

Loss of Bim Allows Precursor B Cell Survival But Not Precursor B Cell Differentiation in the Absence of Interleukin 7

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Abstract

Interleukin (IL)-7 is a stromal cell-derived cytokine required for the survival, proliferation, and differentiation of B cell precursors. Members of the Bcl-2 family of proteins are known to have profound effects on lymphocyte survival, but not lymphocyte differentiation. To distinguish the relative dependence on IL-7 of B cell precursor survival versus B cell differentiation, the combined effects of lack of IL-7 and lack of the proapoptotic Bcl-2 relative, Bim, were studied. Bim is expressed to varying degrees in all B cell precursors and B cells. Lack of Bim compensated for lack of IL-7 in the survival of pro-, pre-, and immature B cells; however, lack of Bim did not substitute for the requirement for IL-7 in B cell precursor differentiation or B cell precursor proliferation. Precursor B cell survival is more dependent on sufficient levels of IL-7 than precursor B cell differentiation because the number of B cells and their precursors were reduced by half in mice heterozygous for IL-7 expression, but were restored to normal numbers in mice also lacking Bim. Hence, Bim and IL-7 work together to control the survival of B cell precursors and the number of B cells that exist in animals.

Key words: Bim • B cell development • IL-7 • B cell survival • Bcl-2 family

Introduction

B cell development from common lymphoid precursors (CLPs) to mature B cells involves a series of differentiation events. Thus, CLPs mature into pre-pro-B cells and then pass sequentially through the pro- and pre-B cell stages, eventually becoming immature B cells expressing, for the first time at this stage, a fully fledged IgM protein (1–3). For some of these events, the external signals that drive these processes are either not known, or the process may simply be programmed into the developing cell. The external signals have been well defined for other steps. Chief amongst these are processes driven by IL-7 (4, 5).

IL-7 is produced by stromal cells and epithelial cells in various organs, including the bone marrow (4). It first came to be recognized as a factor that might be important for B cell development in experiments showing that IL-7 could promote the proliferation and differentiation of B cell pro-

genitors in bone marrow cultures (6–8). Since then, many different types of knockout and transgenic mice have added to our knowledge of the effects of IL-7 on B cell development (9–12). As a result, it is now thought that IL-7 is required during several stages of B cell development. For example, IL-7 induces some of the steps of B cell differentiation. These include the differentiation of CLPs into pre-pro-B cells (13) and, by inducing expression of RAGs with consequent construction of a functional μ chain locus, IL-7 promotes the differentiation of pro-B cells into pre-B cells (14–18). IL-7 also supports the survival of some types of developing B cells and can, in some cases, induce their proliferation (4, 5). Once immature B cells have left the bone marrow they cease to rely on IL-7 for survival or further development. The survival of these more mature B cells is controlled by ligands for the TNF receptor-related protein,

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Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; CLP, common lymphoid precursor; TSLP, thymic stromal lymphopoietin.

BAFF (also known as Tall-1, BLyS, THANK, and zTNF; reference 19).

Some of these conclusions, however, are controversial. This is for several reasons. First, in some cases, IL-7 can be replaced by other factors. For example, in culture and in very young mice, the effects of IL-7 on developing B cells might be replaced by thymic stromal lymphopoietin (TSLP), a cytokine that binds to a receptor related to that for IL-7 (20, 21). However, no specific role for TSLP has been identified and mice lacking the TSLP receptor have a normal B lymphoid compartment (22). Second, in some cases, IL-7 appears to act in conjunction with other stimuli. For example, the effects of IL-7 on very early lymphocyte precursors also involve fms like tyrosine kinase 3 (Flt3) or IL-11 plus steel factor (23–25), and the effects of IL-7 on the pro- to pre-B cell conversion are aided by signaling through the pre-B cell receptor itself (26, 27). Third, because the development of B cell precursors deprived of IL-7-driven signals is substantially blocked at a very early stage, it precludes study of the effects of IL-7 on later stages of B cell development. Finally, it is not always a simple matter to distinguish between the effects of IL-7 on differentiation versus its effects on B cell precursor survival and/or proliferation.

To deal with this last phenomenon, investigators have manipulated factors known to affect lymphocyte survival. These factors include the antiapoptotic Bcl-2 family members Bcl-2, a protein whose expression is increased by IL-7 signaling, and Bcl-xl, as well as their proapoptotic relative, Bim. Overexpression of these two antiapoptotic proteins in B cells has different consequences. Although the number of pre-B cells increases in mice overexpressing Bcl-xl (28), expression of Bcl-2 has no effects on B cell precursor number, but instead results in increased numbers of mature B cells (29). Mice lacking Bim also contain three- to fourfold more mature B cells than normal animals (30, 31). Because of the potential of IL-7 to increase expression of antiapoptotic Bcl-2 family members and the known interaction between these survival factors and Bim, Bim could play a role in mediating the death of B cells deprived of IL-7. Interestingly, it was recently shown that B cell precursors from mice lacking Bim are resistant to death when cultured in the absence of IL-7 (30). However, analysis of the role of Bim in mediating the death of B cells deprived of IL-7 in vivo has not been published.

