

# Interference with Immunoglobulin (Ig) $\alpha$ Immunoreceptor Tyrosine-based Activation Motif (ITAM) Phosphorylation Modulates or Blocks B Cell Development, Depending on the Availability of an Ig $\beta$ Cytoplasmic Tail

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## Abstract

To determine the function of immunoglobulin (Ig) $\alpha$  immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation, we generated mice in which Ig $\alpha$  ITAM tyrosines were replaced by phenylalanines (Ig $\alpha^{FF/FF}$ ). Ig $\alpha^{FF/FF}$  mice had a specific reduction of B1 and marginal zone B cells, whereas B2 cell development appeared to be normal, except that  $\lambda$ 1 light chain usage was increased. The mutants responded less efficiently to T cell-dependent antigens, whereas T cell-independent responses were unaffected. Upon B cell receptor ligation, the cells exhibited heightened calcium flux, weaker Lyn and Syk tyrosine phosphorylation, and phosphorylation of Ig $\alpha$  non-ITAM tyrosines. Strikingly, when the Ig $\alpha$  ITAM mutation was combined with a truncation of Ig $\beta$ , B cell development was completely blocked at the pro-B cell stage, indicating a crucial role of ITAM phosphorylation in B cell development.

Key words: mb-1 protein • B cell antigen receptor • ITAM • B cell subsets • gene targeting

## Introduction

B cell development is a highly ordered and controlled process allowing the formation of a diverse repertoire of B cell receptor (BCR)\* antigen specificities. Dependent on the developmental status and the microenvironment of the B cell, the strength and quality of BCR transmitted signals control survival, differentiation, and proliferation (1, 2).

The BCR is a multimeric protein complex consisting of an antigen binding module that is noncovalently associated with one signal-transducing heterodimer of Ig $\alpha$  (CD79a, mb-1) and Ig $\beta$  (CD79b, B29) (3). An early and essential

feature of the signaling cascade is phosphorylation of the tyrosine residues within immunoreceptor tyrosine-based activation motif (ITAM) by Src family protein tyrosine kinases (PTKs; references 4, 5, and 6). The phosphorylated ITAMs are thought to serve as docking sites for Src homology 2 (SH2) domain-containing proteins. For instance, dual phosphorylation of the BCR ITAMs allows high affinity association of the tandem SH2 domain-containing kinase Syk. Binding of Syk to ITAM leads to its phosphorylation and activation which in turn leads to phosphorylation of downstream cellular substrates. Ig $\alpha$  and Ig $\beta$  both contain single ITAMs that potentially confer distinct signaling functions. In support of this idea, several proteins have been shown to associate differentially with either Ig $\alpha$  or Ig $\beta$  (7, 8). In transfection experiments using chimeric molecules, isolated cytoplasmic domains of either Ig $\alpha$  or Ig $\beta$  were both capable of mediating the mobilization of calcium and tyrosine phosphorylation of cellular molecules. These signaling capacities were critically dependent on ITAM phosphorylation. Further data suggest that Ig $\alpha$  is predominant in activating PTKs (9–16).

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\*Abbreviations used in this paper: BCR, B cell receptor; BM, bone marrow; ES, embryonic stem; ITAM, immunoreceptor tyrosine-based activation motif; MZ, marginal zone; PTK, protein tyrosine kinase; RT, room temperature; SH2, Src homology 2; TD, T cell-dependent; TI, T cell-independent.

Aspects of Ig $\alpha$  and Ig $\beta$  function in B cell development were studied in transgenic and gene-targeted mice. In Ig $\beta$  knockout mice, the lack of Ig $\alpha$ /Ig $\beta$  heterodimer formation presumably also abrogates the surface expression of Ig $\alpha$  and results in a complete block of development at the pro-B cell stage (17). However, the expression of chimeric molecules with cytoplasmic domains of either Ig $\alpha$  or Ig $\beta$  was capable of supporting further steps in B cell maturation, including H chain allelic exclusion (18–20). Critical roles of the cytoplasmic tails of Ig $\alpha$  and Ig $\beta$  in B cell development were revealed in *mb-1<sup>Δc/Δc</sup>* and *Ig $\beta$ <sup>Δc/Δc</sup>* mice (21, 22). In *Ig $\beta$ <sup>Δc/Δc</sup>* mice, the cytoplasmic domain of Ig $\beta$  is lacking, whereas in *mb-1<sup>Δc/Δc</sup>* mice (hereafter termed *Ig $\alpha$ <sup>Δc/Δc</sup>*) a truncated Ig $\alpha$  protein with a cytoplasmic domain consisting of 20 instead of 61 amino acids is expressed. In both mutants, B cell development is severely impaired resulting in a drastic reduction of peripheral B lymphocyte numbers. Functionally, the remaining B cells in the mutants are unable of mounting a measurable immune response against T cell-independent (TI) antigen. Further experiments with *Ig $\alpha$ <sup>Δc/Δc</sup>* mice revealed that the Ig $\alpha$  cytoplasmic truncation causes enhanced negative selection of autoreactive receptor specificities and generally renders immature B cells more sensitive to antigenic contact. Based on these data, an additional negative signaling function for Ig $\alpha$  was suggested (23, 24).

To investigate the specific function of Ig $\alpha$  ITAM phosphorylation in B cell development, we generated a mutant mouse strain by gene targeting in which the Ig $\alpha$  ITAM tyrosines were replaced with phenylalanines (*Ig $\alpha$ <sup>FF/FF</sup>* mice).

## Materials and Methods

**Generation of Targeting Vector and Targeted Embryonic Stem Cell Clones.** The *mb-1* targeting vector was designed as a replacement vector (25). A loxP site was inserted into the Tth1111 site of intronIII. Intron region and exons downstream of this loxP site are exactly repeated 3' to a floxed *neo<sup>r</sup>* gene (see Fig. 1 A). Tyrosine to phenylalanine replacements within the ITAM were achieved by site-directed mutagenesis (Tyr<sub>182</sub>: TAT to TTC; Tyr<sub>193</sub>: TAT to TTT). An additional BamHI restriction site within the 3' UTR, downstream of the mutated exons, was generated by an exchange of two nucleotides.

A 5.2-kb genomic BamHI fragment, containing the *mb-1* gene, was recovered from a murine cosmid (C57BL/6J-pWE15 library; Stratagene; reference 26) and subcloned into the BamHI site of pGEM-7Z+ (Promega) to generate pGEM5.2kbBamHI. Subsequently, a 1.4-kb Tth1111-XhoI fragment was recovered from this vector and subcloned into the EcoRI/XhoI sites of pGEMloxP (unpublished data) to obtain pGEMloxPTth1111-XhoI. Next, the Tth1111-PstI fragment of pGEM5.2kbBamHI was replaced with the NotI-PstI fragment from pGEMloxPTth1111-XhoI to obtain pGEM5.2kbloxP. The 5.2-kb BamHI fragment was then cloned into the BamHI site of pSL1190XX to obtain pSL5.2kbloxP. pSL1190XX was generated from pSL1190 (Amersham Pharmacia Biotech) by deleting the polylinker-region between XhoI and XbaI.

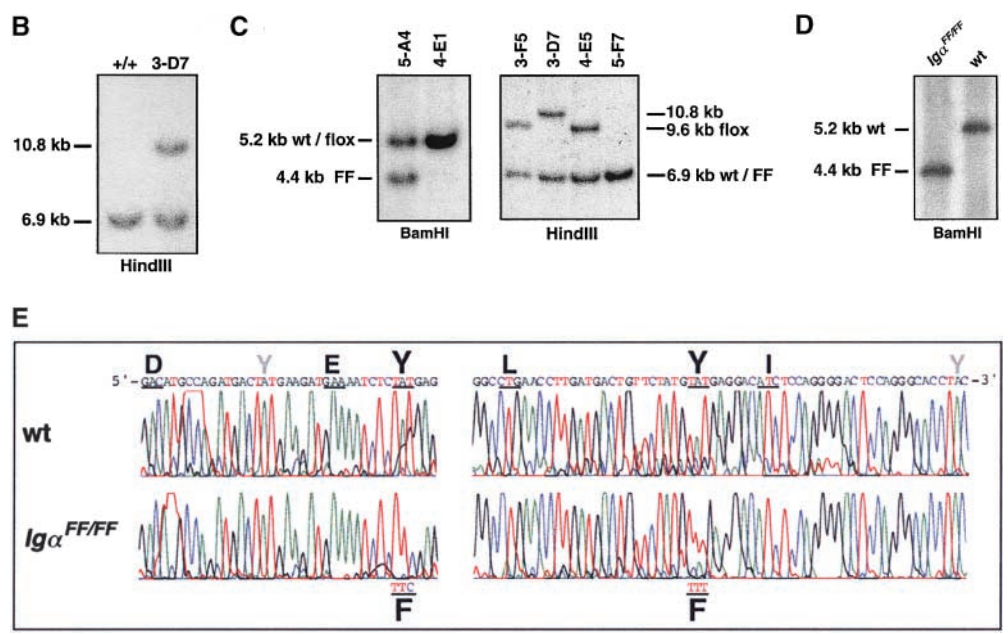
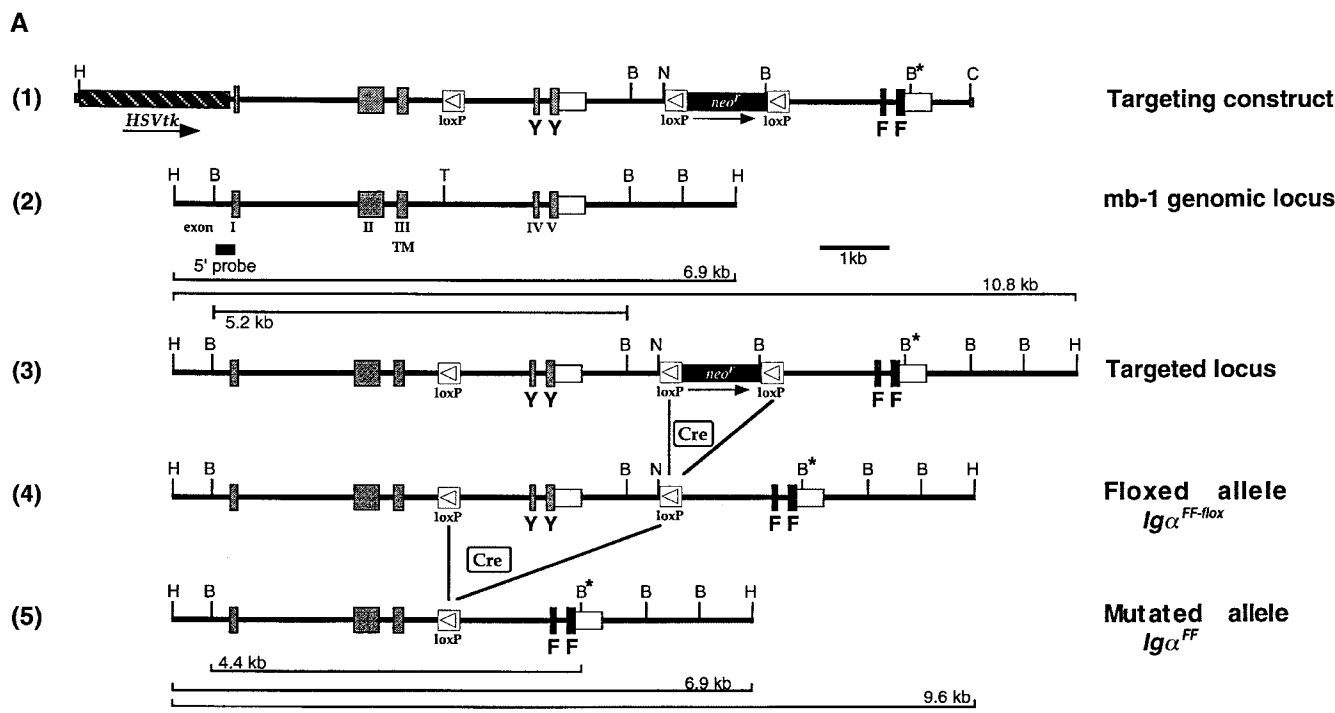
The region downstream of the *mb-1* polyA site was extended 0.4 kb by the following procedure. A 1.2-kb fragment was first

