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A Negative Regulatory Role for Ig-α during B Cell Development

Raul M. Torres* and Katrin Hafen
Basel Institute for Immunology
Grenzacherstrasse 487
CH-4005 Basel
Switzerland

Summary

The development of B cells requires the expression of an antigen receptor at distinct points during maturation. The Ig-α/β heterodimer signals for these receptors, and mice harboring a truncation of the Ig-α intracellular domain (*mb-1*$^{\Delta c/\Delta c}$) have severely reduced peripheral B cell numbers. Here we report that immature *mb-1*$^{\Delta c/\Delta c}$ B cells are activated despite lacking a critical Ig-α-positive signaling motif. As a consequence of abnormal activation, transitional immature IgM^{high}IgD^{low} B cells are largely absent in *mb-1*$^{\Delta c/\Delta c}$ mutants, accounting for the paucity of mature B cells. Thus, Ig-α cytoplasmic tail truncation yields an antigen receptor complex on immature B cells that signals constitutively. These data illustrate a role for Ig-α in negatively regulating antigen receptor signaling during B cell development.

Introduction

The expression of distinct B cell antigen receptor (BCR) complexes is strictly monitored at early and late points during B lymphocyte maturation. These so-called checkpoints for antigen receptor expression ensure that only useful B cells continue to develop and prevent self-reactive lymphocytes from entering into the mature peripheral B cell pool (Rajewsky, 1996).

The early and late forms of the antigen receptor complex have an identical core structure that is composed of an immunoglobulin (Ig) μ heavy chain noncovalently associated with an Ig-α/β heterodimer. The pre-B cell receptor (pre-BCR) is the early form of the antigen receptor complex expressed by B cell progenitors. In the pre-BCR, Ig μ heavy chain pairs with the surrogate light chain; the latter structure composed of the λ*5* and V_{preB} gene products (Karasuyama et al., 1996). At the subsequent precursor-B (pre-B) cell developmental stage, an IgM is produced by the replacement of surrogate light chain with κ or λ conventional Ig light chain. The resulting IgM is deposited on the cell surface with the Ig-α/β heterodimer as a BCR complex. By definition the expression of surface IgM (sIgM) by these cells identifies them now as immature B cells. However, IgM is expressed at variable levels on immature B cells, and those cells that express the highest levels of IgM also coexpress low-to-intermediate levels of IgD. IgM^{high}IgD^{low} immature B cells are transitional immature B cells and have been shown to represent a distinct stage in B cell development (Carsetti et al., 1995; Melamed et al., 1998) from which B cells destined to emigrate to the periphery are selected (Allman et al., 1993). Newly formed IgM$^+$ B cells must express a functional BCR before leaving the bone marrow. This requirement has long been evident by the fact that sIgM-negative pre-B cells are not observed in the periphery under normal circumstances. This late B cell developmental checkpoint has also been demonstrated by a mouse mutant, *mb-1*$^{\Delta c/\Delta c}$, that expresses a signaling impaired BCR. In these mutants, the mature peripheral B cell pool is significantly reduced in cell number (Torres et al., 1996).

The Ig-α/β heterodimer is an integral component of the BCR and is required for both surface expression and signaling (Reth, 1992; Cambier et al., 1993; Williams et al., 1994; DeFranco, 1997). Whether Ig-α and Ig-β make qualitatively different contributions to BCR signaling or are functionally redundant is not yet clear, as evidence exists to support both views. Distinct cytosolic signaling molecules have been found that differentially associate with either Ig-α or Ig-β (Clark et al., 1992), and, consistent with this, studies with cell lines have attributed distinct biochemical events with these molecules (Kim et al., 1993; Sanchez et al., 1993; Choquet et al., 1994; Luisiri et al., 1996; Pao et al., 1998). However, this conclusion has not been supported by all in vitro studies (Law et al., 1993; Taddie et al., 1994; Williams et al., 1994) nor in vivo, with respect to the ability of chimeric Ig-α or Ig-β receptors to drive early B cell development (Papavasiliou et al., 1995a; Teh and Neuberger, 1997). In the latter studies, independent chimeric IgM/Ig-α and IgM/Ig-β receptors were equally capable of promoting B cell development and signaling allelic exclusion when expressed as transgenes.

Irrespective of potential differences in their signaling functions, the signaling capacity of both Ig-α and Ig-β has been shown to reside with an immunoreceptor tyrosine-based activation motif (ITAM) found within the cytoplasmic portion of both molecules. Mutation of the tyrosines in either Ig-α or Ig-β ITAM inhibits early BCR-mediated signal transduction events upon receptor engagement in cell lines (Sanchez et al., 1993; Flaswinkel and Reth, 1994; Taddie et al., 1994; Williams et al., 1994). In accord with these results, mice expressing a chimeric IgM/Ig-β transgenic receptor exhibit normal B cell maturation and allelic exclusion but not when the IgM/Ig-β chimeric receptor harbors mutated ITAM tyrosines (Papavasiliou et al., 1995a, 1995b; Teh and Neuberger, 1997). Thus, as suggested from cell line studies, an intact Ig-β ITAM is apparently also required for appropriate BCR signaling function during development.

The biochemical and molecular events that follow sIg engagement on a B cell has been the subject of intense investigation. Clearly the type of antigen, mode of activation, developmental stage, as well as the environment in which the B cell is activated can influence the resulting signal transduction cascade (DeFranco, 1997). Despite the numerous signaling pathways that may result from sIg engagement, the activation of protein tyrosine kinases and subsequent phosphorylation of the Ig-α/β

*To whom correspondence should be addressed (e-mail: torres@bii.ch).

ITAMs is believed to be an obligate feature in the initiation of virtually all BCR signal transduction scenarios (Weiss and Littman, 1994; DeFranco, 1997; Clements and Koretzky, 1999; Kurosaki, 1999). Tyrosine phosphorylation of Ig-α and Ig-β, presumably by Src family tyrosine kinases, allows SH2-containing proteins including the Syk tyrosine kinase, to bind to phosphorylated ITAMs and is an early step in delivering sIg signals to the nucleus.

To investigate the requirements of antigen receptor signaling in B cell development, we have previously generated a mouse mutant, *mb-1*$^{\Delta c/\Delta c}$, that encodes a truncated Ig-α cytoplasmic domain and that prevents the expression of the ITAM. Consistent with previous studies demonstrating the importance of the Ig-α/β ITAMs in antigen receptor signaling and development, these animals produce very few mature B cells (Torres et al., 1996). The B cell deficiency in these animals has been shown to be the cumulative result of inefficient progression by B cell precursors through at least two points in bone marrow B cell development, both of which coincide with antigen receptor checkpoints. In the present study we have examined the basis underlying the inefficient production of *mb-1*$^{\Delta c/\Delta c}$ peripheral B cells in vivo and in vitro using a recently established reaggregation organ culture system. The results from these experiments demonstrate that *mb-1*$^{\Delta c/\Delta c}$ immature B cells are unexpectedly activated and, as a consequence, are arrested in development at the point at which IgD expression commences in normal development. Thus, in the absence of the Ig-α cytoplasmic domain, immature B cells are activated and B cell development is arrested through a process that appears analogous to that imposed on B cells expressing a self-reactive receptor.

