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The Survival and Differentiation of Pro-B and Pre-B Cells in the Bone Marrow Is Dependent on IL-7Rα Tyr449

Daniel T. Patton,* Adam W. Plumb,* and Ninan Abraham*†

IL-7 is critical for murine T and B cell development and survival and plays a significant role in lymphoblastic leukemia in both humans and mice. We evaluated the role of the IL-7Rα Tyr449 cytoplasmic SH2-binding motif in IL-7–mediated B cell development using a knock-in mouse with a Tyr to Phe mutation (IL-7Rα449F/449F mouse). IL-7Rα449F/449F and IL-7Rα−/− mice showed no defect in the number of pro–pre-B cells, although IL-7Rα449F/449F mice had decreased Ebf1 in pre–pro-B cells and impairment in B cell–committed CLPs. We identified that IL-7Rα Tyr449 was critical for both pro-B and pre-B stages of development in the bone marrow. IL-7Rα449F/449F and IL-7Rα−/− mice had comparable precursor B cell defects, indicating that signaling from the IL-7Rα required this motif. Although the defect in IL-7Rα449F/449F pro-B cells was associated with loss of STAT5 activation and diminished expression of McI1, this was not rescued by overexpression of Bcl-2. IL-7Rα449F/449F and IL-7Rα−/− pre-B cells also showed defective cyto-Igμ and CD25 expression, associated with reduced levels of Rag1, Rag2, and Irf4. Pre-B cells from IL-7Rα449F/449F mice also failed to proliferate, perhaps as a result of the failure to rearrange Igα. Our data suggest that IL-7Rα Tyr449 was essential for IL-7Rα signaling in bone marrow B cell development and survival. The Journal of Immunology, 2014, 193: 3446–3455.

Interleukin-7 is a critical cytokine for the development of B and T cells, the development of antiviral T cell responses and maintenance of memory T cells (1–4). Thymic stromal lymphopoietin (TSLP) is a related cytokine important in Th2 responses and immunity at mucosal sites (5). IL-7Rα pairs with γc or TSLP receptor (TSLPR) to detect IL-7 and TSLP, respectively. Overactive IL-7Rα signaling or high levels of IL-7 resulted in the development of T and B cell lymphoma in mice and humans (6). Despite prior reports suggesting that IL-7 and IL-7Rα signaling were dispensable for human B cell development (7), recent work suggests that, similar to mice, IL-7 is required in adult B cell development (8).

B cell development occurs through a set of stages defined using surface markers. The earliest B cell progenitor is the pro–pre-B cell, which expresses B220 and has germline Ig genes (9). Next, pro-B cells rearrange their H chain Igμ genes, and express CD19 under the control of Pax5 (10). At the pre-B cell stage, cells downregulate CD43, express intracellular Igμ, and then rearrange the L chain and upregulate CD25 in an Irf4-dependent manner.

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D.T.P. designed and performed research, and wrote the manuscript; A.W.P. performed the research; and N.A. designed research, interpreted data, and cowrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CLP, common lymphoid progenitor; HSC, hematopoietic stem cell; IgM, surface IgM; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor; WT, wild-type.

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D.T.P. designed and performed research, and wrote the manuscript; A.W.P. performed the research; and N.A. designed research, interpreted data, and cowrote the manuscript.

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B cell development occurs through a set of stages defined using surface markers. The earliest B cell progenitor is the pro–pre-B cell, which expresses B220 and has germline Ig genes (9). Next, pro-B cells rearrange their H chain Igμ genes, and express CD19 under the control of Pax5 (10). At the pre-B cell stage, cells downregulate CD43, express intracellular Igμ, and then rearrange the L chain and upregulate CD25 in an Irf4-dependent manner.
mutation to block B cell lymphoma in IL-7R$^{a^{-}a^{-}}$ mice (18), we investigated when and how the IL-7R Tyr$^{449}$ motif is critical for B cell development in mice. To undertak this, we examined B cell development in IL-7R$^{a^{-}a^{-}}$ mice. We determined that IL-7R Tyr$^{449}$ is essential for precursor B survival and differentiation at two checkpoints and is required for Rag1, Rag2, and Irf4 induction. To our knowledge, this work represents the first analysis of how IL-7R$^{a}$ signals in vivo to influence B cell development.