To determine the relative effects of IL-7 on B cell precursor survival versus B cell precursor differentiation, we chose to study the requirements for IL-7 in cells that do or do not express Bim. Absence of Bim allows the survival of some B cell precursors in the absence of IL-7 in vitro. However, our results show that lack of Bim does not promote the conversion of lymphocyte precursors to pro-B cells or the induction of IgM expression by pre-B cells. Under conditions of limiting IL-7, B cell differentiation continues, with B cells less likely to survive if they express Bim, but not if they lack Bim. Thus, the number of immature B cells in animals is governed to a large extent by IL-7 and its ability to counteract the death-inducing effects of Bim.

Materials and Methods

Mice. Homozygous Bim^{-/-} mice were bred from mice provided by A. Strasser (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). The IL-7^{-/-} mice were bred from animals provided by V. von Freeden-Jeffry and R. Murray (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA; reference 32). All mice were backcrossed between seven and nine generations to C57BL/6 mice purchased from The Jackson Laboratory. All mice were maintained under pathogen-free conditions in the Biological Resource Center, National Jewish Medical and Research Center, and were 4–16 wk old at the beginning of each experiment unless otherwise noted. The protocols used were in accordance with the guidelines drafted by the National Jewish Medical and Research Center and the Institutional and Animal Care Use Committee.

Cell Sorting and Flow Cytometry Analysis. For surface staining, cell suspensions were incubated for 15 min on ice with anti-mouse FcR- γ 2, 2.4G2 to prevent nonspecific binding of antibodies to the Fc receptors on cells. Cells were then washed and incubated on ice with mixtures of labeled antibodies as noted. Anti-mouse antibodies (BD Biosciences) used for these experiments included anti-CD2-PE, anti-CD21/CD35-FITC, anti-CD23-PE, anti-CD24-biotin, anti-CD5-PE, anti-B220-APC or CyChrome, and anti-IgM-FITC or anti-IgM-APC. The monoclonal hamster anti-mouse Bim antibody, Ham 151-149, was made from a hamster immunized with a complex of mouse Bim and Bcl-XL produced in insect cells. The antibody reacts in Western blots and by immunofluorescence with permeabilized B and T cells from Bim^{+/+}, but not Bim^{-/-}, mice. Cells were permeabilized with 0.03% saponin, incubated with 12.5 μ g/ml Ham 151-149, washed thoroughly, and then stained with PE- or APC-coupled anti-hamster antibodies. After incubation with antibodies, cells were sorted using a MoFlo machine (DakoCytomation) or analyzed on a FACSCalibur machine. Subsequent analyses used CELLQuest software (BD Biosciences) and FloJo (TreeStar).

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Labeling. Cells were labeled with CFSE (Molecular Probes) as described previously (33). In brief, a stock solution of 5 mM CFSE in DMSO was diluted at 1:10 in serum-free Spinner modified Eagle's medium (SMEM; Biosource International) that was prewarmed to 37°C. B cells were diluted to 5 \times 10⁷ cells/ml and incubated with 5 μ M CFSE at 37°C for 10 min. Labeled cells were washed twice with SMEM and placed in culture as described above.

Bone Marrow Culture. Bone marrow cells were isolated and cultured as described previously (34). In brief, bone marrow was obtained from the femurs of 6–12-wk-old mice. A single cell suspension was prepared, depleted of erythrocytes using buffered ammonium chloride, washed twice in SMEM, and cultured at 5 \times 10⁵ cells/ml with 5 ml/well in a six-well Petri dish (7% CO₂, 37°C) in SMEM containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M 2-ME, 10% FBS (Oatianta Biologicals), and 50–100 U of IL-7 derived from culture supernatant of J558L cells transfected with murine IL-7 cDNA (provided by A. Rolink, University of Basel, Basel, Switzerland). Cells were cultured for time points as noted in Fig. 2, washed twice with SMEM, and used in subsequent experiments. Cell viability was assessed using forward scatter and side scatter analysis on the FACSCalibur cytofluorograph.

RNA Purification and Real-Time PCR Analyses. RNA was purified from bone marrow cells using Trizol (Life Technologies) according to the manufacturer's instructions. cDNAs were synthesized using M-MLV reverse transcriptase according to the

manufacturer's instructions (Life Technologies). cDNAs for IL-7 and actin were analyzed by real-time PCR using the TaqMan PCR kit and an ABI 7700 sequence detector thermal cycler according to the manufacturer's instructions (Applied Biosystems), and quantified with Sequence Detection Systems software 1.6.3 (Applied Biosystems). cDNAs were quantified using a real-time PCR probe and primers previously described for IL-7 (35) and actin (36). Analysis of negative control cDNA synthesis in reactions lacking the M-MLV reverse transcriptase did not produce signal within 40 cycles. Additionally, cDNA made from bone marrow of IL-7^{-/-} animals failed to produce a signal within 40 cycles, demonstrating that the IL-7 primers and probe are specific for IL-7 cDNA. RNA levels quantified by these methods were normalized to the levels of actin detected for each sample. All samples were performed in triplicate.

