Published in final edited form as:

Cell Immunol. 2007 ; 250(1-2): 31–39.

Impaired Thymopoiesis in Interleukin-7 Receptor Transgenic Mice Is Not Corrected by Bcl-2

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Abstract

Murine thymocytes down-regulate IL-7 responsiveness following β -selection and reacquire sensitivity after positive selection. To assess the potential consequences of IL-7 signaling during this phase of development, transgenic IL-7 receptor alpha (IL-7R α) mice were evaluated for IL-7 responsiveness as gauged by STAT-5 phosphorylation. Transgenic IL-7R α expression increased the percentage of thymocytes responsive to IL-7 yet resulted in a decrease in total thymic cellularity. Aberrant thymocyte development in transgenic mice was first manifested by a reduction of DN3 thymocytes that correlated with lower Bcl-2 expression. Surprisingly, transgenic restoration of Bcl-2 expression did not correct thymic hypocellularity induced by IL-7R α overexpression. These findings demonstrate that failure to appropriately downregulate IL-7R α expression interferes with thymocyte development past the pro-T stage resulting in significantly lower levels of mature thymocytes.

Keywords

rodent; thymus; transgenic mice; cytokine receptors; cellular differentiation

INTRODUCTION

The requirement for IL-7 in the efficient generation of mature T cells is well documented. The use of animals genetically deficient in molecules necessary for IL-7 signaling has demonstrated the drastic effect loss of IL-7 signaling has on lymphoid development[1–3]. This has also been shown in competitive inhibition studies by use of anti-IL-7 and/or receptor blocking antibodies [4–7]. These studies revealed that disruption of IL-7 signaling severely inhibited thymocyte and T cell development past the DN2 stage. Correction of this defect was achieved by the introduction of a Bcl-2 transgene, thus identifying a causal link between IL-7 survival signaling and continuation of development past the pro-T phase of development[8,9]. These studies demonstrated that while thymocyte maturation to the DN2 stage proceeded relatively normally in the absence of IL-7 signaling, transition to the pre-T cell, DN3 compartment was highly IL-7 dependent. Indeed, in the presence of exogenous IL-7, the DN3 population is the most

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highly influenced developmental phase as measured by relative change in subcompartment cellularity[10]. This effect apparently occurs at the expense of downstream development as generation of mature thymocytes and T cells is compromised by elevated IL-7 levels[6]. These findings demonstrate the delicate balance that must be struck with regard to regulation of thymocyte IL-7 sensitivity during early developmental phases.

We have previously demonstrated a downregulation in IL-7 responsiveness in post- β -selected thymocytes by use of *in vitro* IL-7 response and survival assays[11]. These studies demonstrated that although DN3 and DN4 thymocytes expressed similar levels of IL-7R α , cellular responsiveness was attenuated in DN4 thymocytes, suggesting the presence of intracellular inhibitors of IL-7 signal transduction or loss of one or more IL-7 signaling pathway components. This aspect of post- β -selection development seems to be a reasonable physiologic solution to reduce IL-7 signaling prior to complete loss of the receptor that occurs in the DP compartment. Indeed, insofar as IL-7 signaling can inhibit transition of DN4 thymocytes to the DP compartment via IL-7 mediated repression of the transcription factors, TCF-1, LEF-1 and ROR γ t[12], it is clear that mechanisms must be in place in order to ensure the appropriate downregulation of IL-7 signal transduction during this phase of development. In light of the dominant role IL-7 plays in maintenance of pre-T cells and its potential for inhibiting normal developmental progression, strict enforcement with regards to down modulation of IL-7 signaling appears to be critical to ensure robust T cell development.