Materials and Methods

Mice

All mice were maintained under specific pathogen free conditions in the Centre for Disease Modeling at the University of British Columbia with ethical and procedural approval from the University of British Columbia animal care committee (protocols A07-0417, A07-0415, and A12-0119) in compliance with Canadian Council on Animal Care guidelines. Age- and sex-matched mice were used between 10 and 12 wk old. IL-7R$^{a^{-}a^{-}}$ mice were previously generated (19) by introducing a point mutation into the endogenous IL-7R gene and were backcrossed to C57BL/6 for 15 generations. TSLPR$^{-/-}$ mice were obtained from Dr. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN), and original breeding stocks of IL-7R$^{a^{-}a^{-}}$ (B6.129S7-Itf$^{+}$/+Il2rgb/J), B6.SJL (B6.SJLPiprc$^{-}$ Peprc$^{+/}$ BoyJ), Rag1$^{-/-}$ (B6.129S7-Rag1$^{+/+}$Mom/J) were purchased from The Jackson Laboratory. All mice were bred on site and maintained under identical conditions.

Flow cytometry

A total of 2–10 × 10$^6$ cells were stained for 30 min on ice with Abs as detailed in Supplemental Table I. For intracellular staining, surface-stained cells were fixed and permeabilized using Foxp3 fix/perm kit, as described previously (29). Samples were acquired on a LSRII. For flow-sorting experiments, cells were labeled with Ab mixtures, in some cases depleted of FITC-conjugated cells using anti-FITC beads and an AutoMacs enrichment system (StemCell Technologies). Abs were washed, resuspended, and used for FACS analysis. DNA was stained with propidium iodide (PI) using the齐-97 Staining Kit (Chemicon). Data were acquired on a Beckman-Coulter Epic and analyzed using FlowJo (Tree Star).

Caspase-3/7 activation assessment

Single-cell preparations of organs were incubated for 30 min at 37°C with 1:10 dilution of CellEvent Caspase-3/7 reagent (Invitrogen). Cells were then washed, placed on ice, and labeled with Abs as above then analyzed by flow cytometry.

Bone marrow reconstitution

Rag1$^{-/-}$B6.SJL mice (CD45.1$^{+}$) were irradiated with one or two doses of 6.5 Gy 4 h apart. Twenty-four hours after irradiation, mice were injected via the tail vein with 5 × 10$^6$ total bone marrow cells or 1–5 × 10$^6$ lentivirally infected hematopoietic stem cells (HSCs). Mice were then left for 6–8 wk for lymphocyte reconstitution. The mice were given 2 mg ml$^{-1}$ neomycin for the first 2–4 wk to prevent infection after irradiation.

Real-time PCR

Cells were plated into TRIzol (Invitrogen) and RNA purified as specified previously (30). cDNA was then made from total RNA using SuperScript III (Invitrogen) or Maxima (Thermo) cDNA synthesis kits. Real-time PCR was then performed using SsoFast EvaGreen mastermix (Bio-Rad), CFX96 PCR machine and primers for Rag1 (forward, 5′-CTGGACA-CATTCTGACCTC-3′; reverse, 5′-AACTGAAAGCTCAGGGTAGAC-3′), Rag2 (forward, 5′-TGAATTCTGCGTTGGCGCC-3′; reverse, 5′-TTTCTGGTGTTGAGTGAAATG-3′), Rps29 (forward, 5′-ACGCTG-GATCCGCAAATAC-3′; reverse, 5′-CATGAGCTGCCATCGTG-3′), or Cnd3 (forward, 5′-CTACAGCAGCAGCCTGTG-3′; reverse, 5′-CGCAATTGAGCTTCTTACG-3′). Cycling conditions were as follows: 95°C 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 15 s. Finally, a melt curve and gel was performed to check for presence of a single product of the expected size from each reaction. The expression level of genes-of-interest was then compared with expression of ribosomal protein Rps29 (31).

Lentivirus infection and reconstitution

Human Bcl-2 cDNA (American Type Culture Collection) was cloned into the Pmlne site of the pWP1 lentiviral expression vector (provided by D. Trono, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) upstream of the IRES-GFP. The Bcl-2 and empty vector plasmids were transfected into 293T cells along with the pAX2 and pMDG lentivirus packaging vectors. Virus was harvested from supernatants and concentrated by centrifugation. Hematopoietic progenitors from the bone marrow were enriched using a Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell Technologies) and then infected with lentivirus. Cells were cultured for 3 d before injection.

Statistics

Statistical tests were undertaken using Prism 6 (Graphpad Software, La Jolla, CA). One-way ANOVA were performed to compare genotypes and populations, using Tukey’s multiple comparisons test, except for Figs. 2B, 3D, 4B, 6B, 8B, 8C, 8F, and 8G, where two-tailed Student t tests were used. Error bars reflect 1 SD from the mean, except for quantitative PCR experiments, where they represent 1 SE. Asterisks indicate where results were significantly different, using α = 0.05 as a cutoff.

