Determination of lymphoid cell fate is dependent on the expression status of the IL-7 receptor

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Signaling through the IL-7 receptor (IL-7R) is necessary for the development of the earliest B- and T-lineage cells. IL-7R is first expressed on common lymphoid progenitor cells and is not detected on primitive common myeloid progenitors. In this study, we show that enforced expression of IL-7R on multipotential stem cells does not influence lymphoid versus myeloid cell fate. T cell development was compatible with sustained IL-7R expression; however, we observed a near complete block in B cell development at the onset of B-lineage commitment. Unlike pre-proB cells from control animals, developmentally-arrested IL-7R⁺B220⁺CD19⁺NK1.1⁺Ly-6C⁺ cells failed to express EBF and Pax5. These results suggest that transient downregulation of IL-7R signaling is a necessary event for induction of EBF and Pax5 expression and B-lymphocyte commitment.

Keywords: B cell commitment/EBF/IL-7 receptor/Pax5/T cell development

Introduction

Growth factors and cytokines play vital roles in lineage specification within mammalian developmental systems. For instance, within the mammalian nervous system, multipotent neural crest precursors (NCPs) exposed to glial growth factor differentiate into glial cells while transforming growth factor β stimulates NCP to develop into smooth muscle in a process that is independent of growth factor-mediated cell survival (Anderson, 1997). In the hematopoietic system, growth factor and cytokine signaling pathways have been shown to regulate both proliferation and survival of hematopoietic cells, while their function in lineage specification remains controversial (Enver *et al.*, 1998; Metcalf, 1998). One recent example illustrating the potential instructive role of cytokine receptor signaling in lineage determination within the hematopoietic system was shown when the IL-2R β chain was expressed in common lymphoid progenitor (CLP) cells (Kondo *et al.*, 2000). CLP cells are normally restricted in their developmental potential to the B, T and natural killer (NK) cell lineages. However, when IL-2R β was introduced into CLP cells using either an MHC class I promoter-driven transgene or from a

retroviral vector, CLP were induced to differentiate along the myeloid cell pathway when stimulated at a clonal level with IL-2. Although these findings are informative in showing that lineage determination can be regulated by cytokine receptor signaling, it remains unclear whether IL-2R β has such a function in normal hematopoiesis.

Signaling through the IL-7 receptor (IL-7R) is necessary for the development of the earliest B- and T-lineage cells (Peschon *et al.*, 1994; Miller *et al.*, 2002). IL-7R is comprised of two chains, the IL-7R α ligand-binding chain and the IL-2R γ_c signaling chain (Goodwin *et al.*, 1990; Noguchi *et al.*, 1993), which are both required for proper signaling to occur (Ziegler *et al.*, 1995). The IL-7R α chain also comprises part of the thymic stromal lymphopoietin (TSLP) receptor (Park *et al.*, 2000; Leonard, 2002), while the IL-2R γ_c chain is shared by the receptors for IL-2, IL-4, IL-9, IL-15 and IL-21 (Sugamura *et al.*, 1996; Asao *et al.*, 2001). During hematopoiesis, IL-7R expression is first detected at the CLP stage (Kondo *et al.*, 1997). It is present on developing thymocytes at the CD4⁺CD8⁺ stage, on CD4⁺ and CD8⁺ single positive T cells, and on immature B-lineage cells. It is not expressed on CD4⁺CD8⁺ double positive thymocytes or mature B cells (Sudo *et al.*, 1993; Appasamy, 1999; Fry and Mackall, 2002). Based on findings from IL-7R knockout animals, IL-7R is also required for $\gamma\delta$ T cell development but not for NK and dendritic cell development (He and Malek, 1996; Maki *et al.*, 1996; Fry and Mackall, 2002). Blocking cell death by enforced expression of the anti-apoptotic gene, *bcl-2*, is sufficient to rescue $\alpha\beta$ T cell development in IL-7R^{-/-} animals (Akashi *et al.*, 1997; Maraskovsky *et al.*, 1998) but is not sufficient to rescue the block in early B cell development, suggesting that IL-7R signaling has unique functions within the earliest B- and T-lineage cells.

Although myeloid-lineage cells, including the common myeloid progenitor (CMP), do not express IL-7R (Akashi *et al.*, 2000), several experiments have shown that IL-7 can result in various effects on myeloid lineage cells both *in vitro* and *in vivo*. IL-7 synergistically increases myeloid colony formation of Lin⁻Sca-1⁺ progenitors when combined with various colony-stimulating factors, including GM-CSF and CSF-1 (Jacobsen *et al.*, 1993; Appasamy, 1999). In addition, administration of recombinant IL-7 to mice has been shown to increase the numbers of immature myeloid lineage cells, which further supports a direct or indirect function of IL-7 in myelopoiesis (Faltynek *et al.*, 1992).

A possible role for IL-7R signaling in lymphoid lineage commitment is suggested by a number of observations. First, IL-7R is initially detected on CLP, but not on CMP or multipotent stem cells (Kondo *et al.*, 1997; Akashi *et al.*, 2000). Second, experiments involving the knockout of the B-lineage transcription factor Pax5 showed that Pax5^{-/-} cells could be maintained at the pro-B stage *in vitro* in

media containing IL-7 (Nutt *et al.*, 1999). However, when IL-7 was removed or depleted from the cultures, the cells changed morphology and gave rise to various myeloid lineage cells both *in vitro* and *in vivo* (Nutt *et al.*, 1999; Mikkola *et al.*, 2002). This suggests that both IL-7R signaling and Pax5 activity function to maintain B cell identity at the point of B-lineage commitment. Finally, further analysis of IL-7R^{-/-} animals has indicated that both B and T lymphopoiesis are blocked prior to the earliest committed progenitor stage for both lineages (Peschon *et al.*, 1994; Miller *et al.*, 2002). Collectively, these observations raise the possibility that IL-7R signaling may play an important role in the specification of lymphoid cell fate from multipotent progenitor cells.