Results

B Cell Development with a Truncated Ig-α Results in Fewer Immature B Cells that Are Inefficiently Exported to the Periphery

mb-1$^{\Delta c/\Delta c}$ B lineage cells harbor a truncated Ig-α molecule due to the insertion of a stop codon at residue 181 and only express the first 20 of the 61–amino acid cytoplasmic tail. Importantly, neither of the tyrosines within the Ig-α ITAM is present in *mb-1*$^{\Delta c/\Delta c}$ B lineage cells. Considering the relatively few B lineage cells that exist in *mb-1*$^{\Delta c/\Delta c}$ animals (see below), direct demonstration of the truncated Ig-α was facilitated by the introduction of a *bcl-2* transgene into an *mb-1*$^{\Delta c/\Delta c}$ background. Thus, without overtly protecting any particular developmental stage, *mb-1*$^{\Delta c/\Delta c}$; *bcl-2* mice have an increase in the overall number of bone marrow IgM$^+$ cells (data not shown). Figure 1A shows that anti-Ig-β immunoprecipitation of wild-type bone marrow cells reveals an approximate 34 kDa Ig-α polypeptide and two species of approximately 36 and 39 kDa for Ig-β, as detected after reprobing the membranes with respective anti-Ig-α and anti-Ig-β antibodies (data not shown). In contrast, the Ig-α immunoprecipitated with anti-Ig-β from *mb-1*$^{\Delta c/\Delta c}$; *bcl-2* transgenic bone marrow has a molecular weight of approximately 20 kDa (Figure 1A) and is consistent with the predicted truncation. Interestingly, the Ig-β expressed by *mb-1*$^{\Delta c/\Delta c}$ bone marrow cells is found predominantly as a 34 kDa species and is similar to an

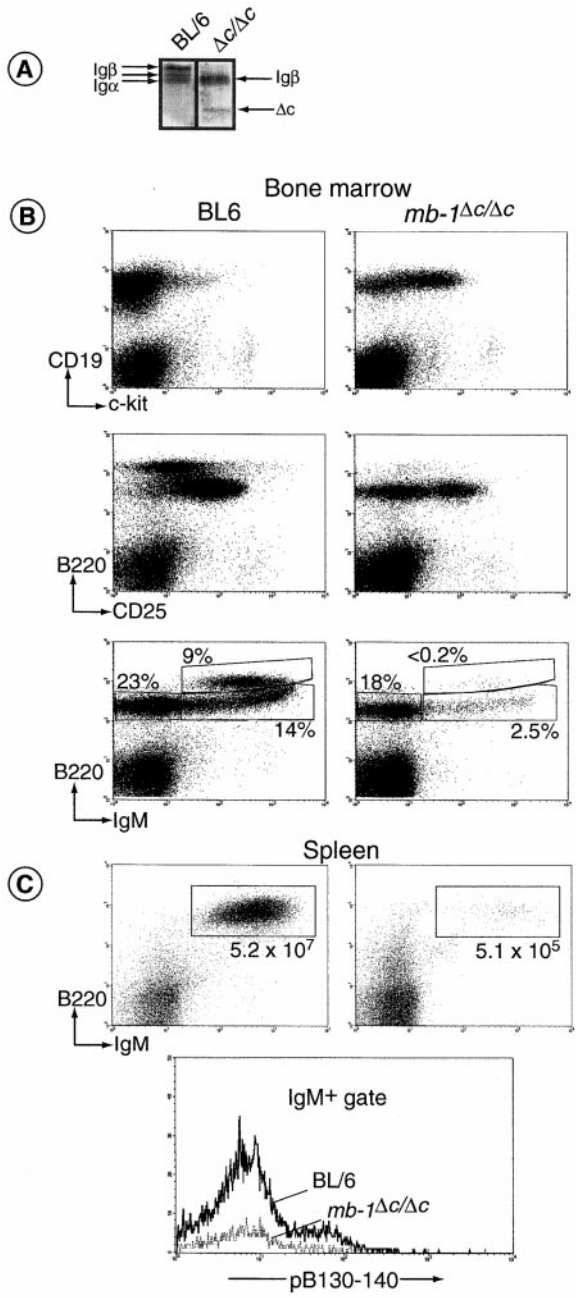

Figure 1. B Cell Development in the Presence of the *mb-1*$^{\Delta c}$ Mutation Results in a Severely Reduced Peripheral B Cell Pool

(A) Immunoprecipitation of Ig-β from surface-biotinylated *mb-1*$^{\Delta c/\Delta c}$; *bcl-2* transgenic total bone marrow reveals a molecular weight of approximately 20 kDa for Ig-α due to the *mb-1*$^{\Delta c}$ mutation.

(B) Flow cytometric analysis of wild-type and *mb-1*$^{\Delta c/\Delta c}$ bone marrow for the developmentally restricted antigens c-kit, CD25, and IgM. Percentages are of total bone marrow cells taken from both femurs ($n = 5$; B6 = $4.1 \times 10^7 \pm 3.1 \times 10^3$; *mb-1*$^{\Delta c/\Delta c}$ = $4.0 \times 10^7 \pm 4.0 \times 10^3$).

(C) Expression of B220 and IgM on B6 and *mb-1*$^{\Delta c/\Delta c}$ splenocytes. Mutant IgM$^+$ B cells are reduced in cell number >100-fold (5.1×10^5 versus 5.2×10^7, respectively) relative to wild type, although they display similar proportions of pB130–140 antigen (15% versus 19%, respectively; bottom histogram).

Ig-β isoform previously found in primary splenic B cells (Friedrich et al., 1993). This 34 kDa Ig-β represents an alternatively glycosylated form of Ig-β that was selectively recovered from low-density splenic B cells, and it was suggested that it may preferentially be associated with the BCR from activated cells (Cambier et al., 1993).

Flow cytometric analysis of wild-type and *mb-1*$^{\Delta c/\Delta c}$ bone marrow and spleen is shown in Figures 1B and 1C. As previously reported (Torres et al., 1996), this representative analysis demonstrates that *mb-1*$^{\Delta c/\Delta c}$ mutants display inefficient bone marrow B lymphopoiesis. In particular, the transition of mutant B220$^+$c-kit$^+$ progenitor B cells to B220$^+$CD25$^+$ pre-B cells is impaired and results in a 3- to 6-fold reduction in the number of pre-B and IgM$^+$ immature B cells. Furthermore, although up to 6-fold fewer in number, these newly generated IgM$^+$ immature B cells migrate with significantly reduced efficiency to the periphery, ultimately resulting in >100-fold reduction in the absolute number of mature peripheral B cells. Inefficient export of newly formed bone marrow immature B cells was initially indicated by the analysis of BrdU incorporation of splenic B cells following a short period of in vivo labeling (Torres et al., 1996). Additional evidence for decreased export of immature B cells from the bone marrow is provided by the expression of the pB130–140 antigen expression on splenic B cells as recognized by monoclonal antibody (mAb) 493. This mAb marks immature B cells in the spleen that have recently emigrated from the bone marrow (Rolink et al., 1998). *mb-1*$^{\Delta c/\Delta c}$ and wild-type mice display a similar frequency of pB130–140$^+$IgM$^+$ cells in the spleen (15% versus 19%, respectively), demonstrating that the number of *mb-1*$^{\Delta c/\Delta c}$ bone marrow immigrants is >100-fold decreased relative to wild-type (7.7×10^4 versus 9.9×10^6, respectively; Figure 1C).

The impaired production of pre-B and mature B cells in mutants coincides with those B cell developmental transitions that are dependent on the expression of a signaling-competent antigen receptor. Specifically, the pre-BCR is necessary for generating pre-B cells and a mature BCR is required for migration to the periphery. We have suggested that the deficiency in *mb-1*$^{\Delta c/\Delta c}$ B cell development is a consequence of an impaired ability to appropriately signal at the known checkpoints for antigen receptor expression (Torres et al., 1996).

Newly Formed *mb-1*$^{\Delta c/\Delta c}$ B Cells Do Not Mature to the Transitional Immature B Cell Stage

To examine the basis for the *mb-1*$^{\Delta c/\Delta c}$ B cell developmental defects in closer detail, we have employed a recently established reaggregation fetal liver organ culture (RFLOC) system. This in vitro cell culture system promotes the maturation of exogenously isolated B220$^+$c-kit$^+$ pro-B cells, when seeded into a fetal stromal microenvironment, to the IgM^{high}IgD^{low} transitional immature B cell stage of development (Figures 2A and 2B). Importantly, the *mb-1*$^{\Delta c/\Delta c}$ developmental blocks observed in vivo are recapitulated upon reaggregation of *mb-1*$^{\Delta c/\Delta c}$ pro-B cells with fetal liver stroma (Figure 2A, right panels). Similar to what is observed in vivo, RFLOC *mb-1*$^{\Delta c/\Delta c}$ B cell development results in an increased proportion of B220$^+$c-kit$^+$ pro-B cells and a concomitant 3- to 6-fold reduction in absolute numbers of B220$^+$CD2$^+$IgM$^-$ pre-B and IgM$^+$ immature B cells compared to controls after 6 days of culture.