Results

IL-7R Tyr$^{449}$ is essential for the development of pro-B cells

To determine the contribution of IL-7R Tyr$^{449}$ to adult B cell development, we compared bone marrow from IL-7R$^{a^{-}a^{-}}$ to wild-type (WT) and IL-7R$^{a^{-}a^{-}}$ mice using flow cytometry. IL-7R$^{a^{-}a^{-}}$ mice showed a dramatic and equivalent defect in the numbers and proportion of bone marrow B220$^{+}$ B cells compared with WT, suggesting that Tyr$^{449}$ is critical for IL-7R$^{a}$-mediated B cell development (Fig. 1A–C). To examine at what stage the defect occurs, we examined the phenotype of the B cell precursors. IL-7R$^{a^{-}a^{-}}$ and IL-7R$^{a^{-}a^{-}}$ mice showed a clear decrease in both the IgM$^{-}$CD43$^{-}$ pre-B cells and IgM$^{+}$CD43$^{-}$ Immature B cells (Fig. 1D). Further subsetting of progenitor IgM$^{+}$CD43$^{+}$ cells showed a developmental block between the pre–pro and pro-Bcell stage of IL-7R$^{a^{-}a^{-}}$ and IL-7R$^{a^{-}a^{-}}$ mice, with a dramatic reduction in the numbers of pro-B cells in IL-7R$^{a^{-}a^{-}}$ mice. IL-7R$^{a^{-}a^{-}}$ mice showed a similar defect, suggesting that the Tyr$^{449}$ motif is the critical signaling residue at the pro-B stage for transition through this checkpoint (Fig. 1F). Neither IL-7R$^{a^{-}a^{-}}$ nor IL-7R$^{a^{-}a^{-}}$ mice showed deficiency in the number of pro–pro-B cells, which suggested B cell development is strongly dependent on IL-7R Tyr$^{449}$ from the pro-B stage onward.

IL-7R$^{a^{-}a^{-}}$ mice possess lower numbers of Ly6D$^{+}$ CLPs and lower expression of Ebf1

Because IL-7R has been reported to have a role in the generation of CLPs (23), we decided to examine this population in IL-7R$^{a^{-}a^{-}}$ mice. We examined CLPs (lineage-negative, CD45$^{+}$ ckit$^{low}$/Sca1$^{lo}$/Flt3$^{low}$IL-7R$^{a^{-}a}$ cells, as previously defined (23)) in WT and IL-7R$^{a^{-}a^{-}}$ mice. Both lines possess similar populations of ckit$^{low}$ Sca1$^{lo}$ and IL-7R$^{a}$ Flt3$^{+}$ cells (Fig. 2A). However, IL-7R$^{a^{-}a^{-}}$ mice have a small, but statistically relevant, decrease in the number of Ly6D$^{+}$ CLP (Fig. 2B), thought to be the precursors of B cells (32). We could not examine IL-7R$^{a^{-}a^{-}}$ mice because they lacked expression of IL-7R, the key marker of CLP in this scheme. However, in IL-7R$^{a^{-}a^{-}}$ mice, we saw fewer Ly6D$^{+}$ cells in the ckit$^{low}$/Sca1$^{lo}$/Flt3$^{+}$ population, which contains CLP cells (data not shown), similar to the lack of Ly6D$^{+}$ CLPs in IL-7R$^{a^{-}a^{-}}$ mice (23). We then examined the expression of Ebf1 in pre–pro- and pro-B cells. Ebf1 is drastically reduced at the pre–pro-B cell stage (Fig. 2C), suggesting that IL-7R Tyr$^{449}$ is critical for its expression. In contrast, Pax5, the other key transcription factor at this stage, was expressed at normal levels in both WT and IL-7R$^{a^{-}a^{-}}$ pre–pro- and pro-B cells (Fig. 2D).

IL-7R Tyr$^{449}$ controls pro-B cell survival

To determine whether the reduction in cells at the pro-B cell stage was due to cell death, we examined markers of cell death in pre–pro-B and pro-B cells from IL-7R$^{a^{-}a^{-}}$ and IL-7R$^{a^{-}a^{-}}$ mice.
Proportions of dead (DAPI+) and apoptotic (Caspase-3/7+) cells were identical between all genotypes at the pre–pro-B stage (Fig. 3A, 3B). However, pro-B cells from IL-7Rα449F/449F and IL-7Rα−/− showed significantly more Caspase-3/7+ apoptotic and DAPI+ dead cells (Fig. 3A, 3B), indicative of higher apoptosis compared with WT. Collectively, these data suggest that in the absence of IL-7Rα Tyr449 signaling, pro-B cells die by apoptosis.