amplified by PCR from the genomic cosmid clone with the primers 3' region-FOR (5'-CTGCCCTCCCCACTCTTCC-3') and 3' region-REV (5'-CCATCGATCCTGCCGCGGCCGCTTACTCT-3'). This PCR product was subcloned into the pCR™ TA Cloning vector II (Invitrogen), and a 1.2-kb EcoRI-XhoI fragment was recovered. This fragment was used to replace the 0.8-kb SmaI-XhoI fragment in pSL5.2kbloxP to obtain pSL5.2kbloxP-3'. Next, pSL5.2kbloxP-3' was cut with EcoRV-XbaI and religated resulting in pSL5.2kbloxP-3'-ΔClaI. Subsequently, the *tk* selection marker was excised as a HindIII fragment from pIC-TKΔCla1, a modified version of pIC19R/MC1-TK (26). This fragment was cloned into the HindIII site of pSL5.2kbloxP-3'-ΔClaI to obtain pSL5.2kbloxP-3'-ΔClaI-TK. For the construction of a loxP-flanked *neo<sup>r</sup>* cassette, a Sall-XhoI fragment was recovered from pMC1neoPolyA (Stratagene) and cloned into the XhoI site of pGEMloxP to obtain pGEM-neo-loxP. From this vector, an XbaI-HindIII fragment was subcloned into NotI-HindIII sites of pGEMloxP, resulting in pMM-neoflox-8. A 1.2-kb XbaI-NsiI fragment from pMMneoflox-8 was cloned into Tth1111-NsiI-digested pGmb1C1C2\*\* (21) to obtain pmb-1C1C2\*\*neoflox. Additional restriction sites were introduced by site-directed mutagenesis. The product of two consecutive PCR reactions (primer: BamHI-FOR 5'-ATGCTGGATCCAGCATCCC-3'; BamHI-REV 5'-GGGATGCTGGATCCAGACAT-3'; FLAG-C1C2\*\*-FOR 5'-AACGGTGCAAAATGAGAAG-3'; FLAG-C1C2\*\*-REV 5'-TTGGGCCCCGATCGATCGCATG-3') was subcloned into the pCR™ TA Cloning vector II (Invitrogen) and retrieved by digestion with Sall and ApaI. The fragment was used to exchange the corresponding 1-kb fragment in pmb-1C1C2\*\*neoflox to generate pmb-1C1C2\*\*neoflox-BamHI.

Culturing and transfection of E14.1 embryonic stem (ES) cells (129/Ola) was performed according to previously published protocols (27). Screening of 596 G418/gancyclovir double resistant colonies by Southern blot analysis led to the identification of three homologous recombinant ES cell clones (Fig. 1).

**Mice.** ES cells carrying the *Ig $\alpha$ <sup>FF-Flox</sup>* allele were injected into C57BL/6 and CB20 blastocysts which were then transferred into foster mothers to obtain chimeric mice. Mice with the *Ig $\alpha$ <sup>FF-Flox</sup>* allele were crossed to the *deleter*-strain (28). Some analyses were performed with *Ig $\alpha$ <sup>FF/FF</sup>* mice of a mixed 129/Ola-C57BL/6 genetic background. To control for a potential influence of the genetic background (e.g., in immunization studies), control groups of 129/Ola and C57BL/6 mice were included and analyzed in parallel with the *Ig $\alpha$ <sup>FF/FF</sup>* mice. In anti-phosphotyrosine immunoblotting experiments, all *Ig $\alpha$ <sup>FF/FF</sup>* mice used had been backcrossed for eight generations to C57BL/6. *Ig $\beta$ <sup>Δc/Δc</sup>* mice (22) are of mixed 129/Sv-C57BL/6 genetic background. *Ig $\alpha$ <sup>Δc/Δc</sup>* mice (21) are of C57BL/6 genetic background. *Ig $\alpha$ <sup>FF/FF</sup>Ig $\beta$ <sup>Δc/Δc</sup>* mice were generated by intercrossing *Ig $\alpha$ <sup>FF/FF</sup>* mice to *Ig $\beta$ <sup>Δc/Δc</sup>* mice (22). All mice, including *Ig $\alpha$ <sup>Δc/Δc</sup>* (21) and *J<sub>H</sub>T/J<sub>H</sub>T* mice (29), were housed in our conventional animal facility.

**Preparation of Cell Suspensions from Lymphoid Organs.** Spleens were minced through a nylon mesh (cell strainer; Falcon) to obtain single cell suspensions in DMEM, 5% FCS, and 2 mM L-glutamine. Bones were flushed with medium to extract bone marrow (BM) cells and the peritoneal cavity was flushed twice with 10 ml of medium to recover cells. Erythrocytes were lysed from spleen and BM preparations by incubating in lysis buffer (140 mM NH<sub>4</sub>Cl, 17 mM Tris-HCl, pH 7.65) for 2 min on ice. To obtain PBLs, mice were bled from the tail vein in the presence of heparin (Liquemin; Roche) and purified by gradient centrifugation with 7% Ficoll 400 (Amersham Pharmacia Biotech).



**Figure 1.** Generation of targeted  $Ig\alpha^{FF/flox/+}$  ES cell clones and  $Ig\alpha^{FF/FF}$  mice. (A) Targeted insertion of a loxP-flanked *neo<sup>R</sup>* gene, mutated cytoplasmic exons and a third loxP site into the *mb-1* locus, followed by the specific deletion of the *neo<sup>R</sup>* selection marker. The configurations of the targeting vector (1), the wild-type *mb-1* locus (2), and the targeted locus (3) after homologous recombination are shown. Cre-mediated deletion of the *neo<sup>R</sup>* selection marker results in the  $Ig\alpha^{FF/flox}$  allele (4). In the mouse, Cre-expression mediates the excision of the wild-type cytoplasmic exons to generate the mutated  $Ig\alpha^{FF}$  allele (5). Wild-type exons are displayed as gray boxes, whereas black boxes represent the mutated exons. The tyrosine encoding triplets in the ITAM are indicated as YY, whereas the mutated ITAM is highlighted by FF. The white box shows the 3'

UTR, and boxes with a triangle represent loxP sites. Arrows underneath the targeting construct indicate the direction of transcription in the selection marker genes. The map is drawn to scale except for the loxP sites and displays the following restriction sites: B, BamHI; C, ClaI; H, HindIII; N, NotI; T, Tth111I. An asterisk marks the additional BamHI restriction site in the 3' UTR of the mutated cytoplasmic exons. The external probe used to verify the targeting event is indicated together with the expected sizes of the restriction fragments. (B) Southern blot analysis of ES cell clones after transfection with the targeting construct. Genomic DNA was digested with HindIII and hybridized with the 5' external probe. The targeted ES cell clone 3-D7, with the characteristic band of 10.8 kb, is shown in comparison to a clone that possesses only the wild-type *mb-1* locus (+/+). (C) Southern blot analysis of targeted, G418 sensitive ES cell clones after transient Cre expression. Genomic DNA was digested with either HindIII or BamHI and hybridized with the 5' external probe. ES cell clones with specific *neo<sup>R</sup>* deletion (3-F5, 4-E5) show a characteristic band of 9.6 kb after HindIII digestion. Complete deletion of all loxP flanked sequences is characterized by the presence of a 4.4-kb fragment after BamHI digestion (5-A4). For comparison, the parental G418 resistant clone 3-D7 is included in the HindIII based analysis. (D) Southern blot analysis with the 5' external probe of BamHI digested genomic DNA from  $Ig\alpha^{FF/FF}$  and wild-type (wt) mice. (E) Sequence analysis of the  $Ig\alpha$  ITAM in  $Ig\alpha^{FF/FF}$  mice. Genomic DNA was extracted from purified splenic B cells of  $Ig\alpha^{FF/FF}$  and wild-type mice. Triplets encoding for the amino acids of the consensus ITAM sequence are underlined and the respective amino acid is indicated. Intronic sequence is not shown (gap). The positions of the two non-ITAM  $Ig\alpha$  tyrosines are indicated by a gray Y.

**Flow Cytometry.** Cells were surface stained with combinations of FITC, PE, Cy-Chrome (Cyc), peridinin chlorophyll protein (PERCP), and/or allophycocyanin (APC)-conjugated monoclonal antibodies for 20 min on ice. Stainings with biotinylated monoclonal antibodies were followed by a secondary staining with either Streptavidin-Cy-Chrome (BD PharMingen) or Streptavidin-PERCP (Becton Dickinson). After staining, the samples were washed and resuspended with PBS, 1% BSA, and 0.01%  $N_3$ . Stained cells were acquired on a FACScan™ or FACSCalibur™ and data were analyzed using CELLQuest™ software (Becton Dickinson). Dead cells were labeled with propidium iodide or Topro-3 (Molecular Probes) and excluded from the analysis.

