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Inositol 1,4,5-Trisphosphate 3-Kinase B Is a Negative Regulator of BCR Signaling That Controls B Cell Selection and Tolerance Induction

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Inositol 1,4,5-trisphosphate 3-kinase B (or Itpkb) converts inositol 1,4,5-trisphosphate to inositol 1,3,4,5-tetrakisphosphate upon Ag receptor activation and controls the fate and function of lymphocytes. To determine the role of Itpkb in B cell tolerance, Itpkb<sup>−/−</sup> mice were crossed to transgenic mice that express a BCR specific for hen egg lysozyme (IgHEL). B cells from Itpkb<sup>−/−</sup> IgHEL mice possess an anergic phenotype, hypoproliferate in response to cognate Ag, and yet they exhibit enhanced Ag-induced calcium signaling. In IgHEL transgenic mice that also express soluble HEL, lack of Itpkb converts anergy induction to deletion. These data establish Itpkb as a negative regulator of BCR signaling that controls the fate of developing B cells and tolerance induction.

A

utoimmune disease occurs when the mechanisms involved in the induction or maintenance of self-tolerance of autoreactive lymphocytes fail to function properly. Studies examining mice expressing transgenic BCRs specific for self-Ags have demonstrated that the avidity of the BCR for self-Ags dictates the fate of developing B cells (1). B cells with receptors that bind with high avidity to self-Ags undergo developmental arrest in the bone marrow and undergo further Ig gene rearrangements. If further rearrangements fail to mitigate self-reactivity or do not produce a functional BCR, these B cells are eliminated by clonal deletion. B cells with minimal self-reactivity proceed to the spleen where the BCR interaction with self-Ags dictates their developmental fate. Highly self-reactive B cells are deleted during the T1 stage, while B cells with less self-reactivity proceed to the T2 stage to undergo positive selection. Here again the “strength” of BCR self-Ag signals drives fate choices; low-affinity BCR signals lead to a marginal zone fate, intermediate-affinity BCR signals give rise to a follicular fate, and higher affinity signals give rise to a B-1 B cell fate (2). Highly self-reactive B cells that escape earlier deletion checkpoints can be induced to become anergic or unresponsive to Ag.

The molecular events that translate BCR signal strength to fate choice remain unresolved. Of the many signals that occur following BCR activation, the elevation in cytosolic calcium is a particularly attractive candidate signal for linking BCR signal strength to fate determination. The degree of intracellular Ca<sup>2+</sup> elevation is proportional to the avidity of the BCR for self-Igs, and several studies have reported correlations in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>)<sub>2</sub> following BCR stimulation with changes in B cell fate (3–8). Unfortunately, most mutations of the BCR or the downstream signaling proteins that alter B cell fate lead to a compendium of alterations in BCR signaling. Thus, determining which signal drives fate choice remains an important open question.

The seminal event in generating elevated [Ca<sup>2+</sup>]<sub>i</sub>, following BCR activation is the activation of phospholipase Cy (PLCy). PLCy activation results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate and generation of the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. Upon binding to its receptors on the endoplasmic reticulum (ER), IP3 induces the release of calcium. Upon depletion of the ER Ca<sup>2+</sup> stores, store-operated calcium (SOC) channels in the plasma membrane are opened, allowing the sustained influx of calcium into the cell and promoting the activation of new gene transcription essential for B lymphocyte development, activation, and function (9).

IP3 can be phosphorylated into higher order inositol phosphates by the action of inositol kinases (10). The calcium-dependent inositol kinase, inositol 1,4,5-trisphosphate 3-kinase B (Itpkb), phosphorylates the 3’ position of the inositol ring to convert IP3 to inositol 1,3,4,5-tetrakisphosphate (IP4) (11). The importance of Itpkb and IP4 in lymphocyte signaling and development is underscored by the phenotype of mice lacking Itpkb. Itpkb<sup>−/−</sup> mice lack peripheral T cells as a result of a complete block at the CD4<sup>+</sup> CD8<sup>+</sup> stage during thymocyte development, indicating an essential role for Itpkb in T cell development (12, 13). More recently, we and others have established that Itpkb is also essential for B cell development, selection, and activation (14, 15).

Mice lacking Itpkb have normal B cell development in the bone marrow, but reduced numbers of all splenic B cell subsets and a shift in the developmental fate toward compartments that are normally selected by strong BCR signals. Mature B cells lacking Itpkb fail to proliferate in response to BCR stimulation but show normal responses to the TLR4 ligand LPS or agonistic Abs to CD40. Additionally, Itpkb-deficient mice display impaired T-independent Ab responses. Selective BCR signaling defects are also found in some models of BCR self-tolerance, suggesting that Itpkb may regulate tolerance induction. However, while most models of B

sHEL, soluble hen egg lysozyme; SOC, store-operated calcium; tg, transgenic; WT, wild type.

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2 Abbreviations used in this paper: [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; ER, endoplasmic reticulum; HEL, hen egg lysozyme; IP3, inositol 1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; Itpkb, inositol 1,4,5-trisphosphate 3-kinase B; mHEL, membrane-bound hen egg lysozyme; PLCy, phospholipase Cy;
cell tolerance show impaired BCR signaling and reduced calcium elevation following Ag stimulation (16–18). B cells from Itpkb−/− mice display enhanced SOCE activity. Enhanced SOCE activity reflects an inhibitory role of IP4 on SOCE. In support of this hypothesis, the addition of cell-permeable IP4 restores normal intracellular calcium levels to Itpkb−/− B cells following BCR stimulation, and treatment of normal B cells with IP4 inhibits the SOCE channels. These data establish Itpkb and its product IP4 as inhibitors of BCR-induced calcium signals, and suggest a model in which the alterations of B cell development and BCR unresponsiveness observed in Itpkb−/− mice result from enhanced BCR-induced calcium signaling during development (14).

