The Interkeukin-7 Receptor Controls Development and Maturation of Late Stages of Thymocyte Subpopulations

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Short title: Roles of IL-7 receptor in thymus
Abstract

Interleukin (IL)-7 is a cytokine essential for T lymphocyte development and homeostasis. However, little is known about the roles of IL-7 receptor α-chain (IL-7Rα) in late stages of T cell development. To address this question, we established IL-7Rα-floxed mice and crossed them with CD4-Cre transgenic mice. Resultant IL-7R conditional knockout (IL-7RcKO) mice exhibited marked reduction in CD8 single positive (SP) T cells, regulatory T cells (Tregs) and natural killer T (NKT) cells in thymus. The proportion and proliferation of both mature CD4SP and CD8SP thymocytes were decreased without affecting Runx expression. In addition, expression of the glucocorticoid-induced TNF receptor (GITR) was reduced in CD4SP and CD8SP thymocytes, and expression of CD5 was decreased in CD8SP thymocytes. IL-7RcKO mice also showed impaired Treg and NKT cell proliferation and inhibition of NKT cell maturation. Bcl-2 expression was reduced in CD4SP and CD8SP thymocytes but not in Tregs and NKT cells, and introduction of a Bcl-2 transgene rescued frequency and CD5 expression of CD8SP thymocytes. Furthermore, IL-7RcKO mice exhibited greatly increased numbers of B cells and, to a lesser extent, of γδ T and dendritic cells in thymus. Overall, this study demonstrates that IL-7Rα differentially controls development and maturation of thymocyte subpopulations in late developmental stages and suggests that IL-7R expression on αβ T cells suppresses development of other cell lineages in thymus.
Introduction

Interleukin (IL)-7 is a cytokine essential for lymphocyte development and survival. The IL-7 receptor (IL-7R) consists of a common cytokine receptor γ-chain (γc) and a unique IL-7R α-chain (IL-7Rα). IL-7Rα also forms the receptor for thymic stromal lymphopoietin (TSLP) by interacting with the TSLP receptor. Mice deficient in either IL-7 or IL-7Rα show markedly reduced numbers of T and B cells. The IL-7R transmits signals for proliferation and survival and for V(D)J recombination of immunoglobulin heavy chain and T cell receptor (TCR) γ-chain loci in early lymphocytes (1, 2). In the periphery, IL-7 regulates T cell homeostasis by enhancing survival and proliferation of naive and memory T cells (3). Upon IL-7 binding, the IL-7R activates JAK1 and JAK3 tyrosine kinases, which then activates STAT5 and phosphatidylinositol 3-kinase. IL-7 signaling promotes cell survival by inducing expression of anti-apoptotic factors such as Bcl-2, Bcl-xL and Mcl-1 in T cells (4). Thus, introduction of Bcl-2 transgenes significantly rescues T cell development in IL-7Rα-deficient mice (5, 6).

The γc cytokine signal controls differentiation of CD8 single positive (SP) thymocytes. The IL-7R is expressed on CD4−8− double negative (DN) thymocytes but then down-regulated in CD4+8+ double positive (DP) thymocytes. After positive selection, transient cessation of TCR signaling induces IL-7Rα re-expression on post-selection thymocytes (7). The IL-7R permits thymocytes to differentiate into the CD8 lineage (8). Both Runx3 and STAT transcription factors function in CD8 T cell differentiation by interacting with CD8 enhancer, which is responsive to IL-7 (7). In contrast, other group reported that CD8 lineage specification occurs normally in the absence of IL-7R signaling by anti-IL-7R antibody (9). In addition, IL-15 also functions in CD8 T cell differentiation (10). However, little is known about precise roles of the IL-7R in CD8 T cell development, since
IL-7Rα-deficient mice show severely impaired expansion of DN thymocytes.

The roles of the IL-7R in development of regulatory T cells (Tregs) and natural killer T (NKT) cells remain controversial. In γc-deficient mice, Tregs are severely reduced in the thymus (11-13). Although the proportion of Tregs to non-Treg CD4 T cells is not altered in the IL-7Rα-deficient thymus, IL-7Rα/IL-2Rβ doubly deficient mice show more severe reduction of Tregs than do IL-2Rβ-deficient mice, and the absolute number of Tregs is reduced in IL-7Rα-deficient mice (11-14). As for NKT cell development, it is reported that γc-deficient mice lack thymic NKT cells (15). In contrast, the proportion of NKT cells is not reduced in the IL-7- or IL-7Rα-deficient thymus, although absolute numbers of thymic NKT cells are drastically reduced (15, 16). The observation that IL-7/IL-15 doubly deficient mice show more severe reduction in NKT cells than do IL-15-deficient mice (16). Nonetheless, the roles of the IL-7R in development of Tregs and NKT cells in the thymus remain unclear.

Early thymocyte development is severely impaired in IL-7Rα-deficient mice, making it difficult to precisely determine IL-7Rα function in late stages of T cell development. To address this question, we used a conditional knockout approach and show that IL-7Rα regulates development and maturation of multiple thymocyte subpopulations in late developmental stages.
Results

Generation of IL-7RcKO mice

To investigate the roles of the IL-7R in the late stages of T cell development, we generated IL-7Rα-floxed (IL-7Rα<sup>fl</sup>) mice (Fig. S1A and B) and bred them with CD4-Cre transgenic (Tg) mice (IL-7RcKO). IL-7Rα expression on DN thymocytes of IL-7RcKO mice was comparable with that seen in control mice (Fig. S1C). Although IL-7Rα is down-regulated on DP thymocytes, re-expression of IL-7Rα on CD4SP, CD8SP, Treg and NKT cells did not occur and IL-7R on γδ T cells was not changed in IL-7RcKO mice (Fig. S1C). Therefore, we conclude that CD4-Cre transgene-mediated deletion of the IL-7Rα gene was successfully achieved.

IL-7R is required for development of CD8 T cells in thymus

First, we compared the total cell number of thymocytes between IL-7RcKO and littermate control mice. Unexpectedly, thymocyte number was slightly increased in IL-7RcKO mice (control: 212 ± 7.1 x 10<sup>6</sup> versus IL-7RcKO: 241 ± 8.4 x 10<sup>6</sup>) (Fig. 1A). IL-7RcKO mice exhibited an almost normal proportion of DP, CD4SP and CD8SP thymocytes, while the proportion and absolute number of DN thymocytes increased by two-fold (Fig. 1 B and C). The proportion and absolute number of TCR<sup>β<hi></sup> (TCR<sup>β<hi></sup>) thymocytes was reduced by 30% in IL-7RcKO mice (Fig. S2A). Gating on TCR<sup>β<hi></sup> thymocytes revealed that the frequency of CD8SP thymocytes was reduced (Fig. 1D). Similar results were obtained by analyzing IL-7RcKO mice crossed with OT-I and H-Y TCR Tg mice, whereas the frequency of CD4SP thymocytes was unchanged in IL-7RcKO mice crossed with OT-II TCR Tg mice (Fig. S2B). The absolute number of TCR<sup>β<hi></sup> CD8SP and CD4SP thymocytes decreased by 40% and 10%, respectively, in IL-7RcKO mice (Fig. 1C). Consistent with previous reports that γ<sub>e</sub> cytokines
influence CD8 T cell commitment (8), our results confirm that IL-7 is not the only factor that regulates development of CD8SP thymocytes. Our data also suggest that the IL-7R is required for CD4SP thymocytes, although to a lesser extent.

