Molecular Immunology, 2Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021
3Department of Medical Chemistry and Molecular Biology, Graduate School of Medicine, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
4Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118
5Center for Blood Research, Harvard Medical School, Boston, MA 02115

Abstract

B cell receptor (BCR) signaling is mediated through immunoglobulin (Igα 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 (IgγC→α mice). IgγC→α 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, IgγC→α B cells showed decreased proliferative and Ca2+ 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, 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 mice 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

Abbreviations used in this paper: BCR, B cell receptor; BrdU, bromodeoxyuridine; CFFA, SE, 5-carboxyfluorescein diacetate succinimidyl ester; CGG, chicken gamma globulin; IP, immunoprecipitation; ITAM, immunoreceptor tyrosine activation motif; MIg, membrane Ig; NP, nitrophenol.
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 Igβ→α mice, but mature B cells had reduced cell surface BCR and were anergic.

**Materials and Methods**

**Mice.** Igβ→α 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. 1A). The long arm of the targeting construct was 1 kb, the short arm was 6 kb, a LoxP-flanked neomycin-resistance gene was used for positive selection, and a diptheria 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 GCC AG 3’. Screening by PCR was performed using the primers, 5’-AGC AGG AAG ATG TTC GAC 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-resistance gene–containing embryonic stem cells were selected in G418, and 10% FCS was included in the medium to maintain the neomycin-resistant cell line. A unique HindIII site was placed into the targeting vector as indicated (see Fig. 1A). The long arm of the targeting construct was 1 kb, the short arm was 6 kb, a LoxP-flanked neomycin-resistance gene was used for positive selection, and a diptheria 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 GCC AG 3’. Screening by PCR was performed using the primers, 5’-AGC AGG AAG ATG TTC GAC 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-resistance gene–containing embryonic stem cells were selected in G418, and 10% FCS was included in the medium to maintain the neomycin-resistant cell line.
ton Dickinson) at 395/510 nm on B220<sup>-</sup>IgM<sup>+</sup> cells. A baseline reading was collected for 60 s before crossing-linking the BCR with 30 μg/ml goat anti–mouse IgM F(ab′)<sub>2</sub> fragments (Southern Biotechnology Associates, Inc.).

*Immunization.* 6–8-wk-old Igβ<sub>-</sub>→α<sub>+</sub> and B6/129F1 controls were immunized intraperitoneally with either 50 μg alum precipitated 4-hydroxy-3-nitrophenylacetetyl 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<sub>16</sub>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 titeres were calculated relative to control sera from un-immunized mice. Three mice were used in each group.

**Results**

*Igβ<sub>-</sub>→α<sub>+</sub> 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, Igβ<sub>-</sub>→α<sub>+</sub>). Igβ<sub>-</sub>→α<sub>+</sub> 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 Igβ<sub>-</sub>→α<sub>+</sub> B cells. Instead we found a new species reactive with the anti-Igα antibody that had the predicted mobility of the Igβ<sub>-</sub>→α<sub>+</sub> protein and no additional Igβ species (Fig. 1 B). We conclude that B cells from Igβ<sub>-</sub>→α<sub>+</sub> mice do in fact express only the chimeric Igβ protein with the cytoplasmic tail of Igα.

*B Cell Development in Igβ<sub>-</sub>→α<sub>+</sub> Mice.* To examine the effects of the Igα cytoplasmic tail on development of B lineage cells we compared B cells from Igβ<sub>-</sub>→α<sub>+</sub> 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, Igβ<sub>-</sub>→α<sub>+</sub> mice showed an increase in IgM<sup>-</sup>B220<sup>+</sup>C<sub>134</sub> pro-B cells, normal levels of IgM<sup>-</sup>B220<sup>+</sup>C<sub>25+</sub> preB cells and a decrease in the number of immature IgM<sup>-</sup>B220<sup>+</sup>C<sub>10</sub>ImmB<sub>21</sub> B cells. In addition, immature Igβ<sub>-</sub>→α<sub>+</sub> 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). Igβ<sub>-</sub>→α<sub>+</sub> differed from IgβΔC in that BCRs with two Igα tails sup-

