Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors

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Interleukin-7 (IL-7) promotes survival and expansion of lymphoid precursors. We show here that, in addition, IL-7 has a fundamental role, as early as the stage of the multipotent (B/T/NK) common lymphoid progenitor (CLP), in maintaining the B cell differentiation program open. CLPs generated in the absence of IL-7 have normal T/NK differentiation potential, but severely impaired B potential. Accordingly, CLPs from IL-7–deficient mice express lower amounts of early B cell factor (EBF) and Pax5 than wild-type CLPs, but similar amounts of GATA-3. Importantly, induced overexpression of EBF is sufficient to restore the B potential in these cells. These results indicate that IL-7 directs commitment of CLPs by modulating EBF expression. This is the first example of a cytokine influencing lymphoid lineage commitment in multipotent progenitors and highlights the relevance of the expression of a functional IL-7 receptor at the CLP stage.

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Abbreviations used: γc, common γ-chain; CLP, common lymphoid progenitor; EBF, early B cell factor; ELP, early lymphocyte precursor; FTOC, fetal thymic organ culture; HSC, hematopoietic stem cell; Lin$^-$, lineage$^-$; TSLP, thymic-stromal lymphopoietin.

Differentiation of hematopoietic stem cells (HSCs) is a stepwise process that begins with the decision to adopt a myelo-erythroid or a lymphoid fate, generating progenitors with a restricted differentiation potential. Common myeloid progenitors (1) and two populations of common lymphoid progenitors (CLPs; references 2, 3), able to generate exclusively myelo-erythroid or lymphoid progeny, respectively, have been described in the adult BM. CLPs are isolated from the lineage (Lin)$^-$ fraction, distinguished from HSCs by the expression of the IL-7Rα and lower levels of c-Kit and Sca-1, and are clonally able to generate B, T, and NK progeny but no myelo-erythroid cells (3). Like conventional CLPs (or CLP-1), CLP-2 express IL-7Rα and have restricted lymphoid potential, but are found among pTα-expressing B220$^+$c-Kit$^-$ cells (2). CLP-2 were proposed to represent thymic immigrants (4).

A molecular understanding of the signals that trigger lineage commitment in the hematopoietic system has been a challenge for >10 yr. According to the instructive model of lineage commitment, signals in the environment determine cell fate, inducing differentiation along a particular pathway. Alternatively, stochastic models predict that asymmetric cell division and/or random fluctuations in the pattern of gene expression lead to heterogeneous distribution of proteins in the progeny of precursor cells, resulting in lineage commitment. We proposed previously that embryonic and adult hematopoiesis obey different rules of commitment, mainly stochastic in the fetus and instructive in the adult (5).

Instructive commitment is exemplified by T cell differentiation in the thymus upon activation of the Notch1 signaling pathway by Delta-like Notch1 ligands (6). Notch1 signaling resolves a binary choice in the lymphoid pathway between the mutually exclusive programs of B and T cell differentiation. This is achieved by regulating RBP/Jκ transcriptional activity (7) and, at least partially, by modulating the turnover of E2A proteins, which are indispensable for B cell differentiation (8). No other instructive signals involved in lymphoid commitment have been positively identified to date, although several transcription factors are known to play key roles in commitment and differentiation. For example, GATA-3 is essential for T cell development, as evidenced by the inability of GATA-3$^{-/-}$ embryonic stem cells to generate T cells while contributing to other hematopoietic lineages in complementa-

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tion chimeras (9). NK cell differentiation is largely dependent on DNA-binding protein inhibitors (Id proteins), Id2 in particular (10, 11), which are negative regulators of transcription that counteract the action of helix-loop-helix transcription factors such as E2A (12). E2A and early B cell factor (EBF) are activators of the B cell differentiation program and have been implicated in the regulation of B cell–specific genes, such as mb-1, λ5, VpreB, and probably Pax5 (13). Targeted disruption of either E2A (14, 15) or EBF (16) abolishes B cell development before D-J_H rearrangements occur. Pax5 is necessary to fix the B-committed status by positively regulating lineage-specific genes such as CD19 and B cell-linker and by repressing nonlymphoid genes such as M-CSFR (13). B cell differentiation is blocked at the pro–B cell stage in Pax5$^{-/-}$ mutants (17), and these precursors retain the ability to differentiate into other hematopoietic lineages (18, 19).

Although a role for cytokines in the commitment of hematopoietic progenitors is suspected, this is still a matter of debate. CLPs ectopically expressing hIL-2Rβ or hGM-CSFR differentiate into myeloid cells upon stimulation with hIL-2 or hGM-CSF, respectively (20, 21), and G-CSF signals were shown to regulate granulocytic versus monocytic differentiation (22). These observations indicate that, in addition to modulation of proliferation and survival, cytokine signals may instruct cell lineage decisions.

No signals upstream of the cascade of events that lead to B cell differentiation have been described yet, but it is well known that cytokines produced by stromal cells are important players in B cell development (23, 24). In particular, IL-7 is known to be essential for the survival, expansion, and differentiation of committed B cell precursors in adult mice (25–29), although no role has been formally demonstrated for this cytokine in the process of B cell commitment itself. It has been shown that IL-7 supports in vitro B cell differentiation from CLPs with a parallel loss of T cell potential (27), but this result is compatible with the notion that IL-7 is a survival factor for progenitors that stochastically entered the B cell pathway and does not demonstrate direct involvement of IL-7 in B cell commitment.