When B cell maturation was directly compared in parallel cultures that were seeded with either mutant or wild-type pro-B cells, it was apparent that *mb-1*$^{\Delta c/\Delta c}$ immature B cells expressed reproducibly lower levels of sIgM relative to wild type (Figure 2A). Whereas wild-type cultures contained an immature B cell population composed of IgM^{low} and IgM^{high} cells, *mb-1*$^{\Delta c/\Delta c}$ immature B cells were predominantly IgM^{low}. Gating on the top one-third of wild-type immature B cells that expressed the highest levels of sIgM results in a 5-fold reduction in the proportion of mutant immature B cells that express similar IgM levels (Figure 2A). The reduction in sIgM expression by *mb-1*$^{\Delta c/\Delta c}$ immature B cells was not an artifact of maturation in RFLOCs. The same observation was made when pro-B cells were cultured on a stromal cell line in the presence of IL-7 and were induced to differentiate to immature B cells upon growth factor withdrawal (data not shown).

The expression of IgM on *mb-1*$^{\Delta c/\Delta c}$ bone marrow immature B cells ex vivo does not necessarily reflect the reduced levels of IgM found on the analogous population generated in vitro (compare Figures 2A and 1B). However, wild-type immature B cells that express the highest levels of IgM also coexpress low-to-intermediate levels of IgD (Figure 2B). IgM^{high}IgD^{low} immature B cells have been characterized as a distinct immature B cell stage in bone marrow B cell development (Melamed et al., 1998) referred to as transitional B cells (Carsetti et al., 1995). Consistent with the reduced expression levels of sIgM by *mb-1*$^{\Delta c/\Delta c}$ immature B cells generated in vitro, only ~7% of these mutant IgM immature B cells coexpress IgD compared with 25%–35% of wild-type immature B cells (Figure 2B). Furthermore, this difference is also observed in vivo. IgD expression by *mb-1*$^{\Delta c/\Delta c}$ immature bone marrow B cells ex vivo is also diminished relative to wild type and similar to that found in vitro (Figure 2B). These data demonstrate that the proportion of IgM^{high}IgD^{low} transitional immature B cells population is significantly reduced in mutant animals.

Reduced surface IgM expression by *mb-1*$^{\Delta c/\Delta c}$ immature B cells results from the truncation of the Ig-α cytoplasmic tail. Since Ig-α is important not only for BCR signaling but also for surface Ig expression, the reduction in IgM expression could conceivably result from either aberrant BCR signaling or an impairment in the assembly and/or transport of a BCR complex to the cell surface. We have previously established that, although reduced in number, splenic *mb-1*$^{\Delta c/\Delta c}$ B cells express equivalent levels of IgM compared to wild-type B cells (Torres et al., 1996). Figure 2C confirms this observation and extends it to IgD expression on peripheral *mb-1*$^{\Delta c/\Delta c}$ and wild-type B cells that carry a functionally rearranged IgH chain at the IgH locus (Pelanda et al., 1997). Thus, mutant B cells are capable of expressing surface IgM and IgD at levels equivalent to wild type.

Together these data show that in vitro and in vivo *mb-1*$^{\Delta c/\Delta c}$ B cell development is characterized by a significant diminution of the IgM^{high}IgD^{low} transitional immature B cell population, and this most likely reflects improper signaling by the mutant BCR.

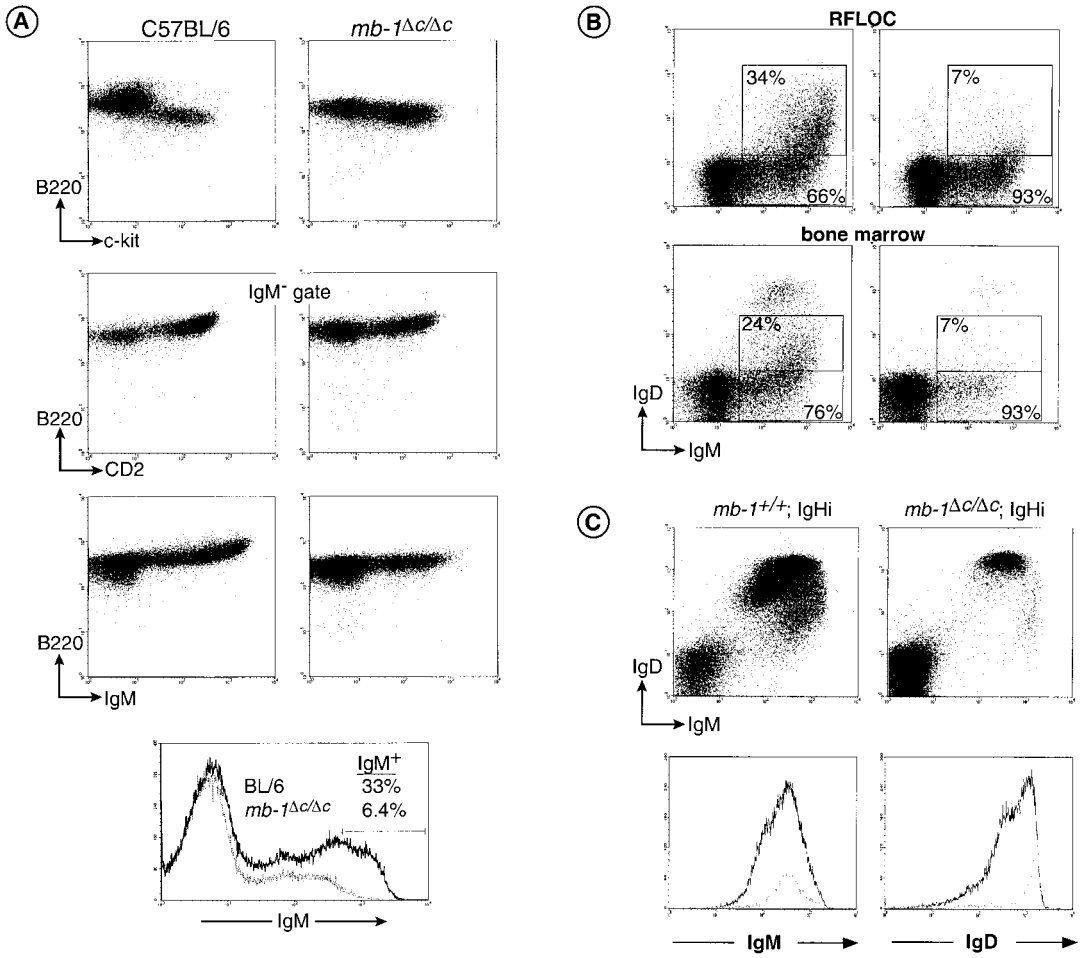

Figure 2. B Cell Development in Fetal Liver Organ Cultures Reveals *mb-1*$^{\Delta c/\Delta c}$ Pro-B Cells Do Not Mature to the IgM^{high}IgD^{low} Transitional Immature B Cell Stage

(A) In vitro B cell development of sorted B220$^+$c-kit$^+$ bone marrow wild-type (left panels) and *mb-1*$^{\Delta c/\Delta c}$ (right panels) pro-B cells reaggregated in fetal liver organ cultures. After 6 days of culture, cells were analyzed by flow cytometry for surface expression of the developmentally associated surface antigens B220, c-kit, CD2, and IgM.

(B) IgM and IgD expression on wild-type (left) and *mb-1*$^{\Delta c/\Delta c}$ (right) immature B cells from RFLOC (top) or bone marrow (bottom) reveals an absence of the IgM^{high}IgD^{low} transitional immature B cell population in mutant animals.

(C) Coexpression of IgM and IgD on splenocytes from wild-type (left) and *mb-1*$^{\Delta c/\Delta c}$ (right) animals that carry a targeted VDJ transgene. Histograms of CD19$^+$ splenocytes reveal comparable sIgM (left) and increased sIgD (right) levels on mutant (gray dotted lines) cells relative to wild type (solid black lines).

Development of Immature B Cells In Vitro in the Presence of a Constitutive BCR Signal

In an attempt to compensate for the presumed defective BCR signaling in *mb-1*$^{\Delta c/\Delta c}$ B cell development, RFLOC B cell maturation was followed in the presence of a constitutive BCR-mediated signal. To achieve this we used a recently described mAb that recognizes an extracellular epitope of Ig-β (Koyama et al., 1997). This mAb, when injected into RAG$^{-/-}$ mice, is capable of relieving the pro-B to pre-B cell developmental arrest that exists in those animals (Nagata et al., 1997).