For intracellular Ig $\mu$  chain staining, cells were first washed in PBS then fixed in a 2% formaldehyde solution for 30 min at room temperature (RT). After three washes with PBS, the cells were stained for 20 min at RT with antibody M41 in 0.05% Saponin in PBS. Monoclonal antibodies R33–24.12 (anti-IgM), 1.3–5 (anti-IgD), RA3–6B2 (anti-B220), M41 (anti- $\mu$  chain), LS.136 (anti- $\lambda$ 1), Cy34.1 (anti-CD22), M5/114 (anti-MHC class II), BP-1 (anti-BP-1), 30F1 (anti-HSA), and Cfo1 (anti-Thy1.2) were prepared and conjugated in our laboratory. Monoclonal antibodies to CD5, CD19, CD21/CD35, CD23, CD25, CD43, CD69, and CD86 were purchased from BD PharMingen. Anti-493 antibody was a gift from A. Rolink (Basel Institute of Immunology, Basel, Switzerland).

**Immunization and Serum Analysis.** Mice were immunized with 5 or 50  $\mu$ g of T cell-dependent (TD) antigen NP-CG (4-hydroxy-3-nitrophenylacetyl chicken- $\gamma$ -globulin) in alum intraperitoneally. Mice were boosted with 5  $\mu$ g/200  $\mu$ l of NP<sub>17</sub>-CG without adjuvant. TI immune response was analyzed by intraperitoneal injections of 5  $\mu$ g/200  $\mu$ l of NP<sub>27</sub>-Ficoll. Mice were bled before and after immunization from tail veins. Ig serum concentrations were determined by ELISA as described previously (30).

Affinities of IgG1 and  $\lambda$  NP-specific antibodies were determined by calculating the association constant as described previously (31). Briefly, relative binding of serum antibodies to NP<sub>5</sub>-BSA versus NP<sub>14</sub>-BSA was determined by ELISA in comparison to anti-NP antibody standards of known affinity.

**Measurement of Ca<sup>2+</sup> Mobilization.** Spleen cells ( $5 \times 10^6$ /ml) were loaded with 2  $\mu$ M Indo-1 AM in RPMI, 2% FCS for 30 min at 37°C (Molecular Probes). Afterwards, the cells were washed and stained with anti-B220-PE (RA3–6B2) monoclonal antibody in RPMI at RT. Fluorescence ratios of Indo-1 emission at 395/510 nm were measured by flow cytometry on a dual laser FACStar™ (Becton Dickinson). Data acquisition was conducted for 50 s without stimulus, followed by the addition of either F(ab)<sub>2</sub> rabbit anti-mouse IgM+G+A fragments (Zymed Laboratories) or rabbit anti-mouse IgM+G+A antibodies (Zymed Laboratories). Data was collected for 512 s and analyzed using CELLQuest™ (Becton Dickinson) and FlowJo (TriStar) software.

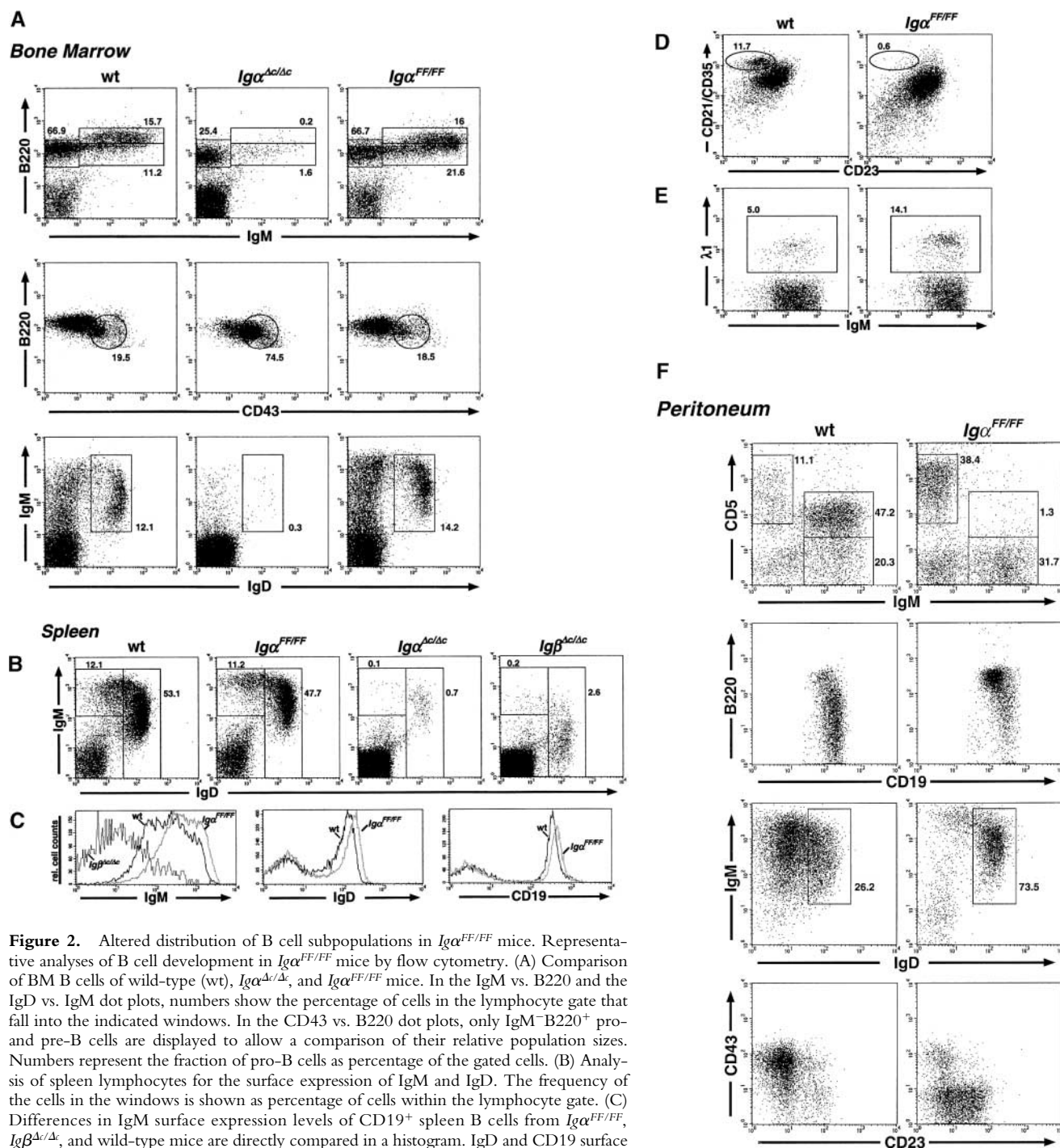
**Analysis of Protein Tyrosine Phosphorylation.** CD19<sup>+</sup> B cell populations were purified from splenic cell suspensions by magnetic beads (MACS; Miltenyi Biotec) according to the manufacturer's instructions. B lymphocytes were resuspended at  $10^7$  cells/ml in RPMI and stimulated with F(ab)<sub>2</sub> rabbit anti-mouse IgM+G+A fragments (Zymed Laboratories) for various time periods at 37°C. The reactions were stopped by addition of ice cold lysis buffer to a final concentration of 1% NP-40, 150 mM NaCl, 0.4 mM EDTA, 10 mM Tris-HCl, pH 8, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml  $\alpha$ 1-antitrypsin, 1 mM PMSF, 10 mM sodium pyrophosphate, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM NaF. After incubation on ice for 10 min, lysates were cleared at 12,000  $g$  for 5 min at 4°C. Ig $\alpha$  was immunoprecipitated from cleared lysates with

anti-Ig $\alpha$  monoclonal antibody 172 directly coupled to Sepharose beads. For the immunoprecipitation of Lyn or Syk, cleared lysates were incubated with either rabbit polyclonal anti-mouse Lyn (sc-15; Santa Cruz Biotechnology, Inc.) or rabbit polyclonal anti-mouse Syk antibodies (13), respectively; followed by addition of protein G-Sepharose beads (Amersham Pharmacia Biotech). Proteins were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (Millipore), and immunoblotted with anti-phosphotyrosine antibody Py54 (Calbiochem), followed by horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG (Amersham Pharmacia Biotech) and developed by chemiluminescence (ECL; Amersham Pharmacia Biotech). To determine the amount of protein loaded in each lane, the same membranes were stripped and reprobed with appropriate antibodies.

## Results

**Generation of Ig $\alpha^{FF/FF}$  Mice.** To allow for conditional mutation of the Ig $\alpha$  ITAM at different stages of B cell development, two *loxP* sites flanking both cytoplasmic exons were targeted into the *mb-1* locus (floxed allele; Fig. 1 A, 4). Downstream of the 3' end of *mb-1* gene, mutated cytoplasmic exons encoding phenylalanine instead of tyrosine within the ITAM were inserted. Transfection of E14.1 ES cells led to the identification of three homologous recombinant ES cell clones (Fig. 1 A, 3, and Fig. 1 B). One clone (3-D7) was shown by PCR analysis to have cointegrated the 5' *loxP* site and the ITAM mutations (data not shown). To remove the floxed *neo<sup>r</sup>* selection cassette from the targeted *mb-1* allele, the clone was transiently transfected with circular pICre plasmid (29). Resulting G418-sensitive ES cell clones were screened by Southern blot (Fig. 1 C) and PCR analysis (not shown) to distinguish clones that carry the Ig $\alpha^{FF-flox}$  floxed allele (Fig. 1 A, 4) from clones where Cre-mediated recombination led also to the loss of the unmutated cytoplasmic exons (Ig $\alpha^{FF}$  mutated allele; Fig. 1 A, 5). ES cells carrying the Ig $\alpha^{FF-flox}$  allele were injected into mouse blastocysts to generate chimeras that subsequently transmitted the floxed allele through the germline. Ig $\alpha^{FF-flox/+}$  mice were bred with the *deleter* mouse strain (28) to obtain Ig $\alpha^{FF/+}$  mice that were then bred to homozygosity. The tyrosine to phenylalanine exchanges in the Ig $\alpha$  ITAM were verified by sequencing. DNA for this analysis was prepared from FACS®-sorted splenic B cells of Ig $\alpha^{FF/FF}$  and wild-type control mice (Fig. 1 E).

