IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF

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Cytokine receptor signals have been suggested to stimulate cell differentiation during hematopoiesis. Such action, however, has not been clearly demonstrated. Here, we show that adult B cell development in IL-7⁻/⁻ and IL-7Rα⁻/⁻ mice is arrested at the pre–pro-B cell stage due to insufficient expression of the B cell–specific transcription factor EBF and its target genes, which form a transcription factor network in determining B lineage specification. EBF expression is restored in IL-7⁻/⁻ pre–pro-B cells upon IL-7 stimulation or in IL-7Rα⁻/⁻ pre–pro-B cells by activation of STAT5, a major signaling molecule downstream of the IL-7R signaling pathway. Furthermore, enforced EBF expression partially rescues B cell development in IL-7Rα⁻/⁻ mice. Thus, IL-7 receptor signaling is a participant in the formation of the transcription factor network during B lymphopoiesis by up-regulating EBF, allowing stage transition from the pre–pro-B to further maturational stages.

B cell development is regulated by multiple B cell–specific transcription factors (1, 2). Both E2A and EBF are necessary for initiating B cell differentiation through the cooperative regulation of B cell–specific genes, such as RAG1, RAG2, Igα, Igβ, λ5, Vpre-B, and CD19 (1, 2). Another transcription factor, PU.1, as well as E2A can up-regulate the promoter activity of the EBF gene (3, 4). Moreover, Pax5 gene expression is regulated by E2A and EBF (5). Once Pax5 is expressed, B cell progenitors are irreversibly committed to B cell lineage (6). Therefore, hierarchical regulation of these transcription factors may be a key for acquisition of B lineage specificity in B cell progenitors. It remains unclear, however, whether the formation of this transcription factor network occurs in a cell autonomous manner or requires extracellular stimuli provided by stromal cells in bone marrow.

Flt3 ligand, stem cell factor (SCF), and IL-7 are important cytokines for B cell development (7–9). Among these cytokines, only IL-7 can promote stage transition from B220⁺CD19⁻ pre–pro-B cells to B220⁺CD19⁺ pro-B cells, whereas Flt3 ligand or SCF enhances the proliferation of B cell progenitors in vitro cultures (10). Studies from IL-7Rα⁻/⁻ mice reported conflicting results of the stage at which B cell development is arrested. One paper suggests a role of IL-7R signaling in regulation of IgH gene rearrangement for transition from pro–B to pre–B stage (11). Other papers indicate that B cell development in IL-7Rα⁻/⁻ mice is arrested at an earlier point than the pro–B/pre–B transition stage, such as the early pro–B stage (using Hardy’s nomenclature, fraction B; references 12, 13) or even earlier at the common lymphoid progenitor (CLP) stage (14). Furthermore, impaired T cell development in IL-7Rα⁻/⁻ or γc⁻/⁻ mice is rescued by enforced Bcl-2 expression, but rescue of B cell development is not observed in the same mice (15–17), suggesting that the primary function of IL-7R signaling is not to maintain cell survival but to promote cell differentiation and/or stimulate cell proliferation during B cell development. Together, these observations imply a role of IL-7 in stage transition during early adult B cell development. Nonetheless, the molecular basis of its function remains totally unknown.

In this study, we clarified that B cell development is arrested at the pre–pro-B cell stage in the absence of IL-7R signaling. We investigated the role of IL-7R in expression of B cell–specific factors at this developmental stage. We found a direct connection between IL-7R signaling and up-regulation of EBF expression. This finding demonstrates an indispens-
able role of IL-7 in the formation of transcription factor networks during adult B cell development.

RESULTS AND DISCUSSION
B cell development is arrested at the pre–pro-B cell stage in IL-7Ra−/− and IL-7−/− mice

To clarify the role of IL-7R signaling in B cell development, we first confirmed the stage at which B cell development is blocked in IL-7R/H9251/H11546/H11546/H11546 and IL-7/H11546/H11546/H11546 mice (see Fig. 1 A for defined stages of B cell development). Consistent with a previous paper (14), we found that there was no difference in the number of CLPs between IL-7/H11546/H11546/H11546 and WT mice (Fig. 1 D). Next, we analyzed the more mature B220+CD43+ population, which is downstream of CLPs and include the pre–pro-B and pro-B fractions (Fig. 1 A). Total number of B220+CD43+BP-1−HSA−cells (fraction A in Hardy’s nomenclature [13]) in IL-7Ra−/− and IL-7−/− mice were also comparable to WT mice (unpublished data). Fraction A contains non-B cell progenitors that express NK1.1 or Ly-6C (18, 19). Therefore, we also compared the number of NK1.1−Ly-6C− cells in fraction A (pre–pro-B cells; Fig. 1 C) and found that the number of these cells were still comparable among WT, IL-7R/H9251/H11546/H11546, and IL-7/H11546/H11546 mice (Fig. 1 D). In contrast, B220+CD43+BP-1−HSAhigh subset (fraction B in Hardy’s nomenclature [13]) in IL-7Ra−/− and IL-7−/− mice was rarely detected (Fig. 1, B and D). Cells in the next

