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 Igα and Igβ function in B cell development were studied in transgenic and gene-targeted mice. In Igβ knockout mice, the lack of Igα/Igβ heterodimer formation presumably also abrogates the surface expression of Igα 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 Igα or Igβ was capable of supporting further steps in B cell maturation, including H chain allelic exclusion (18–20). Critical roles of the cytoplasmic tails of Igα and Igβ in B cell development were revealed in mb-1<sup>−/−</sup> and Igβ<sup>−/−</sup> mice (21, 22). In Igβ<sup>−/−</sup> mice, the cytoplasmic domain of Igβ is lacking, whereas in mb-1<sup>−/−</sup> mice (hereafter termed Igα<sup>−/−</sup>) a truncated Igα 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–induced (TI) antigen. Further experiments with Igα<sup>−/−</sup> mice revealed that the Igα 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 Igα was suggested (23, 24).

To investigate the specific function of Igα ITAM phosphorylation in B cell development, we generated a mutant mouse strain by gene targeting in which the Igα<sup>c</sup>/H9004<sup>c</sup>/H9251<sup>c</sup> or Igβ<sup>c</sup>/H9004<sup>c</sup>/H9251<sup>c</sup> mice were studied in transgenic and gene-targeted mice. In Igα<sup>c</sup>/H9004<sup>c</sup>/H9251<sup>c</sup> or Igβ<sup>c</sup>/H9004<sup>c</sup>/H9251<sup>c</sup> mice, the lack of Igα/Igβ expression against T cell–independent (TI) antigen. Further experiments with Igα<sup>−/−</sup> mice revealed that the Igα 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 Igα was suggested (23, 24).

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 intronII. Intron region and exons downstream of this loxP site are exactly repeated 3′ to a floxed neomycin (neo′ gene (see Fig. 1 A). Tyrosine to phenylalanine replacements within the ITAM were generated by an exchange of two nucleotides.

To investigate the specific function of Igα ITAM phosphorylation in B cell development, we generated a mutant mouse strain by gene targeting in which the Igα ITAM tyrosinines replaced with phenylalanines (Igα<sup>FF/FF</sup> mice).

Mice. ES cells carrying the Igα<sup>FF/FF</sup> allele were injected into C57BL/6 and CB20 blastocysts which were then transferred into foster mothers to obtain chimeric mice. Mice with the Igα<sup>FF/FF</sup> allele were crossed to the delter-strain (28). Some analyses were performed with Igα<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 Igα<sup>FF/FF</sup> mice. In anti-phosphotyrosine immunoblotting experiments, all Igα<sup>FF/FF</sup> mice used had been backcrossed for eight generations to C57BL/6. Igα<sup>−/−</sup> mice (22) are of mixed 129/Sv–C57BL/6 genetic background. Igα<sup>−/−</sup> mice (21) to obtain pmb-1C1C2<sup>−/−</sup>neofox. Additional restriction sites were introduced by site-directed mutagenesis. The product of two consecutive PCR reactions (primer: BamHI-REV 5′-GGATCCAGACAT-3′; FLAG-C1C2<sup>−/−</sup>-REV 5′-TTGGGC-TTCATCGATCCTGCCGCGGCCGC-3′) was subcloned into the pCR™ TA Cloning vector II (Invitrogen) and retrieved by digestion with SalI and ApaI. The fragment was used to exchange the corresponding 1-kb fragment in pmb-1C1C2<sup>−/−</sup>neofox to generate pmb-1C1C2**neofox-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).

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 NH₄Cl, 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 Igα^{FF-flox/+} ES cell clones and Igα^{FF/FF} mice. (A) Targeted insertion of a loxP-flanked neo' gene, mutated cytoplasmic exons and a third loxP site into the mb-1 locus, followed by the specific deletion of the neo' 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' selection marker results in the Igα^{FF-flox} allele (4). In the mouse, Cre-expression mediates the excision of the wild-type cytoplasmic exons to generate the mutated Igα^{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' 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 Igα^{FF/FF} and wild-type (wt) mice. (E) Sequence analysis of the Igα ITAM in Igα^{FF/FF} mice. Genomic DNA was extracted from purified splenic B cells of Igα^{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 Igα 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% N2. Stained cells were acquired on a FACSscan™ 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 IgM 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 on 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-µ chain), LS.136 (anti-CD4), Cy34.1 (anti-CD23), M5/114 (anti-MHC class II), BP-1 (anti-BP-1), 30F1 (anti-HSA), and C601 (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. Rolaik (Basel Institute of Immunology, Basel, Switzerland).