**B Cell Development in Ig $\alpha^{FF/FF}$  Mice.** The requirement of Ig $\alpha$  ITAM phosphorylation for B cell development was analyzed in Ig $\alpha^{FF/FF}$  mice by flow cytometry (32). In contrast to Ig $\alpha^{\Delta c/\Delta c}$  mice, which have reduced fractions of pre-B and immature B cells, Ig $\alpha^{FF/FF}$  mice had normal numbers of pro-B (B220<sup>low</sup>CD43<sup>+</sup>IgM<sup>-</sup>), pre-B (B220<sup>low</sup>CD43<sup>-</sup>IgM<sup>-</sup>), and immature B (B220<sup>low</sup>IgM<sup>+</sup>IgD<sup>-</sup>) cells in the BM (Fig. 2 A, and Table I). Similarly, the earliest stages of B cell development within the pro-B cell compartment, fractions A to C', were not blocked in Ig $\alpha^{FF/FF}$  mice (see Fig. 5). In addition, a striking difference exists between mature B cells in Ig $\alpha^{\Delta c/\Delta c}$  and Ig $\alpha^{FF/FF}$  mutants. Whereas recirculating B



**Figure 2.** Altered distribution of B cell subpopulations in  $Ig\alpha^{FF/FF}$  mice. Representative analyses of B cell development in  $Ig\alpha^{FF/FF}$  mice by flow cytometry. (A) Comparison of BM B cells of wild-type (wt),  $Ig\alpha^{\Delta c/\Delta c}$ , and  $Ig\alpha^{FF/FF}$  mice. In the IgM vs. B220 and the IgD vs. IgM dot plots, numbers show the percentage of cells in the lymphocyte gate that fall into the indicated windows. In the CD43 vs. B220 dot plots, only IgM<sup>-</sup>B220<sup>+</sup> pro- and pre-B cells are displayed to allow a comparison of their relative population sizes. Numbers represent the fraction of pro-B cells as percentage of the gated cells. (B) Analysis of spleen lymphocytes for the surface expression of IgM and IgD. The frequency of the cells in the windows is shown as percentage of cells within the lymphocyte gate. (C) Differences in IgM surface expression levels of CD19<sup>+</sup> spleen B cells from  $Ig\alpha^{FF/FF}$ ,  $Ig\beta^{\Delta c/\Delta c}$ , and wild-type mice are directly compared in a histogram. IgD and CD19 surface expression levels of spleen lymphocytes of  $Ig\alpha^{FF/FF}$  mice are shown in comparison to wild-type controls. (D) Analysis of spleen cells to determine the fraction of MZ B lymphocytes. Shown are CD19<sup>+</sup> spleen B lymphocytes; numbers represent CD21<sup>high</sup>CD23<sup>low</sup> cells from wild-type ( $9.9\% \pm 6.0$ ,  $n = 4$ ; average  $\pm$  SD) and  $Ig\alpha^{FF/FF}$  mice ( $2.1\% \pm 1.5$ ;  $n = 5$ ). (E) Fraction of CD19<sup>+</sup> spleen B lymphocytes expressing a  $\lambda 1$  light chain. (F) Analysis of peritoneal lymphocytes in  $Ig\alpha^{FF/FF}$  mice. In the IgM vs. CD5 dot plot, cells with the forward and side scatter characteristics of lymphocytes are shown. T cells (IgM<sup>-</sup>CD5<sup>+</sup>): wt  $12.3\% \pm 7.3$ ,  $n = 10$ ;  $Ig\alpha^{FF/FF}$   $21.7\% \pm 10.6$ ,  $n = 12$ . B cells (IgM<sup>+</sup>CD5<sup>+</sup>): wt  $35.5\% \pm 11.6$ ,  $n = 10$ ;  $Ig\alpha^{FF/FF}$   $3.7\% \pm 1.6$ ,  $n = 12$ . (IgM<sup>+</sup>CD5<sup>-</sup>): wt  $38.1\% \pm 12.5$ ,  $n = 10$ ;  $Ig\alpha^{FF/FF}$   $51.7\% \pm 21.5$ ,  $n = 12$ . Percentages given are averages  $\pm$  SD. The other dot plots present surface expression levels of CD19<sup>+</sup> peritoneal B lymphocytes from  $Ig\alpha^{FF/FF}$  and wild-type mice.

cells in the BM and splenic B cells were  $\sim 100$ -fold reduced in the former, B cell numbers in  $Ig\alpha^{FF/FF}$  mice were only marginally reduced in comparison to wild-type mice (Table I; Fig. 2, A and B). We analyzed the peripheral B

cells in  $Ig\alpha^{FF/FF}$  mice for the expression of several surface markers to determine their maturation and activation status. As in wild-type controls, splenic B cells from ITAM mutants were CD43<sup>-</sup> and  $<20\%$  of them stained brightly

**Table I.** Relative Size of B Cell Fractions in BM and Spleen

		BM (percentage of all cells)				Spleen (× 10 <sup>6</sup> )
		Pro-B (A–C)	Pre-B (D)	Immature (E)	Recirculating (F)	
wt	(n = 9)	2.4 ± 0.8	8.9 ± 3.0	5.4 ± 0.9	7.2 ± 2.4	27.7 ± 5.1
Igα <sup>FF/FF</sup>	(n = 8)	3.0 ± 1.0	7.7 ± 2.3	5.8 ± 1.5	4.3 ± 1.7	23.0 ± 4.6
Igα <sup>Δc/Δc</sup>	(n = 5)	5.0 ± 0.4	0.82 ± 0.18	0.54 ± 0.07	0.03 ± 0.01	0.21 ± 0.12
Igβ <sup>Δc/Δc</sup>	(n = 5)	4.8 ± 1.7	4.7 ± 2.5	4.6 ± 2.2	0.22 ± 0.07	0.82 ± 0.61

Sizes of BM B cell fractions A to F are indicated as percentage of all live cells. The absolute number of splenic B cells is shown. Values represent the mean ± SD of the indicated number of analyzed mice.

for immature B cell marker 493 (data not shown). Normal surface expression of activation markers CD86 (B7.2), CD69 (data not shown), and MHC class II (Table II) was observed.

Like the Igα<sup>Δc/Δc</sup> mutant (21), Igα<sup>FF/FF</sup> mice displayed allelic exclusion at the IgH locus as verified by the analysis of IgH allotype heterozygous mutant animals (unpublished data).

**Alterations of Surface Receptor Expression Levels in Igα<sup>FF/FF</sup> Mice.** Although the lack of Igα ITAM phosphorylation apparently did not block the generation of a large pool of mature B cells, it caused changes in the surface level expression of BCR and several coreceptors. Fig. 2 C shows a comparison of the surface IgM levels in splenic B cells of wild-type, Igβ<sup>Δc/Δc</sup>, and Igα<sup>FF/FF</sup> mice. In contrast to the extremely low surface IgM levels of B cells in Igβ<sup>Δc/Δc</sup> mice, the Igα ITAM mutation led to increased surface IgM levels. In general, B cells from various lymphoid organs carrying the Igα<sup>FF</sup> allele express on average more surface IgM and IgD than B cells from control mice (Fig. 2, and Table II). This applies also for B1 cells in the peritoneal cavity (Fig. 2 F, and Table II). Furthermore, we consistently observed slightly higher surface expression levels of CD19 (Fig. 2 C, and Table II) and lower surface levels of CD22 and B220 (Table II) in B cells obtained from Igα<sup>FF/FF</sup> compared with wild-type mice.

**Reduction of Marginal Zone and B1 B Cell Populations.** Are the differences in receptor expression levels sufficient to compensate for the Igα ITAM phosphorylation deficiency and to allow for the normal generation of all peripheral B cell subsets? Three different main B cell subpopulations have been described for the spleen: immature, follicular, and marginal zone (MZ) B cells. MZ B cells are large cells that are CD21<sup>bright</sup>CD23<sup>low</sup> and IgM<sup>high</sup>IgD<sup>low</sup>. Analysis of Igα<sup>FF/FF</sup> mice revealed that this population was reduced to 5–25% of wild-type controls (Fig. 2 D).

In the peritoneal cavity, B cell numbers, mainly CD5<sup>+</sup> B1 cells, were drastically reduced in the Igα ITAM mutant mice (Fig. 2 F). B1 cells are phenotypically characterized as IgM<sup>bright</sup>IgD<sup>low</sup> and express lower levels of surface B220. Most B1 cells are also CD43<sup>+</sup> and CD23<sup>-</sup>. As shown in Fig. 2 F, this fraction of peritoneal B cells was reduced in

Igα<sup>FF/FF</sup> mice and most remaining B cells resembled B2 cells in being B220<sup>bright</sup>, IgD<sup>+</sup>, CD23<sup>+</sup>, and CD43<sup>-</sup>. Corresponding to the reduction of B1 cell numbers, the T cell fraction (CD5<sup>+</sup>IgM<sup>-</sup>; Fig. 2 F) of peritoneal lymphocytes was increased.

**Table II.** Comparison of Relative Expression Levels of Various B Cell Surface Molecules in Wild-Type and Igα<sup>FF/FF</sup> Mice

Antigen	B cell population		Surface expression in Igα <sup>FF/FF</sup> mice relative to wild-type (% ± SD)	
IgM	BM	IgM <sup>+</sup> B220 <sup>low</sup>	144.5 ± 35.0	n = 10 *
		IgM <sup>+</sup> B220 <sup>high</sup>	248.2 ± 58.6	n = 15 **
	Spleen		205.5 ± 33.8	n = 11 **
		Blood	222.3 ± 13.9	n = 5 **
IgD	Peritoneum	B1 cells	171.5 ± 37.8	n = 8 *
			126.9 ± 9.3	n = 10 **
	Spleen		132.6 ± 12.0	n = 11 **
		Blood	127.9 ± 17.7	n = 5 #
B220	BM	Pre-B	99.2 ± 5.4	n = 4
		IgM <sup>+</sup> B220 <sup>low</sup>	96.6 ± 5.4	n = 10
	Spleen	IgM <sup>+</sup> B220 <sup>high</sup>	91.1 ± 6.0	n = 12 **
			88.1 ± 6.9	n = 12 **
CD19	Spleen		86.2 ± 6.8	n = 5 #
		Blood	116.6 ± 5.9	n = 15 **
	Peritoneum	B1 cells	122.9 ± 11.4	n = 5 *
			117.7 ± 13.5	n = 6 #
CD22	Spleen		81.8 ± 4.8	n = 10 **
			102.0 ± 28.8	n = 5
MHCII	Spleen			

The levels of surface expression were determined by comparing the mean fluorescence intensities between B cells of wild-type (set as 100%) and Igα<sup>FF/FF</sup> mice. Numbers represent the mean percentages (± SD) based on the number of indicated analyses. In each single analysis, samples from Igα<sup>FF/FF</sup> mutants and wild-type mice were analyzed in parallel. The significance of difference was tested with the paired Student's *t* test. Asterisks indicate the determined *P* values: #*P* < 0.05; \**P* < 0.01; \*\**P* < 0.001.