In order to test whether IL-7R expression would restrict the developmental potential of multipotential cells to the lymphoid lineages, we transduced bone marrow progenitor cells with an IL-7R-expressing retrovirus and examined hematopoiesis derived from these cells in reconstituted mice. Analysis of transplant recipients showed that graded levels of IL-7R expression resulted in altered ratios of B versus myeloid cells in peripheral blood of all reconstituted animals. Mice transplanted with cells expressing a control green fluorescent protein (GFP) retrovirus showed no evidence of lineage skewing at high or low levels of GFP expression. Further analysis of B cell development in bone marrow of mice expressing IL-7R indicated a severe developmental block at the earliest B cell progenitor stage. Unlike their phenotypically equivalent counterparts that do not express IL-7R, developmentally arrested IL-7R^{-/-}B220⁺CD19⁺NK1.1⁻Ly-6C⁻ cells failed to express EBF and Pax5, which suggests that transient down-regulation of IL-7R expression may be a necessary event for induction of EBF and Pax5 expression and B cell commitment. Sustained expression of IL-7R had no inhibitory effect on the development of T or myeloid-lineage cells. This indicates that IL-7R signaling does not influence lymphoid versus myeloid cell fate but rather promotes T versus B cell development in a manner dependent on its expression status.

Results

Retroviral expression of IL-7R rescues B and T cell development in IL-7R^{-/-} cells

In order to test whether enforced expression of IL-7R in multipotent stem cells would promote commitment to the lymphoid lineage, we used murine stem cell virus (MSCV) (Hawley *et al.*, 1994) to coexpress the two components of IL-7R, IL-7R α and IL-2R γ c, along with one of two spectrally distinct GFP variants. Expression of blue-excited GFP (Bex) and violet-excited GFP (Vex) can be detected in a single cell due to their differential excitation properties (Anderson *et al.*, 1996). The two IL-7R chains were individually cloned upstream of an internal ribosome entry site (IRES) within MSCV (Figure 1A). Cells were transduced with both viruses in the event that the IL-2R γ c signaling chain would be limiting for IL-7R assembly or for the proper function of other IL-2R γ c-associated receptors including IL-2R, IL-4R, IL-9R, IL-15R and IL-21R. Stable virus-producing cell lines that produced both MSCV-IL-7R α -Bex and MSCV-IL-2R γ c-Vex, or the control MSCV-Bex and MSCV-Vex viruses, were

Fig. 1. Characterization of IL-7R α and IL-2R γ retroviruses. (A) Schematic diagram of retroviral constructs generated in MSCV. MSCV consists of: LTR (long terminal repeat), IRES, Bex and Vex. (B) Rescue of T cell development in IL-7R^{-/-} cells is shown in a representative FACS stain at four weeks post transplant. Thymocytes were gated on double-positive (Bex and Vex) cells and then analyzed by CD4 and CD8 staining. (C) $\gamma\delta$ T cell development is rescued in IL-7R^{-/-} cells by IL-7R expression. FACS analysis was done at 7 weeks post-transplantation.

generated using the GP+E-86 cell line (see Materials and Methods).

In order to confirm that a functionally competent IL-7R was being assembled on the cell surface *in vivo*, bone marrow was isolated from IL-7R α knockout animals and then cocultured on double virus-producing GP+E-86 cells for 48 h before injection into Ly-5 congenic, lethally

Fig. 2. Increased levels of IL-7R α /IL-2R γ _c expression results in a phenotypic skew to the myeloid lineages in peripheral blood. (A) Peripheral blood analysis at 4 weeks post transplant. Quadrants a, b, c and d, e, f, which gated the three different subpopulations of cells (Vex single positive, Bex/Vex double positive and Bex single positive, respectively), were analyzed for B220 (B cell) and Mac-1 (myeloid) expression. Dead cells were excluded by propidium iodide staining in all samples prior to gating. (B) IL-7R α /IL-2R γ _c-expressing peripheral blood cells were gated for increasing levels of Bex/Vex expression and then analyzed for B220 and Mac-1 expression. (C) A bar graph shows the percentage of B220⁺ and Mac-1⁺ cells for low and high levels of IL-7R expression from five IL-7R-reconstituted animals and five Bex/Vex reconstituted animals from a single experiment at 4 weeks post transplant. Similar observations were made in 15 total mice from three separate reconstitution experiments.

irradiated recipient mice. Four weeks post-transplant, thymi from reconstituted animals were analyzed for rescue of T-lineage development by staining for CD4 and CD8, since IL-7R α knockout animals exhibit a severe developmental block prior to CD4 and CD8 expression (Peschon *et al.*, 1994). T-cell development in animals reconstituted with cells expressing the control Bex/Vex vectors was not rescued and displayed the IL-7R knockout phenotype of arrested $\alpha\beta$ T cell development at the double negative CD4⁻CD8⁻ stage (Figure 1B). In contrast, normal $\alpha\beta$ T cell development and thymic cellularity was completely restored in animals reconstituted with IL-7R-expressing cells as indicated by CD4 and CD8 staining (Figure 1B). T cell development was rescued in the IL-7R knockout cells by IL-7R α /IL-2R γ _c or by IL-7R α alone (Figure 1B; data not shown), which indicates that retrovirally-encoded IL-7R was functional. Constitutive IL-7R expression had no apparent detrimental effects on T cell development in that we continued to observe normal production of T-lineage cells for as long as 14 weeks post-transplantation, which was the longest time point analyzed. In addition, $\gamma\delta$ T cell development was rescued in animals reconstituted with cells expressing the IL-7R, while it was

absent in animals reconstituted with Bex/Vex-expressing IL-7R^{-/-} cells (Figure 1C).

