IL-7R α and E47: independent pathways required for development of multipotent lymphoid progenitors

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Mice that lack the transcription factors encoded by the E2A gene or the receptor for interleukin 7 (IL-7R) have severe overlapping defects in lymphocyte development. Here, we show that E2A proteins are required for the survival of early T-lineage cells; however, they function through a pathway that is distinct from the survival pathway initiated by IL-7R signaling. While E2A proteins are required to suppress caspase 3 activation, ectopic expression of the anti-apoptotic protein Bcl-2 is not sufficient to overcome the lymphopoietic defects observed in the absence of E2A. Remarkably, mice that lack both IL-7R α and E47 display a synergistic decrease in the number of T-cell, NK-cell and multipotent progenitors in the thymus, indicating that these distinct survival pathways converge to promote the development of multipotent lymphoid progenitors.

Keywords: E47/IL-7R α /lymphopoiesis/multipotent lymphoid progenitors

Introduction

The mature cells of the hematopoietic system develop from multipotent stem cells through the progressive restriction in their developmental potential (reviewed in Morrison *et al.*, 1997). Multiparameter flow cytometry, combined with *in vitro* culture systems that support hematopoietic differentiation, have been used to identify an array of multipotent progenitor cell types with lymphopoietic activity. These populations include bipotent B cell/macrophage (Cumano *et al.*, 1992; Montecino-Rodriguez *et al.*, 2001), T cell/natural killer (NK) cell (Rodewald *et al.*, 1992; Carlyle *et al.*, 1997) and common lymphoid progenitors (CLPs), which have B, T, NK and dendritic cell potential (Galy *et al.*, 1995; Kondo *et al.*, 1997b). At the present time, very little is known about the factors regulating the development of these multipotent progenitors or their differentiation into lineage-restricted progenitors.

The CLP population can be identified, in part, by expression of receptors for growth factors and cytokines

that may regulate their growth, survival and differentiation (Kondo *et al.*, 1997b; Tudor *et al.*, 2000). CLPs express the receptor for interleukin (IL)-7, a cytokine that promotes the proliferation and survival of B- and T-lymphocyte progenitors (Namen *et al.*, 1988; Goodwin *et al.*, 1990; Park *et al.*, 1990; Corcoran *et al.*, 1996; Kim *et al.*, 1998). IL-7 is an essential cytokine, as demonstrated by the block in early B- and T-cell development observed in mice that lack IL-7, or its receptor (IL-7R), or in mice administered a neutralizing antibody against IL-7 (Morrissey *et al.*, 1991; Grabstein *et al.*, 1993; Peschon *et al.*, 1994; von Freeden-Jeffrey *et al.*, 1995). IL-7R is composed of two proteins: the α chain and the γ chain (γ c), which is a common component of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (Goodwin *et al.*, 1990; Kondo *et al.*, 1993; Noguchi *et al.*, 1993; Russell *et al.*, 1993). Both IL-7R α ^{-/-} and γ c^{-/-} mice have severe defects in lymphocyte development (Peschon *et al.*, 1994; DiSanto *et al.*, 1995; He and Malek, 1996; Maki *et al.*, 1996). Total thymocyte numbers are reduced in both strains of mice, and there are few CD4⁺CD8⁻ (double-negative, DN) cells that express CD25, a marker associated with commitment to the T-cell lineage (Godfrey *et al.*, 1993). IL-7R α ^{-/-} mice have normal numbers of NK cells and CLPs, but lack $\gamma\delta$ T cells due to an inability to express the T-cell receptor (TCR) γ and δ loci (He and Malek, 1996; Maki *et al.*, 1996; Kondo *et al.*, 1997a; Perumal *et al.*, 1997; Ye *et al.*, 1999). IL-7 has also been implicated in the rearrangement of the TCR α and β loci and the immunoglobulin heavy (IgH) chain locus (Muegge *et al.*, 1993; Corcoran *et al.*, 1996, 1998). However, the major function of IL-7 in T-cell development is to promote cell survival by controlling the ratio of anti-apoptotic to pro-apoptotic members of the Bcl-2 family (Akashi *et al.*, 1997; Kondo *et al.*, 1997a; Maraskovsky *et al.*, 1997). Expression of the anti-apoptotic protein Bcl-2 in DN thymocytes is IL-7 dependent and IL-7 inducible, and ectopic expression of Bcl-2 is sufficient to overcome the defects in T-cell development in IL-7R α ^{-/-} and γ c^{-/-} mice (Akashi *et al.*, 1997; Kondo *et al.*, 1997a; Maraskovsky *et al.*, 1997; von Freeden-Jeffrey *et al.*, 1997; Kim *et al.*, 1998). Surprisingly however, IL-7 is not required for the development of CLPs and other multipotent lymphoid progenitors (MLPs) even though the receptor is expressed on these cells (Kee and Paige, 1996; Kondo *et al.*, 1997a).

IL-7R functions, in part, through activation of the Janus family kinases Jak1 and Jak3, which activate the signal transducers and activators of transcription (STAT) factors STAT1 and STAT5 (reviewed in Leonard and O'Shea, 1998). In addition, IL-7R signaling leads to the activation of numerous proteins involved in signal transduction, such as PI3-kinase, pim-1 and the src related kinases (Leonard and O'Shea, 1998). It has also been hypothesized that IL-7R signaling may regulate the E2A transcription

factors since the phenotypes of $E2A^{-/-}$ and $IL-7R\alpha^{-/-}$ mice are similar (Bain and Murre, 1998; Kee *et al.*, 2000). Alternatively, IL-7R signaling and E2A proteins may function in distinct pathways that are required for the development of the same cell types.