Production of B cells is completely abolished in the BM of adult IL-7$^{-/-}$ mice, with a developmental arrest occurring before D-J_H rearrangement (fraction A; references 25, 28). A small yet persistent peripheral B cell pool is maintained, whose production is supported during fetal and perinatal life by the IL-7–related cytokine thymic-stromal lymphopoietin (TSLP; reference 30). The development of αβ T cells is partially arrested at the DN2 stage in IL-7$^{-/-}$ mice, but a normal percentage of thymic CD4/CD8 double positive cells is always found (31). At the same time, the production of γδ T cells is completely abolished and NK cell development is not compromised by the absence of IL-7 (31).

In this work, we studied the role of IL-7 in lymphoid lineage commitment through quantitative analysis in in vitro limiting dilution assays of the differentiation potential of lymphoid progenitors from IL-7$^{-/-}$ mice. We observed that IL-7$^{-/-}$ CLPs, despite being able to generate T and NK cells at near normal frequency, are largely devoid of B cell potential. Thus, the developmental arrest in B cell development, observed in the absence of IL-7, occurs at the CLP stage, earlier than previously thought (27, 32). Adult IL-7$^{-/-}$ CLPs express lower amounts of the B cell–specific transcription factors EBF and Pax5 than control CLPs. Notably, overexpression of *Ebf* in these cells rescues their ability to give rise to B cell progeny. Therefore, this work discloses a thus far unsuspected role of IL-7 in the modulation of *Ebf* expression, already at the CLP stage, to maintain the B cell differentiation program open.

RESULTS

CLPs in the BM of adult IL-7$^{-/-}$ mice

In the BM of mice deficient for IL-7, B cell production is completely abolished, with a developmental arrest occurring before the first detectable B lineage committed cells (25). CLPs are hierarchically positioned between the HSCs and the earliest committed B cell precursor and are the most immature progenitors expressing the receptor for IL-7 (3). We investigated the BM of IL-7–deficient mice for the presence of multipotent CLPs. Flow cytometry analysis showed that Lin$^-$IL-7Rα$^+$c-Kit^{lo}Sca-1$^+$ cells (3) are present in adult IL-7$^{-/-}$ animals (Fig. 1 A), although reduced in number to 30% of the wild type (not depicted). The expression of IL-7Rα at the cell surface is indistinguishable between IL-7$^{-/-}$ and wild-type CLPs (Fig. 1 A). As is the case for wild-type mice (33), ∼70% of the CLPs from IL-7$^{-/-}$ mice express the Flk-2 receptor (Fig. 1 A). To analyze the in vivo differentiation potential of these cells, we injected 1,000 CLPs from each strain into irradiated allotype-congenic Rag2γc$^{-/-}$ mice. Donor-derived B (IgM$^+$), T (CD3$^+$), and NK (NK1.1$^+$) progeny were detected, 5 wk after transplantation, in both cases and no myeloid reconstitution was observed either in the spleen or BM of any of the recipient mice (unpublished data). These results confirm that CLPs from both mouse strains have a lymphoid-restricted differentiation potential in vivo.

We analyzed the differentiation potential of wild-type CLPs by in vitro limiting dilution assays. We independently sorted CLPs expressing low or high levels of Sca-1 and determined the frequency of precursors in each subpopulation, able to generate B, T, or NK cells (Table S1, available at http://www.jem.org/cgi/content/full/jem.20042393/DC1). We observed that Lin$^-$IL-7Rα$^+$c-Kit^{lo}Sca-1$^+$ cells with different levels of Sca-1 have identical lymphoid differentiation potential, and in no case was myeloid progeny obtained in these cultures. Additionally, we measured the frequency of bipotent precursors among wild-type CLPs by culturing single cells and scoring for precursors that could give rise to B and NK cells in the same well. Because the conditions that allow B or T cell differentiation are mutually exclusive, we used NK differentiation as a surrogate marker for the T cell potential (20). We determined that one out of six CLPs (i.e., 16.7%) can give rise to both B and NK cells (Fig. 1 B).