Recently, multiple labs have re-addressed the role IL-7 plays in regulation of early thymocyte maturational events by evaluating the consequences of elevating IL-7R α during phases of development where it is normally downregulated. In a series of experiments by two different groups, IL-7R α was overexpressed via transgenesis by placing the receptor under control of a human CD2 promoter cassette. Surprisingly, these studies revealed that dysregulation of IL-7R α expression in this manner resulted in thymic hypoplasia. Munitic *et al.* postulated that failure to appropriately downregulate the receptor effectively titrated away a limited thymic supply of IL-7, thus starving pre-T cells of IL-7 needed for their development and/or maintenance [13]. Yu *et al.* illustrated that instigation of IL-7 signaling past the normal point of cessation could inhibit thymocyte maturation at the ISP to DP stage of development[12]. These two explanations for the inhibition of thymocyte development observed in the IL-7R α transgenic mice are apparently in opposition, one suggesting a loss of IL-7 signaling due to inappropriate IL-7 sequestration and the other suggesting enhanced IL-7 signaling due to receptor overexpression and presumed continuation of signaling in normally IL-7 insensitive populations. In order to clarify the role of IL-7R α signaling in early thymocyte development, we assessed multiple aspects of thymocyte IL-7 signaling in backgrounds of ectopic IL-7R α expression achieved by transgenesis. Consequences of enforcing Bcl-2 expression in the transgenic IL-7R α background were also explored. These experiments revealed that both IL-7 depletion and ectopic IL-7R α signaling occur in thymocytes expressing the IL-7R α transgene and demonstrate the necessity of downregulation of IL-7R α expression in the developmental stage between β -selection and positive selection in order for normal thymopoiesis to occur.

MATERIALS AND METHODS

Transgenic construct

Murine IL-7R α cDNA from sort-purified thymocytes was generated using AccuPrime Pfx DNA Polymerase (Invitrogen, Carlsbad, CA) with IL-7R α specific primers similar to those described by Porter *et al.*[14] except we modified the forward primer to include a consensus Kozak sequence (underlined): forward 5'-tccagatctgccaccatgatggctctggtagagctttc-3' and reverse 5'-tccagatcttcattgttttggtaaaaactaga-3'. The PCR product was cloned and verified by sequencing, which indicated that it was 100% identical to that reported for GenBank accession AF078906. The cloned IL-7R α cDNA was isolated after BglII digestion and ligated into the

BamHI site of the p1017 expression vector. The expression vector was checked for proper insertion and then the NotI linearized expression cassette was introduced by pronuclear FVB zygote injection at the Oklahoma Medical Research Foundation Microinjection Core Facility (Oklahoma City, OK).

Mice

Multiple IL-7R α transgenic founders were generated and characterized. The transgene was maintained on the FVB background by heterozygous breeding. FVB female mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) or bred by us in USDA-approved facilities at the University of Tulsa and at the Oklahoma Medical Research Foundation. Bcl-2 transgenic mice have been used previously and were demonstrated to have active transgene expression during both early and late thymocyte development [15–17]. F₁ female progeny from crossing our lck-IL7R α line 10 mice with Bcl-2 transgenic mice were genotyped by evaluation of overexpression of IL-7R α on peripheral T cells and by PCR detection of the Bcl-2 transgene as previously described [15]. All procedures involving mice were performed in accordance with practices outlined in Guide for the Care and Use of Laboratory Animals (National Research Council) under protocols approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center, the University of Tulsa and the Oklahoma Medical Research Foundation.