**IL-7R controls proliferation of mature CD4SP and CD8SP thymocytes**

During positive selection, thymocytes undergo progressive stages of development from TCRβ<sup>int</sup>CD69<sup>+</sup> to TCRβ<sup>hi</sup>CD69<sup>+</sup>. After positive selection, SP thymocytes first acquire a semi-mature phenotype (CD69<sup>+</sup>HSA<sup>hi</sup>Qa-2<sup>low</sup>CD62L<sup>low</sup>) and then differentiate into mature phenotype (CD69<sup>+</sup>HSA<sup>low</sup>Qa-2<sup>hi</sup>CD62L<sup>hi</sup>). While the IL-7R is down-regulated in most of DP thymocytes, its re-expression begins in TCRβ<sup>int</sup>CD69<sup>+</sup> DP cells (17). Although the frequency of TCRβ<sup>int</sup>CD69<sup>+</sup> cells was unchanged in IL-7RcKO mice, the frequency of TCRβ<sup>hi</sup>CD69<sup>+</sup> and TCRβ<sup>hi</sup>HSA<sup>low</sup> cells were more severely reduced than were TCRβ<sup>hi</sup>CD69<sup>+</sup> and TCRβ<sup>hi</sup>HSA<sup>hi</sup> cells in IL-7RcKO mice (Fig. 2A and Fig. S3A). Compared with control mice, thymocytes with HSA<sup>low</sup>Qa-2<sup>hi</sup> and HSA<sup>low</sup>CD62L<sup>hi</sup> mature phenotypes were decreased in both CD4SP and CD8SP thymocytes in IL-7RcKO mice (Fig. 2B and Fig. S3B). These results demonstrate that generation of both mature CD4SP and CD8SP thymocytes is impaired in IL-7RcKO mice.

Because mature thymocytes reportedly proliferate in the thymus (18), we performed BrdU incorporation assay. Twenty-four hours after injection, the frequency of BrdU<sup>+</sup> cells was reduced by 2- to 3-fold in TCRβ<sup>hi</sup>HSA<sup>low</sup> CD4SP and CD8SP thymocytes of IL-7RcKO compared to control mice, although some proliferation still occurred without IL-7R signaling (Fig. 2C). We obtained similar results even 4 hours after a BrdU pulse (Fig. S3C). Consistent with a previous observation (19), these results demonstrate that the IL-7R drives mature SP thymocytes to proliferate in the thymus but that other signals also regulate CD4SP and CD8SP thymocytes.
To determine the role of the IL-7R in acquiring functional competence by SP thymocytes, we analyzed expression of genes characteristic for each population. Transcripts of Runx1 and GATA3, CD4SP-enriched transcription factors, in TCRβ<sup>hi</sup>CD4SP thymocytes were comparable between control and IL-7ReKO mice (Fig. S3D). Next we measured CD8SP-enriched mRNAs encoding perforin-1, granzyme B and Eomes. There was not statistically significant difference between control and IL-7ReKO mice (Fig. 2D). Although it was reported that IL-7 signaling induces Runx3 transcription in post-selected DP thymocytes in vitro (8), Runx3 was not changed in TCRβ<sup>hi</sup>CD8SP thymocytes of IL-7ReKO mice (Fig. 2D).

IL-7 signaling reportedly regulates CD8α co-receptor expression (20). In IL-7ReKO mice, CD8α expression on TCRβ<sup>hi</sup>CD8SP thymocytes was marginally decreased and was not changed in peripheral CD8 T cells (Fig. 2E and S3E). Gating on TCRβ<sup>hi</sup>HSA<sup>hi</sup>, but not TCRβ<sup>hi</sup>HSA<sup>low</sup>, thymocytes (including 4SP, DP and 8SP) revealed that CD8α expression on CD8α<sup>+</sup> population was significantly reduced in IL-7ReKO mice (Fig. S3F). We also found that a co-stimulatory molecule, the glucocorticoid-induced TNF receptor (GITR), was slightly reduced especially in TCRβ<sup>hi</sup> CD4SP thymocytes and peripheral CD4T cells of IL-7ReKO mice (Fig. 2F and S3G). Furthermore, CD5 expression on CD8SP thymocytes was lower in IL-7ReKO compared to control mice, whereas that on CD4SP thymocytes was unchanged (Fig. 2G). These results suggest that the IL-7R plays some role in quality control of both CD4SP and CD8SP thymocytes.

**Bcl-2 expression rescues development and CD5 expression of CD8SP thymocyte**

Enforced expression of Bcl-2 reportedly promotes significant recovery of thymic T cells in IL-7Rα-deficient mice (5, 6), indicating that the IL-7R primarily serves a survival signal. Therefore, we compared Bcl-2 expression in thymocyte subpopulations between control and
IL-7RcKO mice. Like IL-7Rα expression, Bcl-2 levels were almost comparable in DN and DP thymocytes but were significantly reduced in CD4SP and CD8SP thymocytes in IL-7RcKO mice (Fig. 3A). Furthermore, IL-7RcKO thymocytes showed higher caspase-3 activity than control mice with or without IL-7 in vitro (Fig. S4A). In contrast, Bcl-xL and Mcl-1 were unchanged (Fig. S4 B and C).

To test whether Bcl-2 expression rescues SP thymocyte development in IL-7RcKO mice, we bred IL-7RcKO mice with Bcl-2 Tg mice. Introduction of the Bcl-2 transgene completely restored the frequency of TCRβ<sup>hi</sup> thymocytes in IL-7RcKO mice (Fig. 3B). Furthermore, the ratio of CD8SP to CD4SP thymocytes and absolute cell number of CD8SP thymocytes were also recovered in IL-7RcKO mice harboring the Bcl-2 transgene, although they did not reach to those of Bcl-2 Tg mice (Fig. 3B and S4D). In addition, introduction of the Bcl-2 transgene also restored CD5 expression on CD8SP thymocytes in IL-7RcKO mice (Fig. 3C), although Bcl-2 misexpression in control mice did not affect CD5 level on TCRβ<sup>hi</sup>CD8SP thymocytes (Fig. S4E). These results suggest that the IL-7R transmits a survival signal for CD8SP thymocytes primarily through Bcl-2 and that reduced CD5 expression in IL-7RcKO mice may be attributable to reduced survival.