One important unresolved issue is whether the phenotype of B cells from Itpkb−/− mice reflects a positive or negative role for Itpkb in B cell selection and tolerance induction. Since Itpkb−/− mice possess abnormalities in B cell selection, the changes observed in B cells from Itpkb−/− mice could reflect alterations in the repertoire and function of B cells that survive in the absence of Itpkb, or a requirement for Itpkb in mature B cells. Additionally, the signaling defects observed in B cells from Itpkb−/− mice do not match those observed in models of B cell self-tolerance, and thus the influence of Itpkb on tolerance induction remains unclear. To determine the role of Itpkb in B cell selection and tolerance induction, Itpkb−/− mice were bred to mice expressing the MD4 Ig transgene specific for hen egg lysozyme (IgHEL). Nontransgenic Itpkb−/− mice contained fewer B cells that were unable to respond to BCR stimulation. Interestingly, Itpkb−/− IgHEL transgenic (tg) mice contained increased numbers of HEL-specific B cells. For the following re-addition experiments, single-cell suspensions were labeled with the Ca2⁺-sensitive dye Indo-1 (10 μM/mL; Molecular Probes) for 1 h at 37°C. Following incubation, cells were then stained with Abs specific for B220, IgM⁺, and IgD⁺ for 5 min on ice. Lymphocytes were then washed and resuspended in either Ca2⁺-containing or Ca2⁺-free medium as above. Methods for the IP4 re-addition experiments have been previously published (14).

Materials and Methods

Mice

Itpkb-deficient mice (12), backcrossed to C57BL/6 for over eight generations, were crossed to MD4 IgHEL, or the MD4/ML5 IgHEL/sHEL double-tg mice (19, 20) (The Jackson Laboratory) to generate Itpkb−/− IgHEL transgenic (tg) mice. Itpkb-deficient IgHEL tg mice were stimulated with HEL in the absence or presence of exogenous calcium, HEL was added at a final concentration of 10 μg/mL and intracellular calcium flux was measured for 6 min. For the final 2 min, 1 μg of ionomycin was added and intracellular calcium was measured. For cells stimulated in the absence of exogenous calcium, HEL was added for a final concentration of 10 μg/mL. Following 4 min of intracellular calcium measurement, 1 mM of CaCl₂ was added back and intracellular calcium levels were measured for an additional 3 min. As before, 1 μg of ionomycin was added at 5 min and intracellular calcium was measured for the final 2 min. For the analysis of Ca2⁺ influx in the bone marrow of IgHEL mice and for IP4 re-addition experiments, single-cell suspensions were labeled with the Ca2⁺-sensitive dye Indo-1 (10 μM/mL; Molecular Probes) for 1 h at 37°C. Following incubation, cells were then stained with Abs specific for B220, IgM⁺, and IgD⁺ for 5 min on ice. Lymphocytes were then washed and resuspended in either Ca2⁺-containing or Ca2⁺-free medium as above. Methods for the IP4 re-addition experiments have been previously published (14).

Biochemical analysis

Immunoblot analysis was performed as previously described (14). Briefly, 3 × 10⁶ purified B lymphocytes from either WT or Itpkb−/− IgHEL tg mice were stimulated with 10 μg/mL HEL in either the Ca2⁺-free or Ca2⁺-containing media for the indicated time points at 37°C. As a positive control, cells were stimulated with phorbol-12,13-dibutyrate (2.5 μg/mL) and ionomycin (375 μg/mL) for 5 min at 37°C. Lysates were blotted with Abs specific for phospho-Syk (Y525/535), phospho-PLCγ2 (Y789), phospho-PKCµ (S744/748), phospho-Erk (T202/Y204), as well as for the corresponding proteins (Cell Signaling Technology).

In vivo responses to HEL

WT recipient mice were immunized with 200 μg of HEL in alum (Pierce). Seven days postimmunization, 4 × 10⁶ purified B cells from WT or Itpkb−/− IgHEL tg mice were adoptively transferred retro-orbitally in the presence or absence of 200 μg of HEL. Seven days following transfer, spleens and plasma were harvested and assayed for the number of IgHEL B cells by flow cytometry and HEL-specific IgM⁺ by ELISA, respectively.

Measurement of serum anti-HEL IgM⁺

Plates were coated with 5 μg/mL HEL protein at 4°C overnight. After blocking, serum samples were serially diluted and incubated for 1 h at room temperature. Plates were washed three times and incubated with biotinylated anti-IgM⁺. Following incubation, Ab was detected with streptavidin conjugated to HRP. Tetramethylbenzidine substrate was added and the reaction was stopped using 1 N H₂SO₄. The OD at 450 nm was read on a Molecular Devices plate reader.

Intracellular staining

Bone marrow cells and splenocytes were first stained with anti-B220-Pacific Blue and anti-IgM-allophycocyanin, fixed, permeabilized, and then stained intracellularly with a rabbit anti-Bim (Cell Signaling Technology) Ab, followed by staining with a goat anti-rabbit-FITC Ab for detection. For Bim staining control, cells were fixed, permeabilized, and stained with secondary Ab alone. Cells were immediately analyzed by flow cytometry.