Flow cytometry and cell sorting

The various fluorochrome- or biotin-conjugated specific Abs and streptavidin conjugates used for thymocyte surface phenotyping were from BD PharMingen (San Diego, CA) with the exception of the anti-IL-7R α antibody (clone A7R34) which was from eBioscience (San Diego, CA). Sorting and analysis of thymocyte subsets were performed on MoFlo (DakoCytomation, Fort Collins, CO) and FACSCalibur (BD Biosciences, San Jose, CA) instruments, respectively. MoFlo sort logic was achieved with Summit software (version 4.1), while post-sort analysis was performed with CellQuest or CellQuest-Pro software (BD Biosciences, San Jose, CA). Cell preparation, staining and sorting were performed as previously described [11] with the exception of evaluation of DN populations in which biotinylated lineage markers CD4, CD8, CD19, Mac-1, NK1.1, Ter119, Gr-1 and TCR $\gamma\delta$ were used to exclude mature T cells and thymocytes as well as non-T lineage cells from the analyses. For intracellular staining, exclusion of these cells was possible by using streptavidin-Qdot@800 (QuantumDot, Hayward, CA). Use of this reagent is preferred in that it robustly retains its spectral properties throughout the fixation and permeabilization processes used for intracellular staining. For intracellular staining, thymocyte populations were first surface labeled with appropriate antibodies (and if required, treated in the IL-7 response assay) and then fixed with Reagent A (Fix & Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA)). A 10-minute to overnight treatment with ice-cold 90% methanol was performed between fixation and permeabilization of the cells to enhance intracellular staining. Cells were then permeabilized with Fix and Perm Reagent B in the presence of PE-conjugated isotype control antibodies or antibodies recognizing phospho-STAT-5 (Tyr⁶⁹⁴) or Bcl-2 (BD PharMingen). Typically, high antibody titers (1:2 to 1:10 from commercial stocks) diluted in Reagent B were used to intracellularly stain 5–20 $\times 10^6$ cells in a final volume of 100 μ l. For *in vitro* culture of purified DN3 and DN4 thymocyte subsets, total thymocyte preparations were stained with the lineage cocktail described above, CD25-FITC and CD44-APC. This was followed by streptavidin-PE secondary labeling, which allowed for sorting on PE[−], CD44[−], CD25⁺ (DN3) or on PE[−], CD44[−], CD25[−] (DN4) thymocyte subsets. DP TCR[−] thymocytes were sort purified after staining with CD4-FITC, CD8-PE and TCR-APC.

In vitro IL-7 survival assays

Viability of sort-purified wild-type and IL-7R α transgenic DN3, DN4 and DP thymocytes were quantitated based on trypan blue exclusion and hemacytometer enumeration. Cells were cultured in a humidified incubator at 37°C/5% CO₂ in 96-well plates with an initial 1×10^5 cells/well with or without 25 ng recombinant mouse IL-7 (R&D Systems, Minneapolis, MN) and harvested at the indicated time points.

Western blotting

Sort purified DN3 and DN4 thymocytes derived from either wild-type or IL-7R α transgenic mice were lysed and Western blotted as previously described [18–20]. Blots were probed for Bcl-2 using a mouse IgG₁ mAb (clone C-2 from Santa Cruz Biotechnology Inc., Santa Cruz, CA) and horseradish peroxidase (HRP) conjugated goat anti-mouse IgG secondary staining antibody (Santa Cruz Biotechnology, Inc.). Blots were probed for β -actin using a goat polyclonal IgG antibody (I-19 from Santa Cruz Biotechnology Inc.) and donkey anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology, Inc.). Blots were developed with SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology Inc., Rockford, IL) and visualized using a VDS-CL Imaging System (Amersham Pharmacia Biotech, Inc. Piscataway, NJ).

RESULTS

Transgenic IL-7R α expression increases the percentage of thymocytes responsive to IL-7 yet reduces thymic cellularity

Several mouse lines were generated that overexpressed the mouse IL-7R α on developing thymocytes by virtue of placing the transgene under control of the *lck*-proximal promoter. Multiple lines were characterized which varied in IL-7R α expression levels (FIG 1A). As described in previous IL-7R α transgenic mice [12,13] DP thymocytes demonstrated elevated levels of IL-7R α in comparison to the same populations from wild-type littermate control mice (FIG 1B). Ectopic IL-7R α expression in the transgenic mice was first observed on DN2 thymocytes, which extended through all post-DN2 compartments. Additionally, and consistent with other reports for this promoter, expression of the transgene could still be appreciated on peripheral T cells and was markedly diminished upon activation (data not shown). No differences between the lines were observed with respect to IL-7 responsiveness and, unless otherwise noted, all data were obtained with the line 2 mice that express a midrange level of the receptor.

