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Bcl-2 Rescues T Lymphopoiesis in Interleukin-7 Receptor–Deficient Mice

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Summary

Mice lacking functional IL-7 or IL-7Rα genes are severely deficient in developing thymocytes, T cells, and B cells. IL-7 and IL-7 receptor functions are believed to result in lymphoid cell proliferation and cell maturation, implying signal transduction pathways directly involved in mitogenesis and elaboration of developmentally specific new gene programs. Here, we show that enforced expression of the *bcl-2* gene in T-lymphoid cells (by crossing in the Eμ-*bcl-2* transgene) in IL-7Rα-deficient mice results in a significant restoration of thymic positive selection and T cell numbers and function. We propose cell survival signals to be the principal function of IL-7R engagement in thymic and T cell development.

Introduction

Interleukin-7 (IL-7) is a nonredundant cytokine for both T cell and B cell development (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). IL-7 exerts its effect through the interaction of a high affinity receptor complex composed of the IL-7Rα and the common cytokine receptor γ chain (γ_c) (Noguchi et al., 1993a; Kondo et al., 1994a), which is also an indispensable subunit for functional IL-2 (Takeshita et al., 1992), IL-4 (Kondo et al., 1993; Russell et al., 1993), IL-9 (Kimura et al., 1995), and IL-15 (Giri et al., 1994) receptors. Injection with neutralizing antibodies to IL-7 or genetic ablation of either IL-7, IL-7Rα, or γ_c inhibits both T and B cell development (Peschon et al., 1994; Bhatia et al., 1995; Cao et al., 1995; DiSanto et al., 1995; von Freeden-Jeffry et al., 1995; Ohbo et al., 1996). The molecular pathogenesis of X-linked severe combined immunodeficiency (SCID) in humans might be due primarily to γ_c-mediated defects in the IL-7/IL-7R system (Stephan et al., 1997) caused by mutation of the γ_c gene (Noguchi et al., 1993b; Puck et al., 1993).

Thymic maturation from $CD4^-CD8^-$ (DN) primitive progenitors involves rearrangement of TCR genes; expression of TCR with CD4 and CD8 coreceptors; positive selection of $CD4^+CD8^+$ double positive (DP) cells that corecognize self MHC class II molecules with CD4 and TCR, and self MHC class I with CD8 and TCR; negative selection of autoimmune T cells; and commitment to gene expression programs. The survivors of the process become CD4 or CD8 single positive (SP) cells that emigrate to the periphery and participate in immune responses (Adkins et al., 1987; Weissman, 1994). In the thymus, γ_c is expressed in virtually all thymocytes (Kondo et al., 1994b), while IL-7Rα has been reported to be expressed in DN TCR^- thymic precursors and mature TCR^{hi} SP cells, but not in the majority of TCR^{lo} DP cells (Sudo et al., 1993) that contain precursors with selectable TCR. The major role of the IL-7/IL-7Rα interaction has been considered to be the expansion of DN thymic precursors; IL-7- or IL-7Rα-deficient ($IL\text{-}7R\alpha^{-/-}$) mice showed a decrease in the number of thymic precursors (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). IL-7 may have an important role during positive selection, because thymocytes undergoing positive selection express IL-7Rα and/or c-Kit throughout the DP-to-SP transition (Akashi and Weissman, 1996; Akashi et al., submitted). In addition, the rare peripheral T cells in the $IL\text{-}7R\alpha^{-/-}$ mice showed impaired survival and less responsiveness to both TCR-dependent and -independent stimuli, though IL-7/IL-7R signaling is not essential for T cell activation (Maraskovsky, et al., 1996). These data suggest an intrinsic abnormality of $IL\text{-}7R\alpha^{-/-}$ T cells that is possibly due to a lack of IL-7R-mediated signals during positive selection.

Bcl-2 is an antiapoptosis protein (Vaux et al., 1988, 1992; Hockenbery et al., 1991; Sentman, et al., 1991; Strasser et al., 1991; Cory, 1995). Bcl-2 can protect developing and mature T cells against a variety of apoptotic signals such as glucocorticoids and cross-linking of cell surface molecules by anti-CD3 antibody. Bcl-2 is expressed at all stages of cells undergoing positive selection, including DN and SP cells, but not in the majority of the DP cells that have failed positive selection and that will die by default (Gratiot-Deans et al., 1993, 1994; Veis et al., 1993a; Akashi and Weissman, 1996). Bcl-2-deficient mice showed a gradual disappearance of T (and B) cells after the second postnatal week of life (Veis et al., 1993b; Nakayama et al., 1994). This suggests that Bcl-2 may protect thymocytes undergoing positive selection (Linette et al., 1994; Tao et al., 1994; Akashi and Weissman, 1996) as well as peripheral T cells from apoptotic stimuli such as levels of endogenous glucocorticoid reached in the diurnal cycle (Weissman, 1994) or stress.

The enforced expression of Bcl-2 has been shown to maintain the viability of various cytokine-dependent cells upon withdrawal of their dependent cytokines in vitro (for reviews, see Vaux, 1993 and Yang and Korsmeyer, 1996). In these situations, Bcl-2 itself does not stimulate proliferation, but appears to enable cells to undergo intrinsically-determined differentiation through maintenance of the viability of the cells in vitro (Fairbairn et al., 1993). Based on these data, we hypothesized that an important role of IL-7/IL-7R interactions in T cell development was to maintain cell survival. To test this hypothesis, we introduced an Eμ-*bcl-2* transgene that is selectively expressed in T lineage cells into $IL\text{-}7R\alpha^{-/-}$ mice. We show here that enforced expression of Bcl-2 in $IL\text{-}7R\alpha^{-/-}$ mice results in a rescue of T lymphopoiesis.