Results

Bim Is Expressed at All Stages of B Cell Development. We wished to study whether loss of proapoptotic Bim would allow developing B cells to endure IL-7 deprivation. For either of these proteins to affect B cells directly, Bim must be expressed within the cell and the receptor for IL-7 must be expressed on the cell surface. The receptor for IL-7 is expressed on developing B cells from the beginning of lymphoid commitment to the immature B cell stage (37). However, expression of Bim at different stages of B cell de-

velopment has not previously been evaluated. Therefore, we isolated developing and mature B cells from bone marrow and spleens of control Bim^{-/-} mice and their normal Bim^{+/+} littermates, stained them to identify the various B cell developmental subsets, and stained Bim within the cells using a hamster anti-Bim monoclonal antibody, Ham 151-149, which we have produced (Fig. 1).

The anti-Bim antibody has good specificity for Bim because it stained B cells from Bim^{-/-} animals very poorly and mature B cells from Bim^{+/+} animals well. Anti-Bim stained all B cell precursors from Bim^{+/+} animals more intensely than the Bim^{-/-} controls. However, the intensity of staining with anti-Bim varied with the maturational state of the B cell, with pre-pro-B cells barely registering above background. Staining increased as the cells matured from pro- and pre-B cells through the immature stage and was

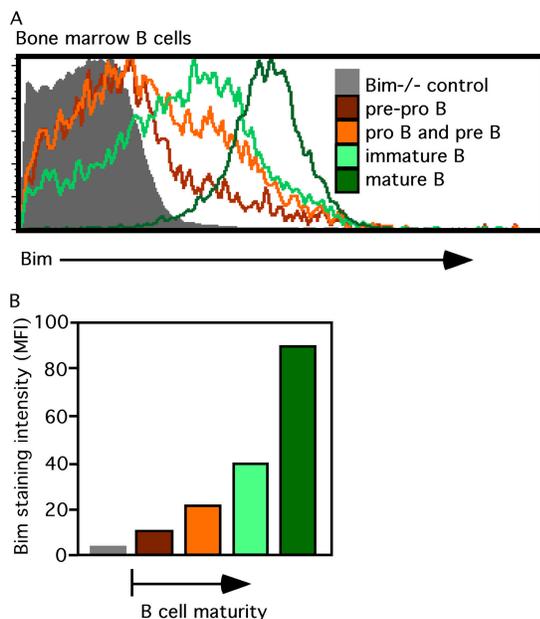


Figure 1. Bim expression in bone marrow B cells. Bone marrow cells isolated from wild-type and Bim^{-/-} mice were stained with antibodies against developmental stage-specific surface markers, and then permeabilized and stained using the anti-Bim antibody Ham 151-149. (A) Bone marrow cells were analyzed by flow cytometry to determine the expression of Bim in pre-pro-B cells (B220⁺ CD19⁻ IgM⁻), pro- and pre-B cells (B220⁺ CD19⁺ IgM⁻), immature B cells (B220⁺ CD19⁺ IgM⁺), and mature recirculating B cells (B220^{hi} CD19⁺ IgM^{int}). Bim^{-/-} B cells were stained as a negative control and are shown in solid gray. (B) Mean fluorescence intensities of cells analyzed above are shown. Data are representative of four individual experiments.