Graded levels of IL-7R expression alter the ratio of B-lymphoid versus myeloid cells in the periphery

To determine the effects of constitutive IL-7R expression on normal hematopoiesis, we analyzed peripheral blood from animals reconstituted with wild-type bone marrow cells transduced with either the control (Bex/Vex) or IL-7R-expressing retroviruses beginning at 3–4 weeks post-transplant. Cells that expressed a single fluorophore (Bex or Vex) or double-expressing cells were independently analyzed (Figure 2A). In all analyses, gates were drawn on Bex/Vex⁺ cells, which were all Ly5.1 (donor-marker) positive (data not shown). While the Bex/Vex control mice (henceforth referred to as control mice) showed a higher frequency of B220⁺ cells in peripheral blood ($n = 15$), mice reconstituted with IL-7R α /IL-2R γ _c-expressing cells (IL-7R mice) displayed a more Mac-1⁺ phenotype ($n = 15$). In IL-7R mice, both double positive IL-7R α /IL-2R γ _c (Bex/Vex) cells and single positive IL-7R α (Bex) cells showed a large increase in the frequency of Mac-1⁺ cells, compared with single positive

Fig. 3. Increased levels of Bex/Vex expression correlate in a non-linear manner with increased IL-7R expression on the cell surface. (A) Bone marrow analysis at 9 weeks post-transplant using IL-7R liposome staining. Representative control Bex/Vex ($n = 2$) and IL-7R α /IL-2R γ_c ($n = 2$) mice were analyzed by staining with IL-7R liposome (see Materials and methods) and Mac-1 (myeloid) antibodies. Non-specific background staining using the liposome approach was assessed by first blocking with 100–1000-fold excess of unlabeled, primary IL-7R antibody prior to staining with the labeled IL-7R α antibody (right panel). (B) IL-7R α /IL-2R γ_c -expressing bone marrow cells were gated at varying levels of Bex/Vex expression and then analyzed for IL-7R expression using IL-7R liposome staining. The mean fluorescence intensity of IL-7R expression represented by each histogram is shown on the right.

IL-2R γ_c (Vex) cells (Figure 2A, d–f). The skewed ratio of B220 $^+$ versus Mac-1 $^+$ cells was only evident in IL-7R α or in IL-7R α /IL-2R γ_c expressing cells, indicating that the Bex/Vex fluorophores do not inherently skew development between lymphoid and myeloid cells (Figure 2A, a–c). Further examination of the data revealed that increasing levels of IL-7R α /IL-2R γ_c expression within a given animal resulted in a statistically significant decrease in the frequency of B220 $^+$ cells along with a pronounced increase in the frequency of Mac-1 $^+$ cells (Figures 2B and C). Cells that expressed increasing levels of Bex (IL-7R α) were gated along the diagonal (Figure 2B) to ensure that approximately stoichiometric levels of IL-7R α and IL-2R γ_c would be expressed within a given cell in the gated populations. It should be noted that the variability seen in the frequencies of individual lineages is due to the inherent variability associated with a reconstitution assay. The same phenotypic shift was observed in 15 mice per construct analyzed from three independent reconstitution experiments.

Increased Bex/Vex levels correlate with increased surface expression of IL-7R

To determine whether increased levels of Bex/Vex expression correlated with increased levels of IL-7R α on

the cell surface, we used an antibody coupled with fluorochrome-filled liposomes as a secondary reagent to detect staining with the IL-7R α primary antibody. In our hands, the commercially available IL-7R antibody generally gave a weak fluorescence signal that did not allow for clear separations of IL-7R $^+$ and IL-7R $^-$ cell populations. The use of the liposome-conjugated reagent resulted in a 10- to 100-fold increase in fluorescence intensity without a significant increase in non-specific background staining (Scheffold *et al.*, 2000). This approach allowed us to detect a significant range of IL-7R α expression on transduced cells, which was critical for establishing a correlation between IL-7R cell-surface expression as a function of increasing Bex/Vex fluorescence intensity. IL-7R α liposome staining of Bex/Vex control bone marrow showed a low percentage of IL-7R α -expressing cells, which was expected since early B cell progenitors express endogenous IL-7R α (Figure 3A, left panel) (Sudo *et al.*, 1993). The liposome staining of bone marrow from animals reconstituted with IL-7R-expressing cells showed a substantial increase in the number of Mac-1 $^+$ cells that expressed IL-7R α (Figure 3A, middle panel). To assess the specificity of the staining, cells were blocked with unconjugated anti-IL-7R α antibody prior to addition of the conjugated anti-IL-7R α liposome secondary antibody

(Figure 3A, right panel). This showed that IL-7R α was specifically detected with the liposome method. Notably, IL-7R expression from the retroviral vectors (Figure 3A, middle panel) was not higher than levels of endogenous IL-7R detected on control bone marrow cells (Figure 3A, compare left and middle panels).

Using the IL-7R liposome stain, we found that IL-7R surface expression increased as levels of Bex/Vex expression increased (Figure 3B), although there was not a direct linear correlation between Bex/Vex fluorescence levels and those observed for IL-7R (as measured by the mean fluorescence intensities of the gated populations and histograms). The highest levels of Bex/Vex expression only represented a 2.5-fold increase in IL-7R surface expression when compared with IL-7R expression in non-transduced cells (Figure 3B). Similar results were obtained when we analyzed expression of cells expressing only the IL-7R α chain (Bex⁺ cells; data not shown). In addition, surface IL-7R expression was not detected above background staining at low levels of Bex/Vex expression even using the highly sensitive liposome staining approach. Collectively, these results indicate that increased surface expression of IL-7R correlates with the phenotypic shift from B220⁺ to Mac-1⁺ cells in the periphery and that levels of IL-7R expression from the retroviral vectors was similar to endogenous IL-7R levels on the cell surface.