![Diagram](http://www.jem.org/cgi/content/full/jem.20050158/DC1)
maturational stage (B220+CD43+BP-1+HSAhigh subset, fraction C/C’ in Hardy’s nomenclature [13]; we simply denote fraction C/C’ as fraction C hereafter in this paper) were almost absent, indicating that IL-7R signaling is required at an earlier stage than the pro-B (fraction B or C) stage in B cell development. The cell surface phenotype of pre-pro-B cells derived from IL-7Rα−/− and IL-7−/− mice was identical to WT pre-pro-B cells (Fig. 1 C).

Expression of B cell–specific genes is impaired in pre-pro-B cells in the absence of IL-7R signaling

A recent study showed that lack of a cell survival factor, Mcl-1, causes severe B cell developmental arrest at the pro-B cell stage (20), although our previous studies suggest that antiapoptotic signals via IL-7R may not be crucial in B cell development (15, 16). We examined the expression of known survival factor genes bcl-2, bcl-xl, and mcl-1 in WT and IL-7Rα−/− pre-pro-B cells (Fig. 2 A). Expression levels of these survival factors were comparable, indicating the dispensable role of IL-7 for expression of these survival factors at the pre-pro-B cell stage. Because the mcl-1 gene flanked with the loxp sites is not deleted in pre-pro-B cells of CD19-CreMcl-1f/fnull mice, it is not clear if Mcl-1 also plays an important role in the pre-pro-B cell stage (20). Regardless, our gene expression profiling suggests that mcl-1 expression is not regulated by IL-7R signaling pathway, at least in pre-pro-B cells.

We next examined the expression of various B cell–specific genes critical for B cell development in WT and IL-7Rα−/− pre-pro-B cells. Notably, expression of EBF, Pax5, and λ5 was absent in IL-7Rα−/− pre-pro-B cells but was present in WT pre-pro-B cells (Fig. 2 B). Igα and Vpre-B expression were also significantly reduced in IL-7Rα−/− pre-pro-B cells. In contrast, E2A and PU.1, which regulate early B cell development (1, 21), were normally expressed (Fig. 2 B). E2A function could be suppressed by direct association with Id2 or Id3 (1), but we did not observe any elevated expression of Id2 or Id3 in IL-7Rα−/− pre-pro-B cells (Fig. 2 B). Because Igα, Igβ, λ5, Vpre-B, RAG1, RAG2, and Pax5 genes are targets of E2A and EBF (1, 2, 5), we hypothesized that the ablated expression of EBF might cause arrested B cell development at the pre-pro-B stage in IL-7Rα−/− mice.

We further examined the expression of EBF at different maturational stages of developing B cells. As shown in Fig. 2 C, we observed strong up-regulation of EBF expression at the pro-B stage. EBF expression peaked at the small, nondividing pre-B cell stage and declined at the immature-B cell stage (Fig. 2 C). Because B cell development is arrested at the B220+CD43+BP-1-HSAhigh (fraction A) stage in EBF−/− mice (22), EBF expression in pre-pro-B cells should be required for the stage transition from the pre-pro-B to pro-B cell stage, despite its lower expression level in the pre-pro-B population compared with the more mature B cell progenitors.

EBF is a downstream target of IL-7R signaling at the pre-pro-B cell stage

Next, we examined the relationship between IL-7R signaling and EBF expression in pre-pro-B cells. We purified pre-pro-B cells from IL-7−/− mice and stimulated them with IL-7 in vitro. EBF expression levels were analyzed by RT-PCR. As shown in Fig. 3 A, there was no EBF expression in pre-pro-B cells derived from IL-7−/− mice, as in the case with IL-7Rα−/− pre-pro-B cells (Fig. 2 B). EBF expression was up-regulated at 12 h after IL-7 stimulation, and its expression was maintained for at least 48 h (Fig. 3 A). We did not observe any change of surface phenotype in these pre-pro-B cells after 24 h of culture in the presence of IL-7 (Fig. 3 A), suggesting that initiation of EBF expression is a direct consequence of IL-7 stimulation in pre-pro-B cells.