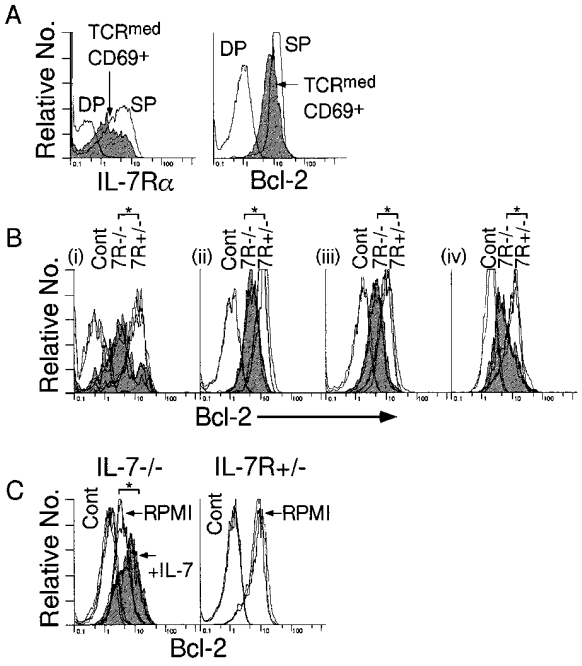

Figure 1. IL-7R Engagement Induces Expression of Bcl-2 during T Cell Maturation

(A) The expression of IL-7Rα and endogenous Bcl-2 in thymocytes. DP cells are negative for both IL-7Rα and Bcl-2, whereas SP are positive for both. The TCR^{med} $CD69^{+}$ cells (closed gray histogram) that are in the process of positive selection have upregulated both IL-7Rα and endogenous Bcl-2.

(B) The expression of endogenous Bcl-2 in DN thymocytes (i), CD4 SP (ii) and CD8 SP thymocytes (iii), and splenic T cells (iv), in either IL-$7R\alpha^{-/-}$ ($7R^{-/-}$; closed gray histogram) or IL-$7R\alpha^{+/-}$ ($7R^{+/-}$) mice

(C) The upregulation of Bcl-2 in splenic $TCR\beta^{+}$ T cells from IL-$7^{-/-}$ mice after culturing for 18 hr with IL-7.

Data from each group of three mice are plotted. (*), $p < 0.05$ by paired t tests compared on each mean value of fluorescence intensity.

Results

IL-7R Engagement Induces High Level Expression of Bcl-2 during T Cell Maturation

The first step of thymic positive selection includes an upregulation of their TCRs to medium–high levels (Guidos et al., 1990; for reviews, see von Boehmer, 1994; Jameson et al., 1995; Guidos 1996). This process begins at the DP stage (Shortman et al., 1991; Akashi and Weissman, 1996). CD69 is an activation marker that is expressed on cells undergoing positive selection (Swat et al., 1993; Yamashita et al., 1993; Brandle et al., 1994). The positively selected cells downregulate either irrelevant CD4 or CD8 coreceptor (Guidos et al., 1990; Lucas and Germain, 1996; von Boehmer, 1996), and complete TCR upregulation. All cells undergoing positive selection express the IL-7R (Akashi et al., submitted).

Figure 1A shows IL-7Rα and Bcl-2 expression in thymocytes in IL-$7R\alpha^{+/-}$ mice. Because virtually all thymocytes express γ_c (Kondo et al., 1994b), thymocytes that express the IL-7Rα chain should possess a functional IL-7R heterodimer on their surface. While the majority of DP (TCR^{lo} $CD69^{-}$) cells did not express IL-7Rα or Bcl-2, the TCR^{med} $CD69^{+}$ population undergoing positive selection had increased the expression of both molecules. Expression of both IL-7Rα and Bcl-2 is maintained in differentiated thymic TCR^{hi} SP cells (Figure 1A) as well as spleen TCR^{hi} T cells. However, in IL-$7R\alpha^{-/-}$ mice, the upregulation and maintenance of Bcl-2 is apparently impaired (Figure 1B); the levels of endogenous Bcl-2 in thymocyte subsets such as DN, CD4 SP and CD8 SP cells, and those in spleen T cells in IL-$7R\alpha^{-/-}$ mice were significantly lower than those in IL-$7R\alpha^{+/-}$ mice. These data suggest a possible link between IL-7/IL-7R interaction and endogenous Bcl-2 expression in T cells. To test this, we evaluated the effect of IL-7R engagement on endogenous Bcl-2 expression in T cells taken from IL-7-deficient (IL-$7^{-/-}$) mice. As shown in Figure 1C, T cells isolated from the spleens of IL-$7^{-/-}$ mice showed a reduction of Bcl-2 levels. However, when IL-$7^{-/-}$ T cells were cultured with recombinant IL-7 for 18 hr, Bcl-2 levels were significantly increased. These data strongly suggest that IL-7R signaling might involve maintenance of Bcl-2 expression in developing thymocytes as well as in peripheral T cells.

IL-7R Signaling Is Required for Positive Selection

To clarify the possible role of IL-7R signaling in positive selection, we evaluated the effect of neutralizing anti-IL-7Rα antibody (A7R34) (Sudo et al., 1993) on the in vivo maturation of thymocytes. For these experiments, we used a model wherein thymic lymphocytes from MHC class I– and II–deficient ($MHC^{-/-}$) mice were injected into the thymi of $MHC^{+/+}$ mice (Akashi et al., submitted), and were followed for their maturation in the presence or absence of A7R34. Since thymocytes from $MHC^{-/-}$ mice could not have received signals for positive selection, the $MHC^{-/-}$ thymocytes should contain precursors that express selectable TCRs in high frequencies. In this experimental system, there was an increase in the numbers of SP progeny 3 days and 7 days after intrathymic injection; the SP (mainly CD4 SP) cells that appeared after 3 days were mainly the progeny of DP (c-Kit^{-}) precursors; and the SP cells (both CD4 and CD8 SP cells) that appeared after 6–7 days were progeny of more immature DN to DP^{lo} c-Kit^{+} IL-$7R\alpha^{+}$ precursors (Akashi et al., submitted). As shown in Figure 2A, the administration of A7R34 suppressed the differentiation of $MHC^{-/-}$ thymocytes along both the c-Kit^{+} and the c-Kit^{-} pathways (Akashi and Weissman, 1996) to both CD4 and CD8 SP cells on days 3 and 7.