Results

Previous data revealed that Itpkb is required for normal B cell selection. However, due to the difficulty of studying B cell selection in a polyclonal background we were unable to determine
whether the phenotypes observed reflected a requirement for Itpkb (and/or IP4) in promoting BCR signaling and selection or an inhibitory role for Itpkb in BCR signaling resulting in tolerance induction. To reduce the inherent complexity observed in a polyclonal repertoire and to more clearly understand the role of Itpkb in B cell selection, we crossed Itpkb−/− mice to mice expressing the MD4 Ig transgene specific for IgHEL, a situation where most B cells express an Ag receptor of one affinity.

To determine whether Itpkb functions during early B cell development and selection in the bone marrow, we examined the bone marrow from WT and Itpkb−/− IgHEL tg mice by flow cytometry. Flow cytometric analysis revealed that B cell development up to the late pre-B cell stage was similar between WT and Itpkb−/− IgHEL tg mice. However, at the immature stage, Itpkb-deficient mice possessed a 60% increase in the numbers of immature B cells (IgM+IgD−) compared with WT mice (Fig. 1a). This increase mostly reflects an increased percentage of immature CD43−B220+ cells in bone marrow from Itpkb−/− IgHEL tg mice. Despite this increase, the numbers of recirculating B cells in the bone marrow were relatively normal (Fig. 1a). Interestingly, we noted a striking 10-fold reduction in the levels of surface IgM on recirculating B cells in the bone marrow of Itpkb−/− IgHEL tg mice (Fig. 1b).

Upon exiting the bone marrow, B cells pass through additional developmental checkpoints in the spleen. Examination of splenic B cell populations in Itpkb−/− IgHEL tg mice revealed that while the total numbers of B220+ cells were relatively normal, the numbers of follicular mature B cells were increased 2.2-fold, while the number of T2 and marginal zone cells were reduced 3- and 3.8-fold, respectively (Fig. 1c). Similar to what was observed in the bone marrow, Itpkb−/− IgHEL tg B cells in the spleen possessed a drastic down-modulation of surface IgM compared with WT (Fig. 1d). Despite the developmental aberrations and the modulation of the surface levels of IgM, Itpkb−/− IgHEL tg B cells maintained the ability to bind HEL and the levels of circulating HEL-specific IgM were unchanged in Itpkb−/− IgHEL tg mice (data not shown).

Recently, Culton et al. described an early pre-plasma cell population of B cells that are enriched in models of autoreactivity. Phenotypically these cells resemble follicular B cells; however, they also express intermediate levels of CD138. Their findings suggest that autoreactive B cells exhibit signs of activation and have begun differentiation toward the plasma cell stage (21). Preceding this differentiation, we noticed that in Itpkb−/− IgHEL tg B cells, the expression of CD138 on WT and Itpkb−/− IgHEL tg B cells, s, p < 0.05; **, p < 0.01. Data are representative of at least four separate experiments.

Recently, Culton et al. described an early pre-plasma cell population of B cells that are enriched in models of autoreactivity. Phenotypically these cells resemble follicular B cells; however, they also express intermediate levels of CD138. Their findings suggest that autoreactive B cells exhibit signs of activation and have begun differentiation toward the plasma cell stage (21). Previously, we found that nearly half of all mature (B220+/IgD−) B cells in non-tg mice lacking Itpkb expressed CD138, consistent with the hypothesis that Itpkb deficiency results in the selection of B lymphocytes that possess high affinity for self-Ags (14). Since the MD4 BCR is thought to possess a very low affinity for self-Ags, we wanted to determine whether the absence of Itpkb induced the differentiation of these cells into the pre-plasma cell lineage as a result of enhanced signaling or altered selection. Indeed, in contrast to Itpkb+/+ IgHEL tg mice, which lack expression of CD138 on IgD+/ follicular B cells, ~35% of IgD− B cells from Itpkb−/− IgHEL tg mice express high levels of CD138.
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Following injection of HEL, the number of transfer, the recipient mice were analyzed for the number of experiment, non-tg WT recipient mice were first primed with 200 g of HEL. In this

proliferative capacity compared with CD138/H11002
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tolerance induction in the polyclonal background. To address
was difficult to assess whether the absence of Itpkb resulted in
response to HEL to the extent observed in WT IgHEL tg B cells
mentally anergic state. Thus, in cells with a fixed BCR affinity, the
proliferate robustly to other BCR stimuli, such as anti-IgM and
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Itpkb-deficient B cells are anergic
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Itpkb defective B cells are anergic
Previously we have shown that B cells from Itpkb−/− mice failed to respond to BCR stimulation. However, in this study it was difficult to assess whether the absence of Itpkb resulted in tolerance induction in the polyclonal background. To address whether Itpkb is required for B cell proliferation in a more controlled system, mature B cells from WT or Itpkb−/− IgHEL tg mice, which had been sorted into CD138− and CD138+ fractions, were stimulated with varying concentrations of the cognate Ag HEL in the presence of exogenous IL-4. In the IgHEL tg system, stimulation with soluble HEL is not sufficient to induce vigorous proliferation in vitro, perhaps reflecting the reduced valency of monovalent HEL or a less mature developmental status as reflected by high levels of surface IgM (16, 22). Interestingly, Itpkb-deficient cells failed to proliferate in response to HEL to the extent observed in WT IgHEL tg B cells (Fig. 2). Additionally, Itpkb-deficient IgHEL tg cells failed to proliferate robustly to other BCR stimuli, such as anti-IgM and anti-IgD, while responses to CD40 were normal. Of note, CD138− Itpkb−/− IgHEL tg B cells displayed an even greater reduction in proliferative capacity compared with CD138− cells, suggesting that these cells may be in a more developmentally anergic state. Thus, in cells with a fixed BCR affinity, the loss of Itpkb results in a selective BCR-mediated proliferation block.