The E2A gene encodes two E-box binding transcription factors (E-proteins): E12 and E47. These proteins contain a basic domain, which is required for binding to DNA, followed by a helix-loop-helix (HLH) protein dimerization domain (Murre *et al.*, 1994). E12 and E47 are identical except for the use of alternatively spliced exons encoding the bHLH domains. However, the bHLH domains of these proteins are >80% identical and both proteins bind to the same DNA sequence, although E47 has a higher affinity for this sequence than E12 (Sun and Baltimore, 1991). In extracts of B-lineage cells, the predominant E-box binding activity is composed of E47 homodimers (Bain *et al.*, 1993; Shen and Kadesch, 1995), whereas in T-cell extracts, E-box binding activity consists of heterodimers of E47 and another E-protein, HEB (Sawada and Littman, 1993).

$E2A^{-/-}$ and $E47^{-/-}$ mice, which lack E47 and have <10% the normal level of E12, lack B-lineage cells and have a number of defects in T-cell development (Bain *et al.*, 1994, 1997a; Zhuang *et al.*, 1994). Thymocyte numbers are decreased 3- to 5-fold in these mice and they exhibit a partial developmental arrest at the transition from $CD44^{+}CD25^{-}$ to the $CD44^{+}CD25^{+}$ stage (Bain *et al.*, 1997a). As in $IL-7R\alpha^{-/-}$ mice, $E2A^{-/-}$ mice have decreased numbers of $CD4^{+}CD8^{+}$ (double-positive, DP) thymocytes compared with wild-type (WT) animals. This phenotype is attributed to both decreased survival of DP cells and increased maturation of DP cells (Bain *et al.*, 1999a). The absence of E2A proteins also leads to alterations in the timing of rearrangement of TCR γ and δ loci, and the absence of some $\gamma\delta$ T-cell populations (Bain *et al.*, 1999b). However, unlike $IL-7R\alpha^{-/-}$ or $\gamma c^{-/-}$ mice, $E2A^{-/-}$ mice have a high incidence of thymic lymphoma, suggesting additional roles for E2A proteins in the regulation of thymocyte homeostasis (Bain *et al.*, 1997a; Yan *et al.*, 1997).

The phenotypic similarities between $IL-7R\alpha^{-/-}$ and $E47^{-/-}$ mice prompted us to examine whether these proteins might function in a common pathway to regulate early lymphocyte development. Herein we demonstrate that DN thymocytes from $E47^{-/-}$ mice show an increased rate of apoptosis and activation of caspase 3 compared with WT DN thymocytes. However, unlike $IL-7R^{-/-}$ mice, Bcl-2 expression does not rescue the thymic defects in $E47^{-/-}$ mice. Therefore, E2A proteins may promote survival through a mechanism that is distinct from IL-7R signaling. In support of this hypothesis we found no evidence for a compound phenotype in mice that are heterozygous for mutations in both $IL-7R\alpha$ and E47. In contrast, mice that lack both $IL-7R\alpha$ and E2A activity lack thymic T-lineage progenitors and NK cells as well as B lymphocytes. Taken together, these findings indicate that $IL-7R\alpha$ and E47 function in distinct pathways that are required for the proper development of MLPs. Our data provide important insights into the mechanisms controlling lymphocyte survival and demonstrate an essential role for $IL-7R\alpha$ and E2A proteins in MLPs.

Results

Bcl-2 does not rescue B- or T-cell development in $E47^{-/-}$ mice

The similar phenotypes in B- and T-cell development in $IL-7R\alpha^{-/-}$ and $E47^{-/-}$ mice led to the hypothesis that these proteins may function in the same signaling pathway (Bain and Murre, 1998; Kee *et al.*, 2000). Alternatively, IL-7R signaling and E47 could function independently to promote the development of the same cell types. IL-7R signaling is required for the expression of the anti-apoptotic protein Bcl-2 in DN thymocytes, and Bcl-2 can rescue T-cell development in $IL-7R\alpha^{-/-}$ and $\gamma c^{-/-}$ mice (Akashi *et al.*, 1997; Kondo *et al.*, 1997a; Maraskovsky *et al.*, 1997). IL-7 also maintains Bcl-2 expression in pro-B lymphocytes, but may have additional functions in either pro-B-cell survival or differentiation (Kondo *et al.*, 1997a; Maraskovsky *et al.*, 1998). To determine whether the lymphopoietic phenotype observed in $E47^{-/-}$ mice is due to loss of a Bcl-2-mediated survival pathway, we tested the ability of the H2K-Bcl-2 transgene to overcome the defects in B- and T-cell development in $E47^{-/-}$ mice. It has been reported that the Bcl-2 transgene driven by the H2K promoter is expressed in hematopoietic stem cells (HSC) as well as multipotent and committed lymphoid progenitors (Kondo *et al.*, 1997a). We found that this Bcl-2 transgene is expressed at similar levels in the majority of lineage-negative (Lin⁻) bone marrow (BM) cells and DN thymocytes from WT, $E47^{-/-}$ and $IL-7R\alpha^{-/-}$ mice, indicating that the transgene is expressed at high levels early during lymphopoiesis (Figure 1A). As reported previously, the H2K-Bcl-2 transgene caused a slight increase in thymocyte number in $IL-7R\alpha^{+/-}$ mice and nearly restored thymocyte numbers in $IL-7R\alpha^{-/-}$ mice to the level observed in $IL-7R\alpha^{+/-}$ mice (Akashi *et al.*, 1997) (Figure 1B). Expression of the Bcl-2 transgene also caused a 1.6-fold increase in the number of $B220^{+}CD43^{-}$ lymphocytes in the BM of $E47^{+/-}$ mice (3.2×10^6 versus 5×10^6 cells per femur, respectively) (Figure 1D). This increase was due, primarily, to an increase in the $B220^{hi}$ mature B-cell population, as observed by Maraskovsky *et al.* (1998). The Bcl-2 transgene did not, however, rescue the development of $B220^{+}CD43^{-}$ cells in $E47^{-/-}$ mice (Figure 1D). In addition, no $CD19^{+}$ cells were found in $E47^{-/-}$ or $E47^{-/-};H2K-Bcl-2$ BM (data not shown). Therefore, as observed in $IL-7R\alpha^{-/-}$ mice, ectopic expression of Bcl-2 does not rescue the development of pro-, pre- or mature B lymphocytes in $E47^{-/-}$ mice.