Anti-Ig-β or control (145–2C11) mAb was added from the onset of wild-type and *mb-1*$^{\Delta c/\Delta c}$ organ cultures and B cell development assessed 7 days later. Addition of the anti-Ig-β mAb resulted in an increase in the number of c-kit$^+$ pro-B cells generated in both wild-type and mutant organ cultures (data not shown). Despite increasing pro-B cell numbers, the maturation of *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ immature B cells was not augmented with respect to either cell number or level of surface IgM expression (Figure 3). On the contrary, RFLOC B cell development in the presence of anti-Ig-β exaggerated the *mb-1*$^{\Delta c/\Delta c}$ developmental phenotype, resulting in a 2- to 4-fold reduction in the number and proportion of *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ B cells. Furthermore, the immature B cells that did develop expressed diminished levels of sIgM relative to control treated cultures (Figure 3E versus 3F).

The same was true for wild-type RFLOCs: in the presence of anti-Ig-β, fewer immature B cells were generated, which expressed reduced levels of surface IgM (Figures 3A and 3B). The reduction in sIgM levels in anti-Ig-β-treated wild-type cultures was also accompanied by a >3-fold decrease in the proportion of IgM$^+$IgD$^+$ immature B cells (Figures 3C and 3D). Interestingly, the presence of continual BCR stimulation in wild-type organ cultures generated IgM$^+$ immature B cells that were

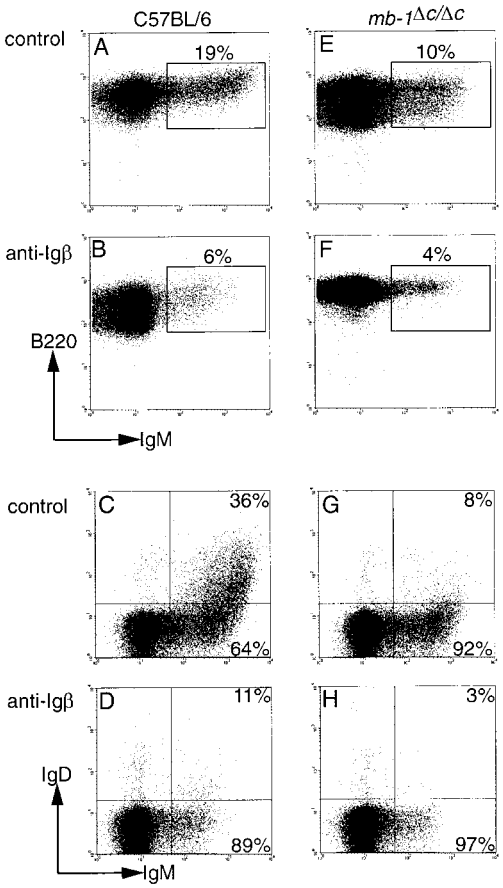

Figure 3. In Vitro B Cell Development in the Presence of Continual BCR Stimulation Yields Newly Formed Wild-Type and *mb-1*$^{\Delta c/\Delta c}$ Immature B Cells Expressing Diminished Levels of sIgM and sIgD

RFLOC maturation of wild-type (A–D) and mutant (E–H) B220^{+}c-kit^{+} pro-B cells in the presence of control (A, C, E, and G) or anti-Ig-β (B, D, F, and H) mAb. After 7 days of in vitro culture, cells were harvested, stained for the indicated antigens, and analyzed by flow cytometry.

remarkably similar to those found in untreated *mb-1*$^{\Delta c/\Delta c}$ organ cultures (compare Figure 3B to Figure 3E and Figure 3D to Figure 3G). The similarities between *mb-1*$^{\Delta c/\Delta c}$ and BCR-augmented wild-type B cell maturation include diminished numbers of immature B cells, decreased levels of surface IgM expression, and a reduced proportion of cells that coexpress IgD. These data suggested to us that the phenotype of *mb-1*$^{\Delta c/\Delta c}$ immature B cells, as well as the diminution in the numbers and proportions of this population, might be the result of hyperactive rather than insufficient BCR signaling during development.

mb-1$^{\Delta c/\Delta c}$ Newly Generated Bone Marrow B Cells Have Increased Levels of Tyrosine-Phosphorylated Proteins

We next directly examined whether *mb-1*$^{\Delta c/\Delta c}$ immature B cells were activated in vivo as might be expected in the presence of constitutive BCR signaling. In initial experiments, wild-type and mutant bone marrow cells were fixed in paraformaldehyde, permeabilized, stained for intracellular phosphotyrosine, and subsequently stained for surface IgM and B220 followed by flow cytometric analysis. As shown in Figure 4A, this analysis revealed that *mb-1*$^{\Delta c/\Delta c}$ immature B cells indeed had increased intracellular phosphotyrosine levels relative to control B220^{low}IgM^{+} immature B cells. The heightened intracellular phosphotyrosine level relative to control was reproducible (n = 4) and appeared specific for *mb-1*$^{\Delta c/\Delta c}$ immature IgM^{+} B cells, since the overall levels of intracellular phosphotyrosine in the B220^{low}IgM^{-} population was decreased in mutants compared to controls.

To confirm that *mb-1*$^{\Delta c/\Delta c}$ bone marrow immature B cells had in vivo elevated levels of tyrosine phosphorylation, immature cells from mutant and control strains were enriched by cell sorting and subjected to Western analysis (Figure 4B). To avoid BCR engagement in the purification of this population, cells were sorted as a B220^{low}c-kit^{-} population and further gated to include only those cells with small forward size scatter. The resulting population of cells from this isolation protocol is devoid of B220high mature B cells but contains a large proportion of sIgM^{-} pre-B cells. However, given that the ratio of pre-B to immature B cells is approximately the same in control and mutant animals, the inclusion of these cells would be expected to influence the analysis of both strains similarly. Consistent with the flow cytometric analysis, Figure 4B directly demonstrates that *mb-1*$^{\Delta c/\Delta c}$ immature B cells display increased levels of tyrosine phosphorylated substrates compared to wild-type immature B cells. These analyses also revealed distinct phosphorylated substrates between mutant and wild-type cells (e.g., ~70 kDa protein in Figure 4B); however, the appearance of these differentially phosphorylated substrates varied in different experiments. We conclude that the Ig-α truncation results in a quantitative increase in tyrosine phosphorylation.

Under normal circumstances the sIg expressed on B lymphocytes is continually internalized and replaced at the surface with newly synthesized Ig. An inability to internalize sIgM could be envisioned to lead to sustained BCR expression, signaling, and, consequently, activation. However, this was considered unlikely, as a deficiency in sIg internalization would be predicted to yield B cells with increased sIgM levels. Nevertheless, this possibility was directly examined, and mutant immature bone marrow B cells were found to downregulate IgM with similar kinetics and to similar levels compared to the same wild-type populations in response to IgM engagement (Figure 4C).

These data together demonstrate that truncation of the Ig-α cytoplasmic tail, including the ITAM, results in activated immature B cells and that this heightened state of activation does not arise from sustained BCR expression. These data further suggest that Ig-α and, specifically, its cytoplasmic tail, have a role in repressing the BCR signaling at the IgM^{+} immature B cell stage of development.

mb-1$^{\Delta c/\Delta c}$ Immature B Cells Express the B7-2 Activation Antigen

We have previously reported that peripheral *mb-1*$^{\Delta c/\Delta c}$ B cells display a resting mature phenotype that includes essentially no expression of the CD86 antigen (Figure