To determine whether elevated IL-7R α expression influenced thymocyte IL-7 responsiveness, we compared results from *in vitro* IL-7 response assays performed on thymocytes from transgenic and wild-type mice. These experiments revealed that the predominant effect of IL-7R α overexpression was to increase the proportion of IL-7 responsive DN4 and DP thymocytes (FIG 2A). Wild-type and transgenic mice contained equivalent percentages of responsive thymocytes within the DN2 and DN3 compartments, as was expected since we previously demonstrated that these populations inherently manifest maximal responsiveness to this cytokine. Within the DN4 compartment, the percentage of responsive thymocytes was elevated, which was mirrored in the transgenic DP population with an increase over the non-responsive state observed in wild-type cells to approximately 30%. This effect was independent of transgene expression levels as demonstrated in the DP response profiles derived from multiple lines of IL-7R α transgenic mice (FIG 2B and data not shown). Evaluation of CD4 and CD8 SP thymocytes revealed that overexpression of IL-7R α only modestly affected these populations compared to the effects observed on the less mature thymocytes. Additionally, evaluation of the relative degree of IL-7 responsiveness in wild-type and transgenic DN CD25

+ thymocytes in dose-response assays revealed enhanced responsiveness (as assessed by the intensity of the P-STAT-5 stain) in transgenic thymocytes (data not shown).

Despite the fact that the proportion of post- β -selected transgenic thymocytes competent to respond to IL-7 was increased, the *in vivo* consequences of this experimental manipulation resulted in overall decreases in thymic cellularity that were most pronounced during or shortly after the phase of maximal thymic expansion which occurs in mice less than 12 weeks of age (FIG 3A) [21]. Although thymic numbers were routinely 30–50% that of aged-matched, wild-type controls, presence of the transgene did not have a dramatic impact on the development of the major peripheral T cell subsets (data not shown). To more precisely define the impact of IL-7R α overexpression, we assessed the cellularity of the DN1-4, DP and CD4 and CD8 SP compartments in thymuses from 6-week-old wild-type and IL-7R α transgenic mice (FIG 3B). Although cellularity in the DN1 and DN2 compartments was similar, there was a marked decrease in DN3 thymocytes (~35% of wild-type control levels) in the transgenic mice. While DN4 cellularity was not significantly different between wild-type and transgenic thymuses, there was an ~50% reduction in the generation of DP thymocytes in the IL-7R α transgenic background. Generation of CD4 SP and CD8 SP thymocytes was also compromised, most likely due to the decrease in precursor populations.

Over-expression of IL-7R α does not enhance survival of β -selected thymocytes

In order to determine whether elevated IL-7R α expression provided a survival advantage for transgenic thymocytes, we evaluated the ability of IL-7 to maintain viability in purified populations of DN3, DN4 and DP from wild-type and transgenic mice (FIG 4). This experiment demonstrated that overexpression of the IL-7R α ultimately provided slightly higher-level survivability upon transgenic DN3 thymocytes in comparison to wild-type DN3s (most likely due to induction of Bcl-2 expression) although survival within the first 24 hours of culture was similar between the two populations. Importantly, in cultures without the addition of IL-7, transgenic DN3 thymocytes fared worse than did their wild-type counterparts. IL-7 afforded little protection for DN4 or DP thymocytes from either wild-type or transgenic mice, although the transgenic DN4 thymocytes appeared to be somewhat harder in their response during the first 24 hours of culture than did wild-type DN4s. Regardless, these data demonstrate that the predominant survival effect of IL-7 is provided to pre- β -selected thymocytes and that this effect is downregulated in the post- β -selected compartments.