To assess whether Itpkb−/− IgHEL tg cells could elicit a functional response in vivo, we next examined IgHEL B cell responses in a B cell transfer and immunization model with HEL. In this experiment, non-tg WT recipient mice were first primed with 200 µg of HEL in alum to elicit a Th response. After 1 wk, either WT or Itpkb−/− IgHEL tg B cells were transferred to the recipient mice with or without an additional 200 µg of HEL. One week post-transfer, the recipient mice were analyzed for the number of IgHEL tg cells in the spleen as well as HEL-specific Ab titers. Following injection of HEL, the number of Itpkb−/− IgHEL tg cells in the spleens of recipient mice were modestly increased compared with mice that received WT IgHEL tg cells (Fig. 3a). Despite this increase, the HEL-specific IgM+ Ab titers between the recipients of WT and Itpkb−/− IgHEL tg cells were comparable (Fig. 3b). Thus, in Ag-primed animals, where T cell help is not limiting, Itpkb-deficient cells are able to function normally. We speculate that in vivo, the normal proliferative responses of Itpkb−/− B cells to helper T cell signals such as CD40L (Fig. 2 and Ref. 14) is able to compensate for the impaired BCR-induced proliferation observed in vitro.

IP4 inhibits SOC entry in B lymphocytes
Previously we reported that polyclonal Itpkb-deficient B cells possessed enhanced SOC entry following stimulation with anti-IgM. This effect was reversed upon provision of IP4 exogenously, suggesting that IP4 functions to inhibit SOC channels. To determine whether monoclonal Itpkb−/− IgHEL tg B cells also possess aberrant calcium responses, we loaded WT and Itpkb−/− IgHEL tg cells with calcium-sensitive dyes and measured their ability to flux calcium by flow cytometry following stimulation with HEL. In the presence of exogenous calcium, Itpkb-deficient B cells displayed greatly enhanced responses following stimulation with HEL (Fig. 4a). To determine whether this effect was specifically a result of enhanced calcium entry through the SOC channels, we stimulated cells with HEL in the absence of calcium to measure ER release, and then provided exogenous calcium to measure SOC channel activity. Consistent with that observed in the polyclonal setting, Itpkb-deficient IgHEL tg B cells possessed enhanced SOC entry following stimulation with their cognate Ag HEL (Fig. 4c). This elevated calcium was not due to the presence of CD138+ cells, as calcium levels were comparable between CD138+ and CD138− cells (data not shown). Lastly, provision of exogenous IP4 rescued the enhanced SOC entry in Itpkb-deficient B cells as observed previously, consistent with our hypothesis that IP4 inhibits SOC entry in B lymphocytes (Fig. 4c).

Previously we discovered that following stimulation with anti-IgM, Itpkb-deficient B cells possessed slightly decreased levels of PLCγ2 phosphorylation while the activation of other key signaling pathways were largely intact (14). Whether this was a result of Itpkb being required for PLCγ2 activation or a consequence of development into an anergic state was unclear. To more clearly understand the function of Itpkb in the activation of other signaling pathways in B lymphocytes, we examined the ability of WT and Itpkb-deficient IgHEL tg cells to activate

FIGURE 2. Itpkb deficiency results in anergy. Purified B220+IgDa+ CD138− or CD138+ cells from both Itpkb−/− and WT IgHEL tg animals were stimulated with either HEL, F(ab′)2 anti-IgM, anti-IgD, or CD40 in the presence of IL-4. Following 2 days of culture, 1H]thymidine was added and proliferation was measured 20 h later. Data are representative of three separate experiments.

FIGURE 3. Itpkb−/− B cells can respond in vivo. Non-tg WT recipient mice were injected with HEL in alum i.p. After 1 wk, purified WT or Itpkb−/− IgHEL tg B cells were transferred to recipient mice in the presence or absence of HEL. One week postimmunization, recipient spleens were analyzed for the presence of transferred B cells and Ab titers. a, Number of IgM+ B cells in the spleen. b, Relative Ab titer of HEL-specific IgM in immunized mice. ++, p < 0.01. Data are representative of three separate experiments.