---

**Figure 1.** Igβ<sub>-</sub>→α<sub>+</sub> mice. (A) Scheme for gene targeting depicts the genomic Ig 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 Igβ<sub>-</sub>→α<sub>+</sub> 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 Igβ<sub>-</sub>→α<sub>+</sub> proteins are indicated to the left of the figure. (C) B cell development in the bone marrow of wild-type, IgβΔC, and Igβ<sub>-</sub>→α<sub>+</sub> mice. Numbers represent the percentages of lymphocytes except for the plots depicting CD25 versus IgM that were gated on B220<sup>+</sup> cells. (D) Spleen B cells in wild-type, IgβΔC, and Igβ<sub>-</sub>→α<sub>+</sub> mice. Numbers represent the percentages of lymphocytes. Rows 2 through 4 were pregated on the B220<sup>+</sup> population. Transitional 1 cells (TI) were HSA<sup>+</sup>CD21<sup>+</sup>, transitional 2 cells (T2) were HSA<sup>+</sup>CD21<sup>+</sup>, and mature (M) B cells were HSA<sup>+</sup>CD21<sup>+</sup>. Peritoneal B cells were gated on lymphocytes as per forward versus side scatter parameters and then fractionated into B1a (B220<sup>+</sup>CD5<sup>+</sup>), B1b (B220<sup>+</sup>CD5<sup>-</sup>), and B2 (B220<sup>+</sup>CD5<sup>-</sup>).
in Fig. 1. Marginal zone B cells were CD21
Ig
number of B1b and B2 cells (Fig. 1 D). Heterozygous development of all B cell subtypes except B1a cells. below. We conclude that BCRs with two Ig
Ig
In the peritoneal cavity Ig
increase in marginal zone B cells (Figs. 1 D and Fig. 2 B). B cells whereas a single Ig
Ig
were CD21
Ig
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
, and mature B cells were IgM+IgD
. (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
B cells. Each symbol shows an individual mouse. Bars represent the means of all mice in a group.
ported development of mature recirculating IgM+B220high 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+B220high B cells in the bone marrow in Igβ−→α− was somewhat lower than wild-type controls (12% Wt vs. 4% Igβ−→α−; Figs. 1 C and Fig. 2 A). The total number of cells in spleens of Igβ−→α− 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 Igβ−→α− mice showed a sevenfold relative decrease in the number of B1a cells but normal number of B1b and B2 cells (Fig. 1 D). Heterozygous Igβ−→α− 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 (IgαFF/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 Igβ−→α− B cells was not due to a change in steady-state levels of Igα mRNA or in Igα protein synthesis as measured by RNase protection and [35S]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). Igβ−→α− B cells showed increased and more rapid receptor internalization than wild-type controls. We conclude that lower levels of cell surface BCR in Igβ−→α− mice are associated with increased BCR internalization.

To determine whether decreased cell surface BCR expression in Igβ−→α− mice is the result of BCR signaling we bred Igβ−→α− and IgαFF/FF mice (Igβ−→α−/IgαFF/FF mice; reference 41). Igβ−→α−/IgαFF/FF B cells differ from Igβ−→α− B cells in that only one of the two cytoplasmic Igα tails in Igβ−→α−/IgαFF/FF B cells carries a functional ITAM. We found that B cells with two functional Igα ITAMs (Igβ−→α−) had the lowest BCR levels, those with no functional Igα ITAMs (IgαFF/FF) had the highest levels and B cells with one functional Igα ITAM (Igβ−→α−/IgαFF/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 Igβ−→α− B cells.

**Table I.** Total Numbers of Nucleated Cells in Bone Marrow and Spleens of 8–12-wk-old Igβ−→α− 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 |
| Igβ−→α−             | 24.2 × 106 SD ± 7.26| 61.5 × 106 SD ± 19.9 |

n, number of mice tested.

Half-Life of Igβ−→α− B Cells In Vivo. Igβ−→α− 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 Igβ−→α− 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 Igβ−→α− B cells in spleen (Fig. 4 A, 58.3 d vs. 23.4 d for the wild type). We conclude that Igβ−→α− 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.
Igβ → α, B Cells Are Relatively Unresponsive to T Cell–dependent and –independent Antigens In Vivo. To determine whether Igβ → α, 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. Igβ → α, 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 Igβ → α, B cells are relatively anergic to stimulation with antigen in vivo.