Ectopic IL-7R α expression causes decreased Bcl-2 expression in DN2 and DN3 thymocytes

In order to determine whether thymocyte IL-7 signaling was enhanced *in vivo* in transgenic thymocytes, we evaluated Bcl-2 levels directly *ex vivo* from 6-week-old wild-type and transgenic mice. Assessment of the four major thymic populations indicated that Bcl-2 levels were ~25% lower in DN thymocytes from the IL-7R α transgenic mice (FIG 5A and similar to what was observed in [13]). Further dissection of this effect indicated that while DN1 thymocytes from both wild-type and transgenic mice contained equivalent Bcl-2 levels, transgenic DN2 and DN3 thymocytes expressed significantly lower amounts (FIG 5B). Passage of thymocytes to the DN4 stage of development in both wild-type and transgenic thymuses resulted in lower overall Bcl-2 expression as assessed by Western blot (FIG 5C). Intracellular staining revealed a bi-modal expression pattern for Bcl-2 in wild-type DN4 thymocytes, both of which were lower than levels observed in the more immature DN3 precursor populations. Expression of Bcl-2 in the transgenic DN4 population was altered insofar as we detected a greater percentage of cells with elevated Bcl-2 levels. Importantly, this level was no higher than the levels observed in the transgenic DN2 and DN3 populations and it was equivalent to the level observed in a portion of the highest Bcl-2 expressing, wild-type DN4 cells. These findings suggest that, in a scenario where IL-7R α is not appropriately downregulated, a situation results where DN2 and DN3 thymocytes experience inadequate IL-7 signaling. This

in turn may result in development of a population of β -selected thymocytes skewed in their Bcl-2 expression pattern potentially caused by either adaptation of thymocytes to an IL-7 depleted thymus or other IL-7 withdrawal-dependent selective pressures. In order to address these possibilities, the IL-7R α effect was isolated by evaluating development of IL-7R α transgenic thymocytes in the presence of constitutive Bcl-2 expression achieved by transgenesis.

A Bcl-2 transgene does not restore thymic cellularity in IL-7R α transgenic mice

Insofar as presence of an IL-7R α transgene resulted in lower thymocyte Bcl-2 expression, we wished to determine whether the observed difference in Bcl-2 expression was the causal link to the decreased thymic cellularity observed in the transgenic animals. We crossed heterozygous female IL-7R α transgenic mice with heterozygous male Bcl-2 transgenic mice and evaluated female F₁ offspring to determine if overexpression of Bcl-2 could correct thymic cellularity in the presence of the IL-7R α transgene. As shown in Figure 5D, total thymic cellularity as well as DN3 compartment cellularity was normal in non-transgenic and Bcl-2 transgenic animals. This was in contrast to the decrease observed in both populations from IL-7R α transgenic mice, regardless of the presence of the Bcl-2 transgene. A direct interpretation of this result indicates that the observed thymic hypocellularity induced by IL-7R α over-expression is not due solely to a loss of Bcl-2. Moreover, since transgenic expression of Bcl-2 has been reported to correct thymocyte development in situations of ablated IL-7 signaling[8, 9], this would argue that the developmental defect instigated by IL-7R α over-expression is not solely due to loss of IL-7 survival signaling. Of note, the bcl2 transgenic mice used in this study express high levels of Bcl-2 throughout development beginning within the DN2 compartment (data not shown) and were previously used to correct thymocyte development at both the β -selection[15] and positive/negative selection[16] checkpoints.

DISCUSSION

The first report concerning IL-7R α transgenesis was made by Porter *et al.*, in which discrete regions of the cytoplasmic tail of the receptor were functionally characterized[14]. Wild-type IL-7R α , as well as IL-7R α deletion mutants, were expressed in IL-7R α $-/-$ hosts to assess the function of the engineered receptors in the absence of endogenous IL-7R α signaling. These analyses revealed distinct functions of various domains of the receptor but the developmental consequences of expressing the wild-type transgene on a wild-type background were not closely examined. Using IL-7 induction of STAT-5 phosphorylation as a functional readout of IL-7 sensitivity, we have found multiple lines of IL-7R α transgenic mice to contain an IL-7-responsive subset of DP thymocytes. We also evaluated DP thymocytes from the mice described by Porter *et al.* and found similar results (data not shown). These results differ from those found in wild-type mice, where DP thymocytes have shut-off IL-7R α expression and show no response to IL-7. The prolongation of IL-7 sensitivity past β -selection into the DP population in the IL-7R α transgenic mice resulted in thymic hypocellularity as was seen in previous models with this transgene[12,13]. The ability of ectopically expressed IL-7R α to promote IL-7 responsiveness in a fraction of DP thymocytes demonstrates that an elevated level of the receptor does not deleteriously effect intracellular signal transduction in transgenic thymocytes. Furthermore, the ability of transgenic thymocytes to respond to IL-7 in the response assays at levels equal to or higher than wild-type thymocytes indicates that receptor desensitization is not a likely explanation for the loss of thymic cellularity in the transgenic mice.