It was important to determine the role of cell division during positive selection in these experiments, because the decrease of day 3 or day 7 progeny could result simply from the suppression of IL-7-dependent proliferation by A7R34. Indeed, the decrease of day 7 progeny by A7R34 might result from the inhibition of IL-7-dependent expansion of the c-Kit^{+} IL-$7R^{+}$ precursors (the c-Kit^{+} pathway; Akashi and Weissman, 1996), since we have found that injection of A7R34 could inhibit the proliferation of c-Kit^{+} IL-$7R^{+}$ precursors in normal mice (Akashi et al., submitted). To understand the role of cell division of thymocytes during positive selection for day 3 CD4 SP progeny, we isolated DP c-Kit^{-} thymocytes from $MHC^{-/-}$ mice, labeled the cells with PKH26, injected the labeled cells intrathymically into normal hosts,

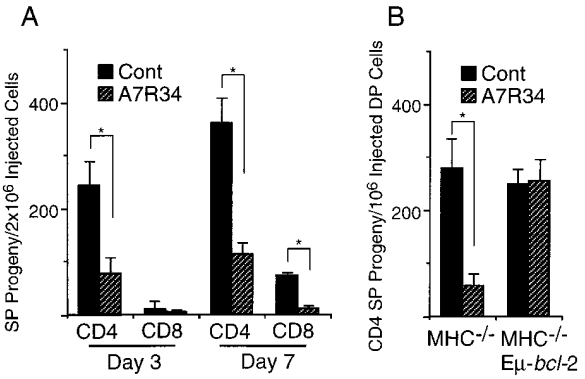

Figure 2. Inhibition of Positive Selection by a Neutralizing Anti-IL-7Rα Antibody (A7R34)

(A) Numbers of CD4 or CD8 SP progeny from unfractionated MHC$^{-/-}$ (Ly5.2) thymocytes, 3 or 7 days after intrathymic injection into MHC$^{+/+}$ (C57BL/6-Ly5.1) thymi with or without intraperitoneal injection of A7R34.

(B) Numbers of CD4 SP progeny 3 days after intrathymic injection of purified DP (2×10^6) cells from either MHC$^{-/-}$ or Eμ-*bcl-2* MHC$^{-/-}$ thymus in the presence or absence of A7R34.

(*), $P < 0.05$ by paired t tests.

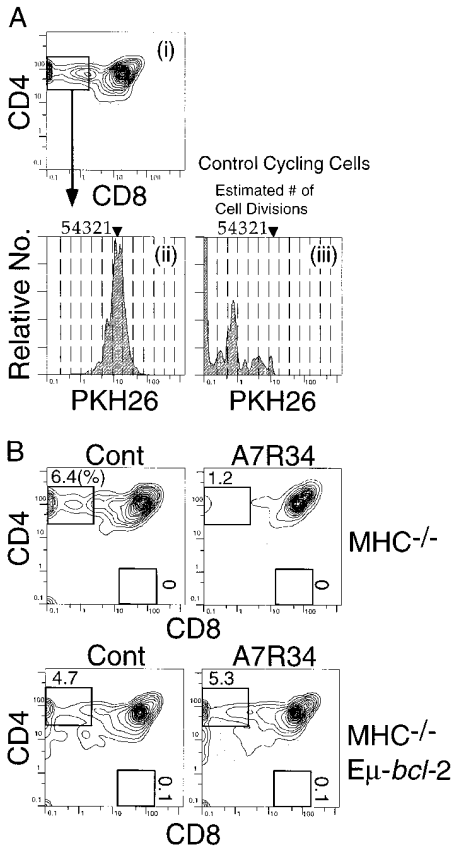

Figure 3. Substitution for IL-7R-Mediated Signals during Positive Selection by Enforced Expression of Bcl-2

(A) Cell cycle status during positive selection determined by cell membrane staining of PKH26. (i), CD4/CD8 profile of day 3 progeny from purified DP (2×10^6) cells stained with PKH26. (ii), the PKH26 fluorescence of day 3 CD4 SP progeny after intrathymic injection of DP cells from MHC$^{-/-}$ thymus to congenic MHC$^{+/+}$ thymus (closed gray line). The mean PKH26 intensity of day 3 CD4 SP progeny is virtually the same as the control value depicted as an inverted closed triangle (see Experimental Procedures), indicating that cell division is not involved in the transition from DP to CD4 SP cells. (iii), the rapid decrease of PKH26 intensity of cells maintaining the TCR$^-$ c-Kit$^+$ phenotype 3 days after intrathymic injection of cycling TCR$^-$ c-Kit$^+$ cells (closed gray line). Broken lines correspond to the 50% decrease of fluorescence intensity of PKH26. The numbers at the top of bottom panels depict the estimated numbers of cell divisions as determined by the decline of PKH26 intensity.

(B) The CD4/CD8 profiles of progeny 3 days after intrathymic injection of purified DP (2×10^6) cells from either MHC$^{-/-}$ or Eμ-*bcl-2* MHC$^{-/-}$ thymus. Absolute numbers of progeny are shown in Figure 2B. A7R34 could inhibited the positive selection from DP to CD4 SP cells, but Eμ-*bcl-2* could antagonize this inhibitory effect of A7R34 on positive selection.

and assessed the change in PKH26 intensity. A decline in the fluorescence signal from PKH26-labeled thymic population should be correlated with the number of cell divisions; each doubling of labeled cells corresponds to a 50% drop of the mean fluorescent signal as the dye present in the plasma membrane of the parent cell was divided between two daughter cells (Kraft et al., 1993).

As shown in Figure 3A, the mean PKH26 intensity of day 3 CD4 SP progeny corresponds to cells that had undergone only <0.1 cell cycles, indicating that the transition from MHC$^{-/-}$ DP c-Kit$^-$ to CD4 SP progeny by positive selection on day 3 post injection occurred mostly without cell division. Accordingly, the effect of A7R34 could be ascribed not only to suppression of thymocyte proliferation, but also to inhibition of positive selection through inhibiting cell survival, differentiation, or both.