Immunization and Serum Analysis. Mice were immunized with 5 or 50 µg of T cell–dependent (TD) antigen NP-CG (4-hydroxy-3-nitrophenylacetyl chicken-γ-globulin) in alum intraperitoneally. Mice were boosted with 5 or 50 mM EDTA, 10 mM Tris-HCl, pH 8, 1 aprotinin, 1 nM leupeptin, and 0.001% N2. Mice were rested and resuspended with PBS, 1% BSA, and 0.01% N2. 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.

Results

Generation of IgαFF/FF Mice. To allow for conditional generation of the Igα 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 reintegrated the 5′ loxP site and the ITAM mutations (data not shown). To remove the floxed neo selection cassette from the targeted mb-1 allele, the clone was transiently transfected with circular pLCcre 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 IgαFF/FF floxed allele (Fig. 1 A, 4) from clones where Cre-mediated recombination led also to the loss of the unmutated cytoplasmic exons (IgαFF mutated allele; Fig. 1 A, 5). ES cells carrying the IgαFF/FF allele were injected into mouse blastocysts to generate chimeras that subsequently transmitted the floxed allele through the germline. IgαFF/FF/+ mice were bred with the deleter mouse strain (28) to obtain IgαFF/− mice that were then bred to homozygosity. The tyrosine to phenylalanine exchanges in the Igα ITAM were verified by sequencing. DNA for this analysis was prepared from FACSort®-sorted splenic B cells of IgαFF/FF and wild-type control mice (Fig. 1 E).

B Cell Development in IgαFF/FF Mice. The requirement of Igα ITAM phosphorylation for B cell development was analyzed in IgαFF/FF mice by flow cytometry (32). In contrast to IgαA+/− mice, which have reduced fractions of pre-B and immature B cells, IgαFF/FF mice had normal numbers of pro-B (B220+/−CD43+ IgM−), pre-B (B220+/−CD43− IgM−), and immature B (B220+/−IgM+ IgD−) 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 IgαFF/FF mice (see Fig. 5). In addition, a striking difference exists between mature B cells in IgαA+/− and IgαFF/FF mutants. Whereas recirculating B
cells in the BM and splenic B cells were ~100-fold reduced in the former, B cell numbers in Igα<sup>FF/FF</sup> 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 Igα<sup>FF/FF</sup> 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 only marginally reduced in comparison to wild-type mice (Table I; Fig. 2, A and B). We analyzed the peripheral B cells in Igα<sup>FF/FF</sup> 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 only marginally reduced in comparison to wild-type mice (Table I; Fig. 2, A and B). 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We analyzed the peripheral B
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 IgαΔkimutant (21), IgαFF/FF 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 IgαFF/FF 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, IgβΔkimutant, and IgαFF/FF mice. In contrast to the extremely low surface IgM levels of B cells in IgβΔkimutant mice, the Igα ITAM mutation led to increased surface IgM levels. In general, B cells from various lymphoid organs carrying the IgαFF 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 IgαFF/FF 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 CD21brighCD23low and IgMbrighIgDlow. Analysis of IgαFF/FF 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+ B1 cells, were drastically reduced in the Igα ITAM mutant mice (Fig. 2 F). B1 cells are phenotypically characterized as IgMbrighIgDlow and express lower levels of surface B220. Most B1 cells are also CD43+ and CD23+. As shown in Fig. 2 F, this fraction of peritoneal B cells was reduced in IgαFF/FF mice and most remaining B cells resembled B2 cells in being B220brigh, IgD+, CD23+, and CD43−. Corresponding to the reduction of B1 cell numbers, the T cell fraction (CD5+IgM−; Fig. 2 F) of peritoneal lymphocytes was increased.