Two other groups[12,13] reported hypocellularity with IL-7R α transgenic mice in which the transgene was expressed under control of the human CD2 promoter rather than the proximal lck-promoter used by us and by Porter *et al*[14]. Use of the CD2 promoter also induced

IL-7R α expression early in thymic development and resulted in similar findings with regard to thymic cellularity and phenotype. Although perhaps counterintuitive, these data demonstrate that overexpression of IL-7R α disrupts thymic homeostasis in a manner reminiscent of developmental models where IL-7 signaling is limited or absent.

In one of the previous reports of IL-7R α transgenic mice, the authors postulated that the failure to appropriately downregulate IL-7R α induced a situation where DP thymocytes “competed away” the limited amounts of IL-7 normally reserved for interaction with DN thymocytes [13]. This hypothesis seems plausible in that IL-7 overexpression mediated by introduction of an IL-7 transgene partially corrected the thymic hypocellularity in IL-7R α transgenic mice. Our results indicate that the DN2 thymocytes are the first population effected by presence of the IL-7R α transgene. Although the numbers of DN2 thymocytes are equivalent in wild-type and transgenic mice, we demonstrated a significant loss of Bcl-2 expression in these cells. This result is consistent with a loss of signaling model with respect to diminished IL-7 levels. Previous studies have demonstrated that in IL-7 signaling deficient animals, there is a decrease in thymic cellularity manifested by a decrease in the absolute number of thymocytes in post-DN2 populations [1–3]. In light of these reports, our data demonstrating a decrease in Bcl-2 levels in transgenic DN2 thymocytes imply that although DN2 thymocytes are responsive to IL-7, it is not critical for their *in vivo* development or maintenance. This is in contrast to the dramatic reduction in DN3 thymocytes observed in the studies with IL-7 signaling deficient animals as well as in the IL-7R α transgenic mice, indicating that in the absence of IL-7 signaling, DN3 thymocytes are compromised either in their ability to develop or survive. Recently IL-7 has been demonstrated to be required for differentiation to the DN3 stage but it is dispensable for, and in some cases, inhibitory to, differentiation to the DP stage [12].

Overexpression of Bcl-2 has been reported to mitigate to some extent the negative developmental consequences due to genetic loss of IL-7 signaling [8,9]. This finding in conjunction with the hypothesis that failure to correctly coordinate downregulation of IL-7R α (and hence IL-7 signaling) creates a situation of thymic IL-7 insufficiency. We evaluated the consequences of overexpression of Bcl-2 in IL-7R α transgenic mice. We reasoned that since development was corrected in the Bcl-2 transgenic $\times$ IL-7 signaling-deficient background then Bcl-2 should mitigate the effect of the IL-7R α transgene if the underlying cause for the observed developmental dysplasia is loss of IL-7 survival signaling. Conversely, if Bcl-2 failed to correct the IL-7R α transgene-induced defect then this would suggest that additional mechanisms are mediating the negative effect. Our results demonstrated that overexpression of Bcl-2 in IL-7R α transgenic mice did not correct the loss of thymic cellularity at the total thymocyte level and more specifically within the DN3 compartment. These data therefore suggest that the mechanism mediating the negative effect due to presence of the IL-7R α transgene was due to presence of inappropriate IL-7 signaling, rather than a lack thereof. Thus, the inability of the Bcl-2 transgene to correct the thymic hypocellularity in the IL-7R α transgenic mice may be due to repressive IL-7 signaling in the DN4/ISP compartment. In contrast, Bcl-2 overexpression is capable of restoring thymic cellularity in IL-7 deficient mice since these mice are devoid of any potentially repressive IL-7 signaling at the ISP to DP transition. The data in Figure 5 demonstrate that Bcl-2 expression is decreased in the DN2 and DN3 compartments of the IL-7R α transgenic thymuses, and that the level of Bcl-2 in the DN4 compartment is shifted in comparison to wild-type DN4 thymocytes. The limited ability of IL-7 to promote survival of cells within the transgenic DN4 population, however, indicates that IL-7 survival signaling has already been downregulated in this population and is not predictive of continuation of IL-7 signaling. Additionally, the overall Bcl-2 levels in the wild-type and transgenic DN4 populations appear to be equivalent based on Western blots for Bcl-2 expression. We propose that these findings are the result of selective pressure placed upon the thymocytes, in the altered environment of the transgenic thymuses. While wild-type DN4 thymocytes demonstrate two sub-populations with respect to Bcl-2 expression, the transgenic