In $E47^{+/-}$ thymus, the Bcl-2 transgene caused a 1.9-fold increase in total cell numbers (Figure 1C). In contrast, ectopic expression of Bcl-2 in the thymus of $E47^{-/-}$ mice led to a modest, but not significant, increase in cell numbers (Figure 1C). Both $E47^{-/-}$ and $E47^{-/-};H2K-Bcl-2$ transgenic mice had a similar number of DP thymocytes (1.6×10^7 versus 1.9×10^7) and DN thymocytes (6×10^5 versus 7×10^5 cells) (Figure 1E). The slight decrease in the percentage of DN thymocytes, and increase in the percentage of $CD4^{+}$ thymocytes, in the $E47^{-/-};H2K-Bcl-2$ transgenic mouse shown in Figure 1E is within the range observed for $E47^{-/-}$ mice. Moreover, the partial arrest in T-cell development at the DN $CD44^{+}CD25^{lo}$ stage was observed consistently in both $E47^{-/-}$ and $E47^{-/-};H2K-Bcl-2$ transgenic mice (Figure 1F). Therefore, ectopic

Fig. 1. Forced expression of Bcl-2 fails to rescue B- or T-cell development in E47^{-/-} mice. (A) Flow cytometric analysis of human Bcl-2 expression in lineage-negative (Lin⁻) BM and DN thymocytes from E47^{+/+} (shaded histogram), WT (dark line) or IL-7R α ^{-/-} (stippled line) Bcl-2 transgenic mice, or E47^{-/-} non-transgenic animals (light line). (B) Graphic representation of the number of cells in the thymus of IL-7R^{+/+} and IL-7R α ^{-/-} mice or (C) E47^{+/+} and E47^{-/-} mice with or without the H2K-Bcl-2 transgene. The mean number of thymocytes and the standard deviation of 3–8 animals analyzed between 5 and 8 weeks of age is shown. (D) FACS analysis of B220 and CD43 expression on BM cells isolated from mice of the genotype indicated. The boxed area represents cells with a pre-B, immature B or mature B-cell phenotype. The percentage of cells within the boxed area is indicated for each plot. FACS analysis of CD4 and CD8 expression on total thymocytes (E), and CD44 and CD25 expression on DN thymocytes (F), from mice with the genotypes indicated. The percentage of cells in each quadrant or each region is indicated in the upper right of each plot.

expression of Bcl-2 did not significantly affect the total number of thymocytes in E47^{-/-} mice and is not sufficient to rescue the lymphopoietic defects observed in these animals.

Spontaneous apoptosis of T-cell progenitors in the absence of E2A proteins

E2A activity is required for survival of pro-B lymphocytes (Kee *et al.*, 2001). However, the inability of Bcl-2 to rescue T-cell development in E47^{-/-} mice led us to question whether E2A proteins are required for survival of DN thymocyte progenitors. To address this question we examined the sensitivity of DN CD25⁺ thymocytes from WT and E47^{-/-} mice to spontaneous apoptosis during *in vitro* culture. At the time of isolation, there were already ~2-fold more annexin V⁺ cells in the DN CD25⁺ population from E47^{-/-} thymus than from WT thymus, indicating an increase in apoptosis in this population *in vivo*

(Figure 2A, upper panels). After *in vitro* culture for 24 h, 25–70% of E47^{-/-} DN thymocytes bound annexin V, whereas only 2–6% of WT thymocytes were annexin V⁺ (Figure 2A, lower panels). In addition, by 24 h, there were significantly more cells with active caspase 3, an executioner of the apoptotic process, in E47^{-/-} thymocyte cultures (26%) compared with WT thymocyte cultures (11%) (Figure 2B). While the overall degree of cell death varied in individual experiments, this pattern of sensitivity was observed consistently. Analysis of the number of viable DN thymocytes 24 and 48 h after the initiation of culture further indicated that E47^{-/-} DN thymocytes were dying more rapidly than their WT counterparts (Figure 2C). Similar to our previous observations in pro-B lymphocytes, addition of IL-7 to the culture media had only a minor effect on the viability of E47^{-/-} DN thymocytes, although it promoted the survival of WT DN thymocytes (Figure 2C). Taken together, our data

Fig. 2. Spontaneous apoptosis of E47^{-/-} DN CD25⁺ thymocytes *in vitro*. (A) Annexin V staining of DN thymocytes from WT (left panels) and E47^{-/-} (right panels) mice at the time of isolation (upper panels) or after 24 h of *in vitro* culture (lower panels). (B) Intracellular staining for active caspase 3 in DN thymocytes from WT (left panel) or E47^{-/-} mice after 24 h of *in vitro* culture. The DN thymocytes shown in (A) and (B) are from different animals. (C) Graphic representation of the relative number of WT (circles) or E47^{-/-} (squares) DN thymocytes after culture for the time indicated in media alone (open) or IL-7 (filled). The mean $\pm$ SD from three independent wells is shown.

indicate that E2A proteins are required to suppress activation of caspase 3 and prevent apoptosis in DN CD25⁺ thymocytes even in the presence of IL-7.