We examined the expression of IL-7Rα in these cells to establish whether levels of the receptor are similar between WT and IL-7Rα449F/449F precursor B cells. Pre–pro-B cells showed a similar expression of IL-7Rα449F/449F compared with the level of IL-7Rα found in WT mice (Fig. 3C), indicating the mutated receptor had no intrinsic defects in expression. Pro-B cells showed bimodal IL-7Rα expression, with two populations in both WT and IL-7Rα449F/449F mice. IL-7Rα449F/449F mice had fewer IL-7Rαint pro-B cells (Fig. 3D). However, the level of expression of IL-7Rα within the IL-7Rαhi pro-B cell population was similar to that in WT (Fig. 3C). IL-7Rα449F/449F pre-B cells had even greater reduction in IL-7Rαhi cells. Because expression of IL-7Rα in pro-B cells correlates with the phase of the cell cycle (33), the lack of IL-7Rαhi cells in IL-7Rα449F/449F mice may represent a failure to differentiate at the pro-B stage. We then examined whether the increase in cell death seen in the pro-B cells is due to a failure of IL-7Rαhi cells to express IL-7Rα. Even IL-7Rα+ cells from IL-7Rα449F/449F mice fail to survive as well as WT (Fig. 3E), suggesting that the increase in cell death in pro-B cells from IL-7Rα449F/449F mice occurs independent of a failure to express the IL-7Rα.

**IL-7Rα Tyr449 controls pro-B cell survival factors**

IL-7 is essential for the upregulation of survival factors in early B cell precursors (34). Survival of B precursors is controlled by the levels of the antiapoptotic Bcl-2 family members Bcl-2, Bcl-xl, and Mcl1 (21). Bcl-2 and Bcl-xl protein expression was similar between WT, IL-7Rα449F/449F and IL-7Rα−/− mice at the pre–pro and pro-B stage (Fig. 4A, 4B). Pre-B cells from IL-7Rα449F/449F and IL-7Rα−/− mice showed a reduction in levels of Bcl-xl, the key Bcl-2 family member at this stage (Fig. 4A) (21). However, expression of Mcl1 was reduced in IL-7Rα449F/449F and IL-7Rα−/− mice at the pro-B cell stage (Fig. 4C). Mcl1 is the key antiapoptotic protein at the pro-B cell stage (21), suggesting that lack of this key antiapoptotic protein may have effects on cell survival.

We asked whether expression of Bcl-2 could rescue the development of IL-7Rα449F/449F pro-B cells by substituting for the absence of Mcl1. We transduced HSCs from WT and IL-7Rα449F/449F mice with a lentivirus containing either a Bcl-2-IRES-GFP construct (pWPI-Bcl-2) or the GFP-expressing vector alone (pWPI-empty). Three days later, the infected cells were then transferred into CD45.1+ B6.SJL mice (either Rag1−/− or Rag-sufficient), and the mice were left for 6 wk to reconstitute. We examined the CD45.2+ B220+ IgM+ BPI− cells, consisting of a mixture of GFP+ and GFP− cells, which represent a mixture of GFP+ and GFP− CD19+ pre–pro-B and CD19+ pro-B cells. We then asked whether pro-B cell development was rescued in Bcl-2-expressing GFP+ Bcl-2–expressing GFP+ pro-B cells compared with either non-Bcl-2 expressing (GFP−) or vector control
suggesting that forced expression of Bcl-2 is unable to rescue IL-7Rα
449F/449F pro-B cell development. Levels of Bcl-2 expression in WT and IL-7Rα
449F/449F splenocytes were similar (data not shown). This suggests that Tyr 449 has a role beyond just inducing the expression of survival factors.

