

# 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 hemato/lymphopoiesis. Such action, however, has not been clearly demonstrated. Here, we show that adult B cell development in *IL-7<sup>-/-</sup>* and *IL-7R $\alpha$ <sup>-/-</sup>* 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<sup>-/-</sup>* pre-pro-B cells upon IL-7 stimulation or in *IL-7R $\alpha$ <sup>-/-</sup>* 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-7R $\alpha$ <sup>-/-</sup>* 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.

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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*, *Ig $\alpha$* , *Ig $\beta$* ,  *$\lambda$ 5*, *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<sup>+</sup>CD19<sup>-</sup> pre-pro-B cells to B220<sup>+</sup>CD19<sup>+</sup> pro-B cells, whereas Flt3 ligand or SCF enhances the proliferation of B cell progenitors in in vitro cultures (10). Studies from *IL-7R $\alpha$ <sup>-/-</sup>* 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-7R $\alpha$ <sup>-/-</sup>* 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-7R $\alpha$ <sup>-/-</sup>* or  *$\gamma_c$ <sup>-/-</sup>* 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-

The online version of this article contains supplemental material.

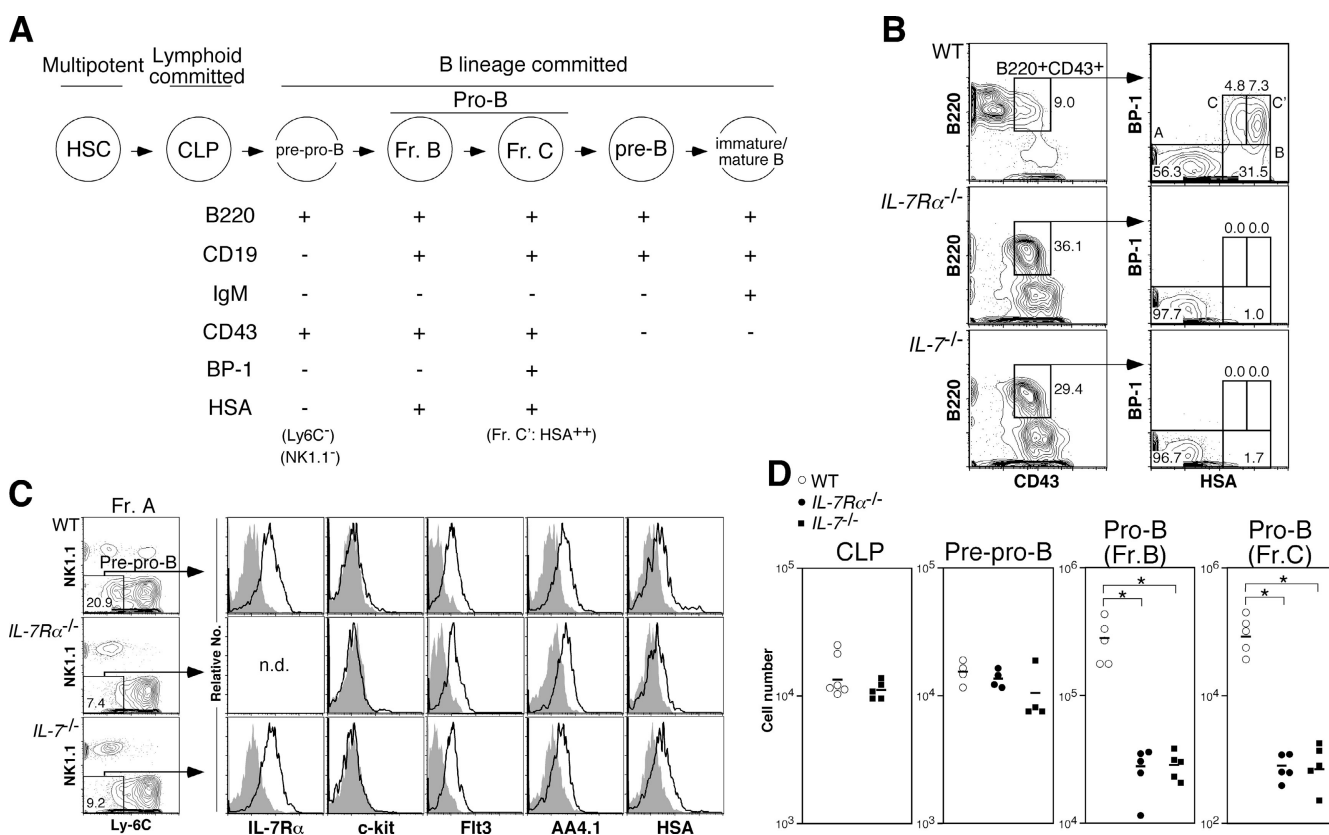
able role of IL-7 in the formation of transcription factor networks during adult B cell development.