IL-7R Signaling during Positive Selection Could Be Substituted by Enforced Expression of the *bcl-2* Transgene

To test whether a survival signal is involved, we provided a survival signal (expression of the human *bcl-2* transgene) for cells undergoing thymic positive selection by using Eμ-*bcl-2* MHC$^{-/-}$ mice as donors. We injected purified DP c-Kit$^-$ cells from either MHC$^{-/-}$ or Eμ-*bcl-2* MHC$^{-/-}$ mice into MHC$^{+/+}$ thymi in the presence or absence of A7R34. After intrathymic injection, the positively selected DP cells immediately upregulated IL-7Rα and TCRβ (data not shown). This process does not involve cell division (as described above). As shown in Figures 2B and 3B, DP c-Kit$^-$ cells lacking Bcl-2 were blocked in their differentiation to SP (CD4) cells by A7R34, whereas the inhibitory effect of A7R34 was not found on Eμ-*bcl-2* MHC$^{-/-}$ DP cells. Based on these data, we propose that the IL-7/IL-7R interaction is unlikely to play a direct role in determining the differentiation of DP progenitors, but is important in permitting DP cells to complete the developmental program specified by positive selection, probably by maintaining their viability.

Enforced Expression of Bcl-2 Restores T Cell Development in IL-7Rα$^{-/-}$ Mice

If IL-7 induces a survival response during thymic positive selection, Bcl-2 expression should rescue thymic cells that had been largely absent in IL-7Rα$^{-/-}$ mice (Peschon

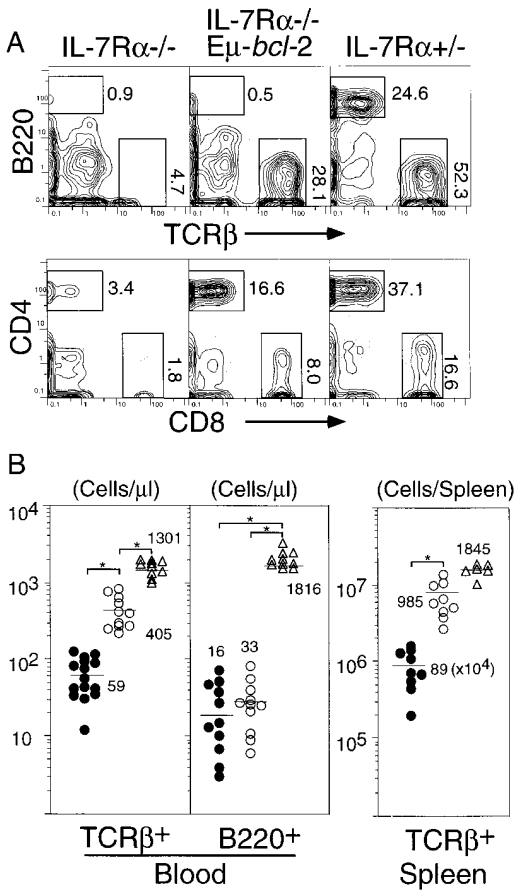

Figure 4. Enforced Expression of Bcl-2 Leads to a Recovery of T Cell Population in IL-7Rα$^{-/-}$ Mice

(A) B220/TCRβ (upper panels) and CD4/CD8 (lower panels) expression profiles of cells from peripheral blood.

(B) Absolute numbers of α/β T (TCRβ^{+}) cells and B (B220^{+}) cells per microliter of peripheral blood, and splenic T (TCRβ^{+}) cells in either IL-7Rα$^{-/-}$ (closed circle), Eμ-*bcl-2* IL-7Rα$^{-/-}$ (open circle), or IL-7Rα$^{+/-}$ (open triangle) mice. Mean values are indicated by the bars and adjacent numbers. (*), $p < 0.05$ by the Bonferroni method on positive results of the Kruskal-Wallis test.

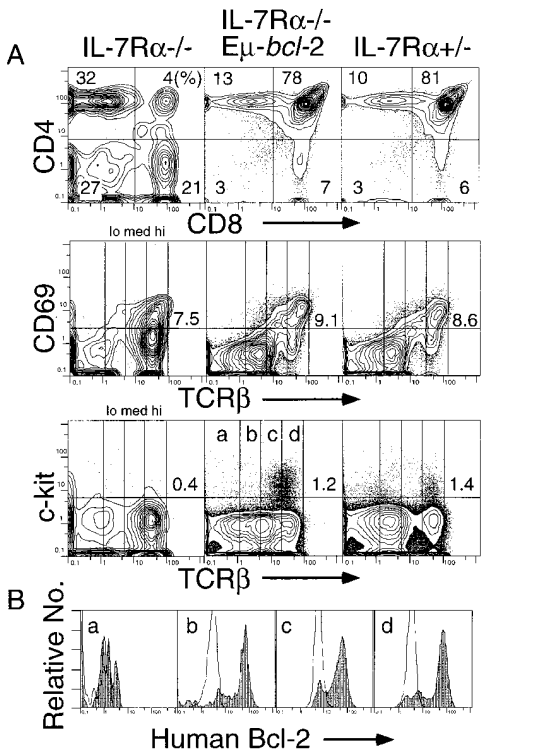

Figure 5. Enforced Expression of Bcl-2 Restores Distribution of Thymocytes Undergoing Positive Selection in IL-7Rα$^{-/-}$ Mice

(A) CD4/CD8, CD69/TCRβ, and c-Kit/TCRβ expression profiles of thymocytes.