Constitutive expression of IL-7R results in an early B cell developmental block

The correlation between IL-7R levels and increased myelopoiesis in peripheral blood led us to consider two possible scenarios to explain our results. First, constitutive IL-7R expression in bone marrow could lead to a block in B cell development, which would result in a perceived increase of mature myeloid cells in the periphery. Second, IL-7R signaling in multipotential cells might stimulate commitment to the myeloid versus lymphoid cell pathway or promote myelopoiesis post-commitment. To investigate these possibilities, we analyzed B cell development and early myelopoiesis in bone marrow. The ordered development of B-lineage cells in bone marrow has been defined by the differential expression of cell-surface markers and by the rearrangement status of immunoglobulin genes (Hardy *et al.*, 1991; Li *et al.*, 1996; Hardy and Hayakawa, 2001). The pre-pro-B cell stage, also termed Fraction (Fr.) A, is B220⁺CD43⁺CD19⁺IgM⁺ (Li *et al.*, 1996; Rolink *et al.*, 1996). Fraction B/C cells are B220⁺CD43⁺CD19⁺IgM⁺ and represent committed B-lineage cells that have undergone rearrangement of D–J gene segments at the immunoglobulin heavy chain locus. Bone marrow analysis of IL-7R mice stained with B220 and Mac-1 showed a similar reduction in B-lineage cells with increasing levels of IL-7R expression (Figure 4A). Further analysis of IL-7R mice revealed a near complete block in B lymphopoiesis at the transition between Fr. A and Fr. B cells as indicated by the absence of B220⁺CD19⁺ IL-7R-expressing cells (Figure 4B). The mean frequency of B220⁺CD19⁺ cells in bone marrow was 0.57% for IL-7R mice ($n = 6$). These cells were also missing among cells that only expressed high levels of the IL-7R α chain (single Bex⁺ cells; data not shown). The frequency of B220⁺CD19⁺ cells was not affected in control mice that expressed high levels of Bex/Vex (Figure 4B, mean

frequency of 13.6%, $n = 6$). Analysis of the thymus by CD4 and CD8 staining showed no difference in T cell development between control and IL-7R animals that expressed high levels of Bex/Vex (Figure 4C, $n = 4$ for each construct). These data show that T cell development, in contrast to B cell development, is compatible with sustained IL-7R expression.

Lymphoid-lineage commitment is not affected by enforced IL-7R expression

We originally questioned whether IL-7R expression in stem cells might promote lymphoid lineage commitment over myeloid development. If there were enhanced commitment to the B cell pathway due to IL-7R expression in stem cells, we might expect to see an increase in the frequency and absolute number of IL-7R-expressing Fr. A cells and a decrease in the frequency of myeloid progenitor cells in bone marrow of IL-7R animals. Bone marrow cells from both control and IL-7R mice were gated for both high and low IL-7R levels and 4000 cells from each gate were then sorted and plated in triplicate into methylcellulose M3434 media supplemented with or without IL-7. Control bone marrow was also plated using the same parameters. Colonies of >50 cells were typed and counted 10 days later. The predominant colony types in all the samples, from both the control and IL-7R animals, were granulocyte–macrophage colony-forming units (CFU-GM). We observed no significant difference in colony type or number between cells from Bex/Vex control animals or IL-7R animals at any level of IL-7R expression, whether in the presence or absence of IL-7 (Table I). Based on this observation, it is likely that IL-7R expression does not promote lymphopoiesis at the expense of myelopoiesis. In addition, the reduced number of Fr. A cells observed among cells expressing higher levels of IL-7R (Figure 4B) is not due to IL-7-mediated promotion of early myelopoiesis, otherwise we would have expected higher numbers of CFU-GM progenitors in platings of IL-7R high cells, which was not the case (Table I).

Constitutive IL-7R expression blocks EBF and Pax5 induction in B220⁺CD19⁺NK1.1⁺Ly-6C⁺ Pre-proB cells

The Fr. A subset has been further characterized for cells that have B-lineage potential by staining for NK1.1 and Ly-6C expression (Tudor *et al.*, 2000). The B220⁺CD19⁺NK1.1⁺Ly-6C⁺ subset of Fr. A contains the only cells that have B-lineage potential. To characterize the developmental block in IL-7R-expressing pre-proB cells, Pax5, EBF and E47 mRNA expression was analyzed by RT-PCR in sorted populations from control or IL-7R mice. Pax5 is required for the maintenance of the B-cell committed state (Nutt *et al.*, 1999; Mikkola *et al.*, 2002) and is sufficient to completely block the earliest stage of T cell development when ectopically expressed in hematopoietic stem cells (Souabni *et al.*, 2002; Cotta *et al.*, 2003). EBF and E47 are also necessary for the earliest differentiation events within the B cell pathway. Targeted disruption of either factor in mice results in a complete block in B cell developmental prior to the onset of immunoglobulin heavy chain D–J rearrangement and CD19 expression (Bain *et al.*, 1994; Zhuang *et al.*, 1994; Lin and Grosschedl, 1995). Five B220⁺CD19⁺NK1.1⁺Ly-6C⁺ cells gated for Bex/Vex expression from control