FIGURE 4. IP4 inhibits SOC entry in B lymphocytes. Previously we reported that polyclonal Itpkb-deficient B cells possessed enhanced SOC entry following stimulation with anti-IgM. This effect was reversed upon provision of IP4 exogenously, suggesting that IP4 functions to inhibit SOC channels. To determine whether monoclonal Itpkb−/− IgHEL tg B cells also possess aberrant calcium responses, we loaded WT and Itpkb−/− IgHEL tg cells with calcium-sensitive dyes and measured their ability to flux calcium by flow cytometry following stimulation with HEL. In the presence of exogenous calcium, Itpkb-deficient B cells displayed greatly enhanced responses following stimulation with HEL (Fig. 4a). To determine whether this effect was specifically a result of enhanced calcium entry through the SOC channels, we stimulated cells with HEL in the absence of calcium to measure ER release, and then provided exogenous calcium to measure SOC channel activity. Consistent with that observed in the polyclonal setting, Itpkb-deficient IgHEL tg B cells possessed enhanced SOC entry following stimulation with their cognate Ag HEL (Fig. 4c). This elevated calcium was not due to the presence of CD138+ cells, as calcium levels were comparable between CD138+ and CD138− cells (data not shown). Lastly, provision of exogenous IP4 rescued the enhanced SOC entry in Itpkb-deficient B cells as observed previously, consistent with our hypothesis that IP4 inhibits SOC entry in B lymphocytes (Fig. 4c).
both BCR proximal and distal signaling molecules, such as Syk, PLCγ2, PKC, and Erk, following stimulation with HEL. All the signaling pathways that we examined were unchanged in the absence of Itpkb (Fig. 4d). To examine ER-mediated Ca\(^{2+}\) release and SOC entry, B cells were stimulated with 10 \(\mu\)g/ml HEL in the absence of exogenous Ca\(^{2+}\), followed by the re-addition of Ca\(^{2+}\) as indicated. Ionomycin stimulation indicated equivalent dye loading. The y-axis represents the ratio of Fluo-4 to Fura Red. c. To examine the effect of IP4 on SOC channel activity in mature IgHEL tg B cells, cells were loaded with the Ca\(^{2+}\)-sensitive dye Indo-1, stained with Abs specific for B220, IgDA, and IgM, and resuspended in Ca\(^{2+}\)-free medium. Cells were then stimulated with HEL, and after the re-addition of exogenous Ca\(^{2+}\), cells were treated with either 200 \(\mu\)M IP4 or DMSO. d, Lysates of purified B lymphocytes stimulated with 10 \(\mu\)g/ml HEL or phorbol 12,13-dibutyrate (PdBu) and ionomycin (P+I) in the presence of exogenous Ca\(^{2+}\) were immunoblotted for the indicated proteins, p-, phosphorylated (letters and numbers indicated the phosphorylated residues). Data represent at least four independent experiments. e, Lysates from WT and Itpkb\(^{−/−}\) IgHEL tg B cells, which had been stimulated in the absence or presence of exogenous calcium with 10 \(\mu\)g/ml HEL or PdBu and ionomycin, were immunoblotted with Abs specific for phosphorylated Erk and Erk protein. Data are representative of at least four separate experiments.

**FIGURE 4.** Enhanced SOC entry but normal activation of other signaling pathways in Itpkb\(^{−/−}\) B cells. Ca\(^{2+}\) responses were examined using the Ca\(^{2+}\)-sensitive dyes Fluo-4 and Fura Red. a, Purified B lymphocytes from Itpkb\(^{−/−}\) and WT animals were stimulated with 10 \(\mu\)g/ml HEL in the presence of exogenous calcium. To examine ER-mediated Ca\(^{2+}\) release and SOC entry, in b, cells were stimulated with 10 \(\mu\)g/ml HEL in the absence of exogenous Ca\(^{2+}\), followed by the re-addition of Ca\(^{2+}\) as indicated. Ionomycin stimulation indicated equivalent dye loading. The y-axis represents the ratio of Fluo-4 to Fura Red. c, To examine the effect of IP4 on SOC channel activity in mature IgHEL tg B cells, cells were loaded with the Ca\(^{2+}\)-sensitive dye Indo-1, stained with Abs specific for B220, IgDA, and IgM, and resuspended in Ca\(^{2+}\)-free medium. Cells were then stimulated with HEL, and after the re-addition of exogenous Ca\(^{2+}\), cells were treated with either 200 \(\mu\)M IP4 or DMSO. d, Lysates of purified B lymphocytes stimulated with 10 \(\mu\)g/ml HEL or phorbol 12,13-dibutyrate (PdBu) and ionomycin (P+I) in the presence of exogenous Ca\(^{2+}\) were immunoblotted for the indicated proteins, p-, phosphorylated (letters and numbers indicated the phosphorylated residues). Data represent at least four independent experiments. e, Lysates from WT and Itpkb\(^{−/−}\) IgHEL tg B cells, which had been stimulated in the absence or presence of exogenous calcium with 10 \(\mu\)g/ml HEL or PdBu and ionomycin, were immunoblotted with Abs specific for phosphorylated Erk and Erk protein. Data are representative of at least four separate experiments.