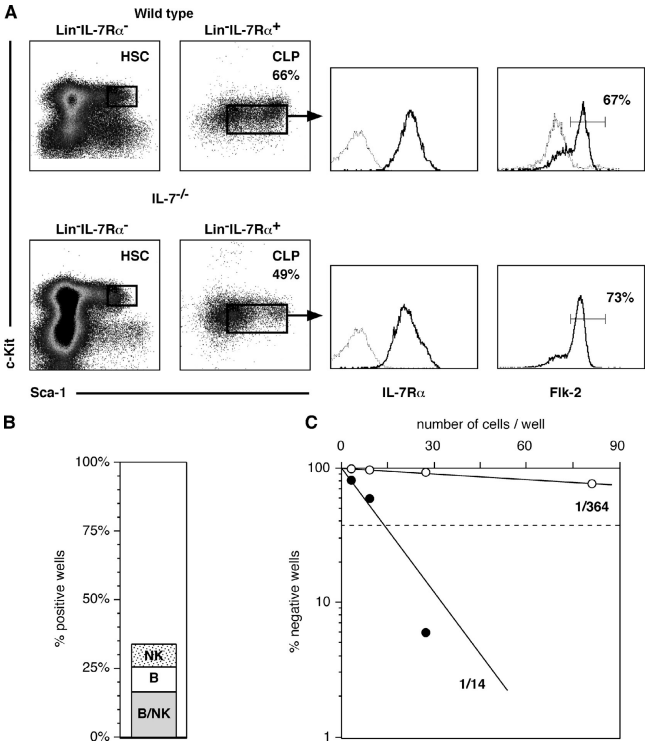

Figure 1. CLPs in adult IL-7$^{-/-}$ mice. (A) Expression of c-Kit and Sca-1 in Lin$^-$ BM cells of adult wild-type (top) or IL-7$^{-/-}$ (bottom) mice. Boxes indicate the gate used to sort HSCs (Lin$^-$IL-7Rα$^-$c-Kit^{hi}Sca-1^{hi} cells; left dot plots) or CLPs (Lin$^-$IL-7Rα$^+$c-Kit^{lo}Sca-1$^+$ cells; middle dot plots). The percentages of CLPs in the Lin$^-$IL-7Rα$^+$ fraction of the BM are indicated. Expression of IL-7Rα at the cell surface of wild-type and IL-7$^{-/-}$ CLPs (left histograms); (solid line) IL-7Rα or (dotted line) streptavidin-only control. Sorted CLPs were restained with Flk-2 (solid line) or isotype control (dotted line) antibodies (right histograms); the percentage of Flk-2$^+$ cells is indicated. (B) Frequency of multipotent progenitors among Lin$^-$IL-7Rα$^+$c-Kit^{lo}Sca-1$^+$ wild-type cells. Single wild-type CLPs were sorted and cultured in conditions optimized for the differentiation of both B and NK cells. The percentages of wells with NK cells only, B cells only, or B and NK cells are indicated. This result is representative of two independent experiments. (C) Frequency of CLPs able to give rise to B cell colonies when isolated from (●) wild-type or (○) IL-7$^{-/-}$ mice, as determined by limiting dilution in vitro (representative experiment). The number of cells plated per well is plotted against the percentage of negative wells for the growth of B cell colonies (CD19$^+$). Full lines represent the best-fit regression and the dashed line intercepts the vertical axis at 37%. The frequency determined for each mouse strain is indicated.

Taking into account the plating efficiency (i.e., reporting to the total number of wells with colonies), this result corresponds to a frequency of 1/2 progenitors (i.e., at least 50% of clonable CLPs qualify as bipotent precursors. These values are in agreement with published estimates on the frequency of multipotent precursors among CLPs (3, 20).

IL-7 deficiency impairs the differentiation potential of CLPs

The differentiation potential of CLPs can only be quantitatively analyzed with in vitro assays. Therefore, we performed limiting dilution assays with CLPs isolated from control and IL-7$^{-/-}$ mice to compare their ability to give rise to lymphocytes in the presence of exogenous IL-7. We determined that wild-type CLPs are at least 25 times more efficient than CLPs from IL-7$^{-/-}$ in generating B lymphocytes (Fig. 1 C and Table I). Wild-type CLPs generated B cells at frequencies ranging from 1/6 to 1/15, in agreement with previous data (3), whereas IL-7$^{-/-}$ CLPs did so at frequencies from 1/364 to 1/756 (Table I). Although generated at a much-reduced frequency, the few clones derived from IL-7$^{-/-}$ CLPs were macroscopically as large as those derived from wild-type CLPs (unpublished data), consistent with the presence of B cells in the spleen of recipient mice reconstituted with CLPs from IL-7$^{-/-}$ mice as mentioned before. To determine the frequency at which CLPs are able to generate T and NK cells, we performed fetal thymic organ cultures (FTOCs) under limiting dilution conditions and observed that CLPs from both strains have equivalent T and NK differentiation potential. Control CLPs give rise to T cells at a frequency of 1/7 and to NK cells at a frequency of 1/8, whereas when IL-7$^{-/-}$ CLPs were used, T and NK cells were generated at frequencies of 1/18 and 1/20, respectively (Table I). We conclude that IL-7 deficiency does not substantially affect the T/NK potential of CLPs, but severely impairs their ability to give rise to B lymphocytes.

Table I. Frequency of progenitors from adult mice able to give rise to B, T, or NK cells

Precursors	Differentiation potential	Experiment	Wild type	IL-7$^{-/-}$
Lin$^-$IL-7α$^+$c-Kit^{lo}Sca-1$^+$	B	1	1/6	ND
		2	1/14[a]	1/364[a]
		3	1/8[a]	1/756[a]
		4	1/15[a]	<1/382[a]
	T		1/7	1/18
	NK		1/8	1/20
Lin$^-$B220$^+$	B		1/316[a]	<1/9,000[a]
	T		1/57	1/137
	NK		1/57	1/119

Cell populations were purified from the BM of adult wild-type or IL-7$^{-/-}$ mice. The frequency of B cell precursors was determined by culture in limiting dilution on OP-9 stromal cells supplemented with cytokines. The frequency of T and NK precursors was determined in FTOCs. The results are representative of at least three independent experiments.

[a]Statistically different, $P < 0.001$. In all experiments, the frequency of myeloid precursors, as determined by the detection of CD11b$^+$ cells, was <1/600.