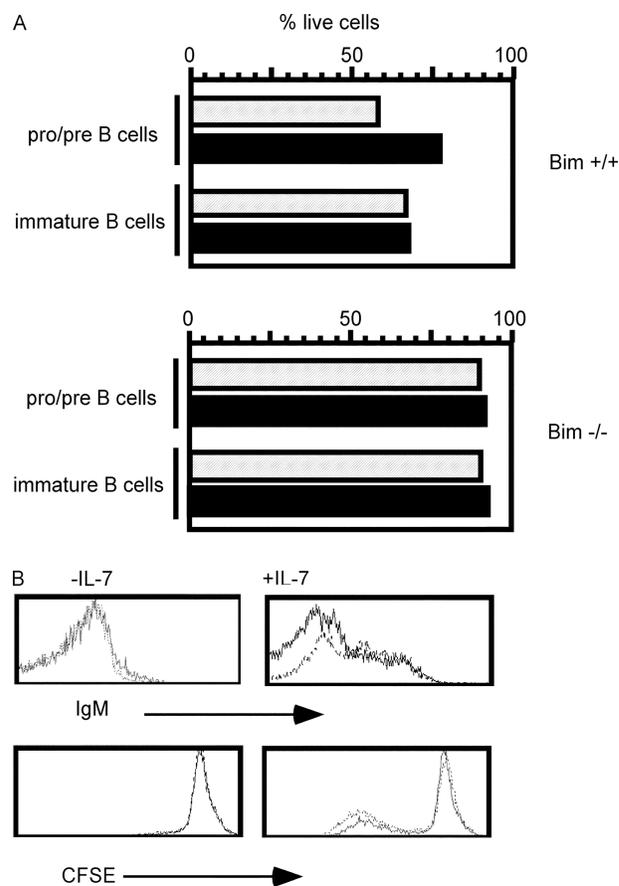


Figure 2. Loss of Bim allows developing B cells to survive in vitro in the absence of IL-7. (A and B) Bone marrow cells were isolated from Bim^{-/-} and Bim^{+/+} mice, sorted into B220⁺ IgM⁻ (pro- and pre-B) and B220^{int} IgM⁺ (immature) populations, and cultured in the absence or presence of exogenous IL-7. (A) Sorted B cells were counted and analyzed at 42 h by flow cytometry. Cells were assessed for percent of live cells (live gated cells/total cell number) by forward versus side scatter analysis. Hatched boxes represent cells cultured in the absence of IL-7, whereas closed boxes represent cells cultured in the presence of IL-7. (B) Sorted B cells were labeled with CFSE and cultured in the absence or in the presence of IL-7 for 3 d. Cells were then analyzed by flow cytometry for differentiation (as determined by expression of IgM) and proliferation (loss of CFSE).

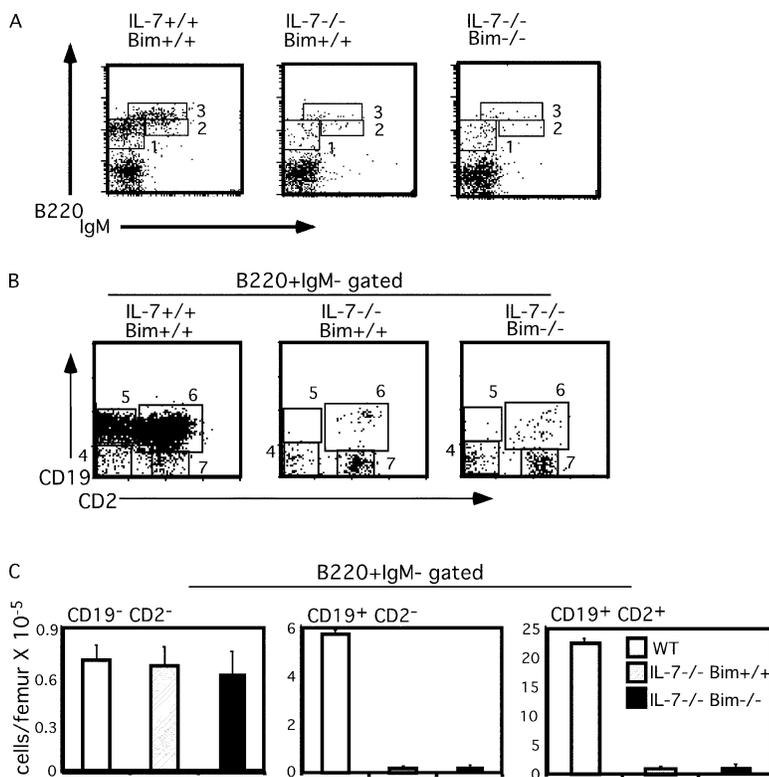


Figure 3. Lack of Bim has no effect on the block in B cell development caused by the absence of IL-7. (A) Bone marrow cells from 6-wk-old wild-type IL-7^{-/-} Bim^{+/+} and IL-7^{-/-} Bim^{-/-} mice were stained with B cell developmental stage-specific markers and analyzed by flow cytometry. Pro- and pre-B cells are indicated by box 1 (B220⁺ IgM⁻), whereas immature B cells and mature recirculating B cells are indicated by boxes 2 (B220⁺ IgM⁺) and 3 (B220^{int} IgM^{int}), respectively. (B) To further delineate the early B cell subsets, cells were gated through box 1 and analyzed for expression of CD19 and CD2. Boxes 4, 5, and 6 depict pre-pro-B, pro-B, and pre-B cells, respectively. Box 7 indicates a population that lacks expression of B cell lineage markers CD19 and IgM, and thus probably represents a B220⁺ B lineage⁻ cell. (C) The total number of B cells in the developmental subfractions was determined for each genotype. Numbers represent cells × 10⁻⁵ collected from one femur per mouse (*n* = 4/genotype). Error bars represent SEM.

highest in mature cells recirculating through the marrow (Fig. 1, A and B). These results show that Bim is expressed to some extent at all stages of B cell development and that expression increases with the maturity of the B cell. Therefore, Bim might affect the survival of all of these subsets.

The Death of Early B Cells Induced by Lack of IL-7 Is Caused by Bim. It has been reported previously that lack of Bim promotes survival of pre-B cells in the absence of IL-7 in vitro (30). To confirm this result and examine the effects of Bim in survival of developing B cells deprived of IL-7, we isolated B220⁺ IgM⁻ pro- and pre-B cells and immature B cell precursors from Bim^{+/+} and Bim^{-/-} mice and cultured them in the absence or presence of IL-7. Cultures were then harvested and B cell survival was monitored (Fig. 2 A).