**IL-7R promotes Treg proliferation in the thymus**

Although γ<sub>c</sub> signaling is critical for thymic Treg development, it is unclear whether IL-7Rα functions in this process. To address this question, we analyzed development of thymic Tregs in IL-7RcKO mice. The frequency and absolute number of both CD25<sup>−</sup> and CD25<sup>+</sup> Foxp3<sup>+</sup> cells were decreased by 20% in TCRβ<sup>hi</sup>CD4SP thymocytes of IL-7RcKO mice (Fig. 4 A and B). Because some Tregs develop from DP thymocytes in the medulla (21), we also examined Tregs in the TCRβ<sup>hi</sup>DP fraction. The frequency and absolute number of Foxp3<sup>+</sup> cells were also reduced by 30% in TCRβ<sup>hi</sup>DP cells (Fig. 4 A and B). These results suggest that the IL-7R
participates in thymic Treg development.

Next we analyzed the basis of Treg reduction in IL-7RcKO mice. Bcl-2 expression in Foxp3^{+} 4SP and DP thymocytes was comparable between control and IL-7RcKO mice (Fig. 4C, top). Bcl-xL expression was also unchanged (Fig. 4C, bottom). Introduction of the Bcl-2 transgene appeared to rescue the frequency of Foxp3^{+} CD4SP thymocytes in IL-7RcKO mice (Fig. S5A). However, CD4SP thymocytes of Bcl-2 Tg mice contain more Foxp3^{+} cells compared with control mice, and the frequency of Foxp3^{+} cells in IL-7RcKO x Bcl-2 Tg mice did not reach the levels seen in Bcl-2 Tg mice (Fig. S5A). These results suggest that factors downstream of the IL-7R other than survival signals regulate Treg development. On the other hand, the frequency of BrdU^{+} cells was reduced in Tregs of IL-7RcKO mice (Fig. 4D). The co-stimulatory molecules CTLA-4 and GITR are reportedly constitutively expressed on Tregs and regulate their activity (22). However, expression of intracellular CTLA-4 and surface GITR was unchanged in Tregs of IL-7RcKO mice (Fig. S5B). Overall, these results suggest that the IL-7R is required for proliferation but not essential for functional maturation of thymic Tregs.

**IL-7R promotes proliferation and maturation of invariant NKT (iNKT) cells in thymus**

iNKT cells are derived from DP thymocytes through interaction of invariant V_{α}14-J_{α}18 TCRα chain with CD1d expressed on DP thymocytes. Although the proportion of iNKT cells was not reduced in IL-7- or IL-7Rα-deficient thymus (15, 16), it is unclear whether the IL-7R participates in NKT cell development. To address this question, we analyzed IL-7RcKO thymocytes by using CD1d-tetramer loaded with α-GalCer. The frequency of CD1d-tetramer^{+} iNKT cells was reduced 2-fold in IL-7RcKO compared to control mice (Fig. 5A). After selection, iNKT cells begin to mature from stage 0 (HSA^{+}) and then proceed to stage 1 (CD44^{−}NK1.1^{+}), stage 2 (CD44^{+}NK1.1^{−}) and stage 3 (CD44^{+}NK1.1^{+}) (23). We found that
frequency of stage 3 iNKT cells was reduced in IL-7RcKO mice (Fig. 5B). Absolute numbers of iNKT cells were decreased in IL-7RcKO mice by 30%, 45% and 50% in stages 0/1, 2 and 3, respectively, suggesting that iNKT cell maturation is partially blocked (Fig. 5C). iNKT cells reportedly differentiate primarily into CD4⁺ NKT cells and to a lesser extent into DN NKT cells. DN iNKT arise from CD4⁺ precursors before stage 3 and DN and CD4⁺ iNKT cells are suggested to be functionally different (24, 25). In IL-7RcKO mice, frequency of DN iNKT cells was reduced compared to control mice (Fig. 5D), suggesting that differentiation or survival of DN iNKT cells depends on IL-7R signaling more than CD4⁺ NKT cells. Similar results were also observed in peripheral tissue (Fig. S6A). These results demonstrate that IL-7R signaling is required for iNKT cell maturation after the DP stage.

To determine the mechanism underlying reduction of iNKT cells in IL-7RcKO mice, we first checked expression of anti-apoptotic factors. Bcl-2 and Bcl-XL expression in iNKT cells was comparable between control and IL-7RcKO mice (Fig. 5E). In contrast to CD8SP thymocytes and Tregs, introduction of the Bcl-2 transgene did not rescue the frequency of iNKT cells in IL-7RcKO mice (Fig. S6B). Since iNKT cells rapidly proliferate after selection (26), we next analyzed proliferation. Twenty-four hours after BrdU injection, BrdU⁺ iNKT cells were greatly decreased in IL-7RcKO compared with control mice (Fig. 5F). These results suggest that the IL-7R is important for proliferation of thymic iNKT cells.

**IL-7RcKO mice show increased numbers of γδ T cells, B cells and dendritic cells (DCs) in thymus**

The frequency and absolute number of DN thymocytes increased 2-fold in IL-7RcKO mice (Fig. 1B and C). To define what cell type had increased, we first checked γδ T cells. Both frequency and absolute number of γδ T cells were increased in IL-7RcKO thymus by 1.5-fold (Fig. 6A). In addition, we found that NK1.1⁺ γδ T cells were increased in IL-7RcKO thymus.
(Fig. S7A). By contrast, the absolute number of CD3\(^+\)NK1.1\(^+\) NK cells was comparable (Fig. S7B).

Although \(\gamma\delta\) T cells were increased, it did not account for the overall increase of DN thymocytes in IL-7ReKO mice (Fig. 1C). Therefore, we assessed CD3\(^-\)CD4\(^-\)CD8\(^-\) triple negative thymocytes based on CD25 and CD44 expression. Interestingly, the frequency of DN1 (CD25\(^-\)CD44\(^+\)) cells was 3-fold higher in IL-7ReKO mice (Fig. 6B). Next, we analyzed the expression of several lineage markers and found that the frequency of B220\(^+\) cells increased by 2- to 3-fold (Fig. 6C). The absolute number of B220\(^+\) DN1 cells increased by 20-fold in IL-7ReKO mice. These cells were confirmed to be B lineage cells, because they expressed both MHC class II and CD19 (Fig. S7C), and about 30% of the B220\(^-\)CD19\(^+\) cells were surface IgM\(^+\) (Fig. S7D). The absolute number of CD11c\(^+\) cells in the DN1 fraction also increased in IL-7ReKO mice (Fig. 6C). In total thymocytes, the frequency and absolute number of CD11c\(^-\)B220\(^+\) plasmacytoid DCs (pDC) increased by 2-fold in IL-7ReKO thymus, while the numbers of CD11c\(^-\)B220\(^-\) conventional DCs (cDC) did not show a statistically significant difference (Fig. 6D and 6E).