IL-7 deficiency impairs the differentiation potential of Lin$^-$B220$^+$ progenitors

The BM of adult IL-7$^{-/-}$ mice is completely devoid of B220$^+$CD19$^+$IgM$^-$ B cell progenitors, but a population of B220$^+$CD19$^-$IgM$^-$ cells can be identified clearly (25). This Lin$^-$B220$^+$ population contains the recently described CLP-2 (pTα$^+$B220$^+$c-Kit$^-$; reference 2). To determine whether the

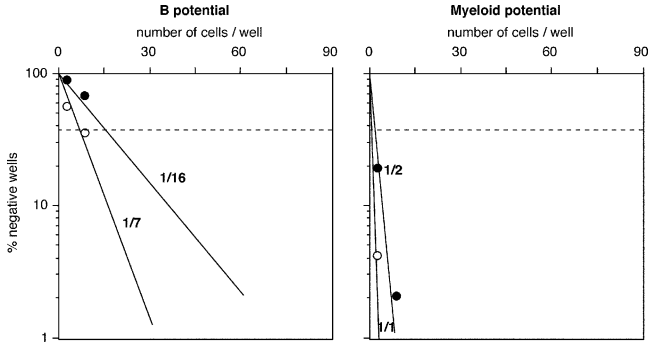

Figure 2. Adult IL-7$^{-/-}$ HSCs have B and myeloid differentiation potential identical to wild-type HSCs. Frequency of (●) wild-type or (○) IL-7$^{-/-}$ HSCs able to give rise to B cell or myeloid colonies, as determined by limiting dilution in vitro. The number of cells plated per well is plotted against the percentage of negative wells for the growth of B cell (CD19$^+$) or myeloid (CD11b$^+$) colonies. Solid lines represent the best-fit regression and the dashed line intercepts the vertical axis at 37%. The frequencies determined for each mouse strain are indicated. The results are representative of two independent experiments.

reduction of the B cell potential, with maintenance of T/NK potential and consequent to IL-7 deficiency, also applied to other multipotent lymphoid progenitors, we analyzed the differentiation potential of Lin$^-$B220$^+$ BM cells by limiting dilution assays. We observed that the frequency at which this population is able to generate T or NK cells in FTOCs is not different in wild-type and IL-7$^{-/-}$ mice. In contrast, <1/9,000 Lin$^-$B220$^+$ cells from IL-7$^{-/-}$ BM were able to generate B cells, whereas wild-type cells did so at a frequency of 1/316 (Table I). The frequency of B cell progenitors among wild-type Lin$^-$B220$^+$ cells is lower than what was reported for CLP-2 (1/20; reference 4) because CLP-2 (i.e., pTα-expressing Lin$^-$B220$^+$) constitute only 10% of the Lin$^-$B220$^+$ BM population (4). We conclude that IL-7 deficiency also impairs the B cell differentiation potential of CLP-2 but only minimally affects their T/NK differentiation potential.

IL-7 deficiency does not alter the differentiation potential of thymic precursors and HSCs

The hierarchical relation between CLP-1 and CLP-2 as well as the nature of the thymic immigrants is still a matter of debate. Whatever thymic precursors exist in adult IL-7$^{-/-}$ mice, they are functional in vivo, as demonstrated by their ability to colonize wild-type thymic lobes grafted under the kidney capsule of IL-7$^{-/-}$ mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20042393/DC1). Such conditions support a normal T cell development (Fig. S1), indicating that any step of T cell commitment and differentiation that may occur prethymically must be IL-7 independent. Thymopoiesis in IL-7$^{-/-}$ mice is reduced due to the role of IL-7 at later stages of T cell development, in the expansion of DN1 and DN2 and at the DN2–DN3 transition (31, 34). Furthermore, we determined that HSCs (Lin$^-$IL-7Rα$^-$ c-Kit^{hi}Sca-1^{hi} cells) from IL-7–deficient mice are functionally indistinguishable from their wild-type counterparts in their ability to generate both B and myeloid cells (Fig. 2). Thus, contrary to previous suggestions (27, 32), B cell differentiation in the adult BM is affected by the absence of IL-7 as early as the CLP stage, but not before.

Table II. Frequency of CLPs from 4-wk-old mice able to give rise to B, T, or NK cells

Differentiation potential	Experiment	Wild type	IL-7$^{-/-}$
B	1	1/2[a]	1/14[a]
	2	1/3[a]	1/10[a]
T		1/38	1/16
NK		1/28	1/8

CLPs were purified from the BM of young wild-type or IL-7$^{-/-}$ mice. The frequency of B cell precursors was determined by culture in limiting dilution on OP-9 stromal cells supplemented with cytokines, and the frequency of T and NK precursors was determined in FTOCs.
[a]Statistically different, $P < 0.001$. The frequency of myeloid precursors, as determined by the detection of CD11b$^+$ cells, was <1/190.

CLPs in the BM of young IL-7$^{-/-}$ mice

During fetal and perinatal life, TSLP is able to drive B lymphopoiesis, thus partially compensating for IL-7 deficiency (30). Accordingly, B220$^+$CD19$^+$IgM$^-$ B cell progenitors can be found in the BM of IL-7$^{-/-}$ mice until at least 4 wk of age (25). At this age, we found a normal number of CLPs in the BM of IL-7$^{-/-}$ (not depicted) and they generated B cells in vitro at a frequency ranging from 1/10 to 1/14, whereas CLPs from wild-type mice differentiated into B cells at a frequency of 1/2 to 1/3 (Table II). The frequency obtained with CLPs from young IL-7$^{-/-}$ is significantly different from adult IL-7$^{-/-}$ CLPs ($P < 0.001$) and also significantly different from the frequency obtained with cells from young wild-type mice ($P < 0.001$). CLPs from young wild-type mice generated T and NK cells at frequencies of 1/38 and 1/28, respectively, whereas cells from IL-7$^{-/-}$ gave rise to T cells with a frequency of 1/16 and NK cells with a frequency of 1/8 (Table II). We conclude that, already at 4 wk of age, CLPs from IL-7$^{-/-}$ mice have impaired B cell differentiation potential, although less pronounced than in adults.