DN4 population is predominantly comprised of thymocytes expressing levels equivalent to that observed in the transgenic DN2 and DN3 populations and similar to the highest levels expressed by wild-type DN4 thymocytes. We interpret the bi-modal distribution of Bcl-2 expression and STAT-5 phosphorylation within this compartment to be indicative of non-synchronous entry of β -selected thymocytes into the DN4 compartment. Specifically, thymocytes that complete TCR β rearrangement on their first attempt most likely enter the DN4 stage of development with higher levels of Bcl-2 than thymocytes that took longer to complete the rearrangement program. As this is an IL-7-dependent event and as IL-7 is predicted to be limiting in the IL-7R α transgenic thymus, only thymocytes that are successful in their first attempt at rearrangement survive and are selected while thymocytes that are unsuccessful have less of a chance to complete this program due to IL-7 insufficiency. This interpretation could account for the 50–70% decrease in thymic cellularity and may explain the bimodality of IL-7 responsiveness observed in the transgenic DP population. During standard developmental progression, thymocytes execute synergistic programs with respect to IL-7 and pre-TCR signaling that influence the successful navigation of β -selection. Thymocytes that will eventually be β -selected, successively pass through IL-7 independent (DN2), dependent (DN3), quasi-dependent (DN4) and non-responsive (DP) phases. Within this framework, the signal that promotes β -selection coincides with multiple cellular changes that eventually down-modulate IL-7 responsiveness. While it is possible to uncouple these two signaling pathways, during normal development it appears that the benefit DN4 thymocytes derive from IL-7 is primarily due to continuation of IL-7 dependent programs initiated prior to or during β -selection with successful transition from DN4 to DP status culminating in the cessation of IL-7 responsiveness. The observed pattern of IL-7 responsiveness suggests then that the initial intrathymic role of IL-7 is to prolong survival of pre- β -selected thymocytes to avail developing thymocytes sufficient time to maximize attempts to successfully generate the requisite TCR β chain needed for continued development. Therefore, the role IL-7 plays during early thymocyte development is to foster and protect thymocytes between the time that the decision to become a T cell is made to the point where the β -selection initiated proliferative burst is under way or completed.

Acknowledgments

Support for this project was received from the Oklahoma Center for the Advancement of Science and Technology (HR03-124) and the National Institutes of Health (COBRE/IDEA P20 RR15577).

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Figure 1.

Characterization of IL-7R α transgene expression in thymocyte subsets. (A) DP thymocytes from wild-type mice and six different lines (lines 9, 6, 8, 2, 4, and 10; from lowest to highest expression level, respectively) of IL-7R α transgenic mice were evaluated for expression of the transgene. Thymocytes were stained for expression of CD4, CD8 and IL-7R α and then gated for analysis of the DP population. (B) IL-7R α expression in the various thymic compartments is shown for wild-type (black) and the line 2 transgenic (gray). The DN1-4 populations were discriminated based on CD44 and CD25 expression after exclusion of lineage positive thymocytes as described in Materials and Methods. The DP and SP expression profiles were discriminated by CD4 and CD8 stains. Wild-type IL-7R α expression levels in each of the compartments are represented by the solid black lines and in the transgenic thymocytes by the solid grey lines. Results are representative of at least two evaluations per transgenic line in (A) and of 5 experiments for (B).