To examine the mechanism underlying the increase in \(\gamma\delta\) T cells, B cells and DCs in IL-7ReKO thymus, we analyzed proliferation. Twenty-four hours after BrdU injection, we detected a greater number of BrdU\(^+\) B cells in IL-7ReKO than in control mice (Fig. 6F). In contrast, the frequency of BrdU\(^+\) cells in \(\gamma\delta\) T cells and plasmacytoid DCs was unchanged in IL-7ReKO mice at 24 hour after injection (Fig. S7E). These results suggest that IL-7R expression on \(\alpha\beta\) T cells suppresses B cell proliferation in thymus.
Discussion

The IL-7R may influence negative selection of CD8SP thymocytes, because the IL-7R transmits survival signal in CD8SP thymocytes. CD5 expression correlates positively with the strength of TCR signal during positive selection (27). IL-7R signaling may be required for resistance against negative selection via Bcl-2 induction. In IL-7RcKO mice, negative selection might be enhanced, deleting CD8SP thymocytes receiving a relatively strong TCR signal, which normally results in survival. Actually, introduction of the Bcl-2 transgene in IL-7RcKO mice restored CD5 expression on CD8SP thymocytes (Fig. 3C). Another possibility is that reduced CD8α expression in IL-7RcKO mice might have attenuated TCR signaling by co-receptor tuning (20), which results in lower CD5 expression. In contrast to our results, it was reported that IL-7R signaling does not play a role in post-selection thymocytes (9). Although the reason for the discrepancy is not clear, it might be that the blocking of IL-7R signaling by antibody was not complete.

The IL-7R may also function in development of conventional CD4 T cells (Fig. 1C). Bcl-2 expression and BrdU incorporation were severely reduced in CD4SP thymocytes. This is mainly attributable to conventional CD4SP cells, since Bcl-2 levels were unchanged in Tregs and NKT cells, and BrdU+CD4SP excluding Foxp3+ and CD1d-tetramer+ cells were still reduced in IL-7RcKO mice (Fig. S8A). Therefore, our study suggests that the IL-7R likely plays multiple roles in proliferation, survival and maturation during conventional CD4 T cell development.

The molecular link between the IL-7R and thymocyte proliferation is still unclear. Degradation of p27Kip1 by its phosphorylation is important for IL-7-induced proliferation (28). The IL-7R induces the transcription of pim-1 (29), and pim-1 can phosphorylate p27Kip1 (30). However, expression of pim-1 was unchanged in SP thymocytes of IL-7RcKO mice (Fig. 2A).
S8B). In another possibility, like IL-2, the IL-7R activates Akt, which phosphorylates Forkhead transcription factors and inhibits the transcription of p27\textsuperscript{Kip1} gene (31). However, p27\textsuperscript{Kip1} mRNA was not increased in IL-7RcKO mice (Fig. S8B). Because Akt can directly phosphorylates p27\textsuperscript{Kip1} (32), p27\textsuperscript{Kip1} degradation mediated directly by Akt might be responsible for IL-7-induced proliferation.

The role of the IL-7R in Treg development has been a matter of debate. Previous studies with IL-7R\textalpha-deficient mice led to varying conclusions (11-14). In this study, we found that IL-7R is partially responsible for intrathymic Treg proliferation (Fig. 4D). Bcl-2 misexpression rescued Tregs in IL-7RcKO mice (Fig. S5A). However, alteration in cell survival might not be the major reason for reduced Treg number, since Bcl-2 and Bcl-xL expression was unchanged in IL-7RcKO mice and the frequency of Tregs in IL-7RcKO mice harboring a Bcl-2 transgene did not reached the levels seen in Bcl-2 Tg mice (Fig. 4C and S5A). We hypothesize that the increased number of Foxp3\textsuperscript{+} cells in Bcl-2 Tg mice are progeny that escape negative selection and that Tregs are not in fact rescued in IL-7RcKO mice carrying the Bcl-2 transgene. The second possibility is that Tregs with lower Bcl-2 expression did not survive in IL-7RcKO mice and that the remaining cells were those with Bcl-2 levels that had reached a certain threshold.

The IL-7R appears to play multiple roles in NKT cell development. IL-7RcKO mice showed markedly reduced numbers of thymic iNKT cells with impaired proliferation (Fig. 5 C and F). The IL-7R seems not to be essential for NKT cell survival, because Bcl-2 and Bcl-xL expression was unchanged in NKT cells and Bcl-2 misexpression in IL-7RcKO mice did not rescue iNKT cells (Fig. 5E and Fig. S6B). Because Bcl-xL, but not Bcl-2, reportedly rescues iNKT cell development in IL-15\textsuperscript{−/−} mice (33), it is possible that iNKT cells with lower Bcl-xL expression did not survive in IL-7RcKO mice and that the remaining cells were those with Bcl-xL levels that had reached a certain threshold by receiving IL-15 signal.
B cell development in the thymus is influenced by several factors. For example, TCRβ-deficient mice show a 10-fold increase in the number of thymic B cells (34), suggesting that disrupting αβ T cell development alone is sufficient to increase the number of thymic B cells. In the IL-7RcKO thymus, B cells were increased 20-fold (Fig. 6C). Although less prominent than B cells, γδ T cells and DCs were also increased in the IL-7RcKO thymus (Fig. 6A, C and E). γδ T cells, some immature B cells and pDCs express IL-7R both in control and IL-7RcKO mice (Fig. S1C and S7F). IL-7R expression in pDCs was slightly reduced in IL-7RcKO mice, as previously suggested that CD4-Cre mice partially deletes floxed allele in CD11c+ cells (35). IL-7 concentrations may be higher in and around the medulla of IL-7RcKO thymus than control thymus, because SP thymocytes do not consume IL-7 produced by thymic epithelial cells. Therefore, we suggest that suppression of B cell development in thymus requires adequate IL-7 concentrations in addition to T cell specification via Notch signaling. This is in agreement with recent report that over-expression of IL-7 promotes extensive B cell development in the thymus (36). TSLP concentrations might also contribute to this mechanism.

Although not analyzed mainly, peripheral T cells of IL-7RcKO mice showed several characteristics as expected from previous studies. First, peripheral T cell numbers were greatly reduced in IL-7RcKO mice (Fig. S9 A-G). Lymphopenia-induced proliferation was also impaired in T cells (Fig. S9H). As previously reported in IL-7R−/− mice (37, 38), TCR-induced proliferation was also reduced in IL-7RcKO mice (Fig. S9I), although TCR expression and early activation, as monitored by CD69 expression, were normal (Fig. S9 J and K). This result suggests that IL-7R is required for expansion of effector T cells, that is, generation of memory T cell precursors. Suppressive activity of Treg from IL-7RcKO mice was also diminished (Fig. S9L), as previously reported in IL-7R−/− mice (13).