Taking into account both the number and the frequency at which CLPs generate lymphocytes in each strain of mice, we calculated the absolute number of CLPs that are able to give rise to lymphocytes in young and adult individuals. The number of progenitors capable of generating T lymphocytes or NK cells is not affected with age by their development in the absence of IL-7 (unpublished data). In contrast, in IL-7$^{-/-}$ mice at 4 wk of age, the number of CLPs able to generate B cells is already reduced to 17% ($P < 0.001$) of the control, and further diminished to <1% ($P < 0.001$) by the time the animals reach adulthood (Fig. 3). We estimate that adult IL-7$^{-/-}$ mice have in the two hind limbs <20 CLPs able to generate B cells (Fig. 3).

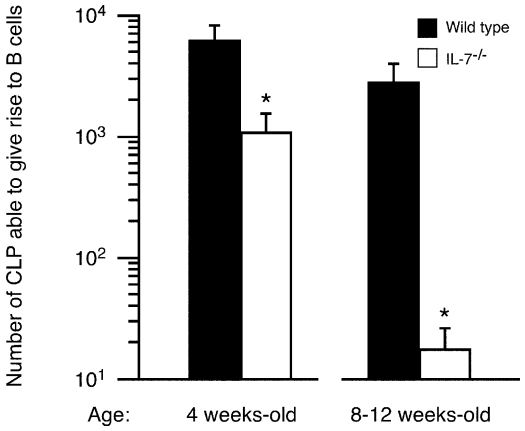

Figure 3. Absolute number of CLPs with B differentiation potential, in the two hind limbs of young and adult mice. The absolute number of progenitors able to generate B cells was calculated by multiplying the total number of CLPs (four mice in each group) by the frequencies calculated in limiting dilution assays. Bars indicate the standard deviation for three independent experiments. *, statistically significant, $P < 0.001$. ■, wild-type mice; □, IL-$7^{-/-}$ mice.

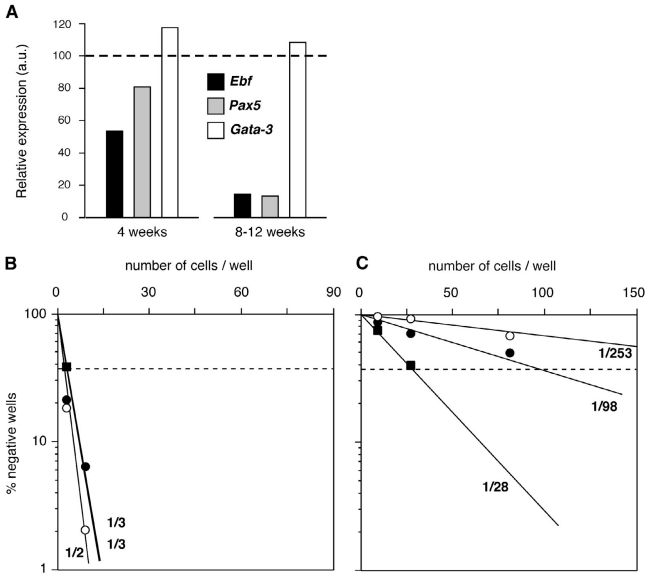

Figure 4. *Ebf*, but not *Pax5*, overexpression in IL-$7^{-/-}$ CLPs restores their B differentiation potential. (A) Relative expression of *Ebf*, *Pax5*, and *Gata-3* mRNA in CLPs from 4-wk-old and adult (8–12 wk old) mice. The amounts of mRNA from each transcription factor were normalized to the amount of *Hprt* mRNA and are plotted as arbitrary units relative to the amounts expressed by wild-type CLPs (100 a.u.). (black) *Ebf*; (gray) *Pax5*; (white) *Gata-3*. a.u., arbitrary units. The results are representative of two independent experiments. Frequency of (B) wild-type or (C) IL-$7^{-/-}$ CLPs able to give rise to B cell colonies after transduction with the retroviral vectors (○) MIEV, (●) MIEV-Pax5, or (■) MIEV-EBF, as determined by limiting dilution assay. The number of cells plated per well is plotted against the percentage of negative wells for the growth of B cell colonies ($CD19^+$). Solid lines represent the best-fit regression and the dashed line intercepts the vertical axis at 37%. The frequency obtained with cells transduced by each vector is indicated.

EBF overexpression rescues the B potential of IL-$7^{-/-}$ CLPs

The transcription factor EBF is specifically expressed during B cell development and, together with Pax5, contributes to the establishment of a B cell characteristic profile of gene expression (13). Both EBF and Pax5 are expressed as early as the CLP stage, although at a lower level than committed B cell precursors (reference 35 and unpublished data). In view of the differential ability of CLPs from adult IL-$7^{-/-}$ and control mice to give rise to B and T lymphocytes, we compared by quantitative RT-PCR their expression of B lineage (*Ebf* and *Pax5*) and T lineage (*Gata-3*)–specific transcription factors. Although CLPs always express comparable amounts of *Gata-3*, at 4 wk of age, CLPs from IL-$7^{-/-}$ mice already have half the amount of *Ebf*. However, in adult IL-$7^{-/-}$ mice the amounts of both *Ebf* and *Pax5* are markedly reduced, to 16 and 13% of the wild type, respectively (Fig. 4 A).