Reduced splenic T-cell numbers in IL-7R α ^{-/-};E47^{-/-} mice

Our data indicate that E2A proteins promote lymphocyte survival through a Bcl-2-independent mechanism or possibly downstream of Bcl-2-related proteins. Given that Bcl-2 is the major target of the IL-7 survival pathway, we questioned whether E2A proteins were functioning in the same pathway as IL-7R signaling or through an alternative pathway. To address this question we created mice that lack both IL-7R α and E47 (IL-7R α ^{-/-};E47^{-/-} mice), and examined the effect of this double mutation on T-cell development. We predicted that if these proteins

function in the same pathway, the phenotype of IL-7R α ^{-/-};E47^{-/-} mice would be similar to that of either single-gene-deficient animal since in the absence of one gene the function or expression of the other would be compromised. In addition, mice that are heterozygous for both mutations might display a phenotype that is more severe than that of either single-heterozygous animal, as seen with other proteins that function in a common pathway, or regulate overlapping targets (Floss *et al.*, 1996; Dunn *et al.*, 1997; O'Riordan and Grosschedl, 1999). However, if these proteins function in distinct pathways, the double-heterozygous animals would not be predicted to display a defect beyond that of either single-heterozygous animal, but we might observe novel defects in the absence of both proteins.

We isolated the spleen from mice of all genotypes generated from IL-7R α ^{+/-};E47^{+/-} $\times$ IL-7R α ^{+/-};E47^{+/-} crosses, and counted the number of viable cells. We found no difference in the phenotype or number of cells from WT thymus compared with any heterozygous combination, and therefore each of these genotypes was considered WT for this analysis. Similarly, the phenotype and number of thymocytes from IL-7R α ^{-/-} mice were identical to IL-7R α ^{-/-};E47^{+/-} mice and E47^{-/-} mice were identical to IL-7R α ^{+/-};E47^{-/-} mice. Therefore, these animals were considered IL-7R α ^{-/-} or E47^{-/-} for the analysis presented here. The average number of splenocytes in WT, IL-7R α ^{-/-} or E47^{-/-} mice was 4.6×10^7 , 1.6×10^7 and 10^7 , respectively (Figure 3A). The spleens from IL-7R α ^{-/-};E47^{-/-} mice were visibly smaller than those of their littermates and contained on average only 6.5×10^6 cells (Figure 3A). In our analysis, an average of 2×10^7 T cells were found in the spleen of WT animals. IL-7R α ^{-/-} spleen contained an average of 6×10^6 T cells, representing a 3.3-fold decrease (Figure 3). E47^{-/-} spleen contained an average of 7×10^6 T cells, a 2.8-fold decrease compared with WT spleen, although the percentage of splenocytes that are T lymphocytes is greatly increased owing to the absence of B lymphocytes in these animals (Bain *et al.*, 1994, 1997a; Zhuang *et al.*, 1994). Remarkably, $<7 \times 10^4$ T lymphocytes were present in the spleen of IL-7R α ^{-/-};E47^{-/-} mice, representing a 286-fold decrease in T-cell numbers (Figure 3). The few cells that remain in IL-7R α ^{-/-};E47^{-/-} spleen also lacked B-lineage markers, and are likely to represent macrophages, granulocytes and nucleated erythroid cells (data not shown). Therefore, IL-7R α ^{-/-};E47^{-/-} mice have a severe reduction in the number of mature T lymphocytes in the spleen.

Reduced thymocyte numbers in IL-7R α ^{-/-};E47^{-/-} mice

The reduced number of T cells in the spleen of IL-7R α ^{-/-};E47^{-/-} mice suggests that T-cell development in the thymus might be compromised in these animals. Analysis of thymocyte numbers and phenotype again revealed no difference between any heterozygous combination and WT thymus, IL-7R α ^{-/-} thymus compared with IL-7R α ^{-/-};E47^{+/-}, or E47^{-/-} thymus compared with IL-7R α ^{+/-};E47^{-/-}. Therefore, for the analysis presented here we considered these animals WT, IL-7R α ^{-/-} or E47^{-/-}, respectively. The average number of thymocytes in WT, IL-7R α ^{-/-} or E47^{-/-} mice was 1.2×10^8 , 4.2×10^6

Fig. 3. T-cell deficiency in the spleen of IL-7R α ^{-/-};E47^{-/-} mice. **(A)** The total number of cells in the spleen is shown for WT, IL-7R α ^{-/-}, E47^{-/-} and IL-7R α ^{-/-};E47^{-/-} mice. Each point represents the spleen of one mouse analyzed between 4 and 8 weeks of age. The average number of splenocytes is indicated by the horizontal bar. **(B)** FACS analysis of CD4 and CD8 expression on splenocytes isolated from mice of each of the genotypes indicated. The percentage of cells in each quadrant is indicated in the upper right of each plot.