To determine the biological importance of EBF up-regulation triggered by IL-7 stimulation, we examined whether or not enforced EBF expression could rescue the impaired B cell development in IL-7Rα−/− mice. IL-7Rα, EBF, and
Pax5, as well as MSCV-IRES-GFP empty vector, were retrovirally introduced into IL-7Rα−/− HSCs (CD45.2+) for in vivo reconstitution. 1.2 × 10⁶ pre-pro-B cells (B220+CD43+CD19−NK1.1−Ly-6C−) derived from IL-7−/− mice were cultured in the presence of 10 ng/ml IL-7 in 96-well plates. The same number of freshly isolated cells was used as a nonstimulated control (time 0). Cells were harvested at the indicated time points and subjected to RT-PCR analysis of EBF expression. We also checked the cell surface phenotype of cells at 24 h after the culture. At this time point, no pro-B cells (CD19+ or BP-1+ cells) were developed from pre-pro-B cells (open histogram). Closed histogram represents background staining with isotype control antibodies.

EBF expression is regulated by IL-7R signaling in B cell development. (A) Restoration of EBF expression in IL-7−/− pre-pro-B cells. 1.2 × 10⁶ pre-pro-B cells (B220+CD43+CD19−NK1.1−Ly-6C−) derived from IL-7−/− mice were cultured in the presence of 10 ng/ml IL-7 in 96-well plates. The same number of freshly isolated cells was used as a nonstimulated control (time 0). Cells were harvested at the indicated time points and subjected to RT-PCR analysis of EBF expression. We also checked the cell surface phenotype of cells at 24 h after the culture. At this time point, no pro-B cells (CD19+ or BP-1+ cells) were developed from pre-pro-B cells (open histogram). Closed histogram represents background staining with isotype control antibodies. (B) IL-7Rα−/− HSCs (CD45.2+) were infected with empty, IL-7Rα, EBF, Pax5, or STAT5 (1*6) retroviruses by spin infection and injected into sublethally irradiated RAG2−/− mice (CD45.1+). 5 wk after injection, mice were killed and spleen cell suspensions were stained with antibodies for CD19, B220, and IgM, as well as for CD45.2. CD19+B220+IgM+ cells are mature B cells. Representative data from more than three samples are shown. We did not observe any immature B cells derived from EBF+ IL-7Rα−/− HSCs in bone marrow at this time point but did observe them at an earlier time point (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050158/DC1). (C) CD19+B220+IgM+ cell numbers of reconstituted mice with IL-7Rα−/− HSCs infected with empty, IL-7Rα, EBF, Pax5, or STAT5 (1*6) retroviruses shown in B were calculated. The cell numbers are expressed as mean ± SD.

All of these CD19+ cells were GFP+ (not depicted) and expressed B cell marker B220 and IgM on the cell surface (Fig. 3 B, bottom). The absolute cell number of the CD19+B220+IgM+ spleen B cells from EBF+ IL-7Rα−/− HSCs (8.8 ± 5.5 × 10⁵) was ~160 times lower than spleen B cells from IL-7Rα−/− HSCs with ectopic IL-7Rα (1.4 ± 0.4 × 10⁶; Fig. 3 C). No mature B cells were observed from IL-7Rα−/− HSCs introduced with Pax5 as well as introduction of empty vectors (Fig. 3 B). These results demonstrated that the enforced expression of EBF, but not Pax5, could rescue B cell differentiation in the absence of IL-7R signaling. The lower yield of mature B cells from IL-7Rα−/− HSC with ectopic EBF reemphasized the importance of IL-7 as a B cell growth factor.
activated extensively in response to IL-7 (25). In addition to the function of IL-7 as a B cell growth factor, IL-7R signaling regulates the accessibility of distal V segments of the IgH genes, which leads to preferential rearrangement of distal V to DJ1 in pro-B cells (11, 26). Also, IL-7 may be necessary for pro-B cell survival through Mcl-1 expression (20). In this study, we have demonstrated that IL-7 is necessary for EBF expression in pro–pre-B cells, as well as for transition to more mature stages during adult B cell development. Initiation of EBF expression before the IL-7Rα+ CLP stage (27). Our preliminary data also suggests that CLPs derived from IL-7−/− mice expressed a comparable level of EBF to WT (unpublished data). Therefore, it is possible that IL-7 stimulation is necessary for the maintenance of EBF expression during the CLP to pre–pro-B cell stage transition. In addition, it is intriguing to know the regulation of EBF gene expression between fetal and adult pre–pro-B cells because fetal B cell development is IL-7 independent (28). Further studies are necessary to elucidate how IL-7 can mediate these different biological processes at different developmental stages of B lymphopoiesis.