The lower amount of *Ebf* and *Pax5* observed in IL-$7^{-/-}$ CLPs would readily explain their impaired B cell differentiation potential. To investigate this possibility, we transduced these cells with retroviral vectors carrying the coding sequence of either Pax5 or EBF, or with an empty vector as control (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20042393/DC1). The frequency at which CLPs from wild-type mice generated B cell colonies in culture was not altered by transduction with either of the vectors (Fig. 4 B). Transduction of IL-$7^{-/-}$ CLPs with the Pax5 vector led to a small increase in the frequency of B cell generation and this in only one out of three experiments (Fig. 4 C). In contrast, overexpression of *Ebf* increased that frequency by a factor of nine (Fig. 4 C). In three independent experiments, transduction of IL-$7^{-/-}$ CLPs with the *Ebf* vector always restored B lymphocyte generation in vitro. The effect of EBF is likely to be even higher because only a fraction of CLPs are in the S/G2/M phases of the cell cycle (3) and, thus, are susceptible to infection by retroviral vectors. Therefore, we conclude that *Ebf* expression restores the B cell differentiation potential of CLPs from adult IL-$7^{-/-}$ mice.

DISCUSSION

IL-7 is well known to be an important cytokine in lymphocyte development. This work now defines IL-7 as a major player in lymphoid lineage commitment. Our results identify the CLP as the earliest developmental stage at which B cell differentiation is impaired in the adult BM by the absence of IL-7, earlier than thought previously (27, 32). We show that in the absence of IL-7 signals, multipotent CLPs do not achieve wild-type levels of expression of B lineage transcription factors and lose their B cell differentiation potential while retaining T and NK potential. Importantly, the fact that only *Ebf* suffices to rescue the B cell potential of IL-$7^{-/-}$ CLPs points to IL-7 as a major regulator of *Ebf* expression already at the uncommitted lymphoid progenitor stage. IL-7

determines the differentiation potential of CLPs by actively maintaining the B cell program open. Our results also indicate that closing the B cell program (i.e., down-modulation of *Ebf*) does not irreversibly engage cells in the T–NK differentiation pathway.

We focused our studies on IL-7$^{-/-}$ mice because B cell differentiation is completely abolished in adult animals. These mice also have severely impaired T cell development, whereas the NK lineage is unaffected. Ligand-deficient mice are particularly suited to study the role of cytokines in lymphopoiesis because precursor populations can be isolated and functionally characterized both in vitro and in vivo. Most conclusions drawn to date about the role of IL-7 signaling in lymphocyte development relied on the study of mice made deficient for IL-7Rα or common γ-chain (γc), the two components of the IL-7 receptor (34, 36). However, the unresponsiveness of those mice to IL-7 has precluded functional analysis of precursor populations. Additionally, the conclusions are limited by the fact that both chains are also components of other cytokine receptor complexes: TSLP binds IL-7Rα–TSLP-R (37, 38) and IL-2, -4, -9, -15, and -21 also signal through γc (39). Thus, in IL-7Rα– or γc-deficient mice, it is difficult to draw firm conclusions about the roles of the individual cytokines.

Mice deficient in γc have a normal percentage of cells with the phenotype of CLPs and it was demonstrated that IL-7 is sufficient to drive wild-type CLP differentiation along the B cell pathway in vitro, with a parallel loss in their ability to generate T cells (27). These results were taken as evidence that IL-7 signaling is dispensable for the generation of CLPs from HSCs, but necessary, after CLP generation, for their survival and differentiation along the B cell pathway (27). By studying IL-7$^{-/-}$ mice, we found that although CLPs can be generated in the absence of IL-7 signals, they are nonfunctional as B cell progenitors, even upon exposure to an excess of IL-7 in vitro. The much-reduced frequency of IL-7$^{-/-}$ CLPs able to generate B cells necessarily reflects the specific loss of B potential in multipotent progenitors (which we determined to be at least 1/6), independently of the possible concomitant loss of already B-committed precursors among Lin$^-$IL-7Rα$^+$c-Kit^{lo}Sca-1$^+$ cells. The requirement for IL-7 to maintain the B cell but not the T/NK potential in CLPs is also observed in Lin$^-$B220$^+$ cells, where the recently described CLP-2 (2, 4) is found. Therefore, our results reveal a novel function for IL-7 signaling in the commitment to the B cell lineage by multipotent lymphoid restricted precursors.

It is striking that CLPs (CLP-1 and CLP-2) are only impaired in their ability to generate B lymphocytes, and not T and NK cells. T lineage–restricted precursors were described recently in the thymus of Ikaros$^{-/-}$ mice, which have no identifiable CLPs in the BM (40). Our analysis concerned the CLP-dependent pathway because we detect T/NK potential in IL-7Rα$^+$ cells with the phenotype of CLPs and also in the B220$^+$ (CLP-2) cells. A population, designated early lymphocyte precursors (ELPs) with an intermediate phenotype between HSCs and CLPs, was also described, which gives rise to all lymphocyte populations and keeps some myeloid potential (41). Again, our analysis did not directly address ELPs. Nevertheless, by being IL-7Rα$^-$, we predict that IL-7 deficiency does not affect the B potential of ELPs.

The thymic precursors found in IL-7$^{-/-}$ mice are able to migrate to the thymus and, together with early T lineage progenitors (40), likely support adult thymopoiesis in IL-7$^{-/-}$ mice. Thymopoiesis in such mice is reduced due to the role of IL-7, at later stages of T cell development, in the expansion of DN1 and DN2 and in the transition from DN2 to DN3 (34). Our finding that IL-7 is essential for the maintenance of the B cell, but not the T cell, potential of CLPs explains why B cell numbers are not rescued the same way as T cell numbers by the overexpression of Bcl-2 (42, 43) or by Bax deficiency (44) in an IL-7 signaling–deficient background.