and 3.2×10^7 cells, respectively (Figure 4A). Therefore, IL-7R α ^{-/-} and E47^{-/-} mice have an average 28- and 3.7-fold decrease in total thymocyte numbers compared with WT animals, respectively. Previous studies have demonstrated that the T-cell phenotype of IL-7R α ^{-/-} mice separates into two categories: those with thymocyte numbers >1% of WT and those with <1% of WT (Peschon *et al.*, 1994; Maraskovsky *et al.*, 1998). IL-7R α ^{-/-} mice with <1% WT thymocyte numbers have been reported to have a more severe phenotype than those with >1%, lacking CD4- and CD8-expressing T-cell populations. However, this distinction has not been observed in our colony and no IL-7R α ^{-/-} mice have been identified that lack DP or single-positive (SP) thymocytes, even in the few cases where thymocyte cellularity was <1% of WT. At the present time, the reason for this difference is not known; one possible explanation is the genetic background of the animals. In contrast to WT, IL-7R α ^{-/-} or E47^{-/-} thymi, those from IL-7R α ^{-/-};E47^{-/-} mice were small and, in most cases, viable cells could not be detected by microscopic examination. Analysis of half of the disrupted thymus by flow cytometry revealed a few cells within the lymphoid gate that we estimated to represent a maximum of 10^4 cells (Figure 4A and B). Therefore, there is a synergistic (> 10^4 -fold) decrease in thymocyte numbers in IL-7R α ^{-/-};E47^{-/-} mice compared with IL-7R α ^{-/-} and E47^{-/-} mice.

To determine which thymic populations are altered in IL-7R α ^{-/-};E47^{-/-} mice we examined the expression of

CD4 and CD8 by flow cytometry. Remarkably, >95% of the cells in the IL-7R α ^{-/-};E47^{-/-} thymus lacked CD4 and CD8 (Figure 4C). However, this represents $<1 \times 10^4$ cells, whereas there are $\sim 3.5 \times 10^6$, 1×10^5 or 4×10^6 DN thymocytes in WT, IL-7R α ^{-/-} or E47^{-/-} thymus, respectively (Figure 4D). The decline in thymocyte numbers is even more severe when the absolute number of DP thymocytes is considered. WT, IL-7R α ^{-/-} or E47^{-/-} mice have an average of 1.2×10^8 , 2.8×10^7 or 2×10^7 DP thymocytes, respectively (Figure 4D). In contrast, IL-7R α ^{-/-};E47^{-/-} mice have an average of only 1.1×10^3 DP thymocytes, representing a 1×10^5 -, 2.5×10^4 - or 1.8×10^4 -fold decrease compared with WT, IL-7R α ^{-/-} or E47^{-/-} mice, respectively (Figure 4D). SP thymocytes were also significantly reduced in IL-7R α ^{-/-};E47^{-/-} compared with WT, IL-7R α ^{-/-} or E47^{-/-} mice. Therefore, the absence of IL-7R α and E47 leads to a severe reduction in all thymocyte subsets.

Absence of T-lineage progenitors in the thymus of IL-7R α ^{-/-};E47^{-/-} mice

In order to determine the stage at which developmental arrest occurs during T-cell development in the absence of IL-7R α and E47, we examined DN thymocytes from IL-7R α ^{-/-};E47^{-/-} mice for expression of CD44, CD25 and Thy1. The most immature cells in WT thymus lack CD4 and CD8, express high levels of CD44 and low levels of Thy1, and lack CD25 (Figure 5A) (Godfrey *et al.*, 1993). These cells acquire CD25 as they commit to the $\alpha\beta$ T-cell lineage, and subsequently downregulate CD44 and then CD25 before they progress to the DP stage. In the absence of IL-7R α , there is a reduced number of DN CD25⁺ cells compared with WT thymus (2×10^4 versus 8.7×10^6 cells), but the majority (85.5%) of IL-7R α ^{-/-} DN cells express Thy1, indicating that they are lymphocyte progenitors (Figure 5A and B). In the absence of E47, thymocyte development is arrested at the CD44⁺CD25^{lo} to CD44⁺CD25⁺ stage, consistent with a role for E2A proteins in commitment of MLPs to the T lineage (Bain *et al.*, 1997a). However, the majority of E47^{-/-} DN thymocytes also express Thy1, demonstrating their T-lineage potential (Figure 5A and B). In contrast, <70 DN cells in the thymus of IL-7R α ^{-/-};E47^{-/-} mice express CD44, CD25 or Thy1, indicating that these cells are not lymphocyte progenitors (Figure 5A and B). Therefore, in the absence of IL-7R α and E47, T-cell development is arrested prior to the development of the first thymic immigrants with lymphopoietic activity.

Absence of NK cells in IL-7R α ^{-/-};E47^{-/-} thymus

The earliest thymic immigrants are thought to be cells that have not yet undergone definitive lineage commitment, and include cells which can give rise to T cells as well as B lymphocytes and NK cells (Galy *et al.*, 1995; Carlyle *et al.*, 1997; Kondo *et al.*, 1997b). Our analysis indicates that the absence of IL-7R α and E47 inhibits the development of this MLP population. If these progenitors fail to develop, the number of NK cells in the thymus should be reduced. Alternatively, if this population develops normally, the few cells that remain in the IL-7R α ^{-/-};E47^{-/-} thymus could be NK cells. In order to identify NK cells, we examined the DN population of IL-7R α ^{-/-};E47^{-/-} thymus for cells that express CD44 and DX5, a pan-NK