Anergy is converted to deletion in the absence of Itpkb
Polyclonal Itpkb-deficient B cells possess the phenotypic and functional characteristics of anergic or tolerant B cells. They fail to respond to BCR signals, possess down-modulated IgM, and express high levels of CD138 (14). Mechanisms of B cell tolerance have been studied in the IgHEL tg line by crossing these mice to the ML5 tg line, which express soluble HEL. The chronic exposure of the HEL-specific B cells to HEL results in anergy, which is characterized by a surface phenotype of IgM low IgD high and an inability to respond to Ag stimulation (19, 20). Interestingly, loss of negative regulators of BCR signaling, such as CD22, SHP-1, and Lyn, leads to deletion rather than anergy in the IgHEL/sHEL tg model (3, 23–26). Given that the data thus far indicate that Itpkb also functions as a negative regulator of BCR signaling, we sought to understand more definitively whether Itpkb plays any role in tolerance induction, and whether the absence of Itpkb also results in deletion rather than anergy. To accomplish this, we bred Itpkb\(^{−/−}\) IgHEL tg mice to the ML5 tg line.
Examination of B cell development in the bone marrow of Itpkb\(^{-/-}\) IgHEL/sHEL tg mice revealed a 2-fold decrease in the number of immature B cells as well as a 2.8-fold decrease in the number of recirculating B cells (Fig. 5a). Interestingly, both the immature B cell and recirculating B cell populations possessed significantly down-modulated levels of surface IgM (Fig. 5b). Examination of splenic B cell development revealed a dramatic 10-fold reduction in total B cell numbers in Itpkb\(^{-/-}\) IgHEL/sHEL tg mice. This reduction in B cell numbers reflected a 10-fold reduction in the number of IgHEL tg B cells (IgM\(^{low}\)IgD\(^{high}\)) in mice lacking Itpkb, while the number of non-tg B cells (IgM\(^{low}\)IgD\(^{low}\)) remained low in all genotypes (Fig. 5c). Total cellularity of the spleen was reduced 20-fold in Itpkb\(^{-/-}\) IgHEL/sHEL tg mice as a result of the absence of T cells and most B cells (data not shown). Thus, while tolerant mature B cells (IgM\(^{low}\)IgD\(^{low}\)) still comprised a significant percentage of the spleen of the Itpkb\(^{-/-}\) IgHEL/sHEL tg mice (Fig. 5d), the total number of Ag-specific IgHEL B cells in the spleens of Itpkb\(^{-/-}\) IgHEL/sHEL tg mice was drastically reduced (Fig. 5c). Interestingly, Culton et al. reported that IgHEL/sHEL tg mice possessed a higher frequency of CD138\(^+\) IgD\(^+\) pre-plasma cells (21). We observed only minimal increases in CD138 expression on IgD\(^+\) B cells from IgHEL/ sHEL tg mice compared with IgHEL/sHEL tg mice lacking sHEL. However, the absence of Itpkb resulted in a dramatic increase in the percentage of CD138\(^+\) cells of the IgD\(^+\) population in the spleen of Itpkb\(^{-/-}\) IgHEL/sHEL tg mice (Fig. 5e). These data indicate that an anergy-inducing signal is interpreted as a deletion signal in the absence of Itpkb.

Itpkb regulates calcium in immature B cells in the bone marrow

The degree of BCR cross-linking, and consequently the levels of calcium signaling, can have qualitatively distinct signaling consequences for lymphocyte development. Prolonged receptor engagement, as is likely the case when an autoreactive B cell encounters self-Ag, may result in enhanced BCR signaling. Since a greater degree of receptor cross-linking can result in greater calcium flux, we reasoned that anergy is converted to deletion in the absence of Itpkb as a result of aberrations in calcium signaling. To determine whether differences in calcium signaling in developing B cells in the bone marrow could explain why an anergy-inducing signal led to deletion in the absence of Itpkb, we compared the ability of immature WT and Itpkb\(^{-/-}\) IgHEL tg cells in the bone marrow to flux calcium in response to HEL. Interestingly, upon exposure to HEL, immature B cells (IgM\(^{low}\)IgD\(^{low}\)) in the bone marrow of Itpkb\(^{-/-}\) IgHEL tg mice displayed elevated peak and sustained levels of [Ca\(^{2+}\)]\(_i\), compared with WT mice as a result of enhanced SOC entry (Fig. 6).

Itpkb is not required for Bim expression in tolerant B cells

Several reports have suggested that the proapoptotic protein Bim can control the fate of anergic B cells in addition to controlling B cell survival and function (27). It has also been demonstrated that anergic B cells express significantly higher levels of Bim compared with naive B cells (28). In the HEL tolerance model, the absence of Bim inhibits deletion and promotes the survival of autoreactive B cells in vivo (29). In accordance with this, in the IgHEL/membrane-bound HEL (mHEL) deletion model, the presence of the antiapoptotic factor Bcl2 impedes the removal of autoreactive B cells (30). Interestingly, in an effort to explain low B cell numbers in Itpkb-deficient mice, Marechal et al. discovered that Itpkb-deficient mice exhibit increased Bim gene expression (15), consistent with the observations that Bim is highly expressed in anergic cells and that Itpkb-deficient B cells possess an anergic phenotype (14).

To understand whether up-regulated Bim in the absence of Itpkb may be causing enhanced deletion in the IgHEL/sHEL tg model, we compared the levels of Bim in the bone marrow and spleen of WT and Itpkb\(^{-/-}\) non-tg, IgHEL tg, and IgHEL/sHEL tg mice by intracellular staining. Consistent with the observations from Marechal et al., we found that non-tg Itpkb-deficient B cells in the bone marrow and spleen express higher levels of Bim (15). Contrastingly, in the IgHEL background, Bim levels were slightly higher in

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**FIGURE 5.** The absence of Itpkb converts tolerance to deletion. Bone marrow and spleen from both Itpkb\(^{-/-}\) and WT IgHEL/sHEL tg animals were harvested and stained for several different developmental markers to analyze B cell development by FACS analysis. a, Total numbers in the bone marrow of each B lymphocyte subset. Populations are defined as previously described using the allotypic Abs specific to IgM\(^{high}\) and IgD\(^{high}\) (14). b, Percentages of late pre-B, immature B, and recirculating B lymphocytes are shown. c, Total numbers of each B lymphocyte population in the spleen. Populations are defined as follows: total B cells (B220\(^{+}\)), mature B cells (B220\(^{+}\)IgD\(^{low}\)IgM\(^{low}\)), and endogenous B cells (B220\(^{+}\)IgD\(^{high}\) IgM\(^{high}\)). d, Gating schemes; numbers in plots indicate the percentages of each of the gated populations. e, Expression of CD138 on WT and Itpkb\(^{-/-}\) IgHEL tg B cells. *p < 0.05; **p < 0.01. Data are representative of four separate experiments.
the bone marrow of Itpkb−/− mice, but exhibited normal levels in the spleen. This observation is consistent with the lack of enhanced negative selection in Itpkb−/− IgHEL tg mice. Similarly, upon examination of IgHEL/sHEL tg mice, the levels of Bim were largely normal in bone marrow and splenic B cells, which lack Itpkb compared with WT (Fig. 7). These data suggest that enhanced calcium signaling rather than enhanced Bim induction is responsible for enhanced deletion observed in Itpkb−/− IgHEL/sHEL tg mice.