(B) Human Bcl-2 expression driven by Eμ-*bcl-2* transgene (closed gray histogram) in c-Kit^{+} populations that express different levels of TCRβ from the Eμ-*bcl-2* IL-7Rα$^{-/-}$ thymus ([a]–[d]; defined in the middle of bottom panels of [A]). Open histograms show control stainings with FITC-conjugated goat anti-hamster IgG in each subset. Human Bcl-2 was expressed in the TCR^{+} c-Kit^{+} populations (b–d), but not in the TCR^{-} c-Kit^{+} early precursors (a).

et al., 1994). To test this possibility, we introduced Eμ-*bcl-2* mice into the IL-7Rα$^{-/-}$ strain by breeding and selection of Eμ-*bcl-2* IL-7Rα$^{-/-}$ progeny. Results from the analysis of Eμ-*bcl-2* IL-7Rα$^{-/-}$ mice are shown in Figures 4, 5, and 6. Eμ-*bcl-2* IL-7Rα$^{-/-}$ mice produced a significant amount of peripheral T cells, approximately 10-fold greater than those severely depleted in IL-7Rα$^{-/-}$ mice (Figure 4); the number of T cells from Eμ-*bcl-2* IL-7Rα$^{-/-}$ mice increased ~7-fold in blood and ~11-fold in spleen. The ratio of CD4 to CD8 SP cells in these mice was almost equal to that in IL-7α$^{+/-}$ mice (Figure 4A). We found no particular Vβ bias in the restoration of peripheral CD4 or CD8 cells in Eμ-*bcl-2* IL-7Rα$^{-/-}$ mice; either CD4 or CD8 SP cells contained similar percentages of Vβ3-, Vβ6-, Vβ8-, and Vβ11-positive cells when compared to normal mice (data not shown). However, B cells were not rescued (Figure 4B), and γ/δ T cells remained undetectable in lymph nodes (data not shown).

In the Eμ-*bcl-2* IL-7Rα$^{-/-}$ thymus, there was a significant increase in the number of thymocytes (~10-fold) as well as in the number of mature TCR^{hi} SP subsets (Figures 5 and 6). The CD4 versus CD8 expression profile of thymic lymphocytes is restored in Eμ-*bcl-2* IL-7Rα$^{-/-}$ mice to the profiles of normal thymus (Figure 5A). In the small number of cells in the IL-7Rα$^{-/-}$ thymus, the ratio of CD4/CD8 SP thymocytes was widely variable. Expression of Bcl-2 in these thymi led to an almost normalized CD4/CD8 SP ratio, confirming that Eμ-*bcl-2* could rescue both CD4 and CD8 lineage maturation (Figures 5A and 6). The small number of cells in the IL-7Rα$^{-/-}$ thymus are also disproportionately skewed to TCR^{hi} SP cells; the ratio of CD69^{+} to CD69^{-} cells in TCR^{med-hi} population in the IL-7Rα$^{-/-}$ thymus was low, indicating that the majority of these cells had not recently been positively selected (Figure 5A). On the other hand, the percentage of TCR^{med-hi} CD69^{+} thymocytes in Eμ-*bcl-2* IL-7Rα$^{-/-}$ mice is similar to that in IL-7Rα$^{+/-}$ mice. The absolute numbers of CD69^{+} cells in Eμ-*bcl-2* IL-7Rα$^{-/-}$ thymus showed an ~10-fold increase compared to those in IL-7Rα$^{-/-}$ thymus (Figure 6).

TCR^{lo-hi} c-Kit^{+} cells represent an efficient progenitor population for receiving positive selection (Akashi and Weissman, 1996). While c-Kit^{+} cells were absent from the IL-7Rα$^{-/-}$ thymus, these populations were also restored in the Eμ-*bcl-2* IL-7Rα$^{-/-}$ thymus both in number

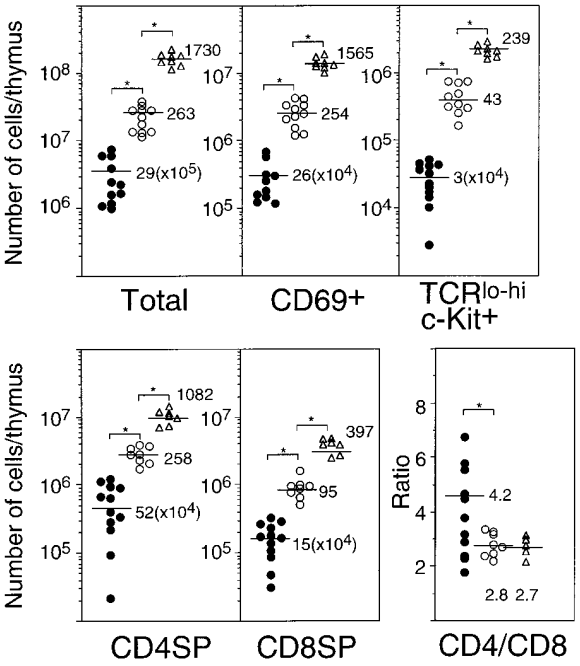

Figure 6. Enforced Expression of Bcl-2 Restores Numbers of Developing Thymocytes in IL-7R$\alpha^{-/-}$ Mice

Absolute numbers of each phenotypically defined thymic population and CD4/CD8 ratio of TCRβ^{hi} SP thymocytes in either IL-7R$\alpha^{-/-}$ (closed circle), Eμ-*bcl-2* IL-7R$\alpha^{-/-}$ (open circle), or IL-7R$\alpha^{+/-}$ (open triangle) mice. Mean values are indicated by the bars and adjacent numbers. (*), $p < 0.05$ by Bonferroni method on positive results of the Kruskal-Wallis test.