Figure 2.

Transgenic expression of the IL-7R α increases the percentage of DN4 and DP thymocytes responsive to IL-7. (A) IL-7 responsiveness, as revealed by STAT-5 phosphorylation induced by 25 ng/ml IL-7 stimulation, in the DN1-4 (left panel), DP and SP populations (right panel) from wild-type (closed) and IL-7R α transgenic (open) are shown. The average percentage of responsive cells within each compartment from 3 wild-type and 3 IL-7R α transgenic mice are shown for the DN1-4 populations while 3 additional wild-type and 3 transgenic mice were used for determination of the DP, CD4 SP and CD8 SP populations ($\pm$ S.D.). Comparison of P-STAT-5 staining profiles from 20 minute IL-7 stimulated and untreated control samples allowed for a clear discrimination of the IL-7-responsive fractions. (B) The P-STAT-5 profile of DP thymocytes from our lowest, midrange and highest IL-7R α expressing transgenic lines is also shown after 20 minutes of *in vitro* IL-7 stimulation. Results are representative of three experiments.

Figure 3.

Transgenic expression of the IL-7R α causes thymic hypocellularity. Total thymic cellularity was determined for embryonic day 17, 1 day old, 6 week old and 12 week old mice (A). Wild-type (closed) and transgenic (open) values are shown $\pm$ S.D. Results are from 5 wild-type and 6 transgenic fetuses, 4 wild-type and 6 transgenic 1 day-old mice, 4 wild-type and 4 transgenic 6 week-old mice and 5 wild-type and 5 transgenic 12 week-old mice. The cellularity $\pm$ S.D. of the various developmentally defined compartments from six-week-old mice is shown in panel B.

Figure 4.

IL-7 promotes survival of pre- β -selected thymocytes. DN3 (*upper*), DN4 (*middle*), and DP (*lower*) sort purified thymocytes from wild-type and IL-7R α Line 2 transgenic mice were treated in the absence (dashed) or presence (solid) of 25 ng/ml IL-7 and evaluated by trypan blue staining to determine percentages of negatively staining cells (surviving thymocytes) at initiation and after 24 and 48 h of culture in 37°C/5% CO₂ humidified incubators. Survival measurements were performed in duplicate, and the results are presented as means $\pm$ SD of three experiments.

Figure 5.

Transgenic expression of the IL-7Rα reduces overall thymocyte Bcl-2 levels, yet transgenic Bcl-2 expression does not correct the thymic hypoplasia induced by IL-7Rα overexpression. (A) Bcl-2 levels were evaluated directly *ex vivo* by intracellular staining in the major thymocyte subsets defined by CD4 and CD8 staining. The average median fluorescent intensities of the Bcl-2 stain over isotype control staining from 3 wild-type (filled) and 3 IL-7Rα line 2 transgenic (open) mice is depicted $\pm$ S.D. (B) Histograms from analysis of Bcl-2 expression in DN1-4 thymocytes from wild-type (black) and IL-7Rα transgenic mice (gray) are shown. Results are representative of three experiments. (C) Western blot analysis for expression of Bcl-2 and β -actin from sort purified DN3 and DN4 thymocytes derived from either wild-type or IL-7Rα

transgenic mice are shown. Results are representative of three experiments. (D) Average total thymic cellularity (upper) and DN3 cellularity (lower) ($\pm$ S.E.M.) from mice derived from breeding heterozygous IL-7R α line 10 transgenic mice by heterozygous Bcl-2 transgenic mice are depicted. Three wild-type, 4 IL-R α tg⁺/Bcl-2 tg⁻, 9 IL-R α tg⁻/Bcl-2tg⁺, and 13 IL-R α tg⁺/Bcl-2tg⁺ mice 6–8 weeks of age were evaluated.