**Table III.** Fraction of  $\lambda$ 1 Light Chain Expressing Spleen B Cells in  $Ig\alpha$  and  $Ig\beta$  Mutant Mice

wt	$4.8 \pm 0.6\%$	$n = 13$
$Ig\alpha^{FF/FF}$	$10.9 \pm 3.1\%$	$n = 10$
$Ig\alpha^{\Delta c/\Delta c}$	$22.1 \pm 3.1\%$	$n = 7$
$Ig\alpha^{\Delta c/\Delta c}$	$20.4 \pm 5.8\%$	$n = 4$

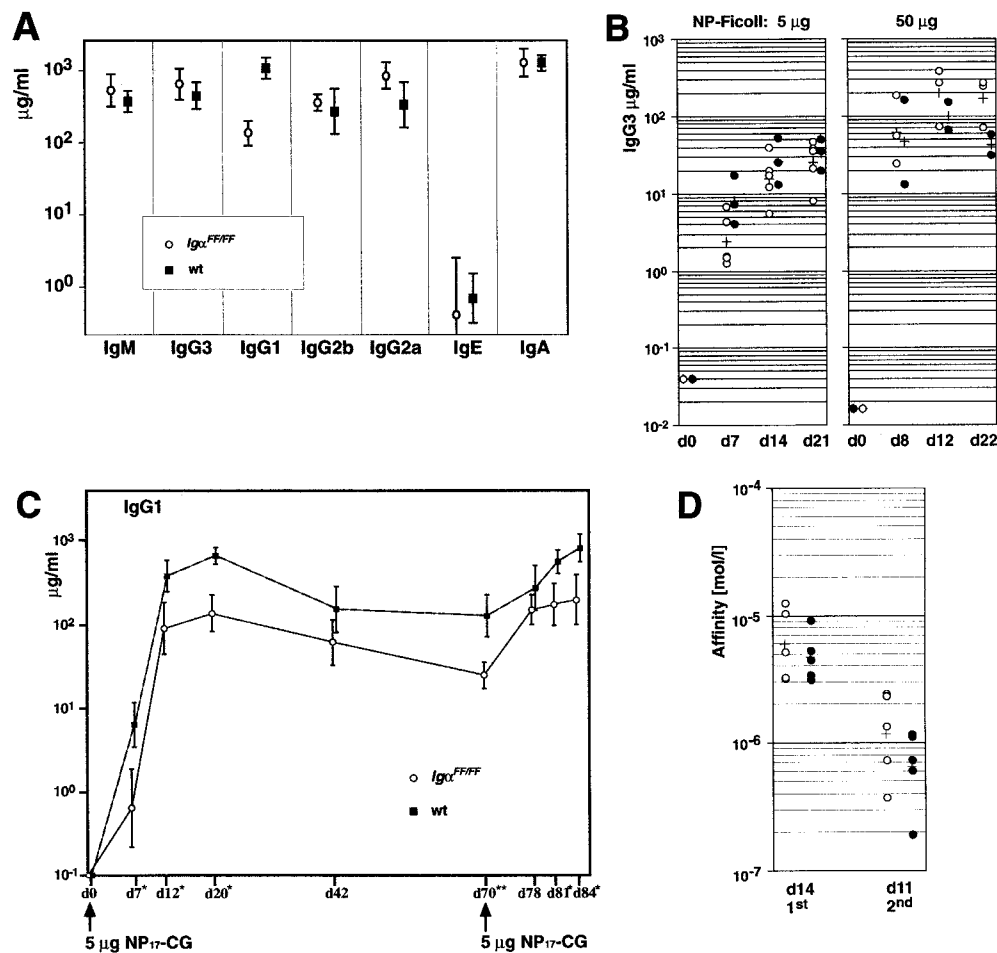
The frequency of B cells expressing a  $\lambda$ 1 light chain was determined by flow cytometry and is shown as percentage of all splenic B cells. Values represent the mean  $\pm$  SD of the indicated numbers of mice.

**Higher Percentage of  $\lambda$ 1 Light Chain Expressing B Cells in  $Ig\alpha/Ig\beta$  Mutants.** As in the BM of  $Ig\alpha^{FF/FF}$  mice, all B cell fractions were present in normal numbers, the lack of  $Ig\alpha$  ITAM phosphorylation does not appear to impose a block in early B cell development (Table I). However, the impact of the ITAM mutation on the rearrangement and/or selection of Ig light chains is apparent from the distortion of the ratio of  $\kappa$  versus  $\lambda$  light chain expressing B cells. Compared with wild-type controls,  $Ig\alpha^{FF/FF}$  mice possessed more  $\lambda$ 1 light chain expressing B cells (Table III, and Fig. 2 E). This phenomenon could also be seen, and appeared even more

pronounced, in the cytoplasmic truncation mutants of  $Ig\alpha$  and  $Ig\beta$  (Table III).

**Reduced IgG1 Serum Levels and Impaired TD Immune Response in  $Ig\alpha^{FF/FF}$  Mice.** Altered BCR signaling in  $Ig\alpha^{FF/FF}$  mice may also affect immune responsiveness. To check whether the ITAM mutation influences the basal levels of Ig titers, Ig isotypes were determined by ELISA in the sera of unimmunized mice. As shown in Fig. 3 A, the total amount of serum Ig in  $Ig\alpha^{FF/FF}$  mice, calculated as a sum of the individual isotypes, was comparable to controls. Although B1 cells are thought to be the main contributors of serum IgM, the reduction in B1 and MZ B cells in  $Ig\alpha$  ITAM mutants did not result in reduced serum IgM concentration. However, the serum concentration of IgG1 in  $Ig\alpha^{FF/FF}$  mice was significantly lower than in wild-type controls.

To analyze humoral immune responses, we immunized  $Ig\alpha^{FF/FF}$  mice with the TI type II (TI-II) antigen NP-Ficoll and TD antigen NP-CG. For two reasons we expected to see an impaired TI-II response. First, in  $Ig\alpha^{\Delta c/\Delta c}$  mice, B cells with a cytoplasmic truncation of  $Ig\alpha$  were unable to mount a measurable response toward NP-Ficoll (21), and second, recent data suggest that MZ B cells are required for a normal TI-II response (33). However, the



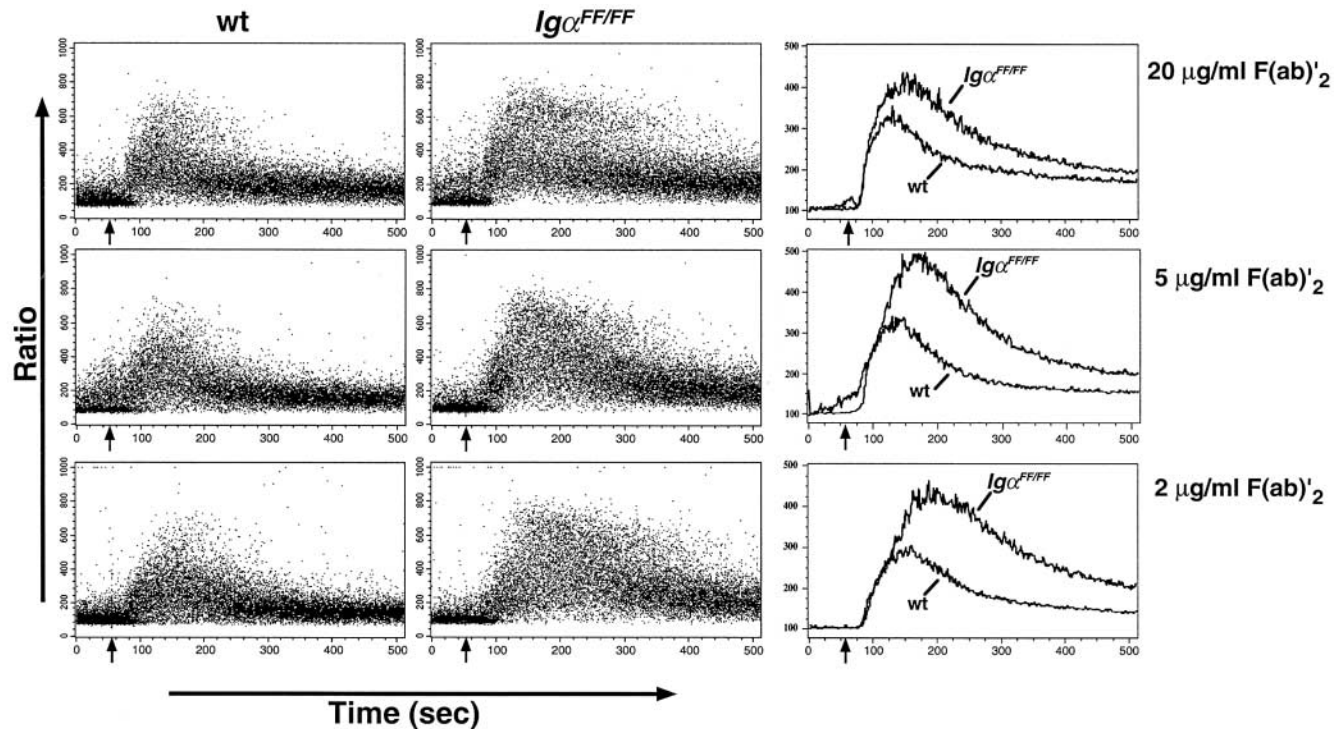
**Figure 3.** Serum Ig levels and humoral immune responses in  $Ig\alpha^{FF/FF}$  mice. Serum Ig isotype levels were determined by ELISA. Filled symbols represent wild-type and open symbols represent  $Ig\alpha^{FF/FF}$  mice. (A) Geometric means and standard deviations of serum levels of Igs in nonimmunized mice ( $n = 5$ ). The data are representative of three independent analyses. (B) Immune response to TI-II NP-Ficoll antigen. Plots show NP-specific IgG3 in sera of mice after immunization with either 5 or 50 µg NP-Ficoll. Crosses indicate geometric mean values. (C) Immune response to a TD antigen. NP-specific IgG1 levels in primary and secondary responses after immunizations with 5 µg NP-CG were determined in  $Ig\alpha^{FF/FF}$  mice ( $n = 5$ ) and wild-type (wt) controls ( $n = 6$ ). Shown are geometric mean values plus standard deviation. Asterisks indicate  $P$  values that were calculated according to the Student's  $t$  test. (D) Affinity maturation of anti-NP IgG1 in sera of  $Ig\alpha^{FF/FF}$  and control mice. Affinities on day 14 after a primary and on day 11 after a secondary immunization with 5 µg NP-CG are shown.