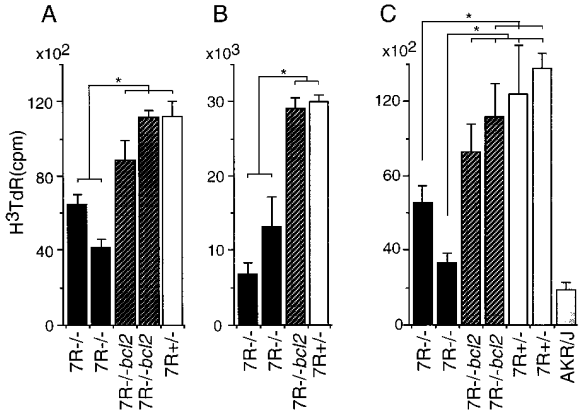

Figure 7. Enforced Expression of Bcl-2 Restores Function of IL-7R$\alpha^{-/-}$ T Cells

Results of activation assays of splenic T cells against nonspecific TCR stimulation by either ConA (A) or anti-CD3 plus anti-CD28 (B), and allogeneic stimulation evaluated by mixed lymphocyte cultures (C). Data was shown as mean ± S.D. (error bars) in quadriplicate cultures. (*), $P < 0.05$ by paired t tests.

and in the various stages of the c-Kit^{+} maturation pathway (Akashi and Weissman, 1996) (Figure 6). These data show significant restoration of thymocyte numbers and regeneration of all maturational stages of positive selection.

The Restored T Cells in Eμ-*bcl-2* IL-7R$\alpha^{-/-}$ Mice Are Functional

It was important to test whether the phenotypic restoration of T cells in the Eμ-*bcl-2* IL-7R$\alpha^{-/-}$ mice signified functional maturation. Although IL-7R signaling was not required for T cell activation, IL-7R$\alpha^{-/-}$ T cells have been shown to be less responsive to alloantigen as well as to receptor-independent stimuli (Maraskovsky et al., 1996). As shown in Figure 7, IL-7R$\alpha^{-/-}$ T cells were significantly less responsive to either concanavalin A (ConA), anti-CD3 plus CD28 stimulations, or allogeneic antigen as evaluated on a mixed lymphocyte reaction. In all of these assays, Eμ-*bcl-2* IL-7R$\alpha^{-/-}$ T cells on a per cell basis could respond significantly better than the residual T cells in IL-7R$\alpha^{-/-}$ mice, and almost as well as T cells from IL-7R$^{+/-}$ mice.

Discussion

Our data show that enforced expression of Bcl-2 in T lineage cells could restore T cell development considerably in IL-7R$\alpha^{-/-}$ mice. Accordingly, it is strongly suggested that the principal role of IL-7R signaling in developing thymocytes and mature T cells might be to maintain viability, at least including upregulation of Bcl-2. The survival signal induced by IL-7 could involve molecules other than Bcl-2, because IL-7 can reinforce the survival of T cells from Bcl-2-deficient mice (Nakayama et al., 1995).

Previous studies showed that Bcl-2 itself had no stimulatory effect on cell proliferation (Vaux et al., 1988; Fairbairn et al., 1993; Mazel et al., 1996), although it is conceivable that Bcl-2 could be involved in preparing cells for mitosis. Bcl-2 could not substitute for signals generated during positive selection driven by TCR–MHC interactions, because no SP maturation could be seen in *lck^{pr}–bcl-2* MHC$^{-/-}$ mice (Linette et al., 1994; Tao et al., 1994) or in the Eμ-*bcl-2* MHC$^{-/-}$ mice used in this study (data not shown). T cells were also not rescued in *lck^{pr}–bcl-2 RAG1*$^{-/-}$ (Linette et al., 1994) or Eμ-*bcl-2* SCID mice (Strasser et al., 1994). Enforced expression of Bcl-2 was reported to increase the number of CD3^{med-hi} CD8 SP cells in the thymus (Linette et al., 1994; Tao et al., 1994), although its effect on positive selection was not clear.

In our study, the Eμ-*bcl-2* transgene could restore thymic development in IL-7R$\alpha^{-/-}$ mice. Developing thymocytes in IL-7R$\alpha^{-/-}$ mice expressed low levels of endogenous Bcl-2. Eμ-*bcl-2* could substitute for the IL-7R-mediated signals associated with positive selection in a subset of progenitors (DP^{hi} c-Kit^{-}) that did not undergo cell division in vivo during their maturation to CD4 (TCR^{hi}) SP cells. These results indicate that enforced expression of Eμ-*bcl-2* did not necessarily lead to positive selection by modifying the cell cycle status of cells on the c-Kit^{-} pathway (Linette et al., 1996; Mazel et al., 1996). Accordingly, the reinforcement of survival by Eμ-*bcl-2* enables thymocytes to complete the differentiation program determined by positive selection and leads to significant increases in both CD69^{+} and TCR^{lo-hi} c-Kit^{+} thymocyte populations.

The survival signal induced by IL-7R might also play a role in the proliferation of thymic precursors. We have

reported that in normal mice, Bcl-2 expression is maintained through all stages of the positive selection process including cycling TCR^{lo-med} c-Kit$^+$ IL-7Rα^+ (DN to CD4^{int}CD8^{int}) cells, TCR^{hi} c-Kit$^+$ IL-7Rα^+ CD4^{lo}CD8$^+$ or CD4$^+$CD8^{lo} cells, and TCR^{hi} c-Kit$^-$ SP cells (Akashi and Weissman, 1996). The c-Kit ligand, steel factor (Slf) is also a factor responsible for expansion of immature thymocytes (Rodewalt et al., 1995), and the proliferation of c-Kit$^+$ IL-7Rα^+ thymocytes is synergistically supported by IL-7 and Slf (Suda and Zlotnik, 1991; Akashi et al., submitted). Accordingly, Eμ-*bcl-2* may maintain the responsiveness to Slf in TCR^{lo-med} c-Kit$^+$ populations in the IL-7R$^{-/-}$ thymus. However, the restoration of thymocyte numbers by Eμ-*bcl-2* was not complete. This may be at least partially because the Eμ-*bcl-2* transgene was not expressed before the developmental stages of TCR$^-$ c-Kit$^+$ precursors (Figure 5B) that has not rearranged TCR genes.