MATERIALS AND METHODS

Mice. IL-7Rα−/− mice were obtained from The Jackson Laboratory. IL-7−/− mice were provided by I.L. Weissman (Stanford University, Stanford, CA), which were originally provided by R. Murray (DNAX Research Institute, Palo Alto, CA). RAG2−/− mice were introduced with CD45.1 as described previously (29). All mice were backcrossed onto C57BL/6 background for more than eight generations. Age-matched C57BL/6 mice were used as WT control. The age of mice used in this study was between 4 and 8 wk old. Specifically, we used 8-wk-old mice in the experiment shown in Fig. 1 D. All mice were bred in a specific pathogen-free environment at the mouse facility of Duke University Medical Center. All experimental procedures related to laboratory mice were done according to guidelines specified by the institution.

Cell sorting and flow cytometric analysis. Antibodies used in this study are listed in the online supplemental material. Preparation of single cell suspension and antibody staining of cells were done as previously described (15). Cell sorting and cell surface phenotyping were performed on a FACSVantage SE with a DeXa option (488 nm argon, 599 nm dye, and 408 nm krypton lasers; BD Biosciences Flow Cytometry Systems), which is available in the FACs facility of Duke University Comprehensive Cancer Center. Data were analyzed with the FlowJo software (Treestar). Dead cells were excluded from analyses and sortings as positively stained cells by propidium iodide.

RT-PCR. Cells were sorted directly into 1.5-ml microcentrifuge tubes with 1 ml TRIzol reagent (Invitrogen). Total RNA was purified based on
the manufactured instruction. First-strand cDNA was synthesized with Superscript III reverse transcriptase and oligo-dT primers (Invitrogen). Verification of the amount of first strand cDNA was done by amplification of β-actin. Genes of interest were amplified by PCR with BD Advantage 2 PCR Enzyme System (BD Biosciences) based on the manufactured protocol. In experiments shown in Fig. 2 C, we quantified EBF expression using the LightCycler system (Roche) after the first strand DNA synthesis. The primers for PCR are listed in the online supplemental material.

**Retrovirus production and infection.** Retroviral vectors used in this study are shown in online supplemental material. STAT5A1*, Mcl-1, Pax5, and Bcl-2 cDNAs were provided by T. Kitamura (University of Tokyo, Tokyo, Japan), S. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA), J. Parnes (Stanford University, Stanford, CA), and K. Sugamura (Tohoku University, Sendai, Japan), respectively. Retroviruses were prepared as described previously (30). For retroviral infection, 2 × 10^5 IL-7Rα−/− HSCs were placed in 200 μl X-VIVO medium (BIO-WHITTAKER) supplemented with 1% BSA, 10 ng/ml IL-11 and TPO, 50 ng/ml SCF (R&D Systems), and 50% of viral supernatants in a well of round bottom 96-well plate. The culture plate was centrifuged at 2,000 rpm at 22°C for 2 h (spin infection). After the spin infection, cells were incubated at 37°C. After 24-h culture, the frequency of GFP+ cells was verified by FACS. The cell numbers for injection were adjusted by the GFP+ cell frequency so that 4 × 10^5 GFP+ cells were injected into a mouse.

**In vivo reconstitution assay.** IL-7Rα−/− HSCs (CD45.1−) infected with retroviruses were injected into retroblast venous sinus of sublethally irradiated (400 rad) RAG2−/− mice (CD45.1−), 5 wk after injection, mice were killed and differentiation of donor-derived cells was assessed by FACS.

**In vitro stimulation of pre–pro-B cells with IL-7.** Pre–pro-B cells derived from IL-7−/− mice were placed in a well of 96-well plates in IMDM with 10% FCS, 50 μM 2-ME, and 10 ng/ml IL-7 (R&D Systems). Cells were harvested at the time points indicated in the figures and subjected to RT-PCR analyses.

**Online supplemental material.** A more detailed characterization of pro–pre-B cells is shown in Fig. S1. Rescue of B precursor compartments by ectopic EBF in IL-7Rα−/− HSCs is shown in Fig. S2. Antibodies, retroviral vectors, and PCR primers used in this study are listed in the online supplemental material. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050158/DC1.

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