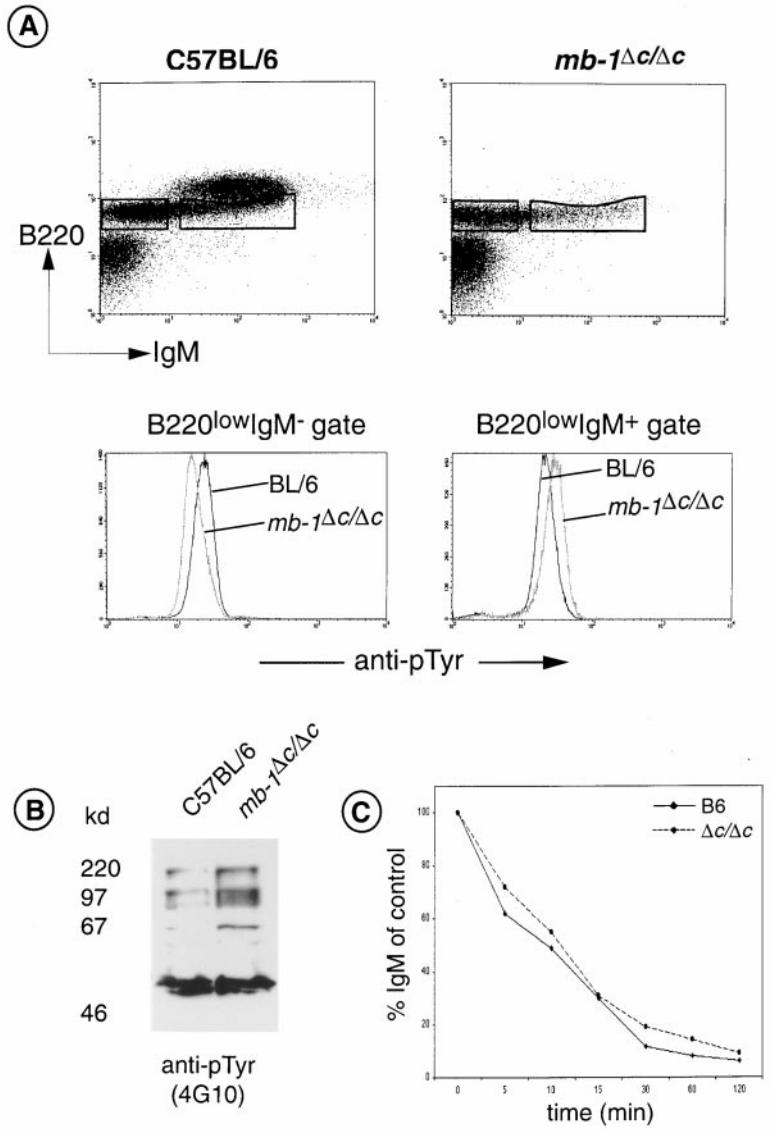

Figure 4. *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ Immature B Cells Are Activated In Vivo as Demonstrated by Elevated Intracellular Phosphotyrosine Levels

(A) Flow cytometric analysis of intracellular phosphotyrosine levels in wild-type and *mb-1*$^{\Delta c/\Delta c}$ B220^{low}IgM$^+$ immature B cells. BL6 and *mb-1*$^{\Delta c/\Delta c}$ total bone marrow cells were fixed, permeabilized, stained intracellularly with a mAb recognizing phosphotyrosine, and subsequently stained for surface B220 and IgM. Cells were gated either on B220^{low}IgM$^-$ cells (left histograms) or B220^{low}IgM$^+$ cells (right histograms). The latter gate is conservative to ensure the exclusion of mature B cells. Wild-type and *mb-1*$^{\Delta c/\Delta c}$ cells are represented by black solid and gray dotted lines, respectively.

(B) Small, B220^{low}c-kit$^-$ bone marrow cells were sorted from control and mutant mice and equivalent protein from whole cell lysates run on an 8% acrylamide gel. After transfer, membranes were probed with an anti-phosphotyrosine antibody and revealed by chemiluminscence.

(C) Wild-type (solid line) and *mb-1*$^{\Delta c/\Delta c}$ (dashed line) immature B cells downmodulate IgM to comparable levels and with similar kinetics.

5A; Torres et al., 1996). However, having shown that bone marrow *mb-1*$^{\Delta c/\Delta c}$ immature B cells are activated in vivo, we sought to determine whether any activation antigens may be present on *mb-1*$^{\Delta c/\Delta c}$ newly generated IgM$^+$ B cells. C57BL/6 and *mb-1*$^{\Delta c/\Delta c}$ bone marrow cells were isolated and the expression of a number of mature B cell activation antigens was examined. While essentially little or no difference was observed in the expression of CD69, class II, and CD80 between mutant or control immature B cells, there was a clear upregulation in the expression of the CD86 antigen on all B220^{low}IgM^{low} B cells in *mb-1*$^{\Delta c/\Delta c}$ bone marrow (Figure 5B; data not shown). In contrast, the equivalent IgM^{low} immature B cell population from C57BL/6 bone marrow did not express appreciable levels of CD86 unless stimulated via the BCR overnight (Figure 5C). CD86 is a well-characterized activation antigen whose expression is upregulated on mature B cells upon BCR engagement (Hathcock et al., 1994; Lenschow et al., 1994). These data provide additional evidence that *mb-1*$^{\Delta c/\Delta c}$ immature IgM$^+$ B cells are activated in vivo and that BCR stimulation of wild-type immature B cells recapitulates the *mb-1*$^{\Delta c/\Delta c}$ phenotype.

Discussion

To investigate how Ig-α contributes to antigen receptor signaling in vivo, we have previously generated a mouse mutant, *mb-1*$^{\Delta c/\Delta c}$, that lacks the carboxy-terminal two-thirds of the Ig-α cytoplasmic tail (Torres et al., 1996). Consistent with the large body of evidence establishing ITAMs as mandatory for antigen receptor signaling, B lymphopoiesis in these mutants is severely compromised. Considering the lack of the Ig-α ITAM, we initially attributed the impaired maturation to inefficient antigen receptor signaling at both early and late developmental transitions. The latter transition involves the migration of newly formed immature B cells to the periphery and their recruitment into the mature B cell pool. Although *mb-1*$^{\Delta c/\Delta c}$ bone marrow harbors up to 6-fold fewer immature B cells compared to controls, the peripheral B cell

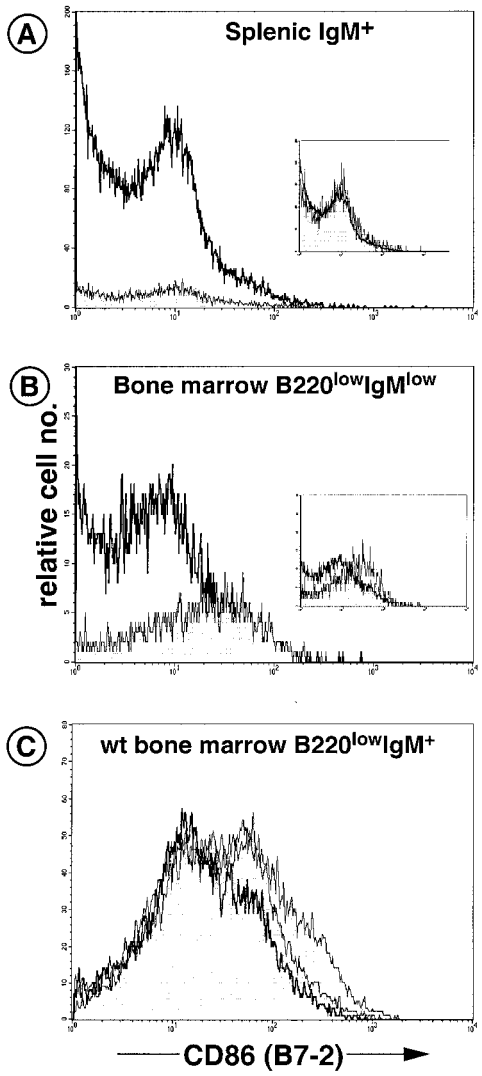

Figure 5. CD86 Expression by *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ Immature but Not Mature B Cells and Induction on Wild-Type Immature B Cells

Expression of CD86 was examined on splenic IgM$^+$ B cells (A) or bone marrow B220^{low}IgM^{low} immature B cells (B) from C57BL/6 (solid black lines) and *mb-1*$^{\Delta c/\Delta c}$ (shaded gray) animals. Histogram insets represent the same data but normalized for cell numbers. (C) Purified C57BL/6 B220^{low}IgM$^+$ immature B cells were sorted and placed in culture overnight with either medium (solid line), 0.05 μg/ml (thin line), or 0.5 μg/ml (shaded gray) of anti-IgM followed by examination of CD86 expression.

pool is diminished >100-fold in cell number. This reduction can be partly explained by an altered lifespan of peripheral B cells as they do not accumulate with age (data not shown). However, bone marrow export of *mb-1*$^{\Delta c/\Delta c}$ immature B cells is also decreased relative to production as indicated by short-term BrdU-labeling experiments (Torres et al., 1996) and proportion of splenic pB130–140$^+$ (Figure 1C) (Rolink et al., 1998). The experiments presented here identify the basis for this inefficient export and indicate that Ig-α has an additional role in B cell development besides positive signaling via its ITAM.