The malfunction of peripheral IL-7R$\alpha^{-/-}$ T cells upon stimulation has been characterized as reduced frequency of clonogenic expansion due to an increased rate of apoptotic cell death and a slower rate of proliferation (Maraskovsky et al., 1996). The lack of IL-7R signaling results in impaired expression of endogenous Bcl-2 in IL-7R$\alpha^{-/-}$ T cells, and might cause their augmented rate of apoptosis. The Eμ-*bcl-2* transgene protects IL-7R$\alpha^{-/-}$ T cells from apoptosis probably before and after activation, and normalizes their in vitro response to either antigen-specific or antigen-independent stimuli.

The hypoplasia of the lymphoid system in IL-7R$\alpha^{-/-}$ mice is probably not simply due to the disruption of IL-7/IL-7R interaction, because IL-7Rα is also used as a receptor for other ligands, e.g., for receptor complexes of thymic stromal cell–derived lymphopoietin (Friend et al., 1994; Ray et al., 1996). We found a similar impairment of endogenous Bcl-2 expression in developing thymocytes in γ_c-deficient ($\gamma_c^{-/-}$) mice (K. A. et al., unpublished data), whose lymphopenia is mostly due to a lack of functional IL-7R (Sadlack et al., 1994). We have observed T cell but not B cell restoration by introducing either the Eμ-*bcl-2* or the H2K-*bcl-2* transgene into the $\gamma_c^{-/-}$ mice, in the latter of which the *bcl-2* transgene was expressed in virtually all hematopoietic cells (Kondo et al., 1997). Therefore, the primary immunopathological mechanism of impaired development of T cells in X-linked SCID might be due to lack of survival signals induced by IL-7R engagement in the thymus.

It is also suggested that the rescue of lymphopoiesis by the *bcl-2* transgene in IL-7R$\alpha^{-/-}$ mice might be restricted to α/β T cells; the Eμ-*bcl-2* transgene appears not to be able to rescue γ/δ T and B cells, although the Eμ-*bcl-2* was expressed in a considerable fraction of the IgM$^-$ B220$^+$CD43$^+$ pro-B cell population in bone marrow (data not shown). Engagement of the IL-7R complex is reported to be critical for the development of γ/δ T cells (Appasamy et al., 1993; He and Malek, 1996; Maki et al., 1996). The IL-7R complex also plays a crucial role in the rearrangement of immunoglobulin heavy chain (IgH) genes (Corcoran et al., 1996). When a rearranged IgH gene is introduced into *RAG*$^{-/-}$ mice, the Eμ-*bcl-2* gene promotes the maturation of pro-B to pre-B cells, resulting in the formation of classical splenic B cell follicles (Gutman and Weissman, 1972) in the spleen (Young et al., 1997). Accordingly, it appears that the particular signal transduction events responsible for the rearrangement of γ/δ TCR and IgH genes cannot be rescued by the survival-promoting *bcl-2* transgene.

It is still unclear whether positively selected thymocytes undergo apoptotic cell death in vivo in the absence of IL-7R-mediated signals, though in vitro survival of IL-7R$^{-/-}$ mature T cells is impaired (Maraskovsky et al., 1996). Hence, there remains a possibility that Bcl-2 may play a role in activating intracellular maturation programs (Chen et al., 1997) as well as in maintaining cell survival during positive selection. Further studies of downstream events associated with the expression of Bcl-2 are required to clarify this issue.

The possibility that other cytokines might have as a significant function the induced survival of their targets, rather than directing differentiation in vivo, may be more general than the IL-7/IL-7R model described here. Elsewhere, we present evidence that M-CSF delivers a survival signal for target monocytes in vivo, and that enforced expression of Bcl-2 in monocytes in *op/op* mice can rescue the differentiation of macrophage subsets and significant reversal of their osteopetrosis (Lagasse and Weissman, 1997 [this issue of *Cell*]). Thus, certain cytokines appear to enforce viability of their target cells in vivo. In the case of IL-7/IL-7R, proliferation and/or maturation of T cells that result from internal gene programs or other external stimuli (including other cytokine receptor/cognate cytokine interactions) could occur on condition that the cells do not die.

Experimental Procedures

Mice

C57BL/6-Ly5.1 mice were bred and maintained in the central animal facility in the Department of Comparative Medicine, Stanford University. The MHC$^{-/-}$ mice were obtained by crossing mice with H-2^b haplotype (Eα^-) (Ly5.2) introduced with a targeted mutation in the β_2-microglobulin gene (MHC class I–deficient mice) and mice with a null mutation in the Aβ^b gene (MHC class II-deficient mice). Eμ-*bcl-2*-25 mice, in which the human *bcl-2* transgene is expressed mainly in T-lineage cells (Strasser et al., 1991), were backcrossed with C57BL/Ka (H-2^b) (Ly5.2) for three generations. The MHC$^{-/-}$ Eμ-*bcl-2* mice were generated by crossing with the Eμ-*bcl-2*-25 mice and the MHC$^{-/-}$ mice. IL-7R$\alpha^{-/-}$ mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). Eμ-*bcl-2* IL-7R$\alpha^{-/-}$ mice were generated by crossing the IL-7R$\alpha^{-/-}$ mice with the Eμ-*bcl-2*-25 mice. All mice were analyzed at 4–5 weeks of age.

Antibodies

The antibodies used in immunofluorescence staining included AL1-4A2 (anti-Ly5.2); 2B8 (anti-c-Kit, CD117) (Ikuta and Weissman, 1992); KT-31 or 2C11 (anti-CD3); H57-579 (anti-TCRβ); GK-1.5 (anti-CD4); 53-6.7 (anti-CD8) and 6B2 (anti-B220) and F23.1 (anti-Vβ8.2). Neutralizing anti-IL-7R (A7R34) antibodies are kind gifts from S. Nishikawa (Kyoto University). Mouse and hamster antibodies were purified by protein A affinity chromatography, and rat antibodies were purified by protein G affinity chromatography. These antibodies are directly conjugated with phycoerythrin (PE) (Cyanotech Corporation, Kailua-Kona, Hawaii), fluorescein-5isothiocyanate (FITC) (Molecular Probes, Incorporated, Eugene, Oregon), allophycocyanin (APC) (Cyanotech Corporation), or Texas Red (Molecular Probes). A7R34 was biotinylated and visualized by avidin-PE (Becton Dickinson and Co., San Jose, California). FITC-conjugated H1.2F3 (anti-CD69), KJ 25 (anti-Vβ3), RR-47 (anti-Vβ6), and RR3-15 (anti-Vβ11) were purchased from PharMingen (San Diego, California). The staining of Bcl-2 was done as follows; after staining for surface antigens, the cells were

stained with purified hamster anti-mouse Bcl-2 antibody (Pharmingen) for 1 hr, then incubated with FITC-conjugated goat anti-hamster IgG (Caltag Laboratories, San Francisco, CA) for 30 min in PBS containing 0.03% saponin and 5% FCS.