Figure 3. Cell surface BCR expression. (A) IgM and IgD surface expression on B cells from bone marrow and spleen of wild-type, Igβ → α, Igβ → α/IgαFF/FF, and IgαFF/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(T0) = % sIgM(Tn)/% IgM(T 0) × 100. The graph shows three separate experiments. Wild type (filled diamonds) and Igβ → α (filled squares).

Figure 4. (A) B cell lifespan in bone marrow and spleen in wild-type and Igβ → α mice measured by BrdU. Diamonds show wild-type B cells and circles show the Igβ → α 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 IgM+IgDlo, mature B cells were IgM−IgDhi, and T cells were CD3+.
Proliferation Responses In Vitro. To determine whether the inability to respond to antigen reflects a B cell autonomous defect we stimulated Igβ→α, 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). Igβ→α B cells responded to anti-IgM stimulation by up-regulating CD69 and CD86 expression (Fig. 6 B). However, Igβ→α 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 Igβ→α/IgαFF/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 Igβ→α/IgαFF/FF B cells (Fig. 6 A). Whereas Igβ→α B cells fail to proliferate in response to anti-IgM treatment, Igβ→α/IgαFF/FF B cells divided, although the average number of divisions was somewhat lower than wild-type or IgαFF/FF B cells (Fig. 6 A). Thus, the lack of responsiveness found in Igβ→α B cells is at least in part due to a process that requires Igα ITAM tyrosine phosphorylation.

Igβ→α 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 [3H]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 Igβ→α B cells show modestly decreased responses to a broad range of mitogenic stimuli in vitro.

To determine whether proximal receptor signaling is decreased in Igβ→α B cells we measured Ca2+ flux responses to BCR cross-linking. We found that Igβ→α B cells showed impaired Ca2+ responses compared with wild-type controls (Fig. 6 F). To further characterize the proximal BCR signaling in Igβ→α 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 Ca2+ signaling in Igβ→α B cells (Fig. 7 A). In addition, Igβ→α B cells showed little Igα phosphorylation in response to BCR cross-linking (Fig. 7 B). Finally, phosphorylation of Syk was substantially reduced in Igβ→α mice when compared with wild-type controls (Fig. 7 C). We conclude that decreased surface BCR levels on Igβ→α 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.
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 IgαITAM was sufficient to reconstitute development (32, 33, 41, 43). Transgenic Fc chimeras fused to the cytoplasmic tails of Igα or Igβ differed from IgαITAM 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 (IgαC and IgβC) were sufficient for B cells to progress to the immature stage of development but not the mature B cell stage (34, 35). IgαC and IgβC differed in that IgβC progressed to the immature stage of development nearly normally whereas IgαC B cells showed a partial block in the transition between preB and immature stage of development (34, 35). These findings

---

**Figure 6.** In vitro responses to mitogens. (A) Histograms show CFSE staining on wild-type, Igβ→αc, IgαFF/FF, and Igβ→α/IgαITAM 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 Igβ→αc B cells. Cell surface expression of CD69 and CD86 after a 24 h incubation with 10 mg/ml goat anti-mouse (Fab’2)2 fragments or CD40L. Solid peaks show unstimulated cells and open peaks represent expression after stimulation. (C) [3H]Thymidine incorporation by wild-type (black bars) and Igβ→αc (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 Igβ→αc (green) B cells. (E) Histograms show CFSE dilution by wild-type (orange) and Igβ→αc (green) CD19+ cells stimulated with CD40L, or Cpg, or LPS, or RP105 after a 72 h culture. (F) Ca2+ flux response to BCR cross-linking in splenic B cells in wild-type and Igβ→αc mice. Contour plots represent Ca2+ 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’1 IgM staining. Baseline fluorescence was acquired for 60 s before cross-linking with goat anti-mouse (Fab’2)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.
were reminiscent of the observation that thymocyte develop-
ment 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 cytoplas-
mic domains suggested that the two transducers bind to dif-
ferent but overlapping sets of kinases (16). However, trans-
fection experiments with chimera 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 physi-
ologic 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 tyro-
sines, 176 and 204, that bind the adaptor molecule BLNK
(20, 21, 26, 47, 48), which links the BCR with phospholi-
pase 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 IgMhighIgDlow and increased expression
of surface IgM (54, 55). These features would lend cre-
dence to the idea that BCRs with two Igα cytoplasmic
domains in Igβ→α B cells may simply recruit abnormally
high levels of BLNK and its signaling partners, however,
we find lower levels of BLNK phosphorylation in Igβ→α
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.