IL-7Rα Tyr449 controls phosphorylation of STAT5 and Akt

We next evaluated the biochemical pathways affected by loss of IL-7Rα Tyr449 in B cell progenitors. Although WT splenic T cells (B220+ cells) induced STAT5 phosphorylation upon stimulation with IL-7, IL-7Rα449F/449F T cells showed complete abrogation at both 30 min and 18 h, consistent with our past findings (19). Bone marrow B cell progenitors from WT but not IL-7Rα449F/449F mice showed small but detectable STAT5 phosphorylation in response to IL-7 (Fig. 5), indicating IL-7Rα Tyr449 is required for activation of STAT5 in B cell precursors. This is similar to our previous observations (18). In contrast, simultaneous staining for phospho-Akt in the same samples showed no evidence for IL-7 induction of Akt phosphorylation over unstimulated controls in either WT or IL-7Rα449F/449F bone marrow B cells. We were able to detect Akt phosphorylation in WT T cells after 18 h stimulation, indicating that IL-7 was able to stimulate Akt phosphorylation but that the levels of IL-7–induced phospho-Akt in bone marrow cells was low in comparison. IL-7Rα449F/449F T cells in the spleen showed reduced pAkt in response to IL-7 after 18 h, suggesting that Tyr 449 may play a role in the activation of PI3K downstream of IL-7Rα.

Adult precursor B cells require IL-7Rα for development

Development of B lymphocytes in the fetal liver has been thought to be IL-7Rα independent (35). To rule out potential contribution of fetal liver hematopoietic cells and specifically assess the requirement of IL-7Rα signaling in adult bone marrow B cell development, we performed adoptive transfer experiments of adult bone marrow cells from CD45.2+ WT or IL-7Rα449F/449F mice into irradiated CD45.1+Rag1−/− mice. WT CD45.2+ cells fully reconstituted the bone marrow B cell compartment of recipients after 8 wk, whereas mice that had received bone marrow from IL-7Rα449F/449F mice had normal numbers of pre–pro-B cells but
reduced numbers of pro-B cells and subsequent B cell stages identical to the phenotype seen in IL-7Rα−/− mice (Fig. 6A, 6B).

To determine whether IL-7Rα Tyr449 is required intrinsically within the B cell compartment, we then generated a series of mixed bone marrow chimeras. Rag2−/− mice were irradiated and injected with a mixture of CD45.1+ WT and either CD45.2+ WT or IL-7Rα−/− bone marrow in a 9:1 CD45.2:CD45.1 ratio. This ratio was used to ensure sufficient reconstitution of the bone marrow B cell compartment by IL-7Rα−/− cells to allow for analysis. In the non-B and pro-B cell populations, there was no difference found between the capacity of WT and IL-7Rα−/− cells to develop, with cells matching the expected 9:1 input ratio found in WT. IL-7Rα−/− pro-B cells were less able to compete than WT, which was reflected in the decreased ratio of CD45.2+IL-7Rα−/− to CD45.1+ WT pro-B cells at this stage and subsequent stages. The defects seen in these chimeras establish a cell-intrinsic requirement for IL-7Rα Tyr449 signaling, which became evident at the pro-pre-B to pro-B transition.

IL-7, not TSLP, is critical for the development of early B cells

Because both TSLP and IL-7 use the IL-7Rα to signal, we used TSLPR−/− mice to examine the relative contribution of TSLP and IL-7 in the development of bone marrow B cells. Similar to previous work, TSLPR−/− mice had no defect in bone marrow B cell development (Supplemental Fig. 1). Furthermore, combination TSLPR−/−IL-7Rα−/− mice were similar to IL-7Rα−/− mice, suggesting that IL-7 but not TSLP is critical in the bone marrow and that TSLP does not compensate for suboptimal IL-7 stimulation in IL-7Rα−/− mice.

Absence of IL-7Rα Tyr449 signaling impairs Ig gene rearrangement and upregulation of Ifn4 and CD25 in pre-B cells

The IL-7Rα−/− mouse at the pre-pro-B to pro-B cell stage was not absolute (Fig. 1F). We therefore examined whether loss of Tyr449 signals had later effects in B cell development. We showed that the reduction in pre-B and immature B cells in IL-7Rα−/− mice compared with WT was even more dramatic than the reduction in pro-B cells (Figs. 1D, 6). Heterozygous IL-7Rα+−/− mice also showed a reduction in pre-B cells (Fig. 1F). We therefore asked how IL-7Rα Tyr449 was required for the pro-B cell transition.

Pre-B cells were originally defined as B cell progenitors that have successfully rearranged their IgH gene chain and begun to express cytoplasmic H chain (‘cyto-Igµ’). Because IL-7 has defined roles in the rearrangement of the Ig chains in pro-B and pre-B cells (36), we compared the development of pro-B cells in WT, IL-7Rα−/−, and IL-7Rα−/− mice. We examined the surface IgM+ (slgM+) B220+CD19+ population, comprising the surviving pro- and pre-B cells in IL-7Rα−/− and IL-7Rα−/− mice. WT mice had a large population of c-kit+ pro-B cells as well as a significant CD25+c-kit+ pre-B compartment (Fig. 7A). However, neither IL-7Rα−/− nor IL-7Rα−/− mice had CD25+c-kit+ pre-B cells.