Culture of Splenic T Cells

Enriched splenic T cells (2×10^6/ml) from either IL-7R$\alpha^{+/-}$ or IL-7$^{-/-}$ mice were cultured in RPMI-1640 containing 10% FCS with or without 20 ng/ml of recombinant mouse IL-7 (Genzyme, Cambridge, MA) at 37°C in 7% CO_2 and 100% humidity for 18 hr. No proliferation of either IL-7$^{-/-}$ or IL-7R$\alpha^{+/-}$ T cells was seen during the culture. After 18 hr, the cells were stained for surface TCRβ and intracellular Bcl-2.

Cell Sorting and Flow Cytometric Analysis

Freshly isolated thymocytes were stained for multicolor flow cytometric analysis and analyzed using a highly modified dual (488-nm argon laser and 599-nm dye laser) or triple laser (plus 390-nm argon laser) FACS (Becton Dickinson Immunocytometry Systems, Mountain View, California) with four-decade logarithmic amplifiers. For sorting, we subjected the sorted cells to a second round of FACS sorting, and the highly purified DP (both DPint and DPhi) populations were used for experiments. Dead cells were identified by their propidium iodide staining profile and excluded from analysis. Fluorescence data were analyzed by FACS/DESK program and presented either in the form of histograms or two-parameter probability plots (5%).

Intrathymic Injection

The details of the surgical procedure have been described elsewhere (Guidos et al., 1990). Thymocytes from MHC$^{-/-}$ mice (Ly5.2) were sorted into 10 μl of saline and injected into a thymic lobe of anesthetized, unirradiated 3- to 5-week-old C57BL/6-Ly5.1 mouse with either intraperitoneal injection of 1 mg of A7R34 or equal volumes of PBS. The same doses of A7R34 or PBS were intraperitoneally administered on days 2 and 4. Individual thymic lobes were harvested and stained with PE-53-6.7, APC-GK1.5, and FITC-Ly5.2. The donor cells were the PI-negative, FITC-positive population. We directly analyzed one-fourth of the cells from each lobe without enriching for donor-derived cells by immunomagnetic beads (Guidos et al., 1990; Akashi and Weissman, 1996), to avoid the loss of donor-derived cells. All events of donor-derived cells were collected by setting electronic gates.

PKH26 Labeling of Thymocytes

Freshly isolated thymocytes were labeled with PKH26 (PKH26 red fluorescent general cell linker kit, Sigma Immuno Chemicals), which is a stable, fluorescent dye that incorporates into the cell membrane. PKH26 has an excitation peak of 551 nm and an emission peak of 567 nm. The PE channel on the FACS was used to measure PKH26 levels. Cells were stained with PKH26 according to the supplier's recommendation with slight modification. Briefly, cells are resuspended in Diluent C (Sigma Immuno Chemicals) at 2×10^6 cells/ml in polypropylene tubes and an equal volume of 2 μM PKH26 in Diluent C was added to the cells. Cells were incubated in the resultant 1 μM PKH26 with continuous mixing for 3 min. The reaction was stopped by adding an equal volume of FCS. Cells were washed three times with HBSS containing 10% FCS. Cells were resuspended in RPMI (without phenol red) supplemented with 10% FCS, and incubated at 37°C for 1–2 hr. The cells were washed twice with HBSS containing 10% FCS; stained with FITC-53-6.7, APC-GK1.5, and PI (1 mg/ml); and purified with the FACS. We noticed some nonspecific decrease in the mean PKH26 signals after intrathymic injection, probably due to the release of PKH26 that may have attached to the cell membrane in a nonspecific manner. A control value for PKH26 was determined by measuring the first peak of PKH26 signal from the donor-derived (injected) DP fraction (in which most cells could not divide). Usually, the first peak of PKH26 became stable within 48 hrs after intrathymic injection. The PMT voltage on each fluorescence channel was set on an automatic computed calibration system on the FACS/DESK program using the same control beads.

Proliferation Assay

Splenocytes were isolated and depleted of erythrocytes with ACK treatment. Splenic T cells were enriched through negative selection of splenocytes by using Gr-1 (8C5), Mac-1 (M1/70) plus B220 (6B2) rat antibodies, and sheep anti-Rat antibody-conjugated immunomagnetic beads (Dynabeads M-450, Dynal A.S., Oslo, Norway). In most cases, T cells were enriched up to ~80%. Enriched splenic T cells (containing 5×10^4 T cells) were incubated for 2 days at 37°C, 7% CO_2 in 96-well plates in RPMI-1640 with 10% FCS, and supplements in the presence or absence of plate-bound anti-CD3 (145.2C11) and CD28 (Pharmingen) (plates were coated with these antibodies at 1 μg/ml in H_2O overnight at 37°C), or 2.5 μg/ml of ConA (Sigma Chemical Co.). For the mixed lymphocyte reaction, spleen cells from AKR/J (H-2^k) mice were treated with γ-irradiation (4000 rad) and were used as stimulating cells. Stimulating cells (10^5 cells) were incubated for 4 days with enriched splenic T cells (containing 5×10^4 T cells). Cultures were pulsed with 1 μCi of [^{3}H]thymidine for 12 hr before harvesting.

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