Fig. 4. Constitutive IL-7R expression results in a B cell developmental block at the Hardy Fr. A to B transition. (A) Bone marrow analysis at four weeks post-transplant of IL-7R α /IL-2R γ_c mice gated for increasing Bex/Vex levels. Data are representative of Bex/Vex and for IL-7R α /IL-2R γ_c mice. (B) Bone marrow analysis of Bex/Vex control mice and IL-7R α /IL-2R γ_c mice to assess early B cell development. Both low and high Bex/Vex levels were gated and then analyzed for B220 and CD19 expression. Fr. A cells are B220 $^+$ CD19 $^-$ while B220 $^+$ CD19 $^+$ cells represent bone marrow Fractions B–F (Hardy *et al.*, 1991). (C) Analysis of control and IL-7R thymi by staining with CD4 and CD8. Cells are gated through high Bex/Vex or IL-7R α /IL-2R γ_c levels and then stained with CD4 and CD8 (Bex/Vex, $n = 4$; IL-7R α /IL-2R γ_c , $n = 4$).

or IL-7R mice (Figure 5A) were directly sorted into wells of a 96-well plate for reverse transcription and subsequent PCR using primers specific for EBF, E47 or Pax5. A β 2 microglobulin primer was included in all reactions as an internal control (Figure 5B). All cells were sorted once and then re-sorted before deposition into the plate to ensure >99% purity of the sorted populations. At least two independent sorting experiments showed that 16/18 samples from control Bex/Vex mice were positive for Pax5 transcript while only 2/28 samples were positive for Pax5 among B220 $^+$ CD19 $^-$ NK1.1 $^-$ Ly-6C $^-$ cells that expressed IL-7R (Figure 5B). EBF was expressed in 11/22 pre-pro-B cell samples sorted from control Bex/Vex animals and in only 4/27 samples from IL-7R mice. E47 expression was detected at approximately equivalent frequencies among control or IL-7R pre-pro-B cell samples (9/22 and 15/27, respectively). These results suggest that down-regulation of IL-7R expression may be a necessary event for EBF and Pax5 induction at the earliest defined stage of B cell development.

Differential expression of endogenous IL-7R in B220 $^+$ CD19 $^-$ NK1.1 $^-$ Ly-6C $^-$ cells from C57BL/6 mice
The observation that constitutive expression of IL-7R blocks the earliest step in B cell development would predict that the normal B220 $^+$ Fr. A population in bone marrow should have cells that lack IL-7R. In previous studies, Fr. A was subsetted into three distinct populations

Table I. Myeloid colony-forming cell assay initiated with 4000 Bex/Vex-positive bone marrow cells FACS-purified from IL-7R and control animals

Mouse	Bex/Vex level	CFU-GM (with IL-7)	CFU-GM (without IL-7)
Bex/Vex	Low	26	13 ^a
Bex/Vex	High	11	17 ^a
IL-7R	Low	21	21
IL-7R	High	9	6

Numbers represent an average of the granulocyte-macrophage colonies from two independent methylcellulose assay experiments.
^aData from one experiment plated in triplicate.

based on CD4 and B220 expression, with the first committed B cell subset (A₁) being IL-7R negative and B220 $^+$ (Allman *et al.*, 1999; Hardy and Hayakawa, 2001). Since FACS staining of IL-7R $^+$ cells in the other Fr. A subsets (A₀ and A₂) was extremely low and since additional markers have been used to further subset Fr. A (Tudor *et al.*, 2000), we wished to examine endogenous IL-7R expression using the IL-7R liposome approach in the B220 $^+$ CD19 $^-$ NK1.1 $^-$ Ly-6C $^-$ cells. As shown in Figure 5C, ~50% of the B220 $^+$ CD19 $^-$ NK1.1 $^-$ Ly-6C $^-$ cells do not express detectable IL-7R levels. Control stains to assess specificity of the liposome reagent using an unconjugated IL-7R antibody to first block binding to the IL-7R showed no background staining (data not shown). This indicates

Fig. 5. Pax5 and EBF are not expressed B220⁺CD19⁺NK1.1⁺Ly-6C⁻ cells that constitutively express IL-7R. (A) High IL-7Rα/IL-2Rγc (or control Bex/Vex) cells were gated as B220⁺CD19⁺NK1.1⁺Ly-6C⁻ and then five cells were sorted directly into a 96-well plate (see Materials and methods). Dead cells were excluded by propidium iodide staining. (B) Representative RT-PCR analysis of Pax5, EBF, E47 and β2 microglobulin. Each lane represents RT-PCR of five cells of the gated phenotype sorted into an individual well from control mice (lanes 1–6) or IL-7R mice (8–14). Lane 7 contained a control reaction that received five cells from control mice without the addition of reverse transcriptase. (C) IL-7R liposome stain of B220⁺CD19⁺NK1.1⁺Ly-6C⁻ cells from the bone marrow of wild-type, C57B/6 mice. (D) IL-7R⁻ or IL-7R⁺ B220⁺CD19⁺NK1.1⁺Ly-6C⁻ cells gated as shown in Figure 5C were sorted once and then resorted before culture on the T220–29 cell line. After 6 days in culture, cells were harvested, counted, and then stained for surface expression of CD19 and μ heavy chain (μHC). The μHC^{lo} cells express the pre-B cell receptor and the μHC^{hi} cells express κ/λ light chains (data not shown and R. P. Stephan, personal observations).

that a significant percentage of B220⁺CD19⁺NK1.1⁺Ly-6C⁻ cells do not express detectable levels of IL-7R. To determine whether the IL-7R⁻ B220⁺CD19⁺NK1.1⁺Ly-6C⁻ cells were able to give rise to committed B-lineage cells, we FACS-sorted both IL-7R⁺ and IL-7R⁻ B220⁺CD19⁺NK1.1⁺Ly-6C⁻ cells and cultured them for 6 days on the T220–29 cell line, which is known to support *in vitro* differentiation of B-lineage progenitors without the addition of exogenous cytokines (Gongora *et al.*, 2000). The data shown in Figure 5D demonstrate that both subsets efficiently differentiated into CD19⁺μHC⁺ B-lineage cells. The only observed difference between the two subsets was less overall cellular expansion in cultures initiated with IL-7R negative cells (15-fold expansion in the IL-7R⁻ cultures versus 21-fold in the IL-7R⁺ cultures). These results indicate that IL-7R⁻ pre-pro-B cells from normal mouse bone marrow have robust B-lineage developmental potential.