CD25 defines a proliferative population of pre-B cells (37). Given the presence of slgM+ immature B cells in IL-7Rα−/− and IL-7Rα−/− mice, we investigated whether pre-B cells were present but not expressing CD25 in IL-7Rα mutant mice. We used a functional definition of pre-B cells as cells that have cyto-Igµ, but lack surface expression of IgM. IL-7Rα−/− mice have reduced proportions of CD19+ slgM− cyto-Igµ+ pre-B cells, whereas cyto-Igµ+ cells are almost entirely absent from IL-7Rα−/− mice (Fig. 7). Confirming our previous observation, even the cells that were expressing cyto-Igµ did not express CD25, suggesting that IL-7Rα−/− pre-B cells that emerge fail to express CD25. This data shows that in the absence of IL-7Rα Tyr449 signals, development of pre-B cells is impaired, but not abrogated as in IL-7Rα−/− mice.

We examined the proportion of Igκ+ and Igλ+ cells on marginal zone B cells in the spleen [which are phenotypically normal and present in normal numbers (38)] to determine the efficiency of IgL chain rearrangement in pre-B cells. B cells rearrange the Igκ L chains first; failure results in rearrangement of Igλ. Hence, an
increase in Igα-expressing cells represents a reduction in the efficiency of L chain rearrangement. IL-7Rα449F/449F mice had a decrease in the ratio of Igκ+ cells to Igλ+ cells in the spleen, because of a decrease in Igκ+ cells and an increase in Igλ+ cells (Fig. 7C). This suggests that rearrangement occurred but that there was a reduction in the frequency of rearrangement of Igκ. Upregulation of CD25 and L chain rearrangement were defective in IL-7Rα449F/449F mice (Fig. 7). Because both processes are dependent on Irf4 (39), we decided to examine Irf4 expression.

Discussion
Elevated IL-7 levels and activating IL-7Rα Cys-substitutions are associated with human leukemia (6, 40, 41). Genetic approaches have shown that blocking IL-7Rα signals from the Tyr449 motif prevent lymphoma development (18). Blocking IL-7Rα signaling to prevent leukemia, without completely removing the function of IL-7 in generating B cells, requires knowledge of what stage of B cell development IL-7 signaling is critical and which signaling motifs within the IL-7R are important.

We report in this study that IL-7Rα requires Tyr449 to prevent progenitor B cell apoptosis and allow B cell development. IL-7Rα signals, through Tyr449, were critical at the pre-pro-B to pro-B transition, as shown by a lack of CD19+ cells. Tyr449 signals were also critical to support pro-B cell survival. Tyr449 was important at the pro-B and pre-B cell stage to upregulate Rag to allow the recombination and expression of intracellular Igμ as well as pro-B cell proliferation. However, Tyr449-independent signals were also important, as shown by IL-7Rα−/− mice showing fewer cells and more apoptosis at pro-B cell stage, when compared with IL-7Rα449F/449F mice. Heterozygous IL-7RαWT/449F mice also show a reduction in pro-B and pre-B cells; however, we cannot discriminate between a dose-dependent effect of reduced signaling or a dominant-negative effect of the mutant receptor competing for binding to IL-7 and yc.

The IL-7Rα449F/449F mutation may affect expression of IL-7Rα. The reduction in numbers of IL-7Rα+/− cells at the pro-B cell stage in IL-7Rα449F/449F mice probably represents a failure of a pro-B cell subset to differentiate (33), rather than a direct regulatory effect on IL-7Rα expression, particularly because expression of the IL-7Rα449F receptor is normal in pro-B cells. Supporting this, IL-7Rα−/− pro-B cells from WT or IL-7Rα449F/449F HSCs transduced with either empty GFP-expressing lentivirus (pWPI-empty) or the same lentivirus expressing human Bcl-2 (pWPI-Bcl-2). Mice were left for 6 wk to reconstitute, bone marrow was taken, and the proportion of CD19+ pro-B cells out of the B220+CD19−BcI2−DAPI− population examined. Experiments were representative of two independent experiments.

To upregulate Rag1 and Rag2 mRNA (Fig. 8D, 8E), both essential steps in Ig rearrangement. Collectively, these data demonstrate an IL-7Rα Tyr449- dependent reduction in pre-B cell development. In the absence of Tyr449 signaling, there is reduced Irf4, CD25, and L chain rearrangement, critical steps at the pre-B cell development checkpoint.