In the absence of IL-7, ~40% of Bim^{+/+} pro- and pre-B cells died within the first 48 h. Cell death was reduced if these cells were cultured in the presence of IL-7 or if they did not express Bim. The rate of death of the Bim^{-/-} pro- and pre-B cells was unaffected by IL-7. The rate of death of immature B cells was unaffected by the presence of IL-7. Bim^{+/+} immature B cells were just as likely to die if IL-7 were present in the culture medium than if it were not. Again, loss of Bim allowed immature B cells to survive without additional cytokine. Thus, our data suggest that Bim is required for death of developing B cells deprived of cytokines. In the case of immature B cells, this is not due to lack of IL-7. It is not surprising that immature B cells do not respond to IL-7 because these cells have low expression of the IL-7Rα (37, 38) and their survival is governed by Baff (19).

Although a straightforward interpretation of the results in Fig. 2 A suggests that IL-7 operates to counteract the death-promoting effects of Bim in pro- and/or pre-B cells, this interpretation is complicated by the fact that IL-7 can promote development of these subsets of B cells. To begin to dissect these events and determine whether lack of Bim could affect the requirement for IL-7 in pro- and pre-B cell development, we isolated B220⁺ IgM⁻ B cells from Bim^{+/+} and Bim^{-/-} mice, labeled the cells with CFSE, and cultured them in the absence or presence of IL-7. After 3 d, we analyzed the cells for proliferation, as assessed by loss of CFSE, and for differentiation into cells expressing IgM, an event that is known to involve IL-7 (13, 14). As shown in Fig. 2 B, the sorted cells did not divide or differentiate unless IL-7 was present. Additionally, the requirements for IL-7 for differentiation and proliferation were unaffected by expression of Bim. Overall, these results show that IL-7 and Bim counteract each other's effects on the survival of pro- and pre-B cells. However, survival alone (promoted by the absence of Bim) is not enough to permit differentiation to IgM expression and proliferation. As reported previously (12–14), IL-7 is needed to induce these latter phenomena.

Absence of Bim Does Not Substitute for IL-7 in the Conversion of CLPs to Pro-B Cells. IL-7 is needed to promote differentiation at very early stages of B cell development, in the conversion of CLPs to pro-B cells and at the conversion of pro- to immature B cells (12–14, 16). To find out whether lack of Bim can substitute for IL-7 in these events, we compared the number of B cells at various developmental stages in the bone marrow of mice expressing or lacking IL-7 and

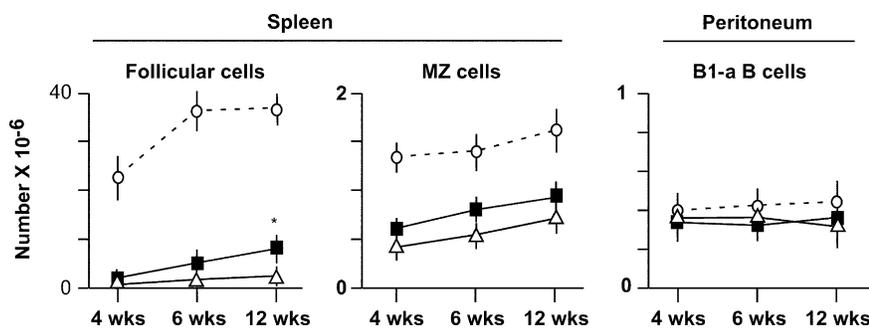


Figure 4. Follicular B cells in IL-7^{-/-} Bim^{-/-} animals accumulate as mice age, but their numbers are fewer than in wild-type mice. Splenic and peritoneal B cells from 4–12-wk-old wild-type (○), IL-7^{-/-} Bim^{+/+} (△), and IL-7^{-/-} Bim^{-/-} (■) mice were analyzed by flow cytometry. B220⁺ IgM⁺ CD5⁺ cells representing B-1a B cells from the various genotypes are depicted on the right. The total number of spleen cells characteristic of marginal zone (B220⁺ CD21^{hi} CD23⁻) or follicular B cells (B220⁺ CD21^{int} CD23⁺) is shown on the middle and left panels, respectively. *n* = 4–6 animals per group and error bars are SEM.

in mice expressing or lacking Bim. Bone marrow cells were isolated from 6-wk-old mice and stained with antibodies against B220, IgM, CD19, and CD2. Immature and mature B cells, identified as cells bearing IgM and intermediate and high levels of B220, respectively (1), could readily be identified in IL-7^{+/+} Bim^{+/+} mice (Fig. 3 A, populations 2 and 3). These cells were found at very low percentages in mice lacking IL-7 whether or not the cells also expressed Bim. Likewise, cells expressing B220 but not IgM, most of which are pro- and pre-B cells (1), were present in IL-7^{+/+} animals and strikingly deficient in mice lacking IL-7 (Fig. 3 A, population 1). Again, absence of Bim had no effect on this deficit.

Several types of bone marrow cells, including several types of B cell precursors, bear B220 but not IgM (1). To find out if lack of Bim affected any of these populations in IL-7-deficient mice, we analyzed the B220⁺ IgM⁻ cells in more detail. B220⁺ IgM⁻ bone marrow cells from IL-7^{+/+} animals contained substantial numbers of CD19⁺ CD2⁻ pro-B cells and CD19⁺ CD2⁺ pre-B cells (Fig. 3 B and C, populations 5 and 6; reference 39). These cells were at very low percentages in mice lacking IL-7 and, again, their numbers were not affected by lack of Bim.