Discussion

In these experiments, we have constitutively expressed a functional IL-7R on hematopoietic progenitor cells using retroviral vectors that expressed spectrally distinct GFP variants. This was done in order to address the function of IL-7R signaling in lymphocyte cell fate determination from multipotential progenitor cells. Bone marrow cells

that expressed IL-7R did so at levels that were nearly identical to levels detected on cells expressing the endogenous IL-7R (Figure 3), which indicates that the retroviral expression system did not result in unusually high IL-7R cell-surface expression. In fact, the non-linear correlation between Bex/Vex expression levels and IL-7R cell-surface expression suggests that surface expression may be regulated by other cellular rate-limiting factors. Retrovirally-expressed IL-7R was detected on multipotential cells of the c-kit⁺Sca-1⁺Lin⁻ phenotype and was sufficient to rescue the early T cell developmental block seen in IL-7R knockout animals (Figure 1B and C; data not shown). Enforced expression of IL-7R at the level of c-kit⁺Sca-1⁺Lin⁻ stem cells did not promote differentiation to the lymphoid (versus myeloid) cell pathway in that we detected normal numbers of *in vitro* myeloid colony-forming progenitor cells among cells that expressed either high or low levels of IL-7R or control Bex/Vex-expressing cells (Table I). We would have anticipated that promotion of lymphoid development at the expense of myelopoiesis would have resulted in reduced numbers of early myeloid progenitors in platings of high IL-7R-expressing cells, which was not the case.

In previous work, it was shown that IL-7R could rescue both B lymphopoiesis and myelopoiesis when expressed in PU.1-deficient fetal liver cells plated on S17 stromal cells in the presence of IL-7 (DeKoter *et al.*, 2002). These

Fig. 6. Model for the role of IL-7R signaling in early lymphocyte development.

results have been reproduced in our laboratory (S.Purohit and C.Klug, unpublished data) and suggest that IL-7R signaling may promote survival and/or potentiate development along both the lymphoid and myeloid pathways, at least in these *in vitro* culture systems. However, based on the phenotype of the IL-7R knockout animals, it is clear that IL-7R signaling is dispensable for myelopoiesis and for the formation of normal numbers of CLP *in vivo* (Peschon *et al.*, 1994; Miller *et al.*, 2002). This indicates that IL-7R is not necessary for lymphopoiesis until the onset of B and T cell development.

Recent work has extended previous findings showing that the absence of IL-7R signaling leads to an incomplete block before the first committed B- or T-lineage cells emerge in the bone marrow and thymus, respectively (Peschon *et al.*, 1994; Miller *et al.*, 2002). The fact that both lineages are critically dependent on IL-7R signaling at this early stage is interesting given observations showing that IL-7R signaling may have distinct functions in early B and T cell development. This conclusion is based on the ability of the anti-apoptotic gene, *bcl-2*, to rescue only the T cell developmental block in IL-7R knockout cells (Akashi *et al.*, 1997). In our gain-of-function experiments, constitutive IL-7R expression permits T cell development but leads to a near complete block in B cell development before the appearance of B-committed cells (B220⁺CD19⁺IgM⁻, Figure 4). Arrest in early B-lineage development in both IL-7R knockout animals and in the constitutive IL-7R expression experiments described in this study would support a model where IL-7R levels are differentially regulated at the onset of B-lineage commitment. Our own observations (Figure 5C and D) and results described previously (Allman *et al.*, 1999) have shown that a subset of the B220⁺CD19⁺NK1.1⁻

Ly-6C⁻ population (or the Fr. A subset, A₁) lacks expression of IL-7R. Transplantation studies and intrathymic injections described previously (Allman *et al.*, 1999) showed that A₁ cells are B-lineage restricted, even though they lack expression of IL-7R. These results provide strong support for endogenous IL-7R down-modulation at the onset of B cell development and also suggest that normal B cell development progresses through an IL-7R negative, B220⁺ stage. Another possible interpretation of our results may be an increase in signaling through the TSLP receptor, rather than the IL-7R. Further support for this possibility will come from future analysis of early B and T lymphocyte development in TSLP knockout animals.

The B220⁺CD19⁺NK1.1⁻Ly-6C⁻ cells in IL-7R mice that continue to express IL-7R fail to progress through B cell development. One explanation for this block is the lack of EBF and Pax5 expression, which is readily seen in phenotypically identical B220⁺CD19⁺NK1.1⁻Ly-6C⁻ cells from control mice that do not express IL-7R (Figure 5B). This may indicate that transcription of either the *EBF* or *Pax5* genes may be coupled with transient down-regulation of IL-7R signaling during B cell commitment. We detect expression of both EBF and Pax5 in all cells that have developmentally progressed to the B220⁺CD19⁺ progenitor B cell stage (Fr. B) (data not shown). An alternative explanation for the developmental arrest observed in IL-7R-expressing Fr. A cells could be due to a requirement for cell-cycle exit at the onset of B cell commitment. IL-7 signaling is known to induce expression of both *c-myc* and *N-myc* in lymphoid cells and is a potent mitogen for immature B cells (Lee *et al.*, 1989; Morrow *et al.*, 1992; Strasser *et al.*, 1996). Interestingly, constitutive expression of both *c-myc* and *bcl-2* in Eμ-*bcl-2-myc*

transgenic mice results in tumor formation and developmental arrest at a similar stage (B220⁺CD19⁻) to what we observe in the IL-7R mice (Strasser *et al.*, 1996).