Igβ→α 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, ppk2,
NF-κB, aiolos, DOCK2, and Lsc (56–61). In addi-
tion, Kraus et al. showed that impaired Igα signaling in a
BCR with point mutations of the Igα ITAM tyrosines re-
sults 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 re-
quires positive selection by low affinity interaction with
self-antigen (62–64). The reduced cell surface BCR and
decreased signaling by Igβ→α B cells may simply be insuffi-
cient 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 IgαFF/FF mice.

Decreased Surface BCR Expression in Igβ→α Mice.
Cell surface expression of mlg requires coexpression of ei-
ther Igα→Igβ or Igβ (6, 38, 65). This requirement is linked
to the presence of polar residues in the transmembrane
domain of mlg 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 cytoplas-
mic 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 hydro-
phobic 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 es-
sential for both signal transduction and antigen presentation
in cells lines transfected with Fc–Igα chimeras (69). In con-
trast Igβ tyrosine residues were dispensable for internaliza-
tion (70). In addition, experiments with PDGFR chimeras
that contained both Igα and Igβ showed that both cyto-
plasmic domains were required to regulate ligand-induced
internalization (47). The implication of these in vitro stud-
ies 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 targetung
(41). B cells with Igα ITAM tyrosines mutated to phe-
nylalanine displayed increased surface BCR expression in
vivo (41). Although the mechanism for increased receptor
expression in IgαFF/FF mice was not determined three alter-
natives 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 mu-
tant BCR (41). Second, Igα signaling might normally in-
hbit Igβ function (36, 71). In this scheme uninhibited Igβ
signaling would account for increased BCR surface expres-
sion in IgαFF/FF B cells and increased BCR signaling in Ig-
aΔC B cells (36, 71). However, IgβΔC mutant B cells that
lack the Igβ cytoplasmic tail showed the same hyper-react-
ve 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
phosphatase 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 IgαFF/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 sur-
face 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. Fur-
thermore, phosphorylation of ITAM tyrosines is critical for
regulating surface BCR levels because Igβ→α/IgαFF/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.

Igβ →α, 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 subimmunogenic 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 observed in anti-double– (77) and anti-single–stranded DNA B cells stimulated with subthreshold BCR signaling. We speculate that Igα may play an important role in this process.

In addition Igβ →α, 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 Igβ →α, 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 Igβ →α/Igα^{FF/FF} B cells in which one of the two BCR ITAMs is inactivated. The major difference between Igβ →α, 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.

We thank Dr. Randolph Noelle for the CD8-gp39 fusion protein derived from insect cells. We thank the Nussenzweig lab for helpful discussions and Eva Besmar for her critical review of the manuscript. We appreciate the expertise of F. Isdell and M. Genova for flow cytometry.

A. Reichlin was supported by a National Institutes of Health grant RO3 and a March of Dimes Investigator award. M.C. Nussenzweig was supported by grants from the National Institutes of Health and is an Investigator at the Howard Hughes Medical Institute.