IL-7Rα Tyr449 supports proliferation at the pre-B cell stage
Pre-B cells undergo a proliferative burst upon rearrangement of the IgH locus. To determine whether the lack of pre-B cells in IL-7Rα449F/449F mice was due to lack of proliferation, we examined the expression of the proliferative marker Ki67 in bone marrow B cells. Pre-pro-B and pro-B cells had no difference in the level of Ki67 protein (Fig. 8F), suggesting that there is no proliferative defect at these stages. At the pre-B cell stage, IL-7Rα449F/449F mice showed a reduction in the proportion of Ki67+ proliferating cells as well as expression of CyclinD3 (encoded by the gene Ccnd3; Fig. 8G). This provides evidence that IL-7Rα Tyr449 aids proliferation of pre-B cells.
underlie the lack of pro-B cells in IL-7Rα449F/449F. Enforced expression of Bcl2 could not rescue pro-B cell development in IL-7Rα449F/449F mice, similar to IL-7Rα2/2 mice (43). This suggests that some factor, most likely reduced Ebf1, rather than the reduced expression of Mcl1, was critical for IL-7Rα449F/449F pro-B cells.

There are thought to be two major signaling pathways downstream of the IL-7Rα Tyr449 motif, STAT5 and PI3K/Akt. We have previously shown that IL-7Rα449F/449F precursor B cells do not phosphorylate STAT5 in response to IL-7 stimulation (18). Ramadani et al. (44) showed that PI3K p110α2/p110αD910A pro-B cells did not proliferate when cultured with IL-7, showing a possible role for PI3K downstream of IL-7Rα. IL-7 induction of phosphorylation of Akt Ser 473 was reduced in IL-7Rα449F/449F cells compared with WT, which suggests that mutation of Tyr449 impairs both STAT5 and PI3K/Akt pathways. This does, however, leave the question whether there are other signaling pathways downstream of IL-7Rα to account for the differences between IL-7Rα449F and IL-7Rα−/− mice or whether the minimal amount of STAT5 and Akt phosphorylation seen is enough to account for the difference. Transgenic Bcl-2 expression partially rescues pro-B cell development in STAT5−/− mice (21). In contrast, Bcl-2 cannot rescue IL-7Rα449F/449F or IL-7Rα−/− pro-B cells (43), reinforcing the idea that IL-7Rα Tyr449 conveys more signals than just STAT5.

**FIGURE 5.** IL-7 stimulates phosphorylation of STAT5 Tyr694 and Akt Ser473 in WT but not IL-7Rα449F/449F cells. Bone marrow B cells and spleen cells from WT and IL-7Rα449F/449F mice were taken and stimulated with IL-7 for either 30 min or 18 h and then fixed, permeabilized, and stained with Abs. Shown is representative of two independent experiments.

**FIGURE 6.** IL-7Rα signaling in B cells is required for adult B cell development. (A) Lethally irradiated (two doses of 6.5gy) CD45.1+Rag1−/− mice were injected with either WT (n = 4) or IL-7Rα449F/449F (n = 5) CD45.2+ bone marrow and left for 8 wk to reconstitute. Shown are B220+CD45.2+DAPI− cells. Pre-pro-B cells are CD43+CD19−IgM−, pro-B CD43+CD19+IgM−, pre-B CD43−CD19+IgM−. (B) Mean numbers of cells per femur in each precursor population. (C) Rag1−/− mice were injected with a 9:1 mixture of WT or IL-7Rα449F/449F to B6.SJL bone marrow and left for 8 wk to reconstitute. Ratio of CD45.1 to CD45.2 cells within pre-pro-B (CD43+B220−CD19−), pro-B (CD43+B220+CD19+), pre-B (CD43−B220+CD19+IgM−), immature (B220+B220+CD19+IgM+IgD−), mature-recirculating (M-R; B220+CD19+IgM+IgD+), and non-B (B220−CD43+) cells are shown. Experiment was representative of four independent sets of chimeras.
We also examined the pre-B cells in IL-7Rα449F/449F mice. Fewer cells successfully expressed cytoplasmic IgM in IL-7Rα449F/449F, with none expressing it in IL-7Rα2/2 mice. Even cyto-IgM+ cells from the IL-7Rα449F/449F mice do not express CD25, suggesting that expression of cyto-IgM alone was insufficient for upregulation of CD25 and that another mechanism was required, potentially through Irf4. Failure to express cytoplasmic-IgM in IL-7Rα449F/449F mice may be due to a failure to upregulate the genes required for recombination, in particular Rag1 and Rag2.