**Discussion**

In this report we have evaluated the role of Itpkb in B lymphocyte selection and tolerance induction using the IgHEL/sHEL tg model system. Our results establish that Itpkb functions as a key negative regulator of BCR signaling that modulates the level of cytoplasmic calcium in a manner proportional to the degree of self-reactivity, thus controlling B cell selection and tolerance induction.

In our previous study we were unable to rectify whether Itpkb was required for proper fate selection of B cells, or whether Itpkb was required for mature B cell function, or both. To clarify this issue and test the hypothesis that Itpkb influences selection, we examined B cell development in a monoclonal repertoire by crossing Itpkb−/− mice to mice expressing a tg BCR with a high affinity for HEL, but with a very weak affinity for self. In contrast to that observed in non-tg Itpkb−/− mice, which have fewer follicular B cells, IgHEL tg mice lacking Itpkb possess an increase in the numbers of follicular mature B cells. Despite these developmental differences, both non-tg B cells and IgHEL tg B cells lacking Itpkb fail to proliferate to BCR stimulation, yet respond normally to CD40 signals (14). In vivo, IgHEL tg Itpkb−/− B cells expand and secrete Ab normally, presumably due to the ability of T cell help to overcome BCR signaling defects, as has been observed in other models of B cell anergy (31, 32). IgHEL tg Itpkb−/− B cells also maintained other defects observed previously in non-tg Itpkb−/− B cells, such as significantly down-modulated surface IgM, an increase in the percentage of IgD−CD138+ B cells, and elevated SOC channel activity in splenic B cells. Additionally, immature Itpkb−/− IgHEL tg B cells in the bone marrow also exhibit enhanced SOC channel activity. We reasoned that the differences observed in development between Itpkb−/− mice possessing either a polyclonal or monoclonal repertoire of B cells can be explained by the degree of self-reactivity whereby elevated SOC channel activity in Itpkb-deficient cells likely translates into a change in the overall signaling threshold required for development into a particular B cell lineage. Thus, in polyclonal non-tg mice, follicular B cells that survive selection have a moderate level of self-reactivity, as reflected by the amount of cell surface IgM down-modulation observed on normal follicular B cells. The absence of Itpkb in this situation converts this moderate level of self-reactivity to a level sufficient to induce an anergic state. In contrast, in the IgHEL tg model the level of self-reactivity is very low, as reflected in the high levels of IgM on follicular B cells. The absence of Itpkb in this situation enhances surface IgM signaling and promotes the development of most B cells into the follicular lineage, as reflected by enhanced down-regulation of slgM.

Despite the enhanced selection, B cells from Itpkb−/− IgHEL tg mice are anergic, as measured by the lack of proliferation in response to cognate Ag. Importantly, the anergic state induced by the lack of Itpkb is distinct from that induced by chronic self-Ag seen...
in B cells from IgHEL/sHEL tg mice. Both possess down-regulation of surface IgM and an inability to proliferate in response to BCR stimulation. However, Itpkb<sup>−/−</sup> IgHEL tg mice contain normal levels of serum HEL-specific IgM, while IgHEL/sHEL tg mice have greatly reduced levels. Itpkb<sup>−/−</sup> IgHEL tg B cells are able to mount a productive Ab response when adaptively transferred into Ag-primed hosts, while anergic IgHEL/sHEL tg B cells do not proliferate or make an Ab response (19). We speculate that the difference in the in vivo Ab responses in these two models may reflect a requirement for proximal BCR signaling, which is largely intact in Itpkb<sup>−/−</sup> IgHEL B cells but absent in IgHEL/sHEL tg B cells, to synergize with signals from T cells and elicit a functional Ab response.

Biochemically, self-Ag-induced tolerance is quite distinct from anergy induced by lack of Itpkb. Aside from the IgHEL/sHEL tg system, several other models of B cell anergy induction have been described (1), including the anti-ssDNA/dsDNA (33), anti-Smith Ag (34), anti-insulin (35), anti-rheumatoid factor (36), and anti-Ars/A1 (37). The mechanisms used to achieve anergy in each of these systems are varied and include a mix of developmental blockade, BCR modulation, BCR desensitization, impaired survival, or trafficking, likely reflecting differences in the affinity/avidity of the autoreactive BCR and the location and form of the autoantigen. The signaling properties of anergic B cells in each of these models are also quite diverse. These range from dramatic reductions in BCR proximal signaling in the IgHEL/sHEL and anti-Ars/A1 system to normal BCR signaling in the anti-ssDNA, anti-insulin, and anti-Smith systems. Of particular relevance to the present work, anergic B cells in the IgHEL/sHEL and anti-Ars/A1 system display constitutive low level elevation of Ca<sup>2+</sup>, elevated basal levels of Erk and Lyn phosphorylation, and dramatic reductions in proximal BCR signaling, leading to impaired Ca<sup>2+</sup> influx (16, 17, 37). In the anti-Ars/A1 model, altered BCR signaling has been attributed to monophosphorylation of the adaptor proteins Igα/Igβ, which preferentially recruit Lyn and the inositol phosphatase SHIP-1 (1). These biochemical phenotypes contrast greatly with the properties of Itpkb<sup>−/−</sup> IgHEL anergic B cells, which have normal proximal signaling and elevated calcium following BCR stimulation. These data suggest that anergy induced by lack of Itpkb represents a distinct pathway. It will be important in future studies to determine how the lack of Itpkb and elevated calcium leads to B cell anergy and the relationship of this pathway to previously described mechanisms.