The reduction in the number of BM cells from IL-7$^{-/-}$ mice that are able to generate B lymphocytes is progressive; at 4 wk, an age when IL-7–independent B cell generation occurs due to the substitutive action of TSLP (30), we found such cells reduced to 17% of controls, whereas in adults, they were reduced to <1%. Consistent with the important role of IL-7 at later stages of B cell development, the reduction in the number of pro–B cells in these animals is even more marked: 0.8% of control at 4 wk and <0.001% at 7 wk as published previously (25).

We showed that already at 4 wk of age, IL-7$^{-/-}$ CLPs have reduced amounts of *Ebf*. The amount of *Ebf* and *Pax5* become drastically reduced in adult CLPs, whereas *Gata-3* expression is unaffected. Thus, IL-7 signaling is necessary to maintain the expression of B lineage–related transcription factors in adult BM cells, as early as the CLP stage. In our studies, *Pax5* overexpression only minimally recovered the B cell potential of CLPs. This is compatible with the notion that the B cell differentiation program is already active before being rendered irreversible by Pax5 (13). The analysis of mice deficient for either EBF or Pax5 shows that Pax5 is necessary in B cell differentiation at a later stage than EBF because EBF$^{-/-}$ cells neither express Pax5 nor undergo heavy chain rearrangements (16), whereas Pax5 deficiency allows B cell development to progress until the D-J_H rearrangement stage (17, 45).

The impairment of the B cell differentiation potential, consequent to IL-7 deficiency in adult mice, is likely due to low expression of *Ebf*, as overexpression of this B cell–specific transcription factor sufficed to fully restore the B cell precursor activity of the BM CLPs. This indicates that *Ebf* expression activates the B lineage program in lymphoid progenitors. Furthermore, closing the B cell program (i.e., down-modulation of *Ebf*), does not irreversibly engage cells in the T–NK differentiation pathway. Our results agree with the observation that *Ebf*, but not *Pax5*, rescues B cell development in E47- or PU.1-deficient fetal liver progenitors (46, 47).

It was recently observed that the expression of a constitu-

tively active form of STAT5b overcomes the B cell differentiation block in IL-7Rα$^{-/-}$ mice (48) and the authors hypothesized that this is achieved by modulation of Pax5 activity. Based on our results, we suggest that EBF is a more likely candidate transcription factor necessary in the very early phases of B cell differentiation. The B cell–specific regulation of recombinase activity that is observed in CLPs (49) may also be secondary to IL-7 signaling. IL-7 activates the JAK/STAT cascade as well as other pathways such as Ras/Raf/Erk (50) or PI3K/AKT (51). Although no direct link between IL-7 signaling and Ebf expression has been described thus far, our results suggest that IL-7 regulates *Ebf* expression in multipotent lymphoid progenitors.

MATERIALS AND METHODS

Mice. C57BL/6 control mice (Ly5.1 or Ly5.2) were purchased from Charles River Laboratories. IL-7$^{-/-}$ mice (28) in the C57BL/6 background (>10 backcrosses) and Rag2γc$^{-/-}$ mice (52) were bred under specific pathogen-free conditions at the Institut Pasteur. We analyzed young (4 wk old) and adult (8–12 wk old) mice. All animal experiments were done in accordance to the guidelines of the Institut Pasteur, which are approved by the French Ministry of Agriculture.

Cell sorting. CLPs (Lin$^-$IL-7Rα$^+$c-Kit^{lo}Sca-1$^+$) and HSCs (Lin$^-$IL-7Rα$^-$c-Kit^{hi}Sca-1^{hi}) were isolated (>97% purity) using a MoFlo CellSorter (DakoCytomation). Freshly isolated BM cells were stained with PE-conjugated monoclonal antibodies for CD3ε (145-2C11), CD4 (RM4-5), CD8α (53–6.7), B220 (RA3-6B2), NK1.1 (PK136), CD11b (M1/70), Gr-1 (RB6-8C5), and Ter-119. Depletion of Lin$^+$ cells was performed by magnetic-activated cell separation using anti-PE MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. After depletion, the Lin$^-$ fraction was treated with Fc-block (CD16/CD32) and stained with anti–Sca-1–FITC (E13-161.7), anti–c-Kit–allophycocyanin (2B8), and with anti–IL-7Rα biotin (A7R34; reference 53). Streptavidin-Cy-chrome was used to reveal the staining with the biotinylated antibody. Dead cells were stained with propidium iodide and excluded by electronic gating. Flk-2 expression was detected by staining sorted cells with anti–Flk-2–PE (A2F10.1). Lin$^-$B220$^+$ cells were isolated after depletion with antibodies for CD3ε, CD8α, CD19 (1D3), IgM (R6-60.2), NK1.1, CD11b, Gr-1, and Ter-119 and staining with anti-B220–FITC. All antibodies were purchased from BD Biosciences except for the biotinylated anti–IL-7Rα, a gift from B. Rocha (CHU Necker-Enfants Malades, Paris, France).

In vivo reconstitution. CLPs were sorted from the BM of adult wild-type or IL-7$^{-/-}$ mice, and 1,000 cells were injected intravenously in irradiated (600 rad) Rag2γc$^{-/-}$ mice. 5 wk later, recipients were analyzed for the presence of donor type cells in the BM, spleen, and thymus, with a FACSCalibur or a LSR flow cytometer and CELLQuest 3.3 software (Becton Dickinson).