Fig. 4. Thymic hypocellularity and impaired T-cell development in IL-7R α ^{-/-};E47^{-/-} mice. (A) Total thymocyte numbers are shown for WT, IL-7R α ^{-/-}, E47^{-/-} and IL-7R α ^{-/-};E47^{-/-} mice. Each point represents the thymus from one mouse analyzed between 4 and 8 weeks of age. (B) Forward (FSC) and side light scatter (SSC) of cells isolated from mice of each of the indicated genotypes. Ten thousand cells within the indicated gate were collected for each genotype with the exception of the IL-7R α ^{-/-};E47^{-/-} thymocytes, for which half of the total cells were acquired. (C) FACS analysis of CD4 and CD8 expression on thymocytes isolated from mice of each of the indicated genotypes. The percentage of cells in each quadrant is indicated in the upper right of each plot. (D) The total number of DN, DP, SP thymocytes from each of the indicated genotypes was determined from the total number of cells and the percentage of cells with the given phenotype. Each point represents the total number of cells in one thymus. The average number of thymocytes is indicated by the horizontal bar.

cell marker. A small population of CD44⁺DX5⁺ cells could be detected among WT DN thymocytes, representing, in the experiment shown, $\sim 10^4$ cells (Figure 5C). The number of CD44⁺DX5⁺ cells was somewhat variable in IL-7R α ^{-/-} and E47^{-/-} mice; however, these thymi invariably had an increase in the percentage of CD44⁺DX5⁺ cells compared with WT DN thymocytes (Figure 5C). In some cases, the absolute number of DX5⁺ cells in E47^{-/-} thymus was increased 3- to 4-fold. In the example shown, there were 1.3×10^4 DX5⁺ cells in the IL-7R α ^{-/-} DN population and 2.4×10^4 DX5⁺ cells in the E47^{-/-} DN population (Figure 5C and data not shown). In contrast, the IL-7R α ^{-/-};E47^{-/-} DN population contained $<10^2$ DX5⁺ cells (Figure 5C). Therefore, the absence of both IL-7R α and E47 affects the development of progenitors of T and NK cells.

Bcl-2 can rescue the development of MLPs in IL-7R α ^{-/-};E47^{-/-} mice

Our data indicate that IL-7R α and E47 function in distinct pathways that converge to promote the survival of MLPs. Therefore, either IL-7R signaling or E47 activity can promote MLP survival. If IL-7R signaling is required to increase the expression of pro-apoptotic Bcl-2-related proteins in MLPs we would predict that Bcl-2 should rescue the development of MLPs in IL-7R α ^{-/-};E47^{-/-} mice. In order to test this prediction we created IL-7R α ^{-/-};E47^{-/-} mice that also express the H2K-Bcl-2 transgene. Since this transgene is detected at high levels in Lin⁻ BM cells we expect that it is expressed in MLPs and their BM-derived precursors (Figure 1A). We found that the IL-7R α ^{-/-};E47^{-/-};H2K-Bcl-2 transgenic mice had significantly more thymocytes than their non-transgenic

Fig. 5. Absence of lymphocyte progenitors and NK cells in the thymus of IL-7R α ^{-/-};E47^{-/-} mice. The DN population of thymocytes from mice of each of the indicated genotypes was analyzed by flow cytometry for the expression of CD44 and CD25 (A), Thy1 (B) and CD44 and DX5 (C). The percentage of cells in each quadrant is indicated in the upper right of each plot. The percentage of Thy1-positive cells is also noted. The plots in (C) are from different mice than those shown in (A) and (B).

IL-7R α ^{-/-};E47^{-/-} littermates (Figure 6A). The IL-7R α ^{-/-};E47^{-/-};H2K-Bcl-2 mice displayed a relatively normal distribution of DN, DP and SP thymocytes, although the total number of these cells was decreased ~9-fold (Figure 6B). Analysis of CD44 and CD25 expression also indicated that the development of T-cell progenitors was rescued in IL-7R α ^{-/-};E47^{-/-} mice carrying the Bcl-2 transgene. However, the accumulation of DN progenitors at the CD44⁺CD25^{lo} stage observed in E47^{-/-} mice was also observed in IL-7R α ^{-/-};E47^{-/-};H2K-Bcl-2 transgenic mice. This observation is consistent with our previous experiments, which demonstrated that Bcl-2 is not able to overcome the requirement for E47 in T-cell development (Figure 1E and F). Taken together, our data demonstrate that either IL-7R signaling, which induces Bcl-2-related survival pathways, or E47 activity, which inhibits caspase activation, is required for the proper development of MLPs (Figure 7).

Discussion

B and T lymphocytes, as well as NK cells and some dendritic cells, develop from MLPs in the BM and thymus (Galy *et al.*, 1995; Carlyle *et al.*, 1997; Kondo *et al.*, 1997b). The development of MLPs and their progeny is dependent, in part, on interactions with factors in the extracellular environment that initiate signaling pathways involved in cell survival, proliferation and differentiation. At the present time, however, very little is known about the signaling pathways or the downstream effector proteins that influence the development of MLPs and their progeny. Our data demonstrate that the bHLH transcription factors encoded by the E2A gene and IL-7R signaling function through distinct mechanisms to promote the survival of multipotent and committed lymphocyte progenitors.

IL-7R signaling induces the expression of the anti-apoptotic protein Bcl-2, whereas E2A proteins are required to suppress the activation of, at least, caspase 3 (Figure 7). Our study reveals a functional role for IL-7R α and E2A in multipotent progenitors, and provides important insight into the mechanisms controlling cell survival in committed lymphoid progenitors and their lineage-unrestricted precursors.