**RESULTS AND DISCUSSION**

**B cell development is arrested at the pre-pro-B cell stage in *IL-7Rα*<sup>-/-</sup> and *IL-7*<sup>-/-</sup> 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-7Rα*<sup>-/-</sup> and *IL-7*<sup>-/-</sup> 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*<sup>-/-</sup> and WT mice (Fig. 1 D). Next, we analyzed the more mature B220<sup>+</sup>CD43<sup>+</sup> pop-

ulation, which is downstream of CLPs and include the pre-pro-B and pro-B fractions (Fig. 1 A). Total number of B220<sup>+</sup>CD43<sup>+</sup>BP-1<sup>-</sup>HSA<sup>-/low</sup> subset (fraction A in Hardy's nomenclature [13]) in *IL-7Rα*<sup>-/-</sup> and *IL-7*<sup>-/-</sup> 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<sup>-</sup>Ly-6C<sup>-</sup> 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-7Rα*<sup>-/-</sup>, and *IL-7*<sup>-/-</sup> mice (Fig. 1 D). In contrast, B220<sup>+</sup>CD43<sup>+</sup>BP-1<sup>-</sup>HSA<sup>high</sup> subset (fraction B in Hardy's nomenclature [13]) in *IL-7Rα*<sup>-/-</sup> and *IL-7*<sup>-/-</sup> mice was rarely detected (Fig. 1, B and D). Cells in the next



**Figure 1. B cell development is arrested at the pre-pro-B stage in the absence of IL-7R signaling.** (A) Schematic maturational relationship of B cell populations used in this study. We excluded Ly-6C<sup>+</sup> and NK1.1<sup>+</sup> cells from Hardy's fraction A (reference 25) except for the experiments shown in Fig. 4. CD19 can be used instead of HSA to distinguish pre-pro-B (fraction A) and pro-B (fractions B and C/C') cells (reference 13; Fig. S1 D, available at <http://www.jem.org/cgi/content/full/jem.20050158/DC1>). Hereafter, we used the fraction C/C' population as fraction C. (B) Bone marrow (BM) cells from 8-wk-old mice were stained with antibodies for lineage markers (Lin: CD3e, Gr-1, Ter119, and Mac-1), B220, CD43, BP-1, and HSA. Propidium iodide<sup>+</sup> dead cells as well as Lin<sup>+</sup> cells were excluded from this cell surface phenotyping. The numbers in FACS plots are percentages of cells in each cell population for each plot. The representative results of at least three independent experiments are shown. (C) The expression of IL-7Rα, c-Kit, Flt3, AA4.1, and HSA in pre-pro-B cells derived

from WT, *IL-7Rα*<sup>-/-</sup>, and *IL-7*<sup>-/-</sup> mice. The definition of pre-pro-B cells here is Lin<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup>Ly-6C<sup>-</sup> cells. Removal of CD19<sup>+</sup> cells from this analysis is critical to exclude the more mature B cell progenitors from the pre-pro-B cell population (see Fig. S1). Open and filled histograms represent the expression level of various markers and the negative control stained with isotype-matched irrelevant antibodies, respectively. We did not stain pre-pro-B cells from *IL-7Rα*<sup>-/-</sup> mice with anti-IL-7Rα antibodies in this analysis. (D) The number of CLP, pre-pro-B, and pro-B cells in bone marrow from bilateral femurs and tibiae derived from WT (open circle), *IL-7Rα*<sup>-/-</sup> (closed circle), and *IL-7*<sup>-/-</sup> (closed square) mice. The absolute number of cells was calculated with total bone marrow cell numbers and the percentage of each cell population. The mean is indicated as a horizontal bar. Because IL-7Rα is a marker to define CLPs, we did not determine the number of CLPs in *IL-7Rα*<sup>-/-</sup> mice. Asterisks denote significant difference by Student's t test; P < 0.05.

maturational stage (B220<sup>+</sup>CD43<sup>+</sup>BP-1<sup>+</sup>HSA<sup>high</sup> 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*<sup>-/-</sup> and *IL-7*<sup>-/-</sup> mice was identical to WT pre-pro-B cells (Fig. 1 C).