The development of committed pro-B cells to the IgM^{high}IgD^{low} transitional immature B cell stage is recapitulated in a recently established in vitro organ culture system. In contrast to the ex vivo analysis of bone marrow, where B cell development is examined at steady state equilibrium, this in vitro system allows the direct comparison of B cell progenitors to mature as a cohort to late stage immature B cells. Direct comparison of wild-type and *mb-1*$^{\Delta c/\Delta c}$ B lymphopoiesis in RFLOCs revealed that mutant cultures were deficient in the population of IgM^{high}IgD^{low} transitional B cells. It is precisely this bone marrow population that emigrates to the periphery, where they are recruited into the mature long-lived peripheral population (Allman et al., 1993). The near absence of IgM^{high}IgD^{low} transitional B cells would severely diminish the export of immature B cells and would account for the paucity of peripheral B cells found in *mb-1*$^{\Delta c/\Delta c}$ animals.

Although the percentage of IgM^{high}IgD^{low} transitional immature B cells is reduced in *mb-1*$^{\Delta c/\Delta c}$ bone marrow, interestingly, the levels of sIgM expression on bone marrow immature B cells is not diminished to the same extent as when generated in vitro. While the basis for this apparent discrepancy is not fully understood, we support the idea that a cell type(s) (Sandel and Monroe, 1999) or factor(s) found in the bone marrow microenvironment preferentially permits the development of IgM^{high} expressing *mb-1*$^{\Delta c/\Delta c}$ immature B cells relative to the fetal microenvironment used in RFLOCs. Consistent with this hypothesis, we do not find IgM^{high} immature B cells in *mb-1*$^{\Delta c/\Delta c}$ newborn fetal liver, although IgM^{high} cells are present in newborn spleen, when compared to analogous wild-type populations (data not shown).

Several lines of evidence independently support the view that deregulated BCR signaling and not an inability to express appropriate levels of sIg accounts for the lack of *mb-1*$^{\Delta c/\Delta c}$ transitional immature B cells. First, mutant B lineage cells do not have an inherent problem in expressing high levels of sIg. Comparable levels of sIgM and sIgD are observed on splenic *mb-1*$^{\Delta c/\Delta c}$ and wild-type B cells (Figure 2D; Torres et al., 1996). Second, providing a surrogate BCR signal by anti-Ig-β treatment during *mb-1*$^{\Delta c/\Delta c}$ B cell development exaggerated the phenotype, i.e., fewer immature B cells were produced that expressed lower levels of sIgM. Similarly, anti-Ig-β treatment of wild-type cultures results in immature B cells that are IgM^{low}IgD$^-$, resembling those found in untreated *mb-1*$^{\Delta c/\Delta c}$ organ cultures (Figure 3). These data demonstrate that a signal(s) transmitted by the BCR on newly formed IgM$^+$ immature B cells arrests development at the IgM^{low} B cell stage. This is also consistent with the similar developmental arrest observed in mice that overexpress an active form of the Btk tyrosine kinase (Maas et al., 1999), which is known to be activated upon BCR engagement (Aoki et al., 1994; Saouaf et al., 1994). Last, in two well-characterized Ig transgenic tolerance models, bone marrow B cell development in the presence of a membrane-bound self-antigen results in the elimination of IgM^{high} immature B cells (and mature B cells) but spares the IgM^{low} immature cells (Nemazee and Burki, 1989; Hartley et al., 1991). Considering that *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ bone marrow B cells are indeed activated (see below), these data provide strong evidence that the decreased number of IgM$^+$ immature B cells, and the virtual absence of *mb-1*$^{\Delta c/\Delta c}$ IgM^{high}IgD^{low} transitional B cells,

is a consequence of deregulated BCR signaling due to the Ig-α truncation.

The phosphorylation of the Ig-α/β ITAMs is one of the earliest membrane-proximal events in BCR signaling and is believed to be an essential feature in the propagation of all signal transduction cascades that result from antigen receptor engagement (Weiss and Littman, 1994; DeFranco, 1997; Reth and Wienands, 1997; Clements and Koretzky, 1999; Kurosaki, 1999). The profound impairment in *mb-1*$^{\Delta c/\Delta c}$ B lymphopoiesis was consistent with this notion. However, in the absence of rigorous biochemical analyses, owing to the paucity of B lineage cells found in the *mb-1*$^{\Delta c/\Delta c}$ mutants, this interpretation was based on the assumption that the absence of the Ig-α ITAM would lead to "inferior" antigen receptor signaling compared to the wild type. It was therefore unexpected to find that IgM$^+$ immature B cells with a truncation of the carboxy-terminal two-thirds of the Ig-α cytoplasmic domain, including the ITAM, have increased levels of intracellular tyrosine phosphorylation and aberrantly express the CD86 activation antigen. That is, *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ immature B cells display an activated phenotype similar to that resulting from BCR engagement on wild-type B cells.

We considered that the activation of mutant immature B cells could potentially result from sustained sIgM expression as a consequence of the Ig-α truncation. In particular, it has previously been shown that constitutive internalization of sIg expressed by a myeloma cell line was dependent on the Ig-α cytoplasmic domain (Cassard et al., 1998). However, *mb-1*$^{\Delta c/\Delta c}$ immature B cells were found to be competent in downmodulating sIgM in a manner comparable to wild type in response to sIg engagement. Thus, sustained BCR expression by mutant immature B cells does not appear to account for the activation of *mb-1*$^{\Delta c/\Delta c}$ immature B cells. Whether the discrepancy between the results presented here and those previously reported reflects the difference between primary and cultured cells or between immature B cells and a terminally differentiated myeloma is not clear but it deserves further investigation. In summary, our data fully support the notion that *mb-1*$^{\Delta c/\Delta c}$ immature B cells are activated as a result of constitutive BCR signaling.

Although the Ig-α ITAM has clearly been shown to fulfill a positive signaling role upon BCR engagement, the development of immature B cells in its absence results in activation. The *mb-1*$^{\Delta c}$ mutation, however, truncates the last 41 residues of the 61–amino acid cytoplasmic tail, and the ITAM, in its minimum configuration, accounts for only 15 residues of this deleted region. It is feasible that the Ig-α cytoplasmic tail can contribute toward BCR signaling in a manner independent of the ITAM, a possibility that has not been thoroughly investigated. Indeed, residues within the Ig-α/β ITAMs and independent of the tyrosines have been shown to serve as docking sites for cytosolic signaling molecules such as protein tyrosine kinases (Clark et al., 1994) that can also influence antigen receptor signaling.

The molecular basis for the activation of mutant IgM$^+$ immature B cells is not yet clear. However, we propose that the Ig-α cytoplasmic domain functions to recruit a cytosolic molecule that attenuates BCR signaling by IgM$^+$ immature B cells. The inability of *mb-1*$^{\Delta c/\Delta c}$ immature B cells to recruit this putative negative regulator results in constitutive BCR signaling upon sIgM expression. Although a number of candidates exist that could potentially negatively regulate BCR activity during B cell development, the SHP-1 protein tyrosine phosphatase has previously been shown to fulfill this role in vivo (Cyster and Goodnow, 1995) and physically associates with Ig-α in resting splenic B cells (Pani et al., 1995). While these data are consistent with a model in which the activation of *mb-1*$^{\Delta c/\Delta c}$ immature B cells results from an inability of SHP-1 to be recruited to the BCR, this remains speculative and requires further investigation. Nevertheless, it is clear that the cytoplasmic truncation of Ig-α results in activated IgM$^+$ immature B cells and implies that Ig-α has a role in repressing constitutive signaling by the antigen receptor on immature B cells.

The development of B lymphocytes in *mb-1*$^{\Delta c/\Delta c}$ mutants demonstrates that IgM^{low} immature B cells can mature in the presence of a constitutive BCR signal. In contrast, the maturation of these IgM^{low} immature B cells to late/transitional IgM^{high}IgD^{low} immature B cells is incompatible with an increased level of constitutive antigen receptor signaling. Thus, B lymphopoiesis in *mb-1*$^{\Delta c/\Delta c}$ mutants is in accord with previous findings gathered from the analysis of B cell development in transgenic mouse models of B cell tolerance (Nemazee and Burki, 1989; Hartley et al., 1991). In addition, those previous observations are extended by these data to include immature B cells that express a more physiological V_H repertoire as opposed to the development of a monoclonal population in transgenic animals. Moreover, the data presented here provide in vivo evidence that IgM^{low} and IgM^{high} immature B cells, as functionally distinct bone marrow developmental populations, respond differentially to an apparently similar BCR signal (Melamed et al., 1998).