These results were perhaps not surprising because lack of IL-7 has recently been reported to interrupt B cell development at the time of conversion of B220⁻ CLPs to B220⁺ pre-pro-B cells. Obviously, a block at this stage would inhibit all downstream events. Therefore, we were particularly interested in the effects of Bim deficiency on the very earliest B cell precursor cells, the B220⁺ IgM⁻ CD19⁻ CD2⁻ cells in bone marrow (Fig. 3 B, population 4). The number of these cells was unaffected by the presence or absence of IL-7 or Bim (Fig. 3, B and C). These cells include non-B lineage cells in addition to pre-pro-B cells (1). So it is difficult to interpret the absence of an effect of IL-7 deficiency in these cells. It appears clear, however, that in the absence of IL-7, there is a block in lymphocyte development during the conversion of CLPs to pro-B cells that it is not influenced by the loss of proapoptotic Bim.

A final population of cells, bearing B220 and CD2 but lacking IgM and CD19, is also evident in Fig. 3 B (population 7). These cells express neither the granulocyte marker, GR1, nor the β chain of the T cell receptor (not depicted), and their numbers are unaffected by the absence of IL-7 or Bim. Taken together with the data presented earlier in this paper, these results suggest that IL-7 promotes early B cell differentiation in at least two stages: during the conversion

of CLPs to pro-B cells and at the conversion of pro-B cells to pre- and immature B cells. Lack of Bim does not substitute for IL-7 in either process.

Lack of Bim Increases the Rate at Which Mature B Cells Accumulate in IL-7^{-/-} Mice. Mature splenic B cells appear in very low numbers in mice lacking IL-7 or lacking the ability to signal via the IL-7 receptor (9–12), whereas B-1-type B cells are unaffected by IL-7 deficiency. To determine whether the absence of Bim would modify the number of cells that could accumulate at the mature B cell stage in the absence of IL-7, we analyzed B cells in the spleens and peritoneal lavage fluid of mice lacking IL-7 with or without Bim, and in the spleens of wild-type animals.

In normal animals, mature, follicular B cells increased somewhat in number as mice aged from 4 to 6 wk and remained constant between 6- and 12-wk-old wild-type mice (Fig. 4). As previously reported (9), the spleens of IL-7-deficient animals contained very few mature B cells. Their numbers increased slightly as the mice aged. In IL-7-deficient mice, the number of mature B cells was increased if the cells also lacked Bim. The differential between IL-7^{-/-} B cells expressing or lacking Bim became more evident with increasing age. Peritoneal B-1a cells were unaffected by loss of either IL-7 or Bim (Fig. 4).

The development of marginal zone B cells has been reported to be relatively resistant to the absence of IL-7 (40, 41). Therefore, we analyzed the number of these cells. As expected, lack of IL-7 had much less effect on the number of marginal zone B cells than on mature B cells (Fig. 4). Even so, marginal zone B cells were reduced by ~75% in number in the spleens of 4-wk-old IL-7-deficient animals, although they gradually increased thereafter (Fig. 4). Absence of Bim increased these numbers somewhat, but did not restore their numbers to those in wild-type animals.

These results indicate that even though there is no demonstrable effect of the absence of Bim on the number of B cells at any stage of development in IL-7^{-/-} mice, lack of Bim can to some extent compensate for lack of IL-7, as far as the number of mature B cells are concerned. This could be due to a very subtle effect of Bim on the number of B cell precursors that can develop independently of IL-7. Alternatively, lack of Bim could prolong the life span of B cells that have managed to develop in the absence of IL-7.

Bim^{-/-} Cells Survive and Develop Better Than Bim^{+/+} Cells When IL-7 Is Limiting. Experiments have shown that availability of IL-7 in normal animals limits the number of