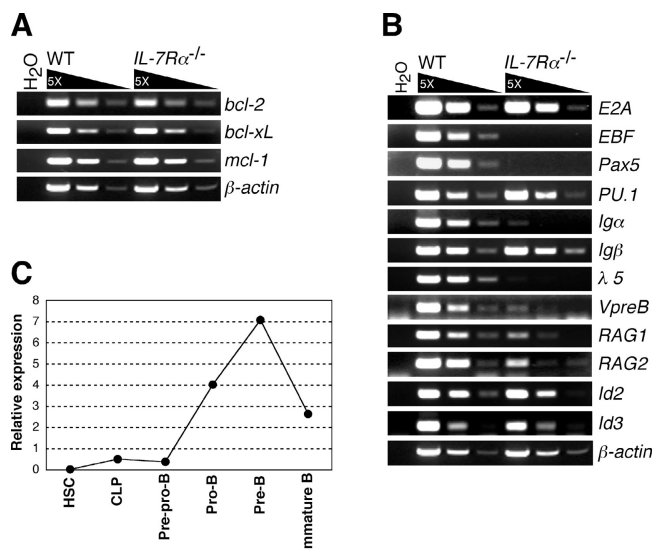
### Expression of B cell-specific genes is impaired in pre-pro-B cells in the absence of IL-7R signaling

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*<sup>-/-</sup> 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-Cre*Mcl-1*<sup>flnull</sup> 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*<sup>-/-</sup> pre-pro-B cells. Notably, expression of *EBF*, *Pax5*, and  $\lambda 5$  was absent in *IL-7Rα*<sup>-/-</sup> pre-pro-B cells but was present in WT pre-pro-B cells (Fig. 2 B). *Igα* and *Vpre-B* expressions were also significantly reduced in *IL-7Rα*<sup>-/-</sup> 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*<sup>-/-</sup> pre-pro-B cells (Fig. 2 B). Because *Igα*, *Igβ*,  $\lambda 5$ , *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*<sup>-/-</sup> 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<sup>+</sup>CD43<sup>+</sup>BP-1<sup>-</sup>HSA<sup>-</sup> (fraction A) stage in *EBF*<sup>-/-</sup> 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.



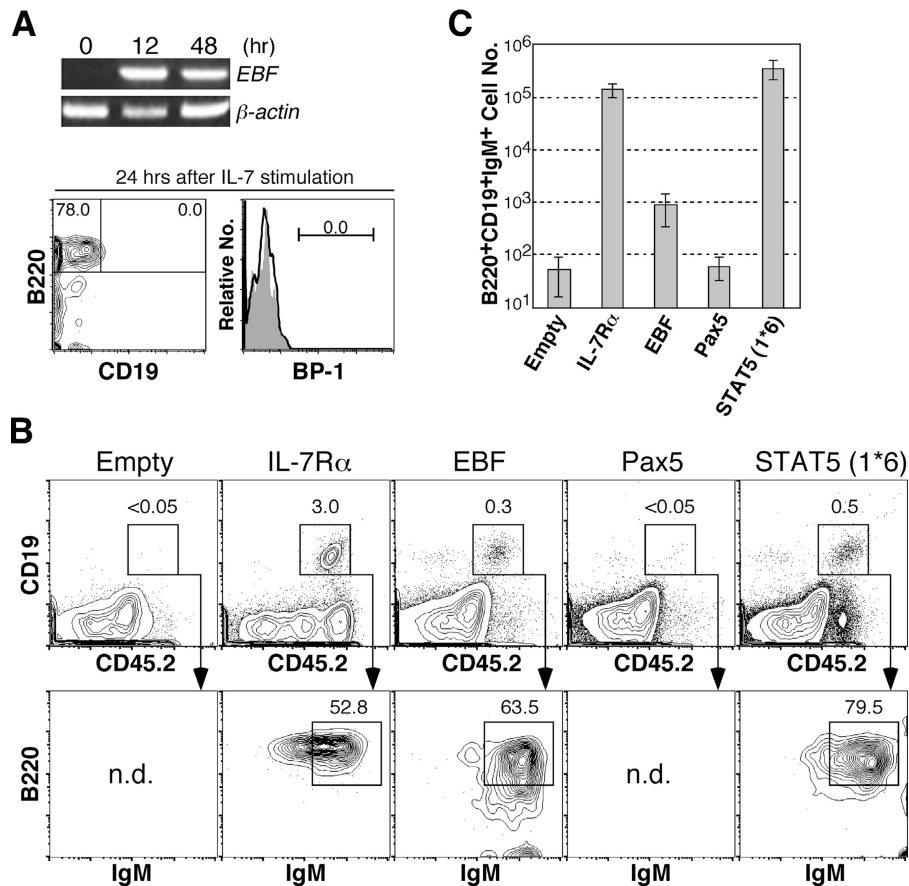
**Figure 2. Gene expression profiles in *IL-7Rα*<sup>-/-</sup> pre-pro-B cells.**