In summary, we demonstrate that *mb-1*$^{\Delta c/\Delta c}$ IgM$^+$ immature B cells are activated and do not efficiently mature to the IgM^{high}IgD^{low} late transitional immature B cell stage. As a consequence, newly formed mutant B cells are not efficiently exported to the periphery and are most likely removed from the developmental pathway via a mechanism similar to that employed in the negative selection of autoreactive bone marrow B cells. These findings are compatible with deregulated constitutive BCR signaling in the absence of the Ig-α cytoplasmic tail and illustrate an additional role for Ig-α in regulating BCR signaling during development.

Experimental Procedures

Mice

C57BL/6 mice were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland). B6-Ly5^a, Eμ-*bcl-2* (Strasser et al., 1991), 383IgHi (Pelanda et al., 1997), and *mb-1*$^{\Delta c/\Delta c}$ (Torres et al., 1996) mice were bred and housed under pathogen-free conditions in the Basel Institute for Immunology animal facilities. The 383IgH chain transgene, which is specific for particular MHC class I molecules when expressed with the appropriate IgL chain, was generated by the insertion of a functionally rearranged VDJ gene segment at the IgH chain locus (Pelanda et al., 1997). It is present as a single copy and is expressed in an appropriate developmental manner exerting efficient allelic exclusion. For timed matings, the initial identification of a vaginal plug was considered day 0.

Reaggregation Fetal Liver Organ Cultures

Day 15 pregnant B6-Ly5^a mice were γ-irradiated with 950 rad, and intact fetal livers were carefully removed from embryos, cut into ~1–1.5 mm^2 pieces, and placed on 25 mm^2 membrane (Corning Nucleopore Track-Etch Membrane 0.8 μm, catalog number 110609O) and floated on IMDM with antibiotics, 2-ME, and 10% FCS. Explants were incubated at 37°C for 6 days during which host hematopoietic progenitors were eliminated. Subsequently, fetal liver explants were transferred to PBS/2% FCS and single-cell suspensions generated. Sorted adult (5–8 weeks) bone marrow B220$^+$ (or CD19$^+$) c-kit$^+$ B lineage progenitor cells were sorted and added to the fetal liver stroma cell suspension at 4×10^4 progenitor cells per fetal liver equivalent (FLE). Twenty microliters of this slurry, containing 2×10^4 progenitors and one half of a fetal liver equivalent, was placed on a 13 mm membrane (Corning 0.8 μm, catalog number 110409) and floated on SF medium (IMDM medium containing penicillin/streptomycin, MEM nonessential amino acids [GIBCO-BRL], 5 μg/ml insulin [Sigma], 50 μM 2-β-mercaptoethanol, and 10% [v/v] Primatone RL [Quest International, Bussum, Netherlands]) supplemented with 2% FCS (Boehringer Mannheim) in the presence of 2.0% (~300 ng/ml) IL-7 conditioned medium. Repopulated fetal liver organ cultures were incubated at 37°C for 6–7 days; cells were harvested, filtered, and viable cells counted using a hematocytometer and Trypan blue.

Antibodies

Monoclonal antibodies used in this study were those recognizing the CD45 allotypes Ly5^a (A20-1.7) and Ly5^b (104-2.1); the pan-B cell antigens B220 (CD45; RA3-6B2) and CD19 (1D3); developmental stage–restricted antigens c-kit (CD117; ACK4), CD25 (IL2rα; 7D4), IgM (R33-24), and IgD (1-3.5); and the activation antigens CD86 (B7-2; GL1), CD80 (B7-1; 1G10), CD69 (H1.2F3), and I-A (AF6-120). Antibodies directly conjugated to phycoerythrin (PE) or allophycocyanin (APC) (B220, CD25, CD86, and CD80) were purchased from Pharmingen; all other antibodies were purified in our lab and directly Fluos- (Boehringer Mannheim), biotin-, or Cy5-labeled using standard protocols. Second-step streptavidin-APC was purchased from Molecular Probes (Leiden, Netherlands).

Purified anti-Ig-β (CD79β; HM79) was purchased from Pharmingen. HM79 and 145–2C11, as control, were added to RFLOC cultures at 5 μg/ml throughout the culture period. Rabbit anti-Ig-β and -Ig-α antiserum were a kind gift from David Mason (Oxford, United Kingdom).

Flow Cytometric Analyses and Cell Sorting

Single-cell suspensions of repopulated fetal liver organ cultures were generated as described above. Bone marrow cells were flushed from both femurs, washed, counted, and stained with appropriate concentrations of the above mAbs labeled with Fluos, PE, APC, or biotin and incubated at 4°C in the dark for 20 min. Biotin-conjugated antibodies were revealed with streptavidin-conjugated PE or APC in a second incubation step. All phenotypic analyses were performed using four-color flow cytometric analysis on a FACScalibur (Becton Dickinson), one parameter which was devoted to the exclusion of dead cells as determined by propidium iodide staining. Percentages and numbers of B lineage cells in RFLOCs were calculated based on B220$^+$CD19$^+$ cells expressing either c-kit (pro-B), CD25 or CD2 (pre-B), or IgM (immature B).

For cell sorting, $5–10 \times 10^7$ adult (5–8 weeks) bone marrow cells were stained with antibodies recognizing B220 (or CD19) and c-kit. A MoFlo cell sorter (Cytomation) was used to sort B220^{low}c-kit$^+$ cells for reaggregation with fetal liver or B220$^+$c-kit$^-$ and further gated on small forward size for biochemical analyses. Reanalysis of sorted populations at all times demonstrated >95% purity.

IgM Downregulation

Wild-type and mutant bone marrow cells were treated with polyclonal goat anti-mouse IgM (10 or 20 μg/ml; Southern Biotechnology) and incubated at 37°C for various times followed by flow cytometric analysis of the remaining sIgM by anti-IgM mAb R33-24.

Phosphotyrosine Analyses

Sorted bone marrow populations were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsufonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml of aprotinin, leupeptin, and pepstatin) and incubated at 4°C for 15 min on an orbital shaker. Lysates were subsequently centrifuged at 14000 × g at 4°C for 15 min and supernatants assayed for protein content (BioRad). Equivalent amounts of protein were heated at 95°C for 5 min and subjected to SDS-PAGE on an 8% acrylamide gel.

For immunoprecipitation, total bone marrow cells from B6 and *mb-1*$^{\Delta c/\Delta c}$ animals were isolated and surface biotinylated by incubating 10^7 cells with 100 μg/ml of Sulfo-NHS-biotin (Pierce) for 10 min at room temperature. After a further 5 min incubation with IMDM, cells were washed three times with PBS and lysed. Lysates were precleared by the addition of control antibody and Sepharose-conjugated protein G (Pharmacia), followed by incubation at 4°C overnight while rotating. Beads were washed twice with lysis buffer and incubated with anti-B29 for 4 hr at 4°C while rotating. Sepharose-conjugated protein G was added for the last 2 hr of incubation, after which beads were washed three times with lysis buffer. Immunoprecipitated proteins were resuspended in lysis buffer and heated to 95°C for 3 min, and samples were subjected to SDS-PAGE on a 12% gel.

Proteins were transferred to a Hybond-P PVDF membrane (Amersham) using semi-dry transfer and filters incubated with TBS-Tween (1%) with 5% chicken serum for 1 hr at room temperature or overnight at 4°C. Filters were incubated with anti-phosphotyrosine (4G10)-HRP or streptavidin-HRP (Upstate Biotechnology) for 1 hr at room temperature and washed four times, and proteins were detected by chemiluminscence according to the manufacturer's protocol (Pierce).

For intracellular phosphotyrosine staining, bone marrow cells were fixed in 2% paraformaldehyde and washed twice with PBS. FITC-anti-pTyr (4G10; Upstate) was appropriately diluted in PBS/2% FCS/0.5% saponin (Sigma) and cells were stained for 30 min at room temperature. After washing twice with saponin buffer, cells were washed once with PBS/2% FCS and stained for surface IgM and B220 using standard protocol.

Immature B Cell Stimulation

B220^{low}IgM$^+$ immature B cells were sorted to >95% purity from total bone marrow and incubated with different concentrations of goat anti-mouse IgM (Southern Biotechnology) at 5×10^5 cells/ml. After overnight incubation (14 hr), cells were harvested, stained with anti-CD86 (B7.2), and examined by flow cytometry.

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References

Allman, D.M., Ferguson, S.E., Lentz, V.M., and Cancro, M.P. (1993). Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. J. Immunol. *151*, 4431–4444.