In summary, we characterized roles of the IL-7R in survival, proliferation and
maturation of different subpopulations of αβ thymocytes. These findings will accelerate the understanding of IL-7R function in thymus and the periphery, and IL-7Rα-floxed mice should serve as a powerful tool to determine the mechanism underlying how the IL-7R controls T cell development and immune responses.
Materials and Methods

Mice.
Generation of IL-7Rα-floxed mice is described in SI Materials and Methods. CD4-Cre Tg mice were reported previously (39). H2K-Bcl-2 Tg mice were described previously (40). All mice were analyzed at 4-5 weeks of age. Control (IL-7R^{++} CD4-Cre or IL-7R^{fl/fl}) and IL-7ReKO (IL-7R^{fl/fl} CD4-Cre) mice were compared. All mice were maintained under specific pathogen-free conditions at the Experimental Research Center for Infectious Diseases at the Institute for Virus Research, Kyoto University. All mouse protocols were approved by Kyoto University.

Antibodies and Flow Cytometry.
The fluorescent dye- or biotin-conjugated antibodies used in this study were described in SI Materials and Methods. Intracellular staining was performed using a Foxp3 staining buffer set (eBioscience) or Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s instruction. Stained cells were analyzed with a FACSCalibur or FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). Values in quadrants, gated area and interval gates indicate the percentages in each population in all figures. Cell sorting was performed using a FACSARia II cell sorter (BD Biosciences).

Real-time RT-PCR.
Total RNA was isolated and treated with RNase-free DNase to remove genomic DNA. After DNase inactivation, total RNA was reverse-transcribed with random primer. cDNA was amplified by using an ABI 7500 Sequence Detector (Applied Biosystems) with QuantiTect SYBR Green PCR Kit (QIAGEN) with ROX reference dye (Invitrogen). PCR was carried out
at 95°C for 15 min, followed by 40 cycles consisting of 95°C for 20 s, 55°C for 30 s and 72°C for 60 s. PCR efficiency was normalized using cDNA of whole thymocytes from wild-type mice. Primer sequences are listed in SI Materials and Methods.

**BrdU Incorporation Assay.**

Mice were injected intraperitoneally with 1 mg of BrdU (Sigma-Aldrich). Four or twenty-four hours after injection, thymocytes were stained with surface markers, fixed and permeabilized with Cytofix/Cytoperm, and treated with DNase at 37°C for 1 h. BrdU was stained with a FITC-conjugated anti-BrdU antibody (BD Biosciences). Values in histogram indicate the percentages of BrdU+ cells defined by interval gates.

**Flow Cytometry for DCs.**

The thymus was cut into small pieces and digested with 1.25 mg/ml collagenase D (Roche) and 0.01% DNase I (Roche) in RPMI 1640 medium for 30 min at 37°C. The cell suspension was gently passed through 24-gauge needles twice, washed twice and used for staining.

**Statistics.**

An unpaired two-tailed Student t test was used for all of the statistical analysis. Asterisks in all figures indicate as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.005; ****, p < 0.001. n.s. means not significant.
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We thank Drs. J. Takeda, G. Kondoh, and K. Yusa for the KY1.1 ES line and targeting system; Dr. N. G. Copeland for the Red recombination system; Drs. I. Saito, Y. Shinkai, M. Tachibana, and Y. Matsumura for AxCAFLP adenovirus; Drs. C. B. Wilson, T. Honjo, and D. Yabe for the CD4-Cre Tg mouse; Dr. J. Domen for the H2K-Bcl-2 Tg mouse; Drs. N. Minato and Y. Agata for OT-I, OT-II and H-Y TCR Tg mice; Dr. M. Satake for Runx primer information; Mr. H. Hayashi and Ms. S. Kamioka for excellent technical assistance; Dr. A. Singer and members of the Ikuta lab for discussion. This work was supported by Grants-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (22790460 and 24790469) and by the BioLegend/Tomy Digital Biology Young Scientist Research Award for 2011.

Author contributions: S.T., T.H., and K.Ikuta designed research; S.T., A.S., and K.W. performed research and analyzed data; H.M., S.K., and K.Imai contributed new reagents/analytic tools; and S.T. and K.Ikuta wrote the paper.

The authors declare no conflict of interest.
References


Figure Legends

Figure 1. IL-7R is required for development of CD8 T cells in thymus.

(A) Absolute numbers of thymocytes from control (IL-7R^{+/+} CD4-Cre or IL-7R^{fl/fl}) and IL-7RcKO (IL-7R^{fl/fl} CD4-Cre) mice of 4-5 weeks old. Cell numbers from individual mice (circles) and means (horizontal bars) are shown (n = 35). (B) CD4 and CD8 expression in total thymocytes. (C) Absolute numbers of indicated populations (mean ± SEM, n = 35). (D) Expression of CD4 and CD8 in thymocytes gated on TCRβ^{hi} cells. Representative data are shown (n > 10) (B and D).

Figure 2. IL-7R controls proliferation of mature CD4SP and CD8SP thymocytes.

(A) Expression of TCRβ and CD69 in thymocytes. (B) Expression of HSA and Qa-2 in TCRβ^{hi}CD4SP excluding CD4^{+}CD8^{low} and TCRβ^{hi}CD8SP thymocytes. (C) BrdU uptake by TCRβ^{hi}HSA^{low} CD4SP and CD8SP thymocytes. Data represent four independent experiments with similar results. (D) Real-time RT-PCR analysis showing expression of indicated genes in TCRβ^{hi}CD8SP thymocytes from control (open bars) and IL-7RcKO (filled bars) mice. Transcripts levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Values are the mean ± SEM of triplicate data points from three to six independent experiments. (E) CD8α expression on TCRβ^{hi}CD8SP thymocytes. Representative data and mean fluorescence intensity (MFI) are shown (n > 10). (F-G), GITR (F) and CD5 (G) expression on TCRβ^{hi}CD4SP and TCRβ^{hi}CD8SP thymocytes. Data represent five independent experiments with similar results.

Figure 3. IL-7R transmits survival signals in CD8SP thymocytes through Bcl-2.

(A) Expression of Bcl-2 in thymocyte fractions. 4SP and 8SP indicate
TCRβhiCD25Foxp3CD4SP and TCRβhiCD8SP, respectively. Data represent five independent experiments with similar results. (B) Histograms (top) indicate expression of TCRβ in total thymocytes from mice of each genotype. Dot plots (bottom) show CD4 and CD8 expression in TCRβhi thymocytes. Bar graph (right) shows the ratio of CD8SP to CD4SP cells in TCRβhi thymocytes (mean ± SEM, n = 8-11). (C) CD5 expression in TCRβhiCD8SP thymocytes from indicated mice. Data represent four independent experiments with similar results.

**Figure 4. IL-7R promotes Treg proliferation in thymus.**

(A) Expression of Foxp3 and CD25 in TCRβhiCD4SP and TCRβhiDP thymocytes. Data represent eight independent experiments with similar results. (B) Absolute numbers of Foxp3+ cells in CD4SP and DP thymocytes (mean ± SEM, n = 8). (C) Bcl-2 and Bcl-xL expression in Foxp3+TCRβhi CD4SP (left panels) and Foxp3+TCRβhi DP (right panels) thymocytes. Data represent five (Bcl-2) and two (Bcl-xL) independent experiments with similar results. (D) BrdU incorporation in Foxp3+TCRβhi CD4SP Tregs. BrdU was injected 24 h before analysis. Data represent three independent experiments with similar results.