NP-specific serum titers at different time points after the immunization revealed that Ig $\alpha$  ITAM phosphorylation is not required for an efficient TI-II response (Fig. 3 B). Ig $\alpha^{FF/FF}$  mice were also able to mount primary and secondary TD responses, which had a kinetics comparable to those of wild-type controls (Fig. 3 C). These data imply that Ig $\alpha$  ITAM phosphorylation is not a prerequisite for B cell memory formation. However, the TD response was significantly lower in Ig $\alpha^{FF/FF}$  mice at all time points examined (Fig. 3 C). To evaluate further the efficiency of the TD response, we determined the averaged relative affinity of the serum antibodies. Affinities of NP-specific antibodies were comparable between Ig $\alpha^{FF/FF}$  mice and control groups, and were equally increased in the course of the immune response, indicating effective affinity maturation (Fig. 3 D).

**Stronger Calcium Response in Ig $\alpha$  ITAM Mutant B Cells.** To analyze the impact of the ITAM mutation on an early BCR-mediated signaling event, we stimulated Indo-1-loaded B cells either with F(ab) $'_2$  fragment of anti-Ig antibody or with anti-Ig antibodies and monitored changes in intracellular calcium by flow cytometry. Previously, we reported that a cytoplasmic truncation of Ig $\alpha$  leads to an elevated and more sustained calcium response in immature B cells of Ig $\alpha^{A_c/A_c}$ Ig $^{HEL}$  mice compared with controls (23). We show here that this effect appears to be correlated with the phosphorylation of the Ig $\alpha$  ITAM, as splenic B cells from

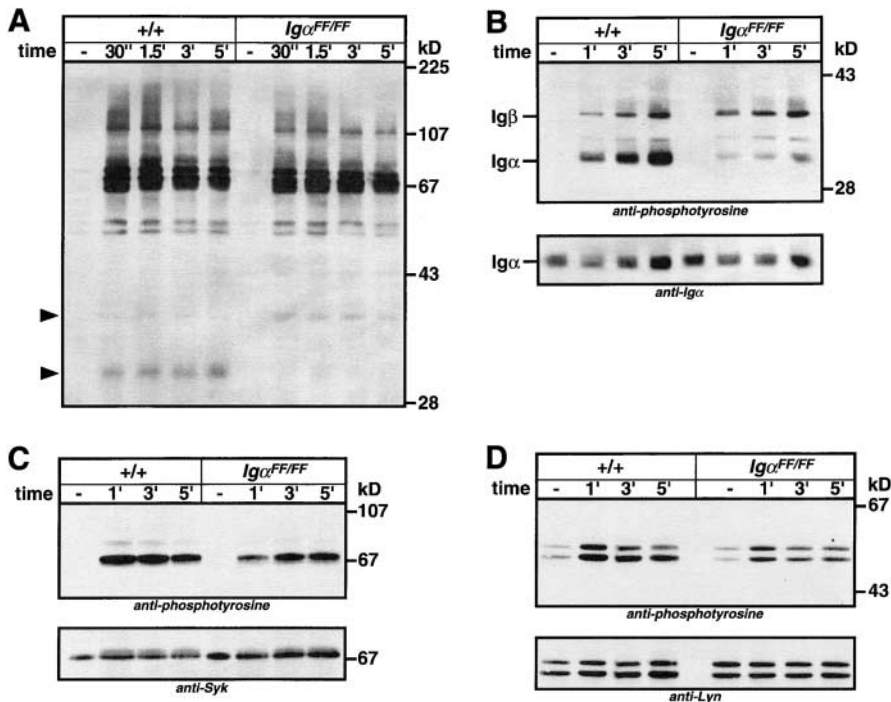
Ig $\alpha^{FF/FF}$  mice also showed a stronger calcium response with a delayed maximum in comparison to wild-type controls (Fig. 4, and data not shown).

**BCR Ligation Leads to Tyrosine Phosphorylation of Ig $\alpha$  in Ig $\alpha^{FF/FF}$  B Cells.** To extend the analysis of the signaling capacity of Ig $\alpha$  ITAM mutant BCRs, we analyzed protein tyrosine phosphorylation after BCR cross-linking. Splenic B cells were isolated from Ig $\alpha^{FF/FF}$  and control mice, and stimulated with F(ab) $'_2$  fragment of anti-Ig antibody for different time durations. Total protein tyrosine phosphorylation was revealed by anti-phosphotyrosine immunoblotting. As shown in Fig. 5 A, BCRs lacking the Ig $\alpha$  ITAM tyrosines were still capable of inducing protein tyrosine phosphorylation to levels comparable to controls. We then specifically examined the tyrosine phosphorylation of Ig $\alpha$  and Ig $\beta$  in anti-Ig $\alpha$  immunoprecipitates before and after BCR cross-linking. As shown in Fig. 5 B, BCR ligation of Ig $\alpha^{FF/FF}$  B cells resulted in a rapid and persistent tyrosine phosphorylation of Ig $\beta$  ITAM. Moreover, BCR-induced tyrosine phosphorylation of Ig $\beta$  in Ig $\alpha^{FF/FF}$  mice was enhanced compared with controls (Fig. 5, A and B). Whereas Ig $\beta$  possesses only the two tyrosines of its ITAM, the cytoplasmic tail of Ig $\alpha$  contains four tyrosine residues (Fig. 1 E). Interestingly, Ig $\alpha$  phosphorylation in Ig $\alpha$  ITAM mutant B cells was detected after BCR ligation (Fig. 5 B). These data suggest that either one or both non-ITAM Ig $\alpha$  tyrosine residues (#176 and #204) can be phosphorylated after



**Figure 4.** Comparison of BCR-induced Ca $^{2+}$  mobilization. Changes in intracellular calcium levels were determined by flow cytometry. Dot plots present the intracellular calcium levels in B220 $^{+}$  gated splenic B cells as the fluorescence ratio of Indo-1 emission at 395/510 nm recorded over time. Baseline fluorescence ratios were acquired for 50 s before F(ab) $'_2$  rabbit anti-mouse Ig antibodies were added (indicated by an arrow). Final antibody concentrations are shown on the right. Histograms on the right show the median values of the analyzed B cells. Results are representative of four independent experiments. wt, wild-type.





**Figure 5.** Analysis of protein tyrosine phosphorylation in splenic B cells of  $Ig\alpha^{FF/FF}$  mice. Cell lysates of purified splenic B cells from  $Ig\alpha^{FF/FF}$  and wild-type mice were prepared from cells that were either mock stimulated with medium or stimulated with  $5 \mu\text{g}/10^6$  cells  $F(\text{ab})'_2$  rabbit anti-mouse Ig for different periods of time. (A) Tyrosine phosphorylation of total cellular proteins ( $5 \times 10^5$  cells/lane) was detected by anti-phosphotyrosine immunoblotting. Arrows indicate the expected positions of  $Ig\alpha$  and  $Ig\beta$ . (B) Phosphotyrosine immunoblot of  $Ig\alpha$  immunoprecipitates. The bottom panel shows the same blot reprobed with an anti- $Ig\alpha$  antibody. (C) Syk tyrosine phosphorylation. Proteins were immunoprecipitated from the cell lysates with anti-Syk antibody, resolved by SDS-PAGE, and analyzed by anti-phosphotyrosine immunoblotting. The blot was reprobed with anti-Syk antibody. (D) Phosphotyrosine immunoblot of Lyn immunoprecipitates. The blot was reprobed with anti-Lyn antibody.

BCR stimulation. This strongly suggests a role for the non-ITAM tyrosines in BCR-mediated signaling, e.g., as a binding site for SH2 domain containing proteins.

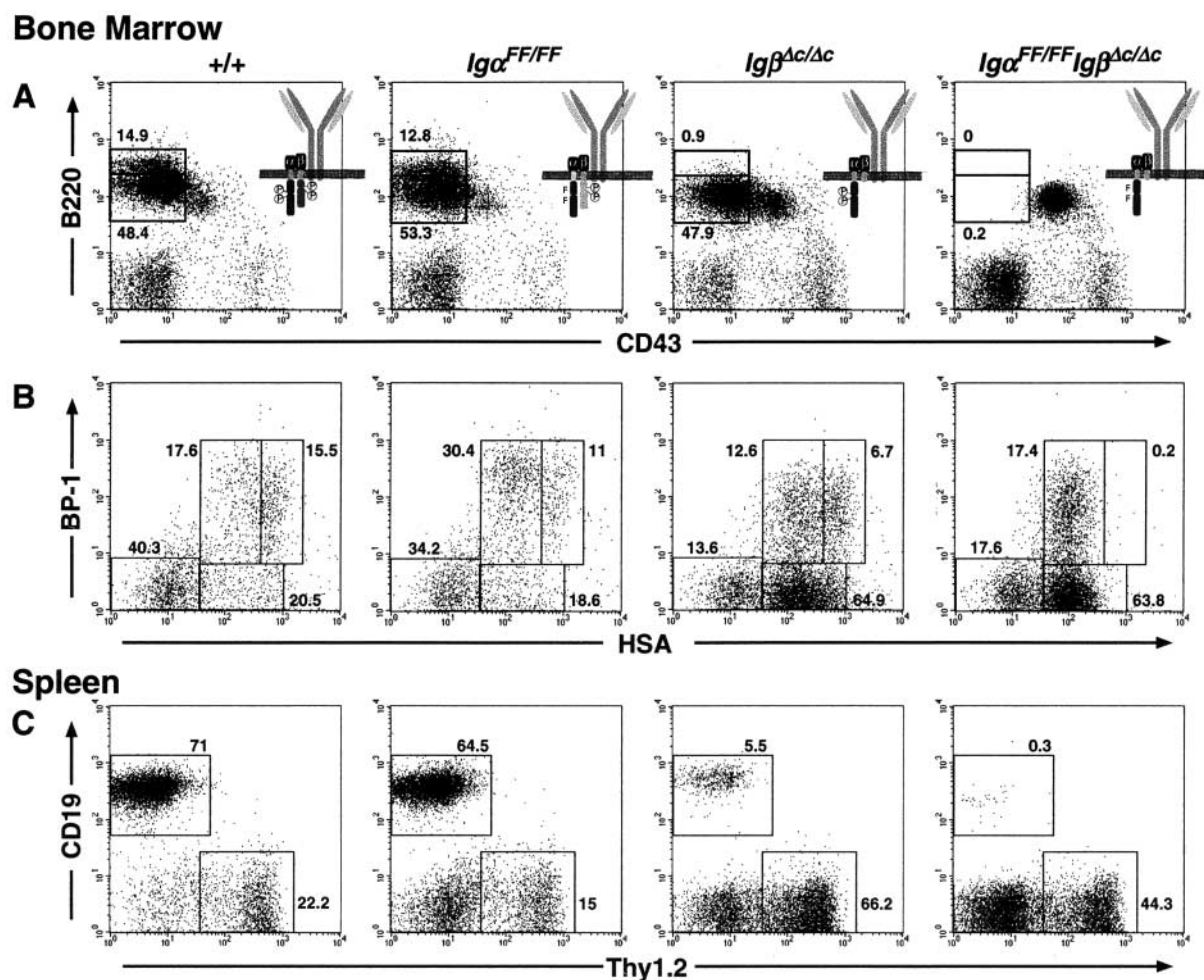
Syk kinase is one of the major signaling components that binds to doubly phosphorylated BCR ITAMs and becomes activated. Ligation of BCRs with the mutant  $Ig\alpha$  ITAM were still able to induce Syk tyrosine phosphorylation, presumably via phosphorylated  $Ig\beta$ . However, maximum phosphorylation was reached at a later time point than in wild-type controls (Fig. 5C). Examination of Lyn phosphorylation showed that basal tyrosine phosphorylation was similar between  $Ig\alpha^{FF/FF}$  and wild-type B cells, but BCR-induced Lyn tyrosine phosphorylation was less than in wild-type controls (Fig. 5D).