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

| BM (percentage of all cells) | Pro-B (A–C) | Pre-B (D) | Immature (E) | Recirculating (F) | Spleen (× 10⁶) |
|-----------------------------|-------------|-----------|--------------|-------------------|----------------|
| wt (n = 9)                  | 2.4 ± 0.8   | 8.9 ± 3.0 | 5.4 ± 0.9    | 7.2 ± 2.4         | 27.7 ± 5.1     |
| IgαFF/FF (n = 8)            | 3.0 ± 1.0   | 7.7 ± 2.3 | 5.8 ± 1.5    | 4.3 ± 1.7         | 23.0 ± 4.6     |
| IgαΔkimutant (n = 5)        | 5.0 ± 0.4   | 0.82 ± 0.18 | 0.54 ± 0.07 | 0.03 ± 0.01       | 0.21 ± 0.12    |
| IgβΔkimutant (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.

The levels of surface expression were determined by comparing the mean fluorescence intensities between B cells of wild-type (set as 100%) and IgαFF/FF mice. Numbers represent the mean percentages (± SD) based on the number of indicated analyses. In each single analysis, samples from IgαFF/FF 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 II. Comparison of Relative Expression Levels of Various B Cell Surface Molecules in Wild-Type and IgαFF/FF Mice

| Antigen | B cell population | Surface expression in IgαFF/FF mice relative to wild-type (% ± SD) |
|---------|-------------------|---------------------------------------------------------------|
| IgM     | BM                | IgM+*B220brigh 144.5 ± 35.0 n = 10 *                           |
|         |                   | IgM+*B220brigh 248.2 ± 58.6 n = 15 **                          |
|         | Spleen            | 205.5 ± 33.8 n = 11 **                                        |
|         | Blood             | 222.3 ± 13.9 n = 5 **                                         |
|         | Peritoneum B1 cells | 171.5 ± 37.8 n = 8 *                                         |
| IgD     | BM                | 126.9 ± 9.3 n = 10 **                                         |
|         | Spleen            | 132.6 ± 12.0 n = 11 **                                        |
|         | Blood             | 127.9 ± 17.7 n = 5 #                                          |
| B220    | BM                | 99.2 ± 5.4 n = 4                                             |
|         | Pre-B             | IgM+*B220brigh 96.6 ± 5.4 n = 10 **                           |
|         |                   | IgM+*B220brigh 91.1 ± 6.0 n = 12 **                           |
|         | Spleen            | 88.1 ± 6.9 n = 12 **                                         |
|         | Blood             | 86.2 ± 6.8 n = 5 #                                           |
| CD19    | Spleen            | 116.6 ± 5.9 n = 15 **                                        |
|         | Blood             | 122.9 ± 11.4 n = 5 *                                         |
|         | Peritoneum B1 cells | 117.7 ± 13.5 n = 6 #                                       |
| CD22    | Spleen            | 81.8 ± 4.8 n = 10 **                                         |
|         | MHCII             | 102.0 ± 28.8 n = 5                                          |

460 Igα ITAM Phosphorylation Mutant Mice
Higher Percentage of $\lambda_1$ Light Chain Expressing B Cells in $\text{Ig}\alpha$ and $\text{Ig}\beta$ Mutant Mice. As in the BM of $\text{Ig}\alpha\text{FF/FF}$ mice, all B cell fractions were present in normal numbers, the lack of $\text{Ig}\alpha$ 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, $\text{Ig}\alpha\text{FF/FF}$ 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 $\text{Ig}\alpha$ and $\text{Ig}\beta$ (Table III).

Reduced IgG1 Serum Levels and Impaired TD Immune Response in $\text{Ig}\alpha\text{FF/FF}$ Mice. Altered BCR signaling in $\text{Ig}\alpha\text{FF/FF}$ 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 $\text{Ig}\alpha\text{FF/FF}$ 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 $\text{Ig}\alpha$ ITAM mutants did not result in reduced serum IgM concentration. However, the serum concentration of IgG1 in $\text{Ig}\alpha\text{FF/FF}$ mice was significantly lower than in wild-type controls.