**Figure 5. IL-7R promotes proliferation and maturation of thymic iNKT cells.**

(A) TCRβ and CD1d tetramer (CD1d-tet) expression in total thymocytes. Representative data are shown (n = 12). (B) Expression of CD44 and NK1.1 in TCRβ+CD1d-tet+ thymocytes. Representative data are shown (n = 11). (C) Absolute numbers of TCRβ+CD1d-tet+ thymocytes in stage 0/1, stage 2 and stage 3. Cell numbers were calculated based on flow cytometry data in (A) and (B) (mean ± SEM, n = 11). (D) Expression of CD4 and CD8 in TCRβ+CD1d-tet+ thymocytes. Data represent of eight independent experiments with similar results. (E) Bcl-2 and Bcl-xL expression in TCRβhiCD1d-tet+ thymocytes. Data represent three
(Bcl-2) and two (Bcl-xL) independent experiments with similar results. 

(F) BrdU incorporation by TCRβ⁺CD1d-tet⁺ thymocytes 24 h after the pulse. Data represent three independent experiments with similar results.

**Figure 6. IL-7RcKO mice show increased numbers of γδ T cells, B cells and DCs in thymus.**

(A) Dot plots (left) show CD3 and γδ TCR expression in thymocytes. Bar graph (right) indicates the absolute numbers of CD3⁺γδ TCR⁺ cells (mean ± SEM, n = 14). (B) Expression of CD44 and CD25 in CD3⁺CD4⁻CD8⁻ thymocytes. Representative data are shown (n = 11). (C) Expression of CD11b, CD11c, B220 and NK1.1 in CD3⁻CD4⁻CD8⁻CD44⁺CD25⁻ DN1 cells was analyzed by flow cytometry. Percentages (left) and absolute cell numbers (right) of each marker positive population are shown (mean ± SEM, n = 7). (D) B220 and CD11c expression of thymocytes isolated with collagenase. Data represent six independent experiments with similar results. (E) Absolute numbers cDC (B220⁻CD11c⁺) and pDC (B220⁺CD11c⁻) (mean ± SEM, n = 6). (F) BrdU incorporation by B220⁺CD11c⁻ B lineage cells 24 h after treatment. Data represent four independent experiments with similar results.
Fig. 1

A

Cell number (x10^8)

Control
IL-7RcKO

B

CD4

Control
7.8
2.8

IL-7RcKO
4.6
6.7

C

Cell number (x10^6)

4SP
***
0
2
4
6
8
10
12
14
16

DN
DP
4SP
8SP
TCRβ

D

Gated on TCRβ^hi

Control
59.2
15.3

IL-7RcKO
61.9
8.9
Fig. 2
Fig. 3

A

Control  IL-7RcKO  IL-7RcKO x Bcl-2 Tg

B

CD8/4 ratio

0.5

0.4

0.3

0.2

0.1

0

% CD8 SP / CD4 SP

Control  IL-7RcKO  IL-7RcKO x Bcl-2 Tg  Bcl-2 Tg

CD4

CD8

11.9

8.2

12.0

13.6

55.6

59.7

49.2

48.8

0.7

1.1

10.5

9.7

15.6

10.5

12.7

15.3

22.5

C

TCRβ hi 8SP

Control  IL-7RcKO  IL-7RcKO x Bcl-2 Tg

CD8

CD6
Fig. 4

A  

B  

C  

D  

Fig. 4

A  

B  

C  

D
Fig. 5

A. Control  IL-7RcKO
B. Control  IL-7RcKO
C. Stage 3  Stage 2  Stage 0/1
D. Control  IL-7RcKO
E. Isotype control  Control  IL-7RcKO
F. Control  IL-7RcKO
Fig. 6

A

Control  IL-7RcKO

B

Gated on CD3-4B-

CD25

C

% of DN

CD11b CD11c B220 NK1.1

D

CD11c

Control  IL-7RcKO

E

Gated on B220+CD11c

F

BrdU
SI Materials and Methods

**Generation of IL-7Rα-floxed Mice.**

The targeting vector was constructed by Red recombination technology with a murine BAC clone containing the IL-7Rα locus (RP23-267M6). One loxP sequence was inserted 97-bp downstream of IL-7Rα exon 2 and the neomycin resistance gene cassette flanked by FRT sequences on both sides and one loxP sequence at the 5' end was inserted 288-bp upstream of exon 2 (Fig. S1A). The targeting vector was isolated with the 2.2-kb 5’-homologous fragment, a neomycin resistance gene cassette, and a 5.6-kb 3’-homologous fragment, flanked by diphtheria toxin A subunit cDNA. The linearized targeting vector was introduced into the KY1.1 embryonic stem (ES) cell line. Homologous recombinants were screened by PCR and confirmed by Southern blot analysis with 5’ and 3’ probes (Fig. S1B). The neomycin resistance gene cassette was removed from the recombinant allele by infecting targeted ES clones in vitro with the adenovirus expressing Flp recombinase, AxCAF LP (1). IL-7Rα-floxed mice obtained were backcrossed into C57BL/6 mice 6 to 9 times.

**Antibodies for Flow Cytometry.**

The following fluorescent dye- or biotin-conjugated antibodies were used: CD3 (145-2C11), CD4 (GK1.5 or RM4-5), CD8 (53-6.7), TCRβ (H57-597), γδTCR (GL-3), CD11c (N418), CD25 (PC61.5), Qa-2 (69H1-9-9), CD62L (MEL-14), MHC class II (M5/114.15.2), CD19 (MB19-1), BP1 (6C3), IL-7Rα (A7R34), Foxp3 (FJK16s), rat IgG2α (eBR2a), hamster IgG isotype control, streptavidin-PE, streptavidin-PE-Cy7, and CaspGLOW TM Fluorescein Active Caspase-3 Staining Kit (purchased from eBioscience); NK1.1 (PK136), CD44 (IM7), GITR (DTA-1), and streptavidin-allophycocyanin (purchased from BioLegend); and CD69 (H1.2F3), Cytotoxic T-lymphocyte antigen (CTLA)-4 (UC10-4F10-11), rat IgG2bκ isotype
control (A95-1), anti-Mouse Bcl-2 Set, and BrdU FITC set (purchased from BD Biosciences). Alexa Fluor 488-conjugated anti-Bcl-xL (54H6) and rabbit IgG isotype control were kindly provided by Beckman Coulter, Tokyo, Japan. PE-conjugated anti-IgM was purchased from Southern Biotechnology. Allophycocyanin-conjugated CD1d-tetramer loaded with α-GalCer was purchased from ProImmune. The following antibodies were purified from hybridoma supernatant and fluorescent dye- or biotin-labeled: CD24 (heat stable antigen) (M1/69), B220 (RA3-6B2), CD11b (M1/70), CD5 (53-7.3).