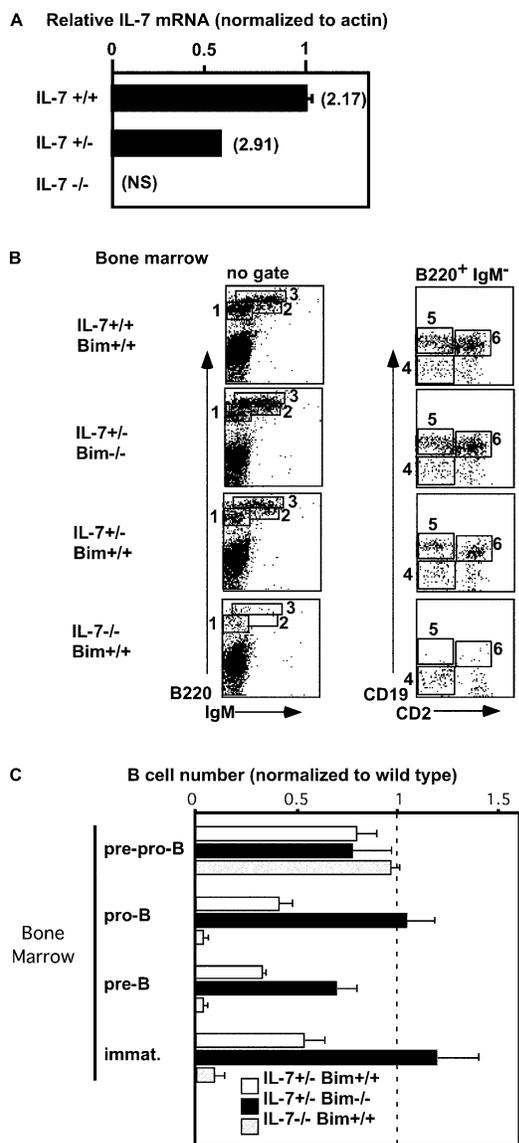


Figure 5. Bim^{-/-} B cells survive and develop better in limiting IL-7 than their Bim^{+/+} counterparts. (A) Limiting dilution real-time PCR analysis of total bone marrow cells from IL-7^{+/+}, IL-7^{+/-}, and IL-7^{-/-} mice. IL-7 mRNA was normalized to levels of actin mRNA for each sample. Samples were run in triplicate from two to four mice per genotype. (B) Bone marrow cells from wild-type, IL-7^{+/-} Bim^{+/+}, IL-7^{+/-} Bim^{-/-}, and IL-7^{-/-} mice were isolated and developing B cell subsets were analyzed by flow cytometry as detailed previously (Fig. 3). (C) Total cell numbers within each subset were determined. Numbers of cells in each subset were then normalized to numbers obtained from wild-type mice. IL-7^{+/-} Bim^{+/+} cells are depicted by open bars, IL-7^{+/-} Bim^{-/-} by closed bars, and IL-7^{-/-} Bim^{+/+} cells are represented by striped bars ($n = 3/$ genotype and error bars show SD).

B cells that are produced. For example, administration of IL-7, or overexpression of the gene for IL-7, increases the number of B cells in mice (42, 43). In old mice, IL-7 levels in the bone marrow decrease and B cell production is diminished (44–45). The experiments described above and elsewhere show that IL-7 is needed both for survival of early B cells and, at several stages, for their differentiation.

Our in vitro experiments also show that IL-7 governs survival of early B cells and that at the stages where this applies, survival in the absence of IL-7 can be achieved by lack of Bim. These results lead us to ask whether differentiation or survival of B cells is more affected by low levels of IL-7.

To answer this question we bred mice expressing or lacking Bim and heterozygous for the IL-7 deficiency. To determine whether IL-7 is more limiting in IL-7^{+/-} mice than in wild-type animals, we performed real-time PCR analysis on bone marrow from IL-7^{+/+}, IL-7^{+/-}, and IL-7^{-/-} mice. We measured IL-7 message and normalized its levels to those of actin. Fig. 5 A shows that the level of IL-7 mRNA in IL-7^{+/-} bone marrow is 60% of that detected in IL-7^{+/+} marrow. Therefore, gene dosage does have an effect on IL-7 mRNA levels and probably (although this is very difficult to measure in vivo) on IL-7 protein levels.

Next, to determine whether limiting IL-7 had a functional consequence in vivo, we analyzed the number of B cells at various developmental stages in the bone marrow and spleens of these animals, and control wild-type mice. The results are shown in Fig. 5 B and summarized in Fig. 5 C. As before, bone marrow from wild-type mice contained B cells at all stages of development, and bone marrow from IL-7^{-/-} Bim^{+/+} mice contained normal numbers only of B220⁺ IgM⁻ cells (Fig. 5 B, population 1). Bone marrow from mice expressing limiting amounts of IL-7 and wild-type levels of Bim, the IL-7^{+/-} Bim^{+/+} animals, contained normal numbers of B220⁺ CD19⁻ CD2⁻ IgM⁻ cells and about half the number of developing B cells at all later stages compared with wild-type animals. Strikingly, however, bone marrow from IL-7^{+/-} Bim^{-/-} animals contained normal numbers of B cells at all stages of development.

Not surprisingly, survival and differentiation in the bone marrow was followed by transport to and accumulation in the spleen. Thus, the spleens of young IL-7^{+/-} Bim^{-/-} animals contained normal numbers of mature B cells, whereas these cells were at half normal numbers in the spleens of IL-7^{+/-} Bim^{+/+} mice (not depicted). Because lack of Bim overcame the partial deficiency of IL-7 in IL-7^{+/-} animals, and Bim affects only the survival of B cells, these results suggested that for early B cells, survival is more dependent on IL-7 levels than on differentiation.

Discussion

IL-7 is clearly very important for many processes that occur during B cell development. It guides the differentiation of B cell precursors through at least two stages, the conversion of CLPs to pro-B cells, and the conversion of pro- to pre-B cells. IL-7 also promotes survival and proliferation at several stages of B cell development. Given the many phenomena associated with IL-7, the interactive effects of the proapoptotic factor Bim and IL-7 were unpredictable.