(A) Expression of antiapoptotic genes in WT and *IL-7Rα*<sup>-/-</sup> pre-pro-B cells were examined by RT-PCR. RNA was purified from  $1.5 \times 10^4$  pre-pro-B cells (Lin<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup>Ly-6C<sup>-</sup> cells), which were sorted twice in sequence (double sorted) from WT and *IL-7Rα*<sup>-/-</sup> BM cells. After double sorting, the purity of cells was >99% in reanalysis. (B) Expression of genes that are involved in B cell development in WT and *IL-7Rα*<sup>-/-</sup> pre-pro-B cells. RT-PCR analyses were done as in A. (C) Expression of *EBF* gene in various developing B cell populations. The cell populations used here are HSC (Lin<sup>-</sup>lowThy-1.1<sup>low</sup>c-Kit<sup>high</sup>Sca-1<sup>+</sup>), CLP (Lin<sup>-</sup>c-Kit<sup>low</sup>Sca-1<sup>low</sup>Thy-1.1<sup>-</sup>IL-7Rα<sup>+</sup>), pre-pro-B (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup>Ly-6C<sup>-</sup>), pro-B (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>+</sup>HSA<sup>high</sup>), pre-B (B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>), and immature B (B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>-</sup>). The lineage cocktail (Lin) used for HSCs and CLPs also contained anti-B220 antibodies. Total RNA was purified from each doubly sorted population and subjected to quantification of *EBF* mRNAs by real-time PCR after first strand synthesis with reverse transcriptase. The amount of the first strand DNA applied was normalized to the expression level of a reference gene, *GAPDH*. *EBF* expression in whole BM was arbitrarily defined as unit one. The mean value of more than three independent samples is shown. Range of error is too small to be displayed in the histogram.

### 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice, as in the case with *IL-7Rα*<sup>-/-</sup> 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*<sup>-/-</sup> mice. *IL-7Rα*, *EBF*, and