Table III. Fraction of $\lambda_1$ Light Chain Expressing Spleen B Cells in $\text{Ig}\alpha$ and $\text{Ig}\beta$ Mutant Mice

| Mice          | Fraction $\lambda_1$ (%) | n  |
|---------------|--------------------------|----|
| wt            | 4.8 ± 0.6%               | 13 |
| $\text{Ig}\alpha\text{FF/FF}$ | 10.9 ± 3.1%              | 10 |
| $\text{Ig}\alpha\Delta/\Delta$ | 22.1 ± 3.1%              | 7  |
| $\text{Ig}\alpha\Delta/\Delta$ | 20.4 ± 5.8%              | 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 ± SD of the indicated numbers of mice.

Figure 3. Serum Ig levels and humoral immune responses in $\text{Ig}\alpha\text{FF/FF}$ mice. Serum Ig isotype levels were determined by ELISA. Filled symbols represent wild-type and open symbols represent $\text{Ig}\alpha\text{FF/FF}$ mice. (A) Geometric means and standard deviations of serum levels of Ig 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 $\text{Ig}\alpha\text{FF/FF}$ 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 $\text{Ig}\alpha\text{FF/FF}$ 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 Igα ITAM phosphorylation is not required for an efficient TI-II response (Fig. 3 B). IgαFF/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 Igα ITAM phosphorylation is not a prerequisite for B cell memory formation. However, the TD response was significantly lower in IgαFF/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 IgαFF/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 Igα 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 Igα leads to an elevated and more sustained calcium response in immature B cells of Igα+/ΔIgHEL mice compared with controls (23). We show here that this effect appears to be correlated with the phosphorylation of the Igα ITAM, as splenic B cells from IgαFF/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 Igα in IgαFF/FF B Cells. To extend the analysis of the signaling capacity of Igα ITAM mutant BCRs, we analyzed protein tyrosine phosphorylation after BCR cross-linking. Splenic B cells were isolated from IgαFF/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 Igα ITAM tyrosines were still capable of inducing protein tyrosine phosphorylation to levels comparable to controls. We then specifically examined the tyrosine phosphorylation of Igα and Igβ in anti-Igα immunoprecipitates before and after BCR cross-linking. As shown in Fig. 5 B, BCR ligation of IgαFF/FF B cells resulted in a rapid and persistent tyrosine phosphorylation of Igβ ITAM. Moreover, BCR–induced tyrosine phosphorylation of Igβ in IgαFF/FF mice was enhanced compared with controls (Fig. 5 A and B). Whereas Igβ possesses only the two tyrosines of its ITAM, the cytoplasmic tail of Igα contains four tyrosine residues (Fig. 1 E). Interestingly, Igα phosphorylation in Igα ITAM mutant B cells was detected after BCR ligation (Fig. 5 B). These data suggest that either one or both non-ITAM Igα tyrosine residues (#176 and #204) can be phosphorylated after

![Figure 4](image-url)
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 Igα ITAM were still able to induce Syk tyrosine phosphorylation, presumably via phosphorylated Igβ. However, maximum phosphorylation was reached at a later time point than in wild-type controls (Fig 5 D). Examination of Lyn phosphorylation showed that basal tyrosine phosphorylation was similar between IgαFF/FF and wild-type B cells, but BCR-induced Lyn tyrosine phosphorylation was less than in wild-type controls (Fig 5 C).

**Figure 5.** Analysis of protein tyrosine phosphorylation in splenic B cells of IgαFF/FF mice. Cell lysates of purified splenic B cells from IgαFF/FF and wild-type mice were prepared from cells that were either mock stimulated with medium or stimulated with 5 μg/10^6 cells F(ab)’2 rabbit anti-mouse Ig for different periods of time. (A) Tyrosine phosphorylation of total cellular proteins (5 x 10^6 cells/lane) was detected by anti-phosphotyrosine immunoblotting. Arrows indicate the expected positions of Igα and Igβ. (B) Phosphotyrosine immunoblot of Igα immunoprecipitates. The bottom panel shows the same blot reprobed with an anti-Igβ 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.