**Complete Block of B Cell Development in  $Ig\alpha^{FF/FF}Ig\beta^{\Delta c/\Delta c}$  Mice.** Targeted mutations of  $Ig\alpha$ , as in truncation of the cytoplasmic tail in  $Ig\alpha^{\Delta c/\Delta c}$  mice (21) or with specific loss of ITAM phosphorylation in  $Ig\alpha^{FF/FF}$  mice, lead to alteration in development and responsiveness of B lymphocytes. Similarly, a cytoplasmic truncation of  $Ig\beta$  in  $Ig\beta^{\Delta c/\Delta c}$  mice compromises B cell development and causes a strong reduction in mature B cells (22; Fig. 6). However, neither the lack of  $Ig\alpha$  nor  $Ig\beta$  ITAM alone leads to a complete block in B cell development. A cytoplasmic domain of either  $Ig\alpha$  or  $Ig\beta$  is still sufficient to signal (22). Based on the phenotypic differences between  $Ig\alpha^{\Delta c/\Delta c}$  and  $Ig\alpha^{FF/FF}$  mice and the finding that  $Ig\alpha$  may be phosphorylated at non-ITAM tyrosines,  $Ig\alpha$  may not mediate signaling exclusively through its phosphorylated ITAM. Whether development of B cells in the  $Ig\alpha$  and  $Ig\beta$  truncation mutants was absolutely dependent on the phosphorylation of the remaining BCR ITAM remained unclear. To answer this question, we generated  $Ig\alpha^{FF/FF}Ig\beta^{\Delta c/\Delta c}$  mice. In comparison to  $Ig\beta^{\Delta c/\Delta c}$

mice, the double mutants possessed no intact ITAM. As shown in Fig. 6A, B cell development in these mice was completely blocked at the pro-B cell stage, as no  $B220^+CD43^-$  B cells were present in the BM. FACS<sup>®</sup> analysis of the  $B220^+CD43^+$  pro-B cell fraction revealed the presence of fractions A to C, but fraction C' ( $BP-1^+HSA^{\text{bright}}$ ) was absent (Fig. 6B). In the periphery of  $Ig\alpha^{FF/FF}Ig\beta^{\Delta c/\Delta c}$  mice, a few  $CD19^+$  cells were still detectable (Fig. 6C), however, these cells did not express surface IgM nor IgD. These  $CD19^+$  cells expressed the immature B cell marker 493 and had low levels of surface CD19 and B220 (data not shown). Thus, these cells might represent peripheral B cell precursors. The lack of any functional mature B cells in  $Ig\alpha^{FF/FF}Ig\beta^{\Delta c/\Delta c}$  mice was further validated by the absence of any detectable serum Igs (data not shown). Collectively, these data indicate an absolute requirement of  $Ig\alpha$  ITAM phosphorylation in  $Ig\beta^{\Delta c/\Delta c}$  mice for B cells to develop beyond the pro-B cell stage.

## Discussion

***Igα ITAM Mutation Affects B Cell Development Less Dramatically than Truncation of the Igα Cytoplasmic Tail.*** The B cell antigen receptor complex relies on its  $Ig\alpha/Ig\beta$  heterodimer for surface expression, internalization, antigen presentation, and signaling. Therefore, a targeted ablation of the cytoplasmic effector domain of either  $Ig\alpha$  or  $Ig\beta$  may result in impaired B cell development and function. This is indeed evident in both  $Ig\alpha^{\Delta c/\Delta c}$  and  $Ig\beta^{\Delta c/\Delta c}$  mice which exhibit a drastic reduction in peripheral B cell numbers (21, 22). To date, the main signaling function of the  $Ig\alpha/Ig\beta$  heterodimer has been ascribed to its two ITAMs.



**Figure 6.** Complete block of B cell development at the pro-B cell stage in the absence of any phosphorylatable BCR ITAM. Representative flow cytometric analyses of lymphocytes from wild-type, *Igα<sup>FF/FF</sup>*, *Igβ<sup>Δc/Δc</sup>*, and *Igα<sup>FF/FF</sup>Igβ<sup>Δc/Δc</sup>* mice. (A) Note the lack of CD43<sup>-</sup>B220<sup>+</sup> cells in the BM of *Igα<sup>FF/FF</sup>* *Igβ<sup>Δc/Δc</sup>* mice. (B) The HSA vs. BP-1 dot plots show CD43<sup>+</sup>B220<sup>+</sup> gated pro-B cells. Numbers indicate the frequency of cells within the windows as percentage of the displayed pro-B cells. (C) CD19 and Thy1.2 surface expression of splenic lymphocytes. Fractions of B and T cells are shown as percentage of cells in the lymphocyte gate.

Moreover, mutational experiments in cell lines have revealed that ITAM functions require phosphorylation at its two tyrosine residues (9, 10, 13, 14, 16, 34). However, the present analysis of *Igα<sup>FF/FF</sup>* mice revealed striking differences in comparison to *Igα<sup>Δc/Δc</sup>* mice. A lack of Igα ITAM phosphorylation did not affect the relative fraction sizes of pro-B, pre-B, and immature B cells in the BM, and the absolute splenic B cell numbers in *Igα<sup>FF/FF</sup>* mice were comparable to those of wild-type controls. Taken together, the differences in phenotypes resulting from either the loss of the Igα cytoplasmic tail or the specific lack of Igα ITAM phosphorylation, imply a role for the non-phosphorylated ITAM and/or other parts of the Igα cytoplasmic tail in Igα-mediated function. This idea is consistent with the finding that Igα ITAM phosphorylation is not a prerequisite for binding of Src family kinases Lyn and Fyn via their N-terminal regions (35) and in agreement with the model that proposes a high affinity, phosphotyrosine-dependent, and a low affinity, phosphotyrosine-independent associa-

tion of Src family kinases with Igα/Igβ (36). In addition, the Igα molecules in *Igα<sup>Δc/Δc</sup>* mice lack the serine/threonine phosphorylation sites which are implicated in the regulation of Igα tyrosine phosphorylation (37). Thus, differences in serine/threonine phosphorylation might also be responsible for the phenotypic differences of *Igα<sup>Δc/Δc</sup>* and *Igα<sup>FF/FF</sup>* mice. Alternatively, lack of the Igα cytoplasmic domain in *Igα<sup>Δc/Δc</sup>* mice might influence the structure of the remaining Igβ tail and distort its ITAM function. The different phenotype of *Igα<sup>FF/FF</sup>* mice could thus also reflect a difference in Igβ ITAM function.

*Altered Surface Antigen Expression in B Cells of *Igα<sup>FF/FF</sup>* Mice Due to Adaptation or Selection.* B cells from *Igα<sup>FF/FF</sup>* mice express higher levels of surface IgM and IgD compared with wild-type controls. Three reasons might account for the higher BCR density. First, lack of efficient protein-kinase activation via phosphorylated Igα ITAMs might be partly compensated by expressing more Igα/Igβ on the cell surface. Second, the increase in Igβ ITAM phosphorylation

might partially substitute for the lack of Ig $\alpha$  ITAM phosphorylation. Third, Ig $\alpha$  ITAM phosphorylation might directly control BCR surface expression or internalization. The latter possibility is supported by the analysis of Ig $\alpha$  mutant molecules in cell cultures. While in reconstitution experiments using the J558L $\mu$ m cell line, neither a cytoplasmic deletion nor Ig–ITAM tyrosine mutations interfered with the surface expression of BCR (10), constitutive receptor endocytosis appeared to be dependent on the cytoplasmic tail of Ig $\alpha$  in a chimeric system (38). Similarly, analyzing Fc $\gamma$ RII–Ig $\alpha$  or Ig $\beta$  chimeric molecules in the A20/IIA1.6 cell line, efficient constitutive BCR internalization was only mediated by the cytoplasmic domain of Ig $\alpha$  and was shown to be dependent on the first Ig $\alpha$  ITAM tyrosine (38).

Interestingly, B cells in Ig $\alpha^{\Delta c/\Delta c}$  mice do not exhibit a similar increase in surface BCR expression levels as in Ig $\alpha^{FF/FF}$  mice, and the cytoplasmic truncation of Ig $\beta$  in Ig $\beta^{\Delta c/\Delta c}$  mice leads to lowered BCR expression (22; Fig. 2, B and C). One might speculate that surface BCR expression levels are controlled by constitutive Ig $\alpha$ /Ig $\beta$ -dependent turnover mechanisms and in addition are adjusted to the specific signaling requirements of the B cells.

In line with the idea that Ig $\alpha$  ITAM phosphorylation is required for efficient activation of Src family kinases are the observations of an increased CD19 and a decreased CD22 coreceptor surface expression in B cells from Ig $\alpha^{FF/FF}$  mice. CD19 was shown to lower the threshold of BCR-mediated cell activation and to be involved in the activation of Src family kinases (39, 40). In contrast, CD22 contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which serve as docking sites for SH2 domain-containing phosphotyrosine phosphatase (SHP)-1, and thus might serve to lower protein kinase activity (41–43). Interestingly, B cells in CD22-deficient mice (44, 45) and transgenic mice overexpressing CD19 (46) possess decreased surface IgM levels. Thus, differences in BCR and coreceptor surface expression levels in Ig $\alpha^{FF/FF}$  mice might be the result of selection and/or adaptation of B cells, in an attempt to cope with weakened intrinsic BCR signaling. To address the issue of selection versus adaptation, we plan to analyze Ig $\alpha^{FF-flox/FF-flox}$  mice in combination with inducible or developmental stage specific Cre-recombinase expression (47). The floxed Ig $\alpha$  allele (Fig. 1 A, 4) allows undisturbed B cell development with a wild-type BCR until Cre-mediated mutation of the Ig $\alpha$  ITAM occurs. Induced mutagenesis should thus allow us to study the adaptive response of mature B cells to a lack of Ig $\alpha$  ITAM phosphorylation.