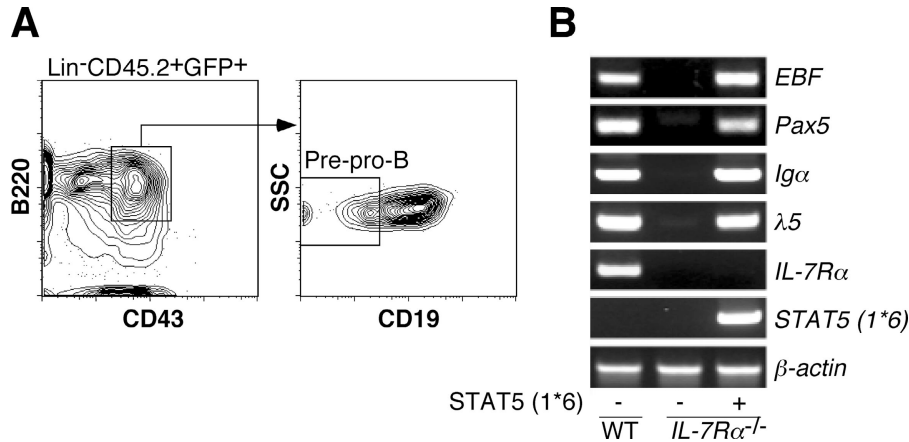


**Figure 3. EBF expression is regulated by IL-7R signaling in B cell development.** (A) Restoration of *EBF* expression in *IL-7*<sup>-/-</sup> pre-pro-B cells.  $1.2 \times 10^4$  pre-pro-B cells (B220<sup>+</sup>CD43<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup>Ly-6C<sup>-</sup>) derived from *IL-7*<sup>-/-</sup> 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<sup>+</sup> or BP-1<sup>+</sup> cells) were developed from pre-pro-B cells (open histogram). Closed histogram represents background staining with isotype control antibodies. (B) *IL-7R $\alpha$* <sup>-/-</sup> HSCs (CD45.2<sup>+</sup>) were infected with empty, IL-7R $\alpha$ , EBF, Pax5, or STAT5 (1\*6) retroviruses by spin infection

and injected into sublethally irradiated *RAG2*<sup>-/-</sup> mice (CD45.1<sup>+</sup>). 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<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup> 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<sup>+</sup> *IL-7R $\alpha$* <sup>-/-</sup> 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<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup> cell numbers of reconstituted mice with *IL-7R $\alpha$* <sup>-/-</sup> HSCs infected with empty, IL-7R $\alpha$ , EBF, Pax5, or STAT5 (1\*6) retroviruses shown in B were calculated. The cell numbers are expressed as mean  $\pm$  SD.

Pax5, as well as MSCV-IRES-GFP empty vector, were retrovirally introduced into *IL-7R $\alpha$* <sup>-/-</sup> HSCs (CD45.2<sup>+</sup>) for in vivo reconstitution.  $4 \times 10^3$  GFP<sup>+</sup> HSCs were injected into sublethally irradiated *RAG2*<sup>-/-</sup> mice (CD45.1<sup>+</sup>). We analyzed spleens in reconstituted mice at 5 wk after injection. The top panels of Fig. 3 B show the frequency of CD19<sup>+</sup> B cells in CD45.2<sup>+</sup> donor-derived cells. In this experimental system, CD19<sup>+</sup> B cells were not detected in reconstituted mice with *IL-7R $\alpha$* <sup>-/-</sup> HSCs introduced with empty vector, but were clearly present in reconstituted mice with *IL-7R $\alpha$* <sup>-/-</sup> HSCs infected with IL-7R $\alpha$  viruses (Fig. 3 B). Although the frequency of B cells was lower than this positive control (*IL-7R $\alpha$* <sup>-/-</sup> HSCs with ectopic IL-7R $\alpha$ ), introduction of EBF clearly rescued B cell development from *IL-7R $\alpha$* <sup>-/-</sup>

HSCs (Fig. 3 B). All of these CD19<sup>+</sup> cells were GFP<sup>+</sup> (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<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup> spleen B cells from EBF<sup>+</sup> *IL-7R $\alpha$* <sup>-/-</sup> HSCs ( $8.8 \pm 5.5 \times 10^2$ ) was  $\sim$ 160 times lower than spleen B cells from *IL-7R $\alpha$* <sup>-/-</sup> HSCs with ectopic IL-7R $\alpha$  ( $1.4 \pm 0.4 \times 10^5$ ; Fig. 3 C). No mature B cells were observed from *IL-7R $\alpha$* <sup>-/-</sup> 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-7R $\alpha$* <sup>-/-</sup> HSC with ectopic EBF reemphasized the importance of IL-7 as a B cell growth factor.



**Figure 4. STAT5, a downstream signaling molecule of IL-7R, mediates *EBF* gene expression.** (A) Donor-derived pre-pro-B cells ( $CD45.2^{+}GFP^{+}B220^{+}CD43^{-}CD19^{-}$ ) were purified from reconstituted mice with  $IL-7R\alpha^{-/-}$  HSCs infected with STAT5 (1\*6) retrovirus. (B)  $1.0 \times 10^4$  pre-pro-B cells derived from WT (left lane),  $IL-7R\alpha^{-/-}$  mice (middle lane), and reconstituted mice with  $IL-7R\alpha^{-/-}$  HSCs expressing STAT5 (1\*6) (right

lane) were used for analysis of B cell-specific gene expression by RT-PCR. *EBF* expression as well as expression of its targets, *Pax5*, *Igα*, and  $\lambda 5$ , were restored in pre-pro-B cells in the presence of constitutive active STAT5. Because *IL-7Rα* expression was not detected in the right lane, there was no contamination of host-derived cells ( $RAG2^{-/-}$  mice) in the sample.