**Collective Block of B Cell Development in IgαFF/FF Igβκ/Δ Mice.** Targeted mutations of Igα, as in truncation of the cytoplasmic tail in Igακ/Δ mice (21) or with specific loss of ITAM phosphorylation in IgαFF/FF mice, lead to alteration in development and responsiveness of B lymphocytes. Similarly, a cytoplasmic truncation of Igβ in Igβκ/Δ mice compromises B cell development and causes a strong reduction in mature B cells (22, Fig 6). However, neither the lack of Igα nor Igβ ITAM alone leads to a complete block in B cell development. A cytoplasmic domain of either Igα or Igβ is still sufficient to signal (22). Based on the phenotypic differences between Igακ/Δ and IgαFF/FF mice and the finding that Igα may be phosphorylated at non-ITAM tyrosines, Igα may not mediate signaling exclusively through its phosphorylated ITAM. Whether development of B cells in the Igα and Igβ truncation mutants was absolutely dependent on the phosphorylation of the remaining BCR ITAM remained unclear. To answer this question, we generated IgαFF/FF Igβκ/Δ mice. In comparison to Igβκ/Δ mice, the double mutants possessed no intact ITAM. As shown in Fig. 6 A, 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® analysis of the B220^+CD43^- pro-B cell fraction revealed the presence of fractions A to C, but fraction C’ (BP^+HSA^bright) was absent (Fig. 6 B). In the periphery of IgαFF/FF Igβκ/Δ mice, a few CD19^+ cells were still detectable (Fig. 6 C), 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 IgαFF/FF Igβκ/Δ mice was further validated by the absence of any detectable serum Igs (data not shown).

Collectively, these data indicate an absolute requirement of Igα ITAM phosphorylation in Igβκ/Δ 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 Igα/Igβ heterodimer for surface expression, internalization, antigen presentation, and signaling. Therefore, a targeted ablation of the cytoplasmic effector domain of either Igα or Igβ may result in impaired B cell development and function. This is indeed evident in both Igακ/Δ and Igβκ/Δ mice which exhibit a drastic reduction in peripheral B cell numbers (21, 22). To date, the main signaling function of the Igα/Igβ heterodimer has been ascribed to its two ITAMs.
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>FF/Fb</sup> mice. A lack of Igα<sup>ITAM</sup> 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 Igα<sup>ITAM</sup> 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 association of Src family kinases with Igα/Igβ (36). In addition, the Igα molecules in Igα<sup>ΔαΔ</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>ΔαΔ</sup> and Igα<sup>FF/Fb</sup> mice. Alternatively, lack of the Igα cytoplasmic domain in Igα<sup>ΔαΔ</sup> mice might influence the structure of the remaining Igβ tail and distort its ITAM function. The different phenotype of Igα<sup>FF/Fb</sup> mice could thus also reflect a difference in Igβ ITAM function.

**Altered Surface Antigen Expression in B Cells of Igα<sup>FF/Fb</sup> Mice Due to Adaptation or Selection.** B cells from Igα<sup>FF/Fb</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 Igα ITAM phosphorylation. Third, Igα ITAM phosphorylation might directly control BCR surface expression or internalization. The latter possibility is supported by the analysis of Igα mutant molecules in cell cultures. While in reconstitution experiments using the J558Lμm cell line, neither a cytoplasmic deletion nor Igα-ITAM tyrosine mutations interfered with the surface expression of BCR (10), constitutive receptor endocytosis appeared to be dependent on the cytoplasmic tail of Igα in a chimeric system (38). Similarly, analyzing FcyRII-Igα or Igβ chimeric molecules in the A20/IIA1.6 cell line, efficient constitutive BCR internalization was only mediated by the cytoplasmic domain of Igα and was shown to be dependent on the first Igα ITAM tyrosine (38).

Interestingly, B cells in IgαΔ/c−/− mice do not exhibit a similar increase in surface BCR expression levels as in IgαFF/FF mice, and the cytoplasmic truncation of Igβ in IgβΔ/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 ligo/Igβ-dependent turnover mechanisms and in addition are adjusted to the specific signaling requirements of the B cells.