FIGURE 7. IL-7Rα Tyr449 aids expression of cytoplasmic-μ and Igk. Expression of c-kit, CD25, and intracellular cytoplasmic IgM was assessed in pro- and pre-B cells from WT (n = 4), IL-7Rα449F/449F (n = 5), and IL-7Rα−/− (n = 4) mice by flow cytometry. (A) Cells are gated on CD19+ slgM− and then on expression of cytoplasmic-IgM (cyto-IgM) to examine expression of CD25 on cells that are slgM− cyto-IgM+. (B) Proportion of CD19+ slgM− cells that are cyto-IgM+; these experiments are representative of three experiments with similar results. (C) Ratio between Igκ+Igλ− and Igκ−Igλ+ cells within the B220+CD19+CD23hiCD21hi DAPI− marginal zone B cells in the spleen.

FIGURE 8. Pre-B cells from IL-7Rα449F/449F mice had lower expression of Irf4 and recombination machinery and fail to proliferate. (A) Expression of intracellular Irf4 in pro-B (B220+CD19+IgM− ckit+) and pre-B ((B220+CD19+IgM− ckit−) cells from WT (n = 3) and IL-7Rα449F/449F (n = 4) mice. (B) Mean fluorescence intensity of Irf4 in pro-/pre-B cell population (B220+CD19+IgM−). Irf4 levels were representative of two independent experiments. (C) Tdt expression in pro-B (CD19+B220+IgM+CD43+) and pre-B (CD19+B220+IgM+CD43−) cells. Expression of Rag1 and Rag2 in cDNA from sorted pro-B (CD19+B220+BP1+CD43−) and pre-B cells (CD19+B220+BP1−CD43−) (Ef). Shown is mean of three cDNA preparations for each genotype. † indicates below detectable levels. (F) Expression of proliferative marker Ki67 in pre-pro-B (B220+IgM+CD34−CD19−), pro-B (B220+IgM+CD34+CD19+), and immature B (B220+IgM−CD19+) bone marrow B cell populations from WT (n = 4) and IL-7Rα449F/449F (n = 3) mice. Repeated three times with similar results. (G) Expression of CyclinD3 (CyclinD3) in sorted pre-pro-, pro-, and immature B cell populations. The mean of three cDNA preparations for each genotype are shown.
Failure to rearrange and express cyto IgM may also prevent cells from going into cell cycle. There were fewer Ki67+ pre-B cells in IL-7Rα−/−, as well as lower expression of CyclinD3, representing a failure to proliferate. IrF4 is expressed at lower levels in pre-B cells in IL-7Rα−/− and IL-7Rα mice; IrF4 is necessary and sufficient for the upregulation of CD25 in pre-B cells as well as rearrangement of the L chain. Given the decrease in Igk expression and increase in Igλ expression in IL-7Rα−/−/− mice, we conclude that the requirement to express WT levels of IrF4, Rag1, and Rag2 may be responsible for the defect in L chain rearrangement. However, it remains to be determined whether the defects seen at the pre-B cell stage are the result of failure of IL-7Rα−/− signaling or are a result of a defect at a previous developmental stage or failure to express the pre-BCR or IL-7Rs.

We have shown that IL-7Rα Tyr449 is essential for the survival, differentiation and proliferation of pro-B and pre-B cells in the mouse bone marrow. Without Ty449 residue, few B cells develop, and a large number of pro-B and pre-B cells undergo apoptosis. This provides compelling evidence for IL-7Rs Ty449 as a potential therapeutic target for future therapies against B cell cancers.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. IL-7, not TSLP, is responsible for the phenotype of IL-7Rα^{449F/449F} mice. (A) Early Precursor B cells in TSLPR^{−/−} (n=4) and TSLPR^{−/−} IL-7Rα^{449F/449F} (n=6) double-mutant mice, compared to WT (n=4) and IL-7Rα^{449F/449F} (n=4) mice. (B) Number of cells per femur of B cell subsets from IL-7Rα and TSLPR^{−/−} mice. Cell populations are as described in Figure 1, with the addition of Immature (B220^{+}CD43^{−}IgM^{+}IgD^{−}) and Mature-Recirculating (M-R; B220^{hi}CD43^{−}IgM^{+}IgD^{+}) Experiment was repeated twice, with similar results.
## Supplementary Table 1

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