Here, we show that Bim probably does not affect any of the differentiation events induced by IL-7 signaling. B cell development is blocked at a very early stage in mice lacking IL-7 and the blockage is not circumvented by the absence

of Bim, even though in wild-type mice, cells at these very early stages express Bim, albeit at very low levels. Thus, either IL-7 does not induce survival signals at this stage, survival alone is not sufficient to drive conversion of CLPs to pro-B cells, or Bim is not the apoptotic protein crucial for death at this stage.

Likewise, IL-7 induces the differentiation of pro- and pre-B cells to the immature B cell stage and consequent IgM expression (14–16). Again, lack of Bim was not sufficient to induce this differentiation event, nor would it allow proliferation of B cell precursors, although lack of Bim did allow survival of the pre-B cells in the absence of IL-7. Thus, simply staying alive is not enough to allow differentiation of the cells. Additional signals are required, and these signals are delivered via the IL-7 receptor.

It is interesting to compare the effects on survival and lack of effects on differentiation and proliferation of Bim and the various antiapoptotic proteins expressed in early B cells. B cell development is fairly normal in at least young mice lacking Bcl-2 (46). Conditional knockout, at the point at which early B cells start to express CD19 (pro-B cells), of the gene coding for the antiapoptotic protein Mcl-1 causes the death of B cells at that stage (47). This is one of the stages at which lack of Bim compensates for lack of IL-7. Hence, it is likely that in pro-B cells, IL-7 allows survival by inducing Mcl-1 (47) and the Mcl-1 counteracts the death-inducing effects of Bim. In the absence of Bim, IL-7 and its downstream effector Mcl-1 are not required for survival. Overall, the results here suggest that Bim and IL-7 balance each other's effects on B cell progenitor survival. However, survival alone is not sufficient to allow B cell precursor differentiation from CLPs to pro-B cells, or from pro- to immature B cells. IL-7 itself is needed for these events.

The data presented here do allow us to draw some conclusions about the relative need for IL-7 signaling for differentiation versus survival. The number of B cells at most maturational stages are about half those in wild-type animals in mice that are heterozygous for IL-7 expression. This suggests that levels of IL-7 are accurately reflected in the number of B cells and their precursors. This effect could be due to control by IL-7 on rates of differentiation, on rates of proliferation, or on survival of B cell precursors. The results presented here suggest that levels of IL-7 affect most predominantly the survival of B cell precursors. This interpretation is suggested by the fact that absence of Bim allows normal numbers of B cell precursors and B cells to appear in mice in which IL-7 is limiting, and Bim is known to affect cell survival, not cell proliferation or, as shown here, cell differentiation.

Why would B cell survival be more sensitive to low levels of IL-7 than B cell differentiation or proliferation? This could be explained in several ways. First, in developing B cells, a brief "hit" by IL-7 might be sufficient to drive differentiation or proliferation signals, whereas survival may require IL-7 to be continuously present. Thus, when IL-7 is limiting, the cells might be intermittently deprived of this life-giving cytokine and thus more likely to die. Alter-

natively, the observations reported here may suggest that low levels of continuous signaling via the IL-7 receptor are sufficient to induce differentiation and proliferation, but not survival. Others have shown that IL-7 acts with other factors at some stages of B cell development. For example, in normal mice, IL-7 and Flt3 ligand act together to stimulate CLP differentiation to pro-B cells (23). Later in B cell development, the pre-B cell receptor and IL-7 act synergistically to promote further differentiation of pre-B cells, and signaling through the pre-B cell receptor increases the ability of the cell to detect signaling via the IL-7 receptor (27). Therefore, perhaps the greater sensitivity of precursor B cell survival versus differentiation to IL-7 reflects the (untested) idea that survival is stimulated solely by IL-7, whereas other factors act together with IL-7 to promote differentiation.

IL-7-deficient mice are profoundly lacking in mature B cells. Lack of Bim counteracts this deficiency to some extent. This could be due to subtle effects of lack of Bim on cell precursors. For example, in the absence of IL-7, Bim^{-/-} pro- and pre-B cells might convert to immature B cells at a slightly higher rate than Bim^{+/+} pro- and pre-B cells, thus slowly giving rise to a larger number of mature B cells as mice age. If this conclusion is correct, it would suggest that survival time is related to the probability that a B cell precursor will convert to the next stage in its development, in the absence of any other signal. However, it is more likely that mature B cells accumulate in the absence of IL-7 if they also lack Bim because Bim is involved in the survival of mature B cells themselves. Isolated mature B cells from normal mice die fairly rapidly in the absence of BlySS/BAFF in culture, whereas mature B cells lacking Bim have a long survival time when cultured in the same manner (unpublished data). It is likely that this could reflect a longer survival time for mature B cells in animals. Hence, in IL-7-deficient mice, mature B cells probably accumulate more rapidly if they also lack Bim because lack of Bim promotes the survival of the mature B cells themselves.

Overall, however, the results presented here show that Bim is expressed at most stages of B cell development and that Bim and IL-7 counteract each other's actions in promoting the death versus the survival of B cell precursors at several stages of development. Bim acts solely to affect B cell survival, however, and does not directly affect B cell proliferation or differentiation. Hence, the balance between Bim and IL-7 is crucial to the control of B cell precursor survival and thus total B cell number.

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