**PTK Activation by Ig $\alpha$  ITAM and Non-ITAM Tyrosine Phosphorylation.** Previous studies with B cell lines have implied a critical requirement for Ig $\alpha$  ITAM tyrosine phosphorylation in the activation of PTKs after BCR ligation (9, 10, 13, 48). However, in some initial signaling studies with splenic B cells of Ig $\alpha^{FF/FF}$  mice we did not observe a drastic reduction in overall protein tyrosine phosphorylation after BCR cross-linking, which could be explained by the presence of a functional Ig $\beta$  ITAM. Overall higher surface expression and more efficient ITAM phosphorylation

of Ig $\beta$  might thus be able to compensate in part for the lack of Ig $\alpha$  ITAM phosphorylation in Ig $\alpha^{FF/FF}$  B cells. We tested the capability of Syk and Lyn to undergo tyrosine phosphorylation upon BCR ligation in B cells of Ig $\alpha^{FF/FF}$  mice. Although Ig $\alpha$  ITAM phosphorylation is not absolutely required to induce tyrosine phosphorylation of Syk, the overall Syk tyrosine phosphorylation was lower and delayed as a result of the Ig $\alpha$  ITAM mutation. Similarly, Lyn was inducibly tyrosine phosphorylated in Ig $\alpha^{FF/FF}$  B cells, but to a lesser extent than in controls. An important novel finding is the demonstration of Ig $\alpha$  tyrosine phosphorylation apart from its ITAM. This suggests an involvement of the Ig $\alpha$  non-ITAM tyrosines in BCR-dependent signaling. These might recruit distinct signaling proteins or alternatively enhance or inhibit the association of molecules with the Ig $\alpha$ /Ig $\beta$  heterodimer. Both non-ITAM tyrosines are indeed in the sequence context of a potential Src family SH2 domain binding site (49, 50). Compared with wild-type controls, the weak tyrosine phosphorylation of Ig $\alpha$  in Ig $\alpha^{FF/FF}$  B cells supports the idea that the ITAM tyrosines are the main tyrosine phosphorylation sites in Ig $\alpha$  (10). However, in the wild-type situation, phosphorylation of the non-ITAM tyrosines could well be linked to and dependent on Ig $\alpha$  ITAM phosphorylation. A clarification of these matters will require a biochemical analysis which is beyond the scope of this paper.

**Ig $\alpha$  ITAM Phosphorylation and Ca<sup>2+</sup> Mobilization.** The enhanced Ca<sup>2+</sup>-flux in B cells of Ig $\alpha^{FF/FF}$  mice after BCR ligation seems to contradict the idea that Ig $\alpha$  ITAM phosphorylation functions to amplify signaling. Previous transfection experiments with Fc $\gamma$ RII–Ig $\alpha$  or Fc $\gamma$ RII–Ig $\beta$  chimeric molecules in the IIA1.6 cell line demonstrated that only the Ig $\alpha$  cytoplasmic tail was capable to mobilize Ca<sup>2+</sup> from intracellular stores and influx of extracellular calcium. In contrast, the Ig $\beta$  cytoplasmic domain stimulated only an oscillatory release from intracellular stores, which overall resulted in a small increase of free intracellular Ca<sup>2+</sup> (9, 51). Further analyses specified an absolute requirement of the proximal Ig $\alpha$  ITAM tyrosine (Y182) for Ca<sup>2+</sup> mobilization (9, 13). Thus, it was surprising to find an even stronger Ca<sup>2+</sup> mobilization in splenic B cells of Ig $\alpha^{FF/FF}$  mice after BCR ligation (Fig. 4). One might interpret the data such that Ig $\alpha$  ITAM phosphorylation is required to contain and terminate Ca<sup>2+</sup> mobilization. While the increased Ca<sup>2+</sup> mobilization in B cells of Ig $\alpha^{FF/FF}$  mice is not caused by overall higher surface BCR expression (data not shown), changes of coreceptor expression might influence the outcome of BCR ligation by resetting signaling thresholds. In support of the latter, overexpression of CD19 (46) or lack of CD22 (44, 45, 52, 53) in mouse B cells also leads to enhanced calcium mobilization, whereas the lack of CD19 lowers it (54).

**Ig $\alpha$ /Ig $\beta$  Mutations Distort Light Chain Rearrangements.** Although we do not yet know whether the Ig $\alpha$  ITAM mutation leads to enhanced editing of autoreactive BCRs like the Ig $\alpha$  truncation (23), it is noteworthy that both mutations distort the  $\kappa$  to  $\lambda$  ratio in favor of  $\lambda$  light chain expression. One might speculate that quantitative and qualita-

tive alterations of signals transmitted by a mutant Ig $\alpha$ /Ig $\beta$  complex might hinder the efficient termination of light chain rearrangements in B cell development, widening the time window in which sequential rearrangements in the light chain loci occur and thus driving more cells to the  $\lambda$  chain expression, if  $\kappa$  rearrangements usually precede  $\lambda$  rearrangements (55). In line with this interpretation are the observations that the shift in favor of  $\lambda$  chain expression is more pronounced in the tail truncation mutants than in Ig $\alpha^{FF/FF}$  mice and that only in the former mutants there is an apparent block of B cell development at the immature B cell stage. Alternatively, altered pre-BCR signaling might influence the order of light chain gene rearrangements and cause an earlier opening of the  $\lambda$  locus. In an extreme scenario this could lead to the exclusive rearrangement of the  $\lambda$  locus in  $\lambda$  chain-expressing cells. We tested this hypothesis by analyzing the rearrangement status of the  $\kappa$  loci in  $\lambda 1^+$  splenic B cells. However, similar to the situation in  $\lambda 1^+$  cells from wild-type mice (56), the  $\kappa$  light chain loci were found to be rearranged in  $\lambda 1^+$  cells of Ig $\alpha^{FF/FF}$  mice (data not shown). Finally, it might be possible that  $\lambda$  light chains confer a selective advantage for B cells in case of distorted BCR signaling.

**Dependence of B1 and MZ B Cell Populations on Ig $\alpha$  ITAM Phosphorylation.** In the peritoneal cavity of Ig $\alpha^{FF/FF}$  mice, the total number of B cells was lower than in the wild-type due to a drastic reduction in B1a cells. The majority of the remaining peritoneal B cells were phenotypically B2 cells. B1 cells often express germline-encoded self-reactive BCRs and appear to have signaling requirements that are different from B2 cells (57). Recent studies have demonstrated that the expression of an autoreactive receptor can drive the generation of B1 cells (58, 59), suggesting that B1 cell development depends on signals mediated by BCR–ligand interaction. The specific reduction of B1 cells in Ig $\alpha^{FF/FF}$  mice could thus be explained by enhanced negative selection of autoreactive BCR specificities, but would also be consistent with the idea that Ig $\alpha$  ITAM phosphorylation functions as a signal amplifier. In support of the latter conclusion, several mouse mutants affecting genes involved in positive regulation of BCR signaling such as CD19 (60, 61), Btk (62, 63), and Vav (64, 65) also show a reduction in the B1 cell population. An opposing phenotype is seen upon mutation of genes thought to negatively regulate BCR-related signaling. SHP-1 deficiency in *me<sup>o</sup>/me<sup>v</sup>* mice (66) and knockouts of CD22 (53, 45) and CD72 (67) all lead to an expansion of the B1 cell population. This is also true for mice in which the SHP-1 gene is selectively inactivated in B cells (data not shown).

In the spleen of Ig $\alpha^{FF/FF}$  mice we observed a strong reduction of MZ B cells. Similar to the B1 population, MZ B cell selection also depends on BCR specificities with a bias for autoreactivity (68). Moreover, both B1 and MZ B cells are CD23<sup>low</sup>IgM<sup>high</sup> and larger in size than B2 cells. These results demonstrate directly that appropriate BCR signaling is required for the normal generation and maintenance of both these subsets.

**Ig $\alpha$  ITAM Phosphorylation Is Required for Efficient TD Immune Responses.** B1 and MZ B cells are thought to be the frontline of an early defense against pathogens and appear to be regulated in a T cell-independent way (69). In a recent study of Pyk-2 knockout mice, which completely lack MZ B cells, an important role for MZ B cells in T cell-independent antibody responses was suggested (33). Therefore and because TI-II responses have been shown to be severely impaired in Ig $\alpha$  and Ig $\beta$  tail truncation mutants (21, 22), we expected an impairment of the anti-NP-Ficolin response in Ig $\alpha^{FF/FF}$  mice. However, the selective elimination of the Ig $\alpha$  ITAM does apparently not interfere with efficient B cell activation upon strong BCR cross-linking. It is unclear at this point whether the difference between the Ig $\alpha$  ITAM and the truncation mutants in this respect reflects a difference in B cell activation or relates to the development of B cell subsets which may be disturbed in the mutants to different extents. That the Ig $\alpha$  ITAM mutation is compatible with an intact TI-II response, but affects the efficiency of a TD antibody response is consistent with the findings in cell lines that Ig $\alpha$  ITAM tyrosines contribute to efficient antigen presentation, but are not absolutely required for the presentation of multivalent antigens (48, 70, 71). Inefficient TD responses may also explain the low IgG1 levels in the sera of Ig $\alpha^{FF/FF}$  mice.

**Absolute Requirement of BCR ITAM Tyrosines for B Cell Development Beyond the Pro-B Cell Stage.** A striking observation is the absolute block of B cell development at the pro-B cell stage in mice lacking the Ig $\alpha$  ITAM and the Ig $\beta$  cytoplasmic tail. This contrasts with an almost unimpaired early B cell development in the Ig $\alpha$  ITAM mutant and significant pre-B cell promotion in mice expressing a truncated Ig $\beta$  or Ig $\alpha$  chain. The simplest interpretation of these results is that there is significant functional redundancy between the Ig $\alpha$  and Ig $\beta$  ITAM (similar to that exhibited by the ITAMs of the T cell receptor complex; reference 72), and that there is an absolute requirement for the presence of an ITAM in the pre-BCR at the pro- to pre-B cell transition which is known from earlier work to depend on pre-BCR expression (17, 29, 73–75). It remains to be established whether this requirement relates to pre-BCR surface expression or more likely, the signaling function of the surface bound receptor. The latter aspect is supported by previous findings showing a requirement of ITAM tyrosines for pre-B cell development and allelic exclusion in transgenic systems using IgM/Ig $\beta$  and IgM/Ig $\alpha$  chimeric receptors (18–20). It will be interesting to determine whether later stages of B cell development, where the BCR rather than the pre-BCR plays a critical role (21, 76–78), exhibit a similar ITAM dependence. Inducible targeted mutagenesis will be instrumental in resolving this issue.

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