Submitted: 10 July 2003
Accepted: 2 February 2004

References

1. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. Nature. 381:751–758.
2. Meffre, E., R. Casellas, and M.C. Nussenzweig. 2000. Antibody regulation in B cell development. Nat. Immunol. 1:379–385.
3. 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.
4. 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 J. 7:3457–3464.
5. 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.
6. Sanchez, M., Z. Misulovin, A.L. Burkhardt, S. Mahajan, T. Costa, R. Franke, J.B. Bolen, and M.C. Nussenzweig. 1993. Signal transduction by immunoglobulin is mediated through Igα and Igβ. J. Exp. Med. 178:1049–1056.
7. Williams, G.T., C.J.G. Peaker, K.J. Patel, and M.S. Neuberger. 1994. The alpha/beta 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. 91:474–478.
8. Reth, M. 1989. Antigen receptor tail clue. Nature. 338:383–384.
9. Kim, K.M., G. Alber, P. Weiser, and M. Reth. 1993. Differential signaling through the Ig-α and Ig-β components of the B cell antigen receptor. Eur. J. Immunol. 23:911–916.
10. Flawsinkel, H., and M. Reth. 1994. Dual role of the tyrosine activation motif of the Ig-α protein during signal transduction via the B cell antigen receptor. EMBO J. 13:83–89.
11. Pao, L.I., S. Fagmiglietti, and J.C. Cambier. 1998. Asymmetric phosphorylation and function of the immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. J. Immunol. 160:3305–3314.
12. Rowley, R., A.L. Burkhardt, H.G. Chao, G.R. Matsueda, and J.B. Bolen. 1995. Syk protein tyrosine kinase is regulated by tyrosine phosphorylated Igα/β and Igα immunoreceptor tyrosine activation motif and autophosphorylation. J. Biol. Chem. 270:11590–11594.
13. Kurosaki, T., S.A. Johnson, L. Pai, K. Sada, H. Yamamura, and J.C. Cambier. 1995. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. J. Exp. Med. 182:1815–1823.
14. Delfranko, A. 1997. The complexity of signaling pathways activated by the BCR. Curr. Opin. Immunol. 9:296–308.
15. Cornall, R.J., A.M. Cheng, T. Pawson, and C.C. Goodnow. 2000. Role of Syk in B-cell development and antigen-receptor signaling. Proc. Natl. Acad. Sci. USA. 97:1713–1718.
16. Clark, M.R., K.S. Campbell, A. Kazlauskas, S.A. Johnson, M. Hertz, T.A. Potter, C. Pleiman, and J.C. Cambier. 1992. The B cell antigen receptor complex: association of Ig-α and Ig-β with distinct cytoplasmic effectors. Science. 258:125–126.
17. Clark, M., S.A. Johnson, and J.C. Cambier. 1994. Analysis of Igα-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-α-stimulation of Fyn activity. EMBO J. 13:1911–1919.
18. Pawson, T.and J.D. Scott. 1997. Signaling through scaffold, anchoring, and adaptor proteins. Science 278:2075-2080.
19. Suethe, R., and R.H. Scheuermann. 2000. Tyrosine kinase activation in the decision between growth, differentiation, and death responses initiated from the B cell antigen receptor. Adv. Immunol. 75:283–316.
20. Fu, C., C.W. Turck, T. Kurosaki, and A.C. Chan. 1998.
BLNK: a central linker protein in B cell activation. *Immunity.* 9:93–103.

21. Wienands, J., J. Schweikert, B. Wollscheid, H. Jumaa, P.J. Nielsen, and M. Reth. 1998. SLP-65 a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J. Exp. Med.* 188:791–795.

22. Saijo, K., C. Schmedt, I.-H. Su, H. Karasuyama, C.A. Lowell, M. Reth, T. Adachi, A. Patke, A. Santana, and A. Tarakhovsky. 2003. Essential role of Src-family protein tyrosine kinases in NF-kB activation during B cell development. *Nat. Immunol.* 4:274–279.

23. Turner, M., P.J. Mee, P.S. Costello, O. Williams, A.A. Price, L.P. Duddy, M.T. Furlong, R.L. Gehlen, and V.L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature.* 378:298–302.

24. Schweighoffer, E., L. Vanes, A. Mathiout, T. Nakamura, and V.L.J. Tybulewicz. 2003. Unexpected requirement for ZAP-70 in pre-B cell development and allelic exclusion. *Immunity.* 18:523–533.

25. Hashimoto, S., A. Iwamatsu, M. Ishiai, T. Okawa, T. Yamadori, M. Matsushita, Y. Baba, T. Kishimoto, T. Kurosaki, and S. Tsukada. 1999. Identification of the SH2 binding protein of Bruton's tyrosine kinase as BLNK-functional significance of btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood.* 94:2357–2364.

26. Engels, N., B. Wollscheid, and J. Wienands. 2001. Association of SLP-65/BLNK with the B cell antigen receptor through a non-ITAM tyrosine of Ig-alpha. *Eur. J. Immunol.* 31:2126–2134.

27. Ishiai, M., M. Kurosaki, R. Pappu, K. Okawa, I. Ronko, C. Fu, M. Shihabi, A. Iwamatsu, A.C. Chan, and T. Kurosaki. 1999. BLNK required for coupling Syk to PLCgamma2 and Rac1-JNK in B cells. *Immunity.* 10:117–125.