#### Activated STAT5 can induce *EBF* expression in pre-pro-B cells

STAT5 is a critical signal molecule downstream of IL-7R (23). In reconstituted mice with  $IL-7R\alpha^{-/-}$  HSCs that were introduced with constitutive active STAT5 (STAT5 (1\*6)), we could see both differentiation and expansion of  $CD19^{+}B220^{+}IgM^{+}$  spleen B cells ( $3.5 \pm 1.4 \times 10^5$ ; Fig. 3, B and C), consistent with a previous observation (24). We isolated pre-pro-B cells from these reconstituted mice (Fig. 4 A) and examined *EBF* expression. As shown in Fig. 4 B, *EBF* as well as its downstream target genes, such as *Pax5*, *Igα*, and  $\lambda 5$ , were expressed in  $IL-7R\alpha^{-/-}$  pre-pro-B cells in the presence of constitutive active STAT5. These data suggest that STAT5 plays a central role in IL-7R signaling pathway to activate *EBF* expression. In pro-B cells, STAT5 has been shown to induce the expression of *cyclin D2*, *pim-1*, and *bc1-x<sub>L</sub>*, which are involved in cell proliferation and survival (24). Hence, the lack of induction of proliferation and/or survival factors may be the reason why we could not observe B cell expansion in mice reconstituted with  $IL-7R\alpha^{-/-}$  HSCs introduced with *EBF* (Fig. 3 C).

IL-7 has been considered to mainly function at the pro-B cell stage in B cell development because pro-B cells proliferate 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 *DJ* 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 pre-pro-B cells, as well as for transition to more mature stages during adult B cell development. Initiation of *EBF* expression before the pre-pro-B cell stage might be IL-7 independent, because *EBF* expression is observed

before the  $IL-7R\alpha^{+}$  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 the *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.**  $IL-7R\alpha^{-/-}$  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 DiVa option (488 nm argon, 599 nm dye, and 408 nm krypton lasers; BD Biosciences Flow Cytometry Systems), which is available in the FAC 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  $\beta$ -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\*6, 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,  $2 \times 10^4$  *IL-7R $\alpha$ <sup>-/-</sup>* HSCs were placed in 200  $\mu$ l X-VIVO medium (BIO-WHITTKER) 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 plates. 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<sup>+</sup> cells was verified by FACS. The cell numbers for injection were adjusted by the GFP<sup>+</sup> cell frequency so that  $4 \times 10^3$  GFP<sup>+</sup> cells were injected into a mouse.

**In vivo reconstitution assay.** *IL-7R $\alpha$ <sup>-/-</sup>* HSCs (CD45.2<sup>+</sup>) infected with retroviruses were injected into retroorbital venous sinus of sublethally irradiated (400 rad) *RAG2<sup>-/-</sup>* mice (CD45.1<sup>+</sup>). 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<sup>-/-</sup>* mice were placed in a well of 96-well plates in IMDM with 10% FCS, 50  $\mu$ M 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 pre-pro-B cells is shown in Fig. S1. Rescue of B precursor compartments by ectopic EBF in *IL-7R $\alpha$ <sup>-/-</sup>* 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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## REFERENCES