Bcl-2 is thought to inhibit the activation of initiator caspases by inhibiting the release of cytochrome *c* from the mitochondria, which can activate the adapter protein Apaf-1, or by interacting directly with adapter proteins (reviewed in Newton and Strasser, 1998; Gross *et al.*, 1999). Initiator caspases cleave and activate downstream effector caspases and other targets involved in executing the apoptotic program (Salvesen and Dixit, 1997; Elkon, 1999) (Figure 7). Many cytokines have been shown to suppress apoptosis by either induction of anti-apoptotic Bcl-2-related proteins or inhibition of their pro-apoptotic relatives such as Bax and the BH3-domain proteins Bad, Bid and Bim (Gross *et al.*, 1999; Huang and Strasser, 2000). The induction of apoptosis following growth factor withdrawal, DNA damage or activation of the tumor suppressor protein p53 can be inhibited by ectopic expression of Bcl-2 (Strasser *et al.*, 1994). Surprisingly, we found that Bcl-2 was not able to rescue the lymphopoietic defects in E47^{-/-} mice even though E2A proteins are required for lymphocyte survival. While it seems likely that E2A proteins play multiple roles in lymphocyte development, we favor the conclusion that E2A activity promotes lymphocyte survival through a Bcl-2-independent pathway for two reasons. First, Bcl-2 transgenic pro-B lymphocytes are sensitive to apoptosis induced by ectopic expression of Id3, an inhibitor of E2A (Kee *et al.*, 2001 and data not shown). Secondly, Bcl-2

Fig. 6. Ectopic expression of Bcl-2 rescues the development of MLP in IL-7Rα^{-/-};E47^{-/-} mice. **(A)** Total thymocyte numbers are shown for WT, IL-7Rα^{-/-};E47^{-/-} and IL-7Rα^{-/-};E47^{-/-};H2K-Bcl-2 transgenic mice. Each point represents the thymus from one mouse analyzed between 5 and 8 weeks of age. **(B)** FACS analysis of CD4 and CD8 expression on thymocytes isolated from mice of the genotypes indicated. **(C)** FACS analysis of CD44 and CD25 expression on DN thymocytes from mice of the genotypes indicated. The percentage of cells in each quadrant is indicated.

Fig. 7. A model for the suppression of apoptosis by IL-7Rα signaling and E2A proteins. Signals initiated through the IL-7R (light blue) lead to an increase in Bcl-2 (red), which can inhibit the activation of initiator caspases (green) through indirect or direct affects on adapter proteins (dark blue) such as Apaf-1. Initiator caspases cleave many cellular targets including effector caspases (yellow), such as caspase 3, which lead to the destruction of the cell. E2A proteins (pink) repress the activation of caspase 3, and probably other caspases involved in the induction of apoptosis. Lines with arrows represent activating events. Lines with blunt ends represent inhibitory events. Dashed lines represent hypothetical associations.

protein levels are not altered in DN thymocytes or pro-B lymphocytes in the absence of E2A. In addition, we were unable to identify any changes in the expression of Bcl-2-related proteins by RNase protection in pro-B lymphocytes transduced with an Id3-expressing retrovirus (data not

shown). These observations suggest that a novel target(s) of E2A proteins is required for the survival of IL-7-dependent B- and T-lymphocyte progenitors.

There are a number of signaling pathways that control lymphocyte survival but do not function through Bcl-2. For example, the survival signals mediated by the pre-T-cell receptor are Bcl-2 independent (Maraskovsky *et al.*, 1997). The development of DP thymocytes can be promoted in rag-1^{-/-} mice by expression of a dominant-negative FADD transgene, indicating a role for death receptor signaling in apoptosis in the absence of pre-TCR signaling (Newton *et al.*, 2000). In addition, apoptosis initiated by the death receptor Fas/APO-1 has been shown to be Bcl-2 independent (Strasser *et al.*, 1995). Therefore, it is possible that E2A proteins are required to protect lymphocytes from apoptosis induced by the activation of alternative death pathways such as those elicited by death domain-containing proteins. It is also interesting to speculate that E2A proteins may function in the feedback pathway that leads to decreased DP thymocyte survival in the absence of Bim, a pro-apoptotic BH3-protein (Bouillet *et al.*, 1999). The decrease in DP thymocytes in Bim^{-/-} mice is not dependent on death receptor signaling, but may reflect alternative pathways leading to caspase activation which may be repressed by E2A. At the present time, the range of caspases activated in the absence of E2A, and the targets of E2A that mediate this anti-apoptotic effect, remain to be determined.

The severe loss of thymic progenitors in IL-7Rα^{-/-};E47^{-/-} mice suggests that IL-7Rα and E47 provide overlapping functions in MLPs. Given that both proteins are required, through distinct mechanisms, to suppress caspase activation we hypothesize that this synergy reflects the set of caspases or apoptotic stimuli that affect MLPs (Figure 7). Alternatively, there may be undetected defects in the development of MLPs in IL-7Rα^{-/-} and E47^{-/-} mice, and the absence of both pathways leads to a more pronounced defect. Interestingly, the thymic phenotype of IL-7Rα^{-/-};E47^{-/-} mice is similar to that observed in γc^{-/-};W/W^x mice (Rodewald *et al.*, 1997). γc^{-/-} mice lack the ability to signal through many