Aoki, Y., Isselbacher, K.J., and Pillai, S. (1994). Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells. Proc. Natl. Acad. Sci. USA *91*, 10606–10609.

Cambier, J.C., Bedzyk, W., Campbell, K., Chien, N., Friedrich, J., Harwood, A., Jensen, W., Pleiman, C., and Clark, M.R. (1993). The B-cell antigen receptor: structure and function of primary, secondary, tertiary and quaternary components. Immunol. Rev. *132*, 85–106.

Carsetti, R., Kohler, G., and Lamers, M.C. (1995). Transitional B cells are the target of negative selection in the B cell compartment. J. Exp. Med. *181*, 2129–2140.

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

Choquet, D., Ku, G., Cassard, S., Malissen, B., Korn, H., Fridman, W.H., and Bonnerot, C. (1994). Different patterns of calcium signaling triggered through two components of the B lymphocyte antigen receptor. J. Biol. Chem. *269*, 6491–6497.

Clark, M.R., Campbell, K.S., Kazlauskas, A., Johnson, S.A., Hertz, M., Potter, T.A., Pleiman, C., and Cambier, J.C. (1992). The B cell antigen receptor complex: association of Ig-α and Ig-β with distinct cytoplasmic effectors. Science *258*, 123–126.

Clark, M.R., Johnson, S.A., and Cambier, J.C. (1994). Analysis of Ig-α-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-α stimulation of Fyn activity. EMBO J. *13*, 1911–1919.

Clements, J.L., and Koretzky, G.A. (1999). Recent developments in lymphocyte activation: linking kinases to downstream signaling events. J. Clin. Invest. *103*, 925–929.

Cyster, J.G., and Goodnow, C.C. (1995). Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. Immunity *2*, 13–24.

DeFranco, A.L. (1997). The complexity of signaling pathways activated by the BCR. Curr. Opin. Immunol. *9*, 296–308.

Flaswinkel, H., and Reth, M. (1994). Dual role of the tyrosine activation motif of the Ig-α protein during signal transduction via the B cell antigen receptor. EMBO J. *13*, 83–89.

Friedrich, R.J., Campbell, K.S., and Cambier, J.C. (1993). The γ subunit of the B cell antigen-receptor complex is a C-terminally truncated product of the B29 gene. J. Immunol. *150*, 2814–2822.

Hartley, S.B., Crosbie, J., Brink, R., Kantor, A.B., Basten, A., and Goodnow, C.C. (1991). Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature *353*, 765–769.

Hathcock, K.S., Laszlo, G., Pucillo, C., Linsley, P., and Hodes, R.J. (1994). Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J. Exp. Med. *180*, 631–640.

Karasuyama, H., Rolink, A., and Melchers, F. (1996). Surrogate light chain in B cell development. Adv. Immunol. *63*, 1–41.

Kim, K.M., Alber, G., Weiser, P., and Reth, M. (1993). Differential signaling through the Ig-α and Ig-β components of the B cell antigen receptor. Eur. J. Immunol. *23*, 911–916.

Koyama, M., Ishihara, K., Karasuyama, H., Cordell, J.L., Iwamoto, A., and Nakamura, T. (1997). CD79 α/CD79 β heterodimers are expressed on pro-B cell surfaces without associated μ heavy chain. Int. Immunol. *9*, 1767–1772.

Kurosaki, T. (1999). Genetic analysis of B cell antigen receptor signaling. Annu Rev Immunol *17*, 555–592.

Law, D.A., Chan, V.W.F., C., Datta, S.K., and DeFranco, A.L. (1993). B-cell antigen receptor motifs have redundant signaling capabilities and bind the tyrosine kinases PTK72, Lyn, and Fyn. Curr. Biol. *3*, 645–657.

Lenschow, D.J., Sperling, A.I., Cooke, M.P., Freeman, G., Rhee, L., Decker, D.C., Gray, G., Nadler, L.M., Goodnow, C.C., and Bluestone, J.A. (1994). Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. J. Immunol. *153*, 1990–1997.

Luisiri, P., Lee, Y.J., Eisfelder, B.J., and Clark, M.R. (1996). Cooperativity and segregation of function within the Ig-α/β heterodimer of the B cell antigen receptor complex. J. Biol. Chem. *271*, 5158–5163.

Maas, A., Dingjan, G.M., Grosveld, F., and Hendriks, R.W. (1999). Early arrest in B cell development in transgenic mice that express the E41K Bruton's tyrosine kinase mutant under the control of the CD19 promoter region. J. Immunol. *162*, 6526–6533.

Melamed, D., Benschop, R.J., Cambier, J.C., and Nemazee, D. (1998). Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. Cell *92*, 173–182.

Nagata, K., Nakamura, T., Kitamura, F., Kuramochi, S., Taki, S., Campbell, K.S., and Karasuyama, H. (1997). The Ig-α/Ig-β heterodimer on μ-negative pro-B cells is competent for transducing signals to induce early B cell differentiation. Immunity *7*, 559–570.

Nemazee, D.A., and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature *337*, 562–566.

Pani, G., Kozlowski, M., Cambier, J.C., Mills, G.B., and Siminovitch, K.A. (1995). Identification of the tyrosine phosphatase PTP1C as a B cell antigen receptor-associated protein involved in the regulation of B cell signaling. J. Exp. Med. *181*, 2077–2084.

Pao, L.I., Famiglietti, S.J., and Cambier, J.C. (1998). Asymmetrical phosphorylation and function of immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. J. Immunol. *160*, 3305–3314.

Papavasiliou, F., Jankovic, M., Suh, H., and Nussenzweig, M.C. (1995a). The cytoplasmic domains of immunoglobulin (Ig) α and Ig β can independently induce the precursor B cell transition and allelic exclusion. J. Exp. Med. *182*, 1389–1394.

Papavasiliou, F., Misulovin, Z., Suh, H., and Nussenzweig, M.C. (1995b). The role of Ig β in precursor B cell transition and allelic exclusion. Science *268*, 408–411.

Pelanda, R., Schwers, S., Sonoda, E., Torres, R.M., Nemazee, D., and Rajewsky, K. (1997). Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification. Immunity *7*, 765–775.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751–758.

Reth, M. (1992). Antigen receptors on B lymphocytes. Annu. Rev. Immunol. *10*, 97–121.

Reth, M., and Wienands, J. (1997). Initiation and processing of signals from the B cell antigen receptor. Annu. Rev. Immunol. *15*, 453–479.

Rolink, A.G., Andersson, J., and Melchers, F. (1998). Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. Eur. J. Immunol. *28*, 3738–3748.

Sanchez, M., Misulovin, Z., Burkhardt, A.L., Mahajan, S., Costa, T., Franke, R., Bolen, J.B., and Nussenzweig, M. (1993). Signal transduction by immunoglobulin is mediated through Ig α and Ig β. J. Exp. Med. *178*, 1049–1055.

Sandel, P.C., and Monroe, J.G. (1999). Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. Immunity *10*, 289–299.

Saouaf, S.J., Mahajan, S., Rowley, R.B., Kut, S.A., Fargnoli, J., Burkhardt, A.L., Tsukada, S., Witte, O.N., and Bolen, J.B. (1994). Temporal differences in the activation of three classes of non- transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. Proc. Natl. Acad. Sci. USA *91*, 9524–9528.

Strasser, A., Whittingham, S., Vaux, D.L., Bath, M.L., Adams, J.M., Cory, S., and Harris, A.W. (1991). Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc. Natl. Acad. Sci. USA *88*, 8661–8665.

Taddie, J.A., Hurley, T.R., Hardwick, B.S., and Sefton, B.M. (1994). Activation of B- and T-cells by the cytoplasmic domains of the B-cell antigen receptor proteins Ig-α and Ig-β. J. Biol. Chem. *269*, 13529–13535.

Teh, Y.M., and Neuberger, M.S. (1997). The immunoglobulin (Ig)α and Ig-β cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice. J. Exp. Med. *185*, 1753–1758.

Torres, R.M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996). Aberrant B cell development and immune response in mice with a compromised BCR complex. Science *272*, 1804–1808.

Weiss, A., and Littman, D.R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Williams, G.T., Peaker, C.J., Patel, K.J., and Neuberger, M.S. (1994). The α/β sheath and its cytoplasmic tyrosines are required for signaling by the B-cell antigen receptor but not for capping or for serine/threonine-kinase recruitment. Proc. Natl. Acad. Sci. USA *91*, 474–478.