28. Taddie, J., T.R. Hurley, B.S. Hardwick, and B.M. Seffon. 1994. Activation of B- and T-cells by the cytoplasmic domains of the B-cell antigen receptor proteins Ig-alpha and Ig-beta. *J. Biol. Chem.* 269:13529–13535.

29. Luisier, 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.

30. 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.

31. Papavasiliou, F., Z. Misulovin, H. Suh, and M.C. Nussenzweig. 1995. The role of IgB in precursor B cell transition and allelic exclusion. *Science.* 268:408–411.

32. Papavasiliou, F., M. Jankovic, H. Suh, and M.C. Nussenzweig. 1995. The cytoplasmic domains of Igα and Igβ can independently induce the pre-B cell transition and allelic exclusion. *J. Exp. Med.* 182:1389–1394.

33. Teh, Y., and M.S. Neuberger. 1997. The immunoglobulin Igα and Igβ cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice. *J. Exp. Med.* 185:1753–1768.

34. 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.* 272:1804–1808.

35. Reichlin, A., Y. Hu, E. Meffire, H. Nagaoka, S. Gong, M. Kraus, K. Rajewsky, and M.C. Nussenzweig. 2001. B cell development is arrested at the immature B cell stage in mice carrying a mutation in the cytoplasmic domain of immunoglobulin B. *J. Exp. Med.* 193:13–23.

36. Torres, R.M., and K. Hafen. 1999. A negative regulatory role for Ig-alpha during B cell development. *Immunity.* 11:527–536.

37. Yagi, Y., Y. Ikawa, K. Yoshida, Y. Shigetani, N. Takeda, I. Morimura, K. Uetsuka, K. Doi, S. Tsuji, and D. Kitamura. 1999. Identification of the SH2 binding protein of Bruton's tyrosine kinase as BLNK-functional significance of btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood.* 94:2357–2364.

38. Foy, T.M., F.H. Durie, and R.J. Noelle. 1994. The exapansive role of CD40 and its ligand, gp39, in immunity. *Semin. Immunol.* 6:259–266.

39. Schreiber, A., T. Costa, Z. Misulovin, B. Stealy, J.B. Bolen, and M.C. Nussenzweig. 1994. Ig alpha and Ig beta are functionally homologous to the signaling proteins of the T cell receptor. *Mol. Cell. Biol.* 14:1095–1103.

40. Kraus, M., L. Pao, A. Rechlin, Y. Hu, B. Canono, J.C. Cambier, M.C. Nussenzweig, and K. Rajewsky. 2001. Interference with immunoglobulin (Ig) alpha immunoreceptor tyrosine activation motif (ITAM) phosphorylation modulates or blocks B cell development, depending on the availability of an Ig beta cytoplasmic tail. *J. Exp. Med.* 194:455–469.

41. Hao, Z., and K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J. Exp. Med.* 194:1151–1164.

42. 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.

43. Love, P., E.W. Shores, M.D. Johnson, M.E. Tremblay, J. Lee, A. Grinberg, S.P. Huang, A. Singer, and H. Westphal. 1993. T cell development in mice that lack the z chain of the T cell receptor complex. *Science.* 261:918–921.

44. Shores, E.W., M. Ono, T. Kawabe, C.L. Sommers, T. Tran, K. Lui, M.C. Udey, J. Ravetch, and P.E. Love. 1998. T cell development in mice lacking all t cell receptor z family members (z,n, and FceRIg). *J. Exp. Med.* 187:1093–1101.

45. Shores, E.W., T. Tran, A. Grinberg, C.L. Sommers, H. Shen, and P.E. Love. 1997. Role of the multipurpose T cell receptor (TCR)-z chain signaling motifs in selection of the t cell repertoire. *J. Exp. Med.* 185:893–900.

46. Siemasko, K., B.J. Eisfelder, C. Stebbins, S. Kabak, A.J. Sant, W. Song, and M.R. Clark. 1999. Ig alpha and Ig beta are required for efficient trafficking to late endosomes and to enhance antigen presentation. *J. Immunol.* 162:6518–6525.

47. Siemasko, K., B.J. Skaggs, S. Kabak, E. Williamson, B.K. Brown, W. Song, and M.R. Clark. 2002. Receptor-facilitated antigen presentation requires the recruitment of B cell linker protein to Igα. *J. Immunol.* 168:2127–2138.