Taken together with the IL-7R knockout phenotype, our data support a model for the regulation of T versus B cell fate that depends on the expression status of the IL-7R (Figure 6). Lymphoid progenitor cells that continue to express the IL-7R would differentiate and/or emigrate to the thymus where T cell development would progress upon activation of T cell specification factors. The transient downregulation of IL-7R would lead to induction of EBF and Pax5 and re-expression of IL-7R, which would be a primary event in establishing the B cell fate. This model is consistent with observations that enforced expression of either EBF or Pax5 in hematopoietic stem cells completely blocks T cell development and allows efficient differentiation to the B cell lineage (Souabni *et al.*, 2002; Cotta *et al.*, 2003; Zhang *et al.*, 2003). Alternatively, it is possible that B and T cell development does not progress through a common intermediate that is sensitive to the expression status of IL-7R. Recent studies have indicated that an IL-7R⁻ early thymic progenitor (ETP) cell that efficiently generates T lymphocytes both *in vitro* and *in vivo* may be derived from a cell that is more primitive and distinct from the CLP (Allman *et al.*, 2003). These results suggest that early steps in T cell development may progress through an IL-7R⁻ stage. Our results would argue that enforced expression of IL-7R on all ETP cells has little consequence on early thymic development. Based on the IL-7R knockout phenotype in the thymus, expression of IL-7R would be essential by the DN2 stage of thymopoiesis. It may be that both bone marrow-derived CLP and thymic ETP may contribute to thymopoiesis depending on the developmental needs of the animal. For instance, if a mutation blocked the development of ETP, then CLP may provide an alternative pathway for the generation of T lymphocytes. Further studies will be necessary to integrate the roles of other signaling molecules (like Notch1) and transcription factors (like GATA3 and c-myc) into a more comprehensive picture of B and T lymphocyte commitment. Our studies of the IL-7R arguably show, for the first time, a physiologic role for cytokine receptor signaling in lymphocyte-lineage determination within the hematopoietic system.

Materials and methods

Generation of retroviruses

cDNAs for IL-7R α (a kind gift from Dr Linda Park, Immunex Corporation) and IL-2R γ_c (a kind gift from Dr Warren Leonard, NIH) were cloned into the parental vectors MSCV IRES Bex and MSCV IRES Vex, respectively. Both genes were cloned into the *EcoRI* site upstream of the IRES sequence. The retroviruses were each transiently transfected into Phoenix-ampho retroviral packaging cells and supernatant was collected. GP+E-86 cells, a stable ecotropic retrovirus-producing line, were co-infected with the supernatants of cells producing MSCV IL-7R α IRES Bex and MSCV IL-2R γ_c IRES Vex in the presence of polybrene. Transduced cells were then sorted for double-expressing IL-7R α -Bex/IL-2R γ_c -Vex or control Bex/Vex expressing cells.

Retroviral transduction of stem and progenitor cells

C57B/6-Ly5.2 mice were treated with 5-fluorouracil (i.p. injection of 150 mg/kg body weight) and bone marrow was harvested 4 days later. To lyse red blood cells, the bone marrow was treated with ACK (0.15 M NH₄Cl and 0.01 M KHCO₃) for 5 min on ice and subsequently prestimulated for 24 h at 37°C in the presence of IL-6 (5 ng/ml), LIF (1X,

ESGRO, Chemicon), and SCF (50 μ g/ μ l). Prestimulated bone marrow cells were then cocultured on irradiated (30 Gy) GP+E-86 stable producer cells for 48 h in the presence of polybrene (4 μ g/ml).

Transplantation

Recipient C57B/6-Ly5.1 congenic mice were lethally irradiated (10 Gy) in two equivalent sublethal doses 3 h apart. The transduced bone marrow was harvested from coculture dishes and then intravenously injected into anesthetized mice at a dose of $\sim 1 \times 10^6$ cells/animal. Transplanted mice were maintained on acidified water containing neomycin sulfate (1.1 g/l), polymyxin B sulfate (10⁶ U/l) and sulfamethoxazole (400 mg/l) for 3 weeks.

Peripheral blood analysis

Three to four weeks post-transplant, mice were tail bled into 0.5 ml phosphate-buffered saline (PBS) containing 10 mM EDTA. Peripheral blood was sedimented for 30 min at 37°C after addition of 1 ml of 2% dextran in PBS. Non-sedimented cells were transferred to a new tube, pelleted, and then treated with ACK to lyse the remaining red blood cells. Cells were washed and stained for 25 min at 4°C with an antibody cocktail of B220 (6B2; Pharmingen) and Mac-1 (M1/70; Pharmingen). Cell analysis was performed on a triple laser Mo-Flo cell sorter (Cytomation) configured with argon, krypton and rhodamine dye lasers. Dead cells were excluded from the analysis by propidium iodide staining.