Transplantation of fetal thymic lobes. Fetal thymic lobes from C57BL/6 Ly5.1 E15 embryos were irradiated (3,000 rad) and grafted under the kidney capsule of adult IL-7$^{-/-}$ or control mice (four lobes per mouse). Mice were killed four wk later and the lobes were analyzed for the presence of recipient Ly5.2 T cells by flow cytometry. The absolute number of cells per transplant was determined using a Neubauer chamber, excluding dead cells with trypan blue. T cell subsets were analyzed by staining with monoclonal antibodies for CD4, CD8, TCRγδ (GL3), CD25 (7D4), and CD44 (IM7). Staining with monoclonal antibodies for CD3ε, NK1.1, and CD19 was also used to gate on DN T cells.

Limiting dilution assays. The frequency of lymphoid precursors was determined in limiting dilution assays as described previously (54) with some modifications. OP9 stromal cells, a gift from T. Nakano (Osaka University, Osaka, Japan) were seeded in 96-well plates (2,500 cells/well). A minimum of 48 wells were analyzed per cell dilution. The culture medium was supplemented with saturating amounts of c-Kit ligand, Flk-2 ligand, IL-2, and IL-7. For the clonal analysis of B/NK potential, we used purified hIL-2 (provided by J.P. Di Santo, Institut Pasteur, Paris, France). These conditions also allow myeloid differentiation from HSCs. The frequency of T and NK precursors was determined by limiting dilution in FTOCs as described previously (55). Scores were assigned to B cell (CD19$^+$) or myeloid colonies (CD11b$^+$) on days 10–12 or reconstituted FTOCs (CD4$^+$/CD8$^+$/TCRγδ$^+$ or NK1.1$^+$) on days 14–15 by flow cytometry. The frequency of responding cells in each population (estimated by fitting a generalized linear model and finding the maximum likelihood using the Newton-Raphson method) and the significance of the reported differences between populations (assessed by relying on the asymptotic normality of the maximum likelihood estimates) were calculated using L-Calc software (StemSoft Software Inc.).

Quantitative RT-PCR analysis. Cells (10^4–10^5) were directly sorted on TRIzol (Invitrogen) or RNA-Plus (Qbiogene) and RNA was extracted following the manufacturer's instructions. Total cDNA was prepared using Oligo-d(T)12-18 (Amersham Biosciences) with M-MLV Reverse Transcriptase (Invitrogen) at 37°C for 50 min. 1/10 of the cDNA was used in each quantitative assay using SYBR green PCR core reagents (Applied Biosystems) and detection was done using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). For each target cDNA, the pair of primers permits the amplification of a multi-exon sequence: Hprt, forward, 5′-GTTCTTTGCTGACCTGCTGG-3′ and reverse 5′-TCCAACACTTCGAGAGGTCC-3′; Ebf, forward 5′-AACTGGCTGTGAATGTCTCG-3′ and reverse 5′-TCACATGGGAGGGACAATCA-3′; Pax5, forward 5′-TCCTCGGACCATCAGGACAG-3′ and reverse 5′-CCTGTTGATGGAGCTGACGC-3′; and Gata-3, forward 5′-TCGGCCATTCGTACATGGAA-3′ and reverse 5′-GAGAGCCGTGGTGGATGGAC-3′. Cycling conditions were as follows: 1 cycle of 10 min at 94°C; 45 cycles of 30 s at 94°C; 30 s at 60°C (Pax5, Gata-3) or 63°C (Hprt, Ebf); and 45 s at 72°C. Product size was confirmed by gel electrophoresis. Primer-dimer formation was monitored by gel electrophoresis and with the dissociation protocol. The amounts of Ebf, Pax5, or Gata-3 in each sample were calculated with ABI Prism 7000 Sequence Detection System software (Applied Biosystems) and normalized to the amount of Hprt.

Retroviral transduction. The MSCV-pgk.1-IRES-eGFP (MIEV) retroviral vector, a gift from G. Wu (University of Toronto, Toronto, Canada), has been described previously (56). The coding regions of murine Pax5 and rat Ebf (provided by B. Kee, University of Chicago, Chicago, IL) were blunt-end cloned into the SnaBI site of MIEV, the orientation was confirmed by restriction analysis to generate MIEV, MIEV–Pax5, and MIEV–EBF vectors. The ecotropic packaging cell line Plat-E (provided by T. Kitamura, Institute of Medical Science, Tokyo, Japan; reference 57) was transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Viral supernatant was harvested 48 h after transfection, filtered through a 45-μm membrane (Sartorius), and incubated for 5 min on ice with 2.5 μg/ml of polybrene (Sigma-Aldrich). Sorted cells were cultured overnight with saturating concentrations of c-Kit ligand, Flk-2 ligand, and IL-7, followed by two serial spin-infections (1,800 revolutions/min at 32°C, for 45 min) with a 24-h interval. After infection, cells were counted and used in limiting dilution assays as described before. We estimated the efficiency of transduction by analyzing GFP expression, 48 h after infection, in Lin$^-$ BM cells. GFP expression was detected by flow cytometry in 52, 18, and 10% of the cells transduced with MIEV, MIEV-Pax5, or MIEV-EBF, respectively.

Online supplemental material. Fig. S1 shows that adult IL-7$^{-/-}$ thymic precursors are able to migrate and repopulate a thymus. Fig. S2 shows a schematic representation of the retroviral vectors. Table S1 shows the fre-

quency of Sca-1^{low} and Sca-1^{high} CLPs able to give rise to B, T, or NK cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042393/DC1.

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