Peripheral T Cell Isolation and Cell Culture.
Lymph nodes (LNs) were isolated from cervical, axillary, brachial, inguinal, and mesenteric regions. Naive T cells were sorted from LNs and spleen as TCRβ⁺CD25⁻NK1.1⁻CD44lowCD4⁺ or TCRβ⁺CD44⁺CD8⁺ cells by FACSAria II. Cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, 50 µM 2-mercaptoethanol, and antibiotics in 5% (vol/vol) CO₂ at 37°C. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed by using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) at OD490 nm.

Adoptive Transfer.
Naive CD4 or CD8 T cells from LNs (4 x 10⁵) and spleen (2 x 10⁵) were mixed and labeled with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) and then intravenously injected to sex-matched Rag2⁻/⁻ mice.

Treg Suppression Assay.
Naive CD4 and CD25⁺CD4 T cells were purified by FACSAria II. Naive CD4 T cells (5 x
$10^4$) from wild-type mice were seeded in triplicate in 96 well round bottom plate with erythrocytes-removed splenocytes (1 x $10^5$) from Rag2$^{-/-}$ mice (used as antigen-presenting cells) and 0.5 µg/ml of anti-CD3. CD25$^+$CD4 T cells from control or IL-7RcKO mice were added to each well at the indicated ratio. After 72 h culture, proliferation was measured by the MTS assay.

**Primer Sequences.**

Perforin-1/For, 5’-CCTATCAGGACCAGTACAAC-3’;
Perforin-1/RV, 5’-GAGATGAGCCTGTGGTAAGC-3’;
GranzymeB/For, 5’-TTTGTGCTGACTGCTGCTCA-3’;
GranzymeB/RV, 5’-TCTAGTCTCCTTTGGCCTTAC-3’;
Eomes/For, 5’-GCACCAAACGTAGATGATCA-3’;
Eomes/RV, 5’-TGATGTTATTGTCCGCTTTGC-3’;
Distal Runx3/For, 5’-AACAGCAGCCAACAAAGTGG-3’;
Distal Runx3/RV, 5’-TGCTCGGGTCTCGGTATGAAG-3’;
Distal Runx1/For, 5’-ATGGCTTCCAGACAGCATTTTTGAGTCATT-3’ (2);
Runx1/RV, 5’-ACTGTCATTGATGGCTCTATGGTAGGT-3’ (2);
GATA3/For, 5’-TACCGGGGTTGGATGTAAGTC-3’;
GATA3/RV, 5’-CCTCGTTGCGGCTTGATAAG-3’;
Mcl-1/For, 5’-TCAAGGATGGCGTAACAAACTGG-3’;
Mcl-1/RV, 5’-CCCCTTCCGCCTTACAAGAAC-3’;
HPRT/For, 5’-GTTGGATAACAGCCAGACTTTGGTTG-3’;
HPRT/RV, 5’-GATTCAACTTGCCTCAGTCTTTAGGC-3’;
pim-1/For, 5’-CACAGTCTACACGCCTTTTGG-3’;
pim-1/RV, 5’-AGAACACTTGGCCTTTGATG-3’;
p27\textsuperscript{kip1}/For, 5'-CTCAGGCAAACTCTGAGGAC-3';

p27\textsuperscript{kip1}/RV, 5'-TTCGGGGAACCGTCTGAAAC-3'.
SI References


Supplemental Figure Legends

Fig. S1. Conditional targeting of the mouse IL-7Rα locus.

(A) Strategy for targeting of the mouse IL-7Rα locus. B, BamHI; E, EcoRI; Neo, neomycin resistant cassette; DT-A, diphtheria toxin. Open boxes indicate exons. Open and filled triangles indicate Flp recombinase target (FRT) sequences and loxP sequences, respectively. Numbered filled boxes indicate probes used for Southern blot analysis. (B) Genomic DNA from Flp recombinase-treated ES cells were digested with BamHI and analyzed by Southern blotting. Probes used for detection are shown in (A). (C) IL-7R expression in thymocyte subpopulations. Data represent eight independent experiments.

Fig. S2. Positive selection of CD8SP thymocytes is impaired in IL-7RcKO mice.

(A) TCRβ expression in total thymocytes. Bar graphs show frequency and absolute numbers of TCRβ^hi^ cells in total thymocytes (n > 10). (B) CD4 and CD8 expression in CD69^+^ thymocytes from IL-7RcKO or control mice crossed with indicated transgenes on a Rag2^−/−^ background. Data represent 3-4 independent experiments.

Fig. S3. Mature CD4SP and CD8SP thymocytes are reduced in IL-7RcKO mice.

(A and B) Expression of TCRβ and HSA in total thymocytes (A) and HSA and CD62L in TCRβ^hi^CD4SP and TCRβ^hi^CD8SP thymocytes (B). Data represent five independent experiments. (C) BrdU uptake by TCRβ^hi^HSA^low^ CD4SP and CD8SP thymocytes at 4 h after injection. (D) Distal-Runx1 and GATA3 mRNA in TCRβ^hi^CD4SP thymocytes. Transcripts levels were normalized to HPRT mRNA. Values are the mean ± SEM of triplicate data points from six independent experiments. (E and F) Expression of CD8α in TCRβ^+^CD8^+^ cells in LNs and spleen (E) and in TCRβ^hi^HSA^hi^ and TCRβ^hi^HSA^low^ thymocytes (F). Data represent
more than ten experiments. (G) Expression of GITR in TCRβ⁺CD4⁺CD25⁻ and TCRβ⁺CD8⁺ cells in LNs. Data represent two independent experiments.

Fig. S4. Expression of anti-apoptotic factors in IL-7RcKO thymocytes.

(A) Total thymocytes were cultured with or without 5 ng/ml IL-7 for 20 h. Active caspase-3 expression in TCRp⁴⁺DP, TCRβ⁺⁴SP, and TCRβ⁺⁸SP thymocytes were shown. Data represent two independent experiments. (B) Expression of Bcl-xL in thymocytes. Data represent two independent experiments. (C) Mcl-1 mRNA expression in TCRβ⁺ SP thymocytes. Values are the mean ± SEM of triplicate data points. Data represent three independent experiments. (D) Absolute number of TCRβ⁺CD4SP and TCRβ⁺CD8SP thymocytes from each genotype (n = 5-11). (E) CD5 expression in TCRβ⁺CD8SP thymocytes from indicated mice. Data represent three independent experiments.

Fig. S5. Effect of Bcl-2 overexpression on Treg development in IL-7RcKO mice.

(A) Expression of Foxp3 and CD25 in TCRβ⁺⁴CD4SP thymocytes from indicated mice (left). Data represent four independent mice of each genotype. Absolute numbers of Foxp3⁺TCRβ⁺⁴CD4SP Tregs from indicated genotypes (n = 4-8) (right). (B) GITR and intracellular CTLA-4 expression in Foxp3⁺TCRβ⁺⁴CD4SP Tregs. Data represent three independent experiments.

Fig. S6. Bcl-2 overexpression fails to rescue iNKT cell development in IL-7RcKO mice.