In line with the idea that Igα ITAM phosphorylation is required for efficient activation of Src family kinases are the observations of an increased CD19 and a decreased CD22 ITAM phosphorylation in the activation of PTKs after BCR ligation (Fig. 4). One might interpret the data such that Igα 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 Igα ITAM mutation. Similarly, Lyn was inducibly tyrosine phosphorylated in IgαFF/FF B cells, but to a lesser extent than in controls. An important novel finding is the demonstration of Igα tyrosine phosphorylation apart from its ITAM. This suggests an involvement of the Igα non-ITAM tyrosines in BCR-dependent signaling. These might recruit distinct signaling proteins or alternatively enhance or inhibit the association of molecules with the Igα/Igβ 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 Igα in IgαFF/FF B cells supports the idea that the ITAM tyrosines are the main tyrosine phosphorylation sites in Igα (10). However, in the wild-type situation, phosphorylation of the non-ITAM tyrosines could well be linked to and dependent on Igα ITAM phosphorylation. A clarification of these matters will require a biochemical analysis which is beyond the scope of this paper.

Igα ITAM Phosphorylation and Ca2+ Mobilization. The enhanced Ca2+−flux in B cells of IgαFF/FF mice after BCR ligation seems to contradict the idea that Igα ITAM phosphorylation functions to amplify signaling. Previous transfection experiments with FcyRII-Igα or FcyRII-Igβ chimeric molecules in the IIA1.6 cell line demonstrated that only the Igα cytoplasmic tail was capable to mobilize Ca2+ from intracellular stores and influx of extracellular calcium. In contrast, the Igβ cytoplasmic domain stimulated only an oscillatory release from intracellular stores, which overall resulted in a small increase of free intracellular Ca2+ (9, 51). Further analyses specified an absolute requirement of the proximal Igα ITAM tyrosine (Y182) for Ca2+ mobilization (9, 13). Thus, it was surprising to find an even stronger Ca2+ mobilization in splenic B cells of IgαFF/FF mice after BCR ligation (Fig. 4). One might interpret the data such that Igα ITAM phosphorylation is required to contain and terminate Ca2+ mobilization. While the increased Ca2+ mobilization in B cells of IgαFF/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).

Igα/Igβ Mutations Distort Light Chain Rearrangements. Although we do not yet know whether the Igα ITAM mutation leads to enhanced editing of autoreactive BCRs like the Igα truncation (23), it is noteworthy that both mutations distort the k to λ ratio in favor of λ light chain expression. One might speculate that quantitative and qualita-
tive alterations of signals transmitted by a mutant Igα/Igβ 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 λ chain expression, if κ rearrangements usually preceed λ rearrangements (55). In line with this interpretation are the observations that the shift in favor of λ chain expression is more pronounced in the tail truncation mutants than in IgαFF/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 λ locus. In an extreme scenario this could lead to the exclusive rearrangement of the λ locus in λ chain–expressing cells. We tested this hypothesis by analyzing the rearrangement status of the κ loci in λ1+ splenic B cells. However, similar to the situation in λ1+ cells from wild-type mice (56), the κ light chain loci were found to be rearranged in λ1+ cells of IgαFF/FF mice (data not shown). Finally, it might be possible that λ light chains confer a selective advantage for B cells in case of distorted BCR signaling.

**Dependence of B1 and MZ B Cell Populations on Igα ITAM Phosphorylation.** In the peritoneal cavity of IgαFF/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 IgαFF/FF mice could thus be explained by enhanced negative selection of autoreactive BCR specificities, but would also be consistent with the idea that Igα 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 B1 cell population. An opposing phenotype is seen for mice expressing truncated Igα or Igα chain. The simplest interpretation of these results is that there is significant functional redundancy between the Igα and Igβ ITAMs (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-B cell stage 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/Igα and IgM/Igβ 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.

Igα 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 Igα and Igβ tail truncation mutants (21, 22), we expected an impairment of the anti-NP-Ficoll response in IgαFF/FF mice. However, the selective elimination of the Igα 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 Igα 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 Igα 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 Igα 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 IgαFF/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 Igα ITAM and the Igβ cytoplasmic tail. This contrasts with an almost unimpaired early B cell development in the Igα ITAM mutant and significant pre-B cell promotion in mice expressing a truncated Igβ or Igα chain. The simplest interpretation of these results is that there is significant functional redundancy between the Igα and Igβ 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-B cell stage 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/Igα and IgM/Igβ 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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