Enhanced immunofluorescent assay

The enhanced indirect immunofluorescence system (Scheffold *et al.*, 2000) employed fluorochrome-filled liposomes conjugated to Fab fragments of sheep anti-digoxigenin antibodies as a second-step reagent. Viable cells pre-incubated with 200 μ g/ml rat IgG and 20 μ g/ml anti-CD16/CD32 antibody (BD Pharmingen) in PBS containing 0.5% BSA for 10 min at 4°C were incubated with digoxigenin-conjugated A7R34 (anti-IL-7R α chain mAb) (Sudo *et al.*, 1993) for 15 min before washing and incubation with anti-digoxigenin conjugated Cy5-fluorescent liposomes for 1 h on ice with agitation, followed by washing and analysis by flow cytometry. Staining specificity was assessed by pre-incubation with 100–1000-fold excess of the unlabeled primary antibody and by analysis of cells from IL-7R^{-/-} mice (data not shown).

Organ analysis

Organs were harvested from sacrificed mice and cells were strained through a 70 μ m filter to obtain a single cell suspension in PBS. ACK was added to the bone marrow cells to lyse red blood cells. CD4 (GK1.5; Pharmingen), CD8 (53–6.7; Pharmingen) and $\gamma\delta$ (GL3; Pharmingen) were used to stain thymocytes. The antibodies used to examine bone marrow were B220, Mac-1, IgM and CD19 (1D3; Pharmingen). Bone marrow pre-pro-B cells were stained with B220^{CyChrome} (Pharmingen), CD19^{PE}, NK1.1^{PE}, Ly-6C^{bio} (a kind gift from Dr Paul Kincade).

Myeloid colony-forming assay

Bone marrow cells were harvested, ACK lysed, and resuspended in PBS containing propidium iodide to exclude dead cells. Two thousand cells were then sorted for low or high levels of Bex/Vex-expression from either control or IL-7R α /IL-2R γ_c reconstituted animals into 1 μ l of DMEM, which was then added to 3 ml of 3434 methylcellulose media (Stem Cell Technologies) and mixed before plating into three 1 cm dishes. Colonies were typed for morphology and size and were also picked for cytospin preparations and Wright–Giemsa staining. Colonies consisting of >50 cells were enumerated and characterized after 5–7 days. All methylcellulose cultures were supplemented with GM-CSF (50 ng/ml) and some samples were also supplemented with IL-7 (300 U/ml; R&D Systems).

RT-PCR

RT-PCR was performed on double-sorted cell populations isolated from bone marrow stained and gated for B220⁺CD19⁻Ly6C⁺NK1.1⁻. Reverse transcription was done using gene-specific primers for Pax5, EBF, E47 and β 2 microglobulin. Pax5 RT primer: 5'-CATCCCTGTACGCGT-CGG-3'; Pax5 internal primer 1: 5'-CTACAGGCTCCGTGACGCAG-3'; Pax5 internal primer 2: 5'-TCGCCAAGTCTCGGCCTGTG-3'; Pax5 external primer 3: 5'-GCAGAGTAGCTGCCCTGTCC-3'; Pax5 external primer 4: 5'-TTCCAGTCACAGCATAGTGT-3'; EBF RT primer: 5'-ATGTTATCAGAGACTGCCAG-3'; EBF internal primer 1: 5'-CTC-ACTTTGAGAAGCAGCCGC-3'; EBF internal primer 2: 5'-CATGT-CACGTGGGTTTCTGC-3'; EBF external primer 1: 5'-CCAACACAG-CGGCCAGAGC-3'; EBF external primer 2: 5'-ACATGGCCATC-CACGTTGAC-3'; E47 RT primer: 5'-GTTCTCGACCTGCTGCTCC-

3'; E47 internal primer 1: 5'-GAAGCCACGCTGTTGGCACC-3'; E47 internal primer 2: 5'-GGATGAGCAGCTTGGTCTGC-3'; E47 external primer 1: 5'-CAGGATGACCTGCACCGCCT-3'; E47 external primer 2: 5'-ACCGCTTGGACGAGGCCATC-3'; β 2-microglobulin RT primer: 5'-CGATCCAGTAGACGGTCTTG-3'; β 2-microglobulin internal primer 1: 5'-TTCAGTCGTCAGCATGGCTC-3'; β 2-microglobulin internal primer 2: 5'-CATGCTTAACCTTCGACGGC-3'; β 2-microglobulin external primer 3: 5'-TTCAGTGGCTGCTACTCGGC-3'; β 2-microglobulin external primer 4: 5'-GCTCGGCCATACTGTCATGC-3'. PCRs were performed using two rounds of nested PCR of 30 cycles in the first round and 33 cycles in the second round using external and then internal primer sets. Cycle conditions were 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Ten percent of the RT reaction was used for first round PCR and 15% of the first round PCR was used as template for second round PCR. β 2-microglobulin was used as an internal control for all reactions. PCR products were resolved on a 1.2% agarose gel.

Ex vivo B lymphopoiesis assay

BM cells from 4–6 week old C57BL/6 mice were depleted of myeloid and κ/λ^+ B cells by magnetic cell sorting (Miltenyi Biotec, Auburn, CA). Immunofluorescent staining was done using the enhanced immunofluorescent assay for staining of IL-7R expression along with costaining for B220, CD19, NK1.1 and Ly-6C expression. The indicated cell populations were sorted once and then resorted on a Mo-Flo cell sorter to ensure purity of the populations used to initiate the cultures. Sorted cells were cultured with the non-irradiated T220-29 cell line (Borzillo *et al.*, 1992; Gongora *et al.*, 2000) at a concentration of 10^5 cells/ml in RPMI 1640 medium with 10% FBS, L-glutamine, penicillin/streptomycin and 50 μ M β -mercaptoethanol. A total of 10^4 cells was used to initiate each culture. After 6 days, cultured cells were harvested by treatment with 0.02% EDTA and viable lymphoid cells were enumerated by phase microscopy on the basis of trypan blue exclusion. FACS phenotyping of the harvested cells was performed as described above.

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