cytokine receptors, including the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15, and have a more profound defect in lymphoid development than IL-7R $\alpha^{-/-}$ mice (Kondo *et al.*, 1993; Noguchi *et al.*, 1993; Russell *et al.*, 1993; DiSanto *et al.*, 1995). The W mutation deletes 78 amino acids, including the transmembrane domain, of the *c-kit* tyrosine kinase receptor, leading to the absence of the receptor for *c-kit* ligand, also called mast cell growth factor, stem cell factor or Steel (Zsebo *et al.*, 1990). In $\gamma c^{-/-}$;W/W^x mice, thymus cellularity is reduced 10⁴-fold and >95% of the remaining thymic cells lack CD4 and CD8 (Rodewald *et al.*, 1997). NK cells are also absent from these animals since γc is a component of the receptor for IL-15, a cytokine which is required for NK cell development (DiSanto *et al.*, 1995; Kennedy *et al.*, 2000). It is possible that E2A proteins function in the same pathway as *c-kit*, which promotes the survival of immature T cells and multipotent progenitors (Rodewald, 1995; Rodewald *et al.*, 1995; Domen and Weissman, 2000). In this regard, it is interesting that *c-kit* and Bcl-2 promote the survival of HSC through distinct pathways (Domen and Weissman, 2000). Surprisingly, it was reported that Bcl-2 does not overcome the progenitor cell defect in $\gamma c^{-/-}$;W/W^x mice (Rodewald *et al.*, 2001). This is in contrast to our observation that Bcl-2 can rescue the function of IL-7R $\alpha^{-/-}$;E47^{-/-} mice. The reason for this discrepancy is not known; however, it may involve differences between IL-7R α and γc , in the genetic background of the animals studied, or in the expression patterns of the Bcl-2 transgenes used in each study. It is also possible that E47 functions in an independent pathway from any of the cytokine, or growth factor, receptors that are known to regulate lymphocyte development. It will be important to determine the targets of E47, IL-7R and *c-kit* that are essential for lymphocyte survival, and to determine how these target proteins interact in MLPs. Our study demonstrates the independent nature of the survival pathways regulated by IL-7R α and E47 during the generation of committed lymphoid cells, and provides important insights into the mechanisms of MLP cell regulation.

Materials and methods

Mice and genotyping

Mice were housed in the vivarium at the University of California at San Diego. E47^{-/-} mice were described previously (Bain *et al.*, 1997a). E47^{-/-} mice are unable to produce E47 protein, and produce very low levels of E12, leading to a phenotype in B- and T-cell development that is indistinguishable from that of E2A^{-/-} mice (Bain *et al.*, 1994, 1997a,b). However, the embryonic lethality observed in E47^{-/-} mice is less severe than that of E2A^{-/-} mice. The E47 mutation was identified by PCR using the primers E47bas for (5'-CAGATGAGGTGCTGTCCCGGA-3') and E47bas rev (5'-CAGGATCACCTGCACCGCCT-3') in the presence of 10% DMSO. These primers amplify a 220 bp fragment from DNA of WT animals and a 1.4 kb fragment from DNA of mice carrying the E47-mutation. IL-7R $\alpha^{-/-}$ mice were purchased from Jackson Laboratories and genotyped by PCR using the primers IMR119 (5'-CTTTTACGAGTGAAATGCCTAACTC-3') and IMR120 (5'-CAGGTATGATTCAGATGCAATACA-3'), which detects an 850 bp fragment from DNA of WT animals and a 2 kb fragment from DNA of mice carrying the IL-7R α -mutation (Peschon *et al.*, 1994). The presence of the mutant allele was confirmed by PCR using the primers IMR119 and IMR121 (5'-CACGGCTAGCCAACGCTATGTC-3'), which amplify a 950 bp fragment from DNA of mice carrying the neo insertion in the IL-7R α gene. H2K-Bcl-2 transgenic mice were genotyped by PCR using H2Kp

(5'-ATCAGAACTCGGAGACGACAGCAC-3') and hBcl2p (5'-CAGAGACAGCCAGGAGAAATCAAAC-3'), which amplify a 924 bp fragment from the H2K-Bcl-2 transgene in the presence of 10% DMSO. Alternatively, the transgene was detected by Southern blot analysis.

FACS analysis

Thymocytes and splenocytes were harvested by disruption of the tissue with frosted glass slides in 5 ml of phosphate-buffered saline (PBS)/10% fetal bovine serum (FBS). Cell numbers were determined using a hemacytometer after resuspending the cells in 0.25% Trypan Blue to exclude non-viable cells. The cells were washed 1 $\times$ in PBS/10% FBS and stained with the appropriate antibodies for 30 min on ice. The antibodies used include anti-mouse Bcl-2, anti-CD4 (RM4-5), anti-CD8 α (53-6.7), anti-CD44 (IM7), anti-CD25 (7D4), anti-Thy1.1 (OX-7), anti-Thy1.2 (30-H12), anti-DX5 and anti-activated caspase 3 (PharMingen, La Jolla, CA). Cells were stained with annexin V at room temperature according to the manufacturer's instructions (PharMingen).

In vitro cell cultures

Thymocytes were isolated from WT or E47^{-/-} mice and disrupted with sterile frosted glass slides. The cells were counted and plated at 3 $\times$ 10⁵ cells/well in a round bottom 96-well dish. All cells were cultured in OPTI-MEM containing 10% FBS (Gibco), 5 $\times$ 10⁻⁵ M 2-mercaptoethanol and 100 μ g/ml penicillin/streptomycin/glutamine. IL-7 supernatant, produced from the J558-IL-7 cell line (a gift of Dr F. Melchers), was used at a dilution of 1:100.

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