- Quong, M.W., W.J. Romanow, and C. Murre. 2002. E protein function in lymphocyte development. *Annu. Rev. Immunol.* 20:301–322.
- Busslinger, M. 2004. Transcriptional control of early B cell development. *Annu. Rev. Immunol.* 22:55–79.
- Kee, B.L., and C. Murre. 1998. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J. Exp. Med.* 188:699–713.
- Medina, K.L., J.M. Pongubala, K.L. Reddy, D.W. Lancki, R. DeKoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev. Cell.* 7:607–617.
- O'Riordan, M., and R. Grosschedl. 1999. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity.* 11:21–31.
- Nutt, S.L., B. Heavey, A.G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature.* 401:556–562.
- Mackarehtschian, K., J.D. Hardin, K.A. Moore, S. Boast, S.P. Goff, and I.R. Lemischka. 1995. Targeted disruption of the *flk2/flt3* gene leads to deficiencies in primitive hematopoietic progenitors. *Immunity.* 3:147–161.
- von Freeden-Jeffry, U., P. Vieira, L.A. Lucian, T. McNeil, S.E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J. Exp. Med.* 181:1519–1526.
- Sitnicka, E., C. Brakebusch, I.L. Martensson, M. Svensson, W.W. Agace, M. Sigvardsson, N. Buza-Vidas, D. Bryder, C.M. Cilio, H. Ahlenius, et al. 2003. Complementary signaling through *flt3* and interleukin-7 receptor  $\alpha$  is indispensable for fetal and adult B cell genesis. *J. Exp. Med.* 198:1495–1506.
- Kouro, T., K.L. Medina, K. Oritani, and P.W. Kincade. 2001. Characteristics of early murine B-lymphocyte precursors and their direct sensitivity to negative regulators. *Blood.* 97:2708–2715.
- Corcoran, A.E., A. Riddell, D. Krooshoop, and A.R. Venkataraman. 1998. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. *Nature.* 391:904–907.
- Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Marsevskiy, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180:1955–1960.
- Hardy, R.R. 2003. B-cell commitment: deciding on the players. *Curr. Opin. Immunol.* 15:158–165.
- Miller, J.P., D. Izon, W. DeMuth, R. Gerstein, A. Bhandoola, and D. Allman. 2002. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. *J. Exp. Med.* 196:705–711.
- Kondo, M., K. Akashi, J. Domen, K. Sugamura, and I.L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. *Immunity.* 7:155–162.
- Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray, and I.L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell.* 89:1033–1041.
- Maraskovsky, E., J.J. Peschon, H. McKenna, M. Teepe, and A. Strasser. 1998. Overexpression of Bcl-2 does not rescue impaired B lymphopoiesis in IL-7 receptor-deficient mice but can enhance survival of mature B cells. *Int. Immunol.* 10:1367–1375.
- Rolink, A., E. ten Boekel, F. Melchers, D.T. Fearon, I. Krop, and J. Andersson. 1996. A subpopulation of B220<sup>+</sup> cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J. Exp. Med.* 183:187–194.
- Tudor, K.S., K.J. Payne, Y. Yamashita, and P.W. Kincade. 2000. Functional assessment of precursors from murine bone marrow suggests a sequence of early B lineage differentiation events. *Immunity.* 12:335–345.
- Opferman, J.T., A. Letai, C. Beard, M.D. Sorcinelli, C.C. Ong, and S.J. Korsmeyer. 2003. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature.* 426:671–676.
- Singh, H., R.P. DeKoter, and J.C. Walsh. 1999. PU.1, a shared transcriptional regulator of lymphoid and myeloid cell fates. *Cold Spring Harb. Symp. Quant. Biol.* 64:13–20.
- Lin, H., and R. Grosschedl. 1995. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature.* 376:263–267.
- Lin, J.X., T.S. Migone, M. Tsang, M. Friedmann, J.A. Weatherbee, L.

- Zhou, A. Yamauchi, E.T. Bloom, J. Mietz, S. John, and W.J. Leonard. 1995. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity*. 2:331–339.
24. Goetz, C.A., I.R. Harmon, J.J. O’Neil, M.A. Burchill, and M.A. Farrar. 2004. STAT5 activation underlies IL7 receptor-dependent B cell development. *J. Immunol.* 172:4770–4778.
25. Hardy, R.R., and K. Hayakawa. 2001. B cell development pathways. *Annu. Rev. Immunol.* 19:595–621.
26. Chowdhury, D., and R. Sen. 2003. Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. *Immunity*. 18:229–241.
27. Igarashi, H., S.C. Gregory, T. Yokota, N. Sakaguchi, and P.W. Kin-  
cade. 2002. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity*. 17:117–130.
28. Vosshenrich, C.A., A. Cumano, W. Muller, J.P. Di Santo, and P. Vieira. 2003. Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development. *Nat. Immunol.* 4:773–779.
29. Kondo, M., I.L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 91:661–672.
30. Kondo, M., D.C. Scherer, T. Miyamoto, A.G. King, K. Akashi, K. Sugamura, and I.L. Weissman. 2000. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature*. 407:383–386.