Our studies have revealed that in the IgHEL/sHEL tg model system, the absence of the negative regulator Itpkb leads to deletion rather than to anergy induction. This is manifested by down-modulated surface IgM in the immature B cell population, a dramatic reduction in the numbers of both the immature B cells and recirculating B cells in the bone marrow, as well as a 10-fold reduction in the numbers of mature B cells in the spleen. As discussed previously, the levels of intracellular calcium elicited by BCR signaling alter B lymphocyte fate decisions (7). Thus, we reasoned that a tolerance signal is converted to a deletion signal in the absence of Itpkb as a result of elevated calcium. In support of this, we found that immature B cells in the bone marrow of Itpkb<sup>−/−</sup> IgHEL tg mice exhibit significantly enhanced calcium responses upon exposure to HEL.

Previously, Marechal et al. discovered that Itpkb-deficient non-tg B cells possess elevated levels of the proapoptotic protein Bim (15). While we also found this to be true in our Itpkb<sup>−/−</sup> mice, both IgHEL tg and IgHEL/sHEL tg mice, which lack Itpkb, exhibited normal levels of Bim in the bone marrow and spleen. These data indicate that in a polyclonal repertoire, lack of Itpkb results in elevated Bim levels and deletion while in IgHEL tg mice, enhanced BCR-induced calcium signaling promotes selection and fails to up-regulate Bim. We speculate that affinity of the BCR for self-Ags may dictate the dependency on Itpkb for Bim expression. Thus, in the absence of Itpkb, the selection of B cells with weakly self-reactive BCRs is enhanced. In contrast, in B cells containing moderately self-reactive BCRs the lack of Itpkb leads to B cell induction and deletion.

The phenotype of Itpkb<sup>−/−</sup> IgHEL/sHEL tg mice is similar to IgHEL/mHEL tg mice where the HEL Ag is expressed as a multivalent membrane-bound Ag. In this model, the IgHEL B cells are deleted in the bone marrow as a result of greater cross-linking of the BCR, indicating that the degree of cross-linking can result in dramatically different cellular consequences. While the IgHEL/mHEL tg mice possess normal numbers of immature B cells in the bone marrow, surface IgM is noticeably reduced compared with IgHEL tg cells, indicating that these cells have clearly encountered self-Ag (38). This observation is similar to what is found in immature B cells from Itpkb<sup>−/−</sup> IgHEL/sHEL tg bone marrow, where the levels of surface IgM are clearly diminished.

Other negative regulators of B lymphocytes, such as CD22, SHP-1, and Lyn, are part of a common biochemical signaling pathway that involves the regulation of calcium. Like Itpkb-deficient mice, mice deficient in any of these negative regulators also display alterations in tolerance induction leading to deletion rather than anergy of IgHEL tg B cells in the presence of HEL (3, 23–26). Since elevated calcium entry is the only known common signaling defect observed between mice that lack Itpkb and mice that lack any of these negative regulators, these studies provide strong support for the hypothesis that the levels of intracellular Ca<sup>2+</sup> following BCR triggering determine the fate of developing B cells and control tolerance induction.

Interestingly, mice lacking Itpkb do not show signs of autoimmune disease. This is in contrast to deletion of other negative regulators of BCR signaling (SHP-1, Lyn, CD22) that manifest autoimmune pathology. The lack of autoimmunity in Itpkb<sup>−/−</sup> animals may reflect additional phenotypes present in Itpkb<sup>−/−</sup> mice such as the absence of T cells and reduced neutrophil viability that may limit induction of autoimmunity (12, 13, 39). However, mixed bone marrow chimeras, which contain normal numbers of WT T cells and neutrophils, did not show evidence of autoimmunity (M. P. Cooke and A. T. Miller, unpublished observation). The use of tissue-specific deletions of Itpkb and the impact of these deletions on autoimmune models will be required to determine whether loss of Itpkb in mature leukocytes promotes or prevents autoimmune disease.

The present studies using the IgHEL/sHEL system have allowed us to formulate a more comprehensive model for how Itpkb controls B cell selection and tolerance induction. Our model proposes that Itpkb functions to suppress tolerance induction by extending the repertoire of B cells that survive selection. Thus, self-reactive BCRs induce sufficient [Ca<sup>2+</sup>], to activate the calcium-dependent kinase Itpkb. Itpkb, once activated, produces IP<sub>4</sub>, which dampens SOC activity, reduces [Ca<sup>2+</sup>]<sub>i</sub>, and salvages B cells that would otherwise be deleted. In the absence of Itpkb, Ca<sup>2+</sup> responses are augmented, causing cells that would normally be selected to be anergized and cells that would normally be anergic to be deleted. Importantly, the current model does not exclude a role for IP<sub>4</sub> or other higher order inositol phosphates at later stages of B cell development or a function for IP<sub>4</sub> outside of Ca<sup>2+</sup> regulation. Given the importance of Itpkb for the development and function of both B and T lymphocytes, further studies directed at understanding the molecular signals that regulate the production, metabolism and function of inositol phosphates in lymphocytes should provide...
important insights toward understanding and treating autoimmune diseases.

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References