(A) iNKT cells in spleen. Data represent two independent experiments. (B) Percentages (left) and absolute number (right) of iNKT cells in thymus from indicated mice (n = 3-6).

Fig. S7. γδ T cells, B cells and dendritic cells are increased in IL-7RcKO mice.
(A) NK1.1⁺γδ T cells in thymus. Data represent three independent experiments. (B) Absolute NK cell numbers in thymus (mean ± SEM, n = 9) (C) Expression of CD19, MHC class II and B220 in thymocytes. Values indicate the percentages of each area. Data represent five independent experiments. (D) IgM expression on thymic B220⁺CD19⁺ cells. Bar graph indicates absolute numbers of IgM⁻ and IgM⁺ thymic B cells (mean ± SEM, n = 5). (E) BrdU uptake by γδ T cells and pDCs in thymus at 24 h after injection. Data represent three independent experiments. (F) IL-7R expression on thymic B cells and pDCs. Data represent four independent experiments.

Fig. S8. Proliferation of conventional CD4SP thymocytes is impaired in IL-7RcKO mice.

(A) BrdU uptake by TCRβ⁺HSA⁺ CD4SP thymocytes excluding Foxp3⁺ and CD1d-tet⁺ cells at 24 h after injection. (B) Pim-1 and p27kip1 mRNA expression in TCRβ⁺SP thymocytes. Values are the mean ± SEM of triplicate data points. Data represent two independent experiments.

Fig. S9. Reduced numbers and impaired functions of peripheral T cells in IL-7RcKO mice.

(A) Absolute cell numbers in LNs and spleen (n = 13). (B-E) Absolute numbers of CD3⁺ and CD19⁺ cells (n = 5) (B), TCRβ⁺CD4⁺ and TCRβ⁺CD8⁺ cells (n = 8) (C), CD3⁺γδTCR⁺ cells (n = 5) (D), and TCRβ⁺NK1.1⁺ cells (n = 7) (E), in LNs and spleen. (F) Expression of Foxp3 and CD25 in TCRβ⁺CD4⁺ cells in LNs and absolute number of Foxp3⁺CD4⁺ Tregs in LNs and spleen (n = 7). (G) Absolute number of cDCs and pDCs in spleen (n = 5). (A-G) All mice were analyzed in 4-5-week-old. (mean ± SEM). (H) Naive CD4 or CD8 T cells were CFSE-labeled and transferred to Rag2⁻/⁻ mice. Five days after transfer, donor populations (CD3⁺CD4⁺ or CD3⁺CD8⁺) in host Rag2⁻/⁻ mice were analyzed. (I) Naive CD4 or CD8 T cells
(5 x 10⁴) were cultured in 96-well plate with or without 5 µg/ml of plate-bound anti-CD3ε and 1 µg/ml of soluble anti-CD28. Proliferation was measured by the MTS assay after 72 h. Values are the mean ± SEM of duplicate data points. (J) CD3 and CD28 expression of freshly isolated naive T cells from LNs. (K) CD69 expression of T cells 18 h after stimulation as described in (I). (L) Treg suppression activity. (H-K) All data represent at least two independent experiments.
Fig. S1.

A. Schematic representation of the targeting vector for IL-7Rα floxed mice, showing the targeting cassette (Neo and DT-A) inserted into the 3' end of the IL-7Rα gene. The targeting vector is flanked by homology arms (HA) that align with the endogenous IL-7Rα sequence. The targeting will result in a frameshift mutation, disrupting the IL-7Rα gene.

B. Southern blot analysis of genomic DNA from WT and targeted mice using probes specific for the WT and targeted alleles. The left panel shows the expected bands for WT and targeted alleles using Probe 1, and the right panel shows the bands using Probe 2.

C. Flow cytometry analysis of T cell populations gated on TCRβ+ CD8SP and CD4SP, showing the expression of CD1d-tet, CD4 Foxp3, and IL-7Rα in WT and IL-7RαCKO mice. The histogram shows the distribution of T cells in each population, with shaded bars indicating isotype control and solid lines indicating control or IL-7RαCKO conditions.
Fig. S2.

A

- Histogram showing TCRβ^hi cells with frequency and cell number.
- Control and IL-7RcKO conditions.

B

- Graphs showing cell number (x10^7) and frequency (%) for Control and IL-7RcKO conditions.
- Gated on CD69^hi and TCRβ^hi cells.
- Data for OT-I and H-Y (female) conditions.

Legend:
- Control
- IL-7RcKO

- TCRβ^hi cells
- Frequency (%)
- Cell number (x10^7)

- Gated on CD69^hi
- Gated on TCRβ^hi
- Gated on CD8
**Fig. S3.**

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Fig. S4.

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B

TCRβhiCD4SP  TCRβhiCD8SP

Isotype control  Control  IL-7RcKO

C

Mcl-1

Relative mRNA level

D

Cell number

E

CD5
Fig. S5.

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<th>IL-7RcKOxBcl2 Tg</th>
<th>Bcl-2 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25</td>
<td>2.4</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Foxp3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Cell number

0
2
4
6
8
10
12
14

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IL-7cKO</th>
<th>IL-7RcKOBcl2 Tg</th>
<th>Bcl2 Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* n.s.
**
***

(>10^5)

Foxp3^CD4SP

CTLA4

GITR

Isotype control
	Control
	IL-7RcKO
Fig. S6.

A  Spleen  Control  IL-7RCKO  IL-7RCKO

Gated on CD1d-tet

CD1d-tet+  TCRβ  CD44  CD8

0.33  0.22  0.15  0.22

B  Control  IL-7RCKO  IL-7RCKO

Gated on CD1d-tet

CD1d-tet+  TCRβ  CD44  CD8

0.15  0.08  0.07  0.05

Cell number (x10^5)

Control  IL-7RCKO  Bcl-2 Tg  Bcl-2 Tg

0  1  2  3

n.s.

n.s.

n.s.

n.s.
Fig. S7.

A

Control  IL-7RcKO
NK1.1  γδ TCR
0.07  0.05
0.28  0.31

B

CD3 NK1.1^ (NK cells)
Control  IL-7RcKO
Cell number (x10^5)
0  2  4  6  8  10  12

C

Control  IL-7RcKO
CD19
Cell number (x10^5)
0  2.2  0.12  0.46

D

Control  IL-7RcKO
B220
Cell number (x10^5)
0  3.5  59  23

E

γδ T cells
Control  IL-7RcKO
23.7  24.5

pDC

Control  IL-7RcKO
BrdU
9.7  10.3

F

B cell (B220^CD19^)
Control  IL-7RcKO
67.6  27.3

pDC (B220^CD11c^)
Control  IL-7RcKO

IL-7R
Fig. S8.

A Gated on TCRβHSAlow

Control IL-7RcKO

CD4 CD4SP

B Relative level of pim-1 and p27 mRNA

Control IL-7RcKO

CD4SP CD8SP CD4SP CD8SP

n.s. n.s. n.s.