48. Goitsuka, R., Y. Fujimura, H. Mamada, A. Umeda, T. Morimura, K. Uetsuka, K. Doi, S. Tsuji, and D. Kitamura. 1998. BASH, a novel signaling molecule preferentially expressed in B cells of the bursa of Fabricius. *J. Immunol.* 161:5804–5808.

49. Tan, J., S.C. Wong, S.K. Gan, S. Xu, and K.P. Lam. 2001. The adaptor protein BLNK is required for B cell antigen receptor-induced activation of nuclear factor-κB and cell cycle entry and survival of B lymphocytes. *J. Biol. Chem.* 276:20055–20063.
51. Tsuji, S., M. Okamoto, K. Yamada, N. Okamoto, R. Goitsuka, R. Arnold, F. Kefer, and D. Kitamura. 2001. B cell adaptor containing src homology 2 domain (BASH) links B cell receptor signaling to the activation of hematopoietic progenitor kinase 1. *J. Exp. Med.* 194:529–539.

52. Hayashi, K., R. Nittono, N. Okamoto, S. Tsuji, Y. Haru, R. Goitsuka, and D. Kitamura. 2000. The B cell-restricted adaptor BASH is required for normal development and antigen receptor-mediated activation of B cells. *Proc. Natl. Acad. Sci. USA.* 97:2755–2760.

53. Jumaa, H., B. Wollscheid, M. Mitterer, J. Wienands, M. Reth, and P.J. Niesl. 1999. Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity.* 11:547–554.

54. Flemming, A., T. Brummer, M. Reth, and H. Jumaa. 2003. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. *Nat. Immunol.* 4:38–43.

55. Xu, S., J.E. Tan, E.P. Wong, A. Manickam, S. Ponniah, and A. Flemming. 2003. Cariappa, A., H.C. Liou, B.H. Horwitz, and S. Pillai. 2000. Nuclear factor kappa B is required for the development of marginal zone B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity.* 11:547–554.

56. Martin, F., and J.F. Kearney. 2000. Positive selection from newly formed to marginal zone B Cells depends on the rate of clonal production, CD19, and Btk. *Immunity.* 12:39–49.

57. Gunamard, R., M. Okigaki, J. Schlessinger, and J.V. Cariappa, A., M. Tang, C. Parng, E. Nebelitskiy, M. Carroll, K. Georgopoulos, and S. Pillai. 2001. The follicular versus marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol.* 1:31–36.

58. Carriapa, A., H.C. Liou, B.H. Horwitz, and S. Pillai. 2000. Nuclear factor kappa B is required for the development of marginal zone B lymphocytes. *J. Exp. Med.* 192:1175–1182.

59. Carriapa, A., M. Tang, C. Parng, E. Nebelitskiy, M. Carroll, K. Georgopoulos, and S. Pillai. 2001. The follicular versus marginal zone B lymphocyte fate decision is regulated by Aiolos, Btk, and CD21. *Immunity.* 14:603–615.

60. Fukui, Y., O. Hashimoto, T. Sanui, T. Oono, H. Koga, M. Abe, A. Inayoshi, M. Noda, M. Otke, T. Shirai, and T. Sazuki. 2001. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. *Nature.* 412:826–831.

61. Girkontaite, I., K. Missy, V. Sakk, A. Harenberg, K. Tedford, T. Potzel, K. Pfeffer, and K.D. Fischer. 2001. Lsc is required for marginal zone B cells, regulation of lymphocyte motility and immune responses. *Nat. Immunol.* 2:855–862.

62. Fagarasan, S., N. Watanabe, and T. Honjo. 2000. Generation, expansion, migration and activation of mouse B1 cells. *Immunity.* Rev. 176:205–215.

63. Lam, K.P., and K. Rajewsky. 1999. B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. *J. Exp. Med.* 190:471–477.

64. Hayakawa, K., M. Asano, S.A. Shinton, M. Gui, D. Allman, C.L. Stewart, J. Silver, and R.R. Hardy. 1999. Positive selection of natural autoreactive B cells. *Science.* 285:113–116.

65. Hombach, J., T. Tsubata, L. Leclercq, H. Stappert, and M. Reth. 1990. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature.* 343:760–762.

66. Siemasko, K., B.J. Eifelder, E. Williamson, S. Kabak, and M.R. Clark. 1998. Signals from the B lymphocyte antigen receptor regulate MHC class II containing late endosomes. *J. Immunol.* 160:5203–5208.

67. Jing, S., T. Spencer, K. Miller, C. Hopkins, and I.S. Trowbridge. 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. *J. Cell Biol.* 110:283–294.

68. Chen, W., J.L. Goldstein, and M.S. Brown. 1990. NPXY a sequence often found in cytoplasmic tails is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265:3116–3123.

69. Cassard, S., J. Salamero, D. Hanau, D. Spehner, J. Davoust, W.H. Fridman, and C. Bonnerot. 1998. A tyrosine-based signal present in Ig alpha mediates B cell receptor constitutive internalization. *J. Immunol.* 160:1767–1773.

70. Patel, K., and M.S. Neuberger. 1993. Antigen presentation by the B cell antigen receptor is driven by the a/b sheath and occurs independently of its cytoplasmic tyrosines. *Cell.* 74:939–946.

71. Kraus, M., K. Sajo, R.M. Torres, and K. Rajewsky. 1999. Ig-alpha cytoplasmic truncation renders immature B cells more sensitive to antigen contact. *Immunity.* 11:537–545.

72. Macián, F., F. García-Cózar, S.H. Im, H.F. Horton, M.C. Byrne, and A. Rao. 2002. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell.* 109:719–731.

73. Dolmetsch, R., R.S. Lewis, C.C. Goodnow, and J.I. Healy. 1997. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature.* 386:855–858.

74. Pike, B., A.W. Boyd, and G.J. Nossal. 1982. Clonal anergy: the universally anergic B lymphocyte. *Proc. Natl. Acad. Sci. USA.* 79:2013–2017.

75. Rudich, S., K.H. Roux, R.J. Winchester, and P.K. Mongini. 1988. Anti-IgM-mediated B cell signaling. Molecular analysis of ligand binding requisites for human B cell clonal expansion and tolerance. *J. Exp. Med.* 168:247–266.

76. Goodnow, C., J. Crosbie, H. Jorgensen, R.A. Brink, and A. Basten. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature.* 342:340–341.

77. Mandik-Nayak, L., S.J. Sea, A. Eaton-Bassiri, D. Allman, R.R. Hardy, and J. Erikson. 2000. Functional consequences of the developmental arrest and follicular exclusion of anti-double-stranded DNA B cells. *J. Immunol.* 164:1161–1168.

78. Noorchashm, H., A. Bui, L. Hsiu-Ling, A. Eaton, L. Mandik-Nayak, C. Sokol, K.M. Potts, E. Pure, and J. Erikson. 1999. Characterization of anergic anti-DNA B cells: B cell anergy is a T cell-independent and potentially reversible process. *Int. Immunol.* 11:765–776.

79. Healy, J., R.E. Dolmetsch, L.A. Timmerman, J.G. Cyster, M.L. Thomas, G.R. Crabtree, R.S. Lewis, and C.C. Goodnow. 1997. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity.* 6:419–428.

80. Weintraub, B., J.E. Jun, A.C. Bishop, K.M. Shokat, M.L. Thomas, and C.C. Goodnow. 2000. Entry of B cell receptor into signaling domains is inhibited in tolerant B cells. *J. Exp. Med.* 191:1443–1448.

81. Mecklenbrauker, I., K. Sajo, N.Y. Zheng, M. Leitges, and K.I. Nakayama. 2002. Increased proliferation of B cells and auto-immunity in mice lacking protein kinase C. *Nature.* 416:860–864.

82. Miyamoto, A., K. Nakayama, I. Hiroyuki, S. Hirose, Y. Jiang, A. Masaaki, T. Tadasuke, H. Nagahama, S. Ohno, H. Hatakeyama, and K.I. Nakayama. 2002. Increased proliferation of B cells and auto-immunity in mice lacking protein Kinase C. *Nature.* 416:865–869.

83. Fulcher, D., and A. Basten. 1994. Reduced lifespan of anergic self reactive B cells in a double-transgenic model. *J. Exp. Med.* 179:125–134.