Conditional deletion of cytokine receptor chains reveals that IL-7 and IL-15 specify CD8 cytotoxic lineage fate in the thymus

Tom M. McCaughtry,[1] Ruth Etzensperger,[1] Amala Alag,[1] Xuguang Tai,[1] Sema Kurtulus,[2] Jung-Hyun Park,[1] Alex Grinberg,[3] Paul Love,[3] Lionel Feigenbaum,[4] Batu Erman,[2] and Alfred Singer[1]

[1]Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
[2]Biological Sciences and Bioengineering Program, Faculty of Engineering and Natural Sciences, Sabanci University, Istanbul 34956, Turkey
[3]Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, Bethesda, MD 20892
[4]SAIC-Frederick, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702

The thymus generates T cells with diverse specificities and functions. To assess the contribution of cytokine receptors to the differentiation of T cell subsets in the thymus, we constructed conditional knockout mice in which IL-7Rα or common cytokine receptor γ chain (γ_c) genes were deleted in thymocytes just before positive selection. We found that γ_c expression was required to signal the differentiation of MHC class I (MHC-I)–specific thymocytes into $CD8^+$ cytotoxic lineage T cells and into invariant natural killer T cells but did not signal the differentiation of MHC class II (MHC-II)–specific thymocytes into $CD4^+$ T cells, even into regulatory $Foxp3^+CD4^+$ T cells which require γ_c signals for survival. Importantly, IL-7 and IL-15 were identified as the cytokines responsible for $CD8^+$ cytotoxic T cell lineage specification in vivo. Additionally, we found that small numbers of aberrant $CD8^+$ T cells expressing *Runx3d* could arise without γ_c signaling, but these cells were developmentally arrested before expressing cytotoxic lineage genes. Thus, γ_c-transduced cytokine signals are required for cytotoxic lineage specification in the thymus and for inducing the differentiation of MHC-I–selected thymocytes into functionally mature T cells.

CORRESPONDENCE
Alfred Singer:
singera@nih.gov

Abbreviations used: cKO, conditional KO; DN, double negative; DP, double positive; γ_c, common gamma chain; iNKT cell, invariant NK T cell; Int, intermediate; SP, single positive.

T cell development in the thymus is an intricate process that requires coordinate integration of TCR and cytokine receptor signaling. Signals transduced by TCR components regulate the transition of thymocytes through two critical developmental checkpoints in the thymus. At the first checkpoint, signaling by pre-TCR induces $CD4^-CD8^-$ double-negative (DN) stage 3 (DN3) thymocytes to differentiate into DN4 thymocytes and, at the second checkpoint, signaling by fully assembled αβ-TCR induces $CD4^+CD8^+$ double-positive (DP) thymocytes to differentiate into mature single-positive (SP) T cells (Starr et al., 2003). In contrast, the importance of cytokine receptors for transduction of differentiative signals in αβ thymocytes is less certain and remains controversial, even though IL-7 signaling is known to be required for γδ T cell development (Maki et al., 1996; Candéias et al., 1997). Although it has been a long held perspective that essentially all aspects of αβ thymocyte development are a result of the quality, quantity, or duration of TCR signaling (Singer et al., 2008), we have proposed that cytokine receptor signals, not TCR signals, specifically induce MHC-I–selected DP thymocytes to adopt the CD8 cytotoxic lineage fate (Brugnera et al., 2000; Yu et al., 2003; Park et al., 2010).

T.M. McCaughtry's present address is Dept. of Respiratory, Inflammation, and Autoimmunity, MedImmune, LLC, Gaithersburg, MD 20878.

S. Kurtulus' present address: Division of Immunobiology, Dept. of Pediatrics, University of Cincinnati College of Medicine and Children's Hospital Medical Center, Cincinnati, OH 45229.

The Rockefeller University Press $30.00
J. Exp. Med. 2012 Vol. 209 No. 12 2263-2276
www.jem.org/cgi/doi/10.1084/jem.20121505

Because preselection DP thymocytes do not express IL-7Rα and are highly refractory to cytokine stimulation (Yu et al., 2006), survival of DP thymocytes exclusively depends on signaling by their TCR which initiates a sequence of developmental steps referred to as positive selection. Positive selection restores cytokine responsiveness in signaled DP thymocytes by inducing both up-regulation of IL-7Rα and down-regulation of SOCS1 (suppressor of cytokine signaling 1; Chong et al., 2003; Yu et al., 2006). Most positively selected thymocytes then differentiate into either MHC-II–selected $CD4^+$ T helper lineage cells or MHC-I–selected $CD8^+$ cytotoxic lineage cells before emigrating out of the thymus. Our understanding of CD4 versus CD8 lineage commitment has been enhanced by the discoveries of Th-POK and Runx3 as key transcription factors, with Th-POK expression promoting differentiation into CD4 T cells and Runx3 promoting differentiation into CD8 T cells (Taniuchi et al., 2002; He et al., 2005; Sun et al., 2005; Egawa et al., 2007; Egawa and Littman, 2008; Wang et al., 2008). However, it is important to know which cell surface receptors induce positively selected thymocytes to express these different transcription factors and to pursue different lineage fates.

Based on data obtained from multiple experimental approaches, we have suggested that γ_c-dependent cytokines, such as IL-7, can signal MHC-I–selected thymocytes to differentiate into CD8 cytotoxic lineage T cells but are not involved in differentiation of MHC-II–selected thymocytes into CD4 helper lineage T cells (Brugnera et al., 2000; Yu et al., 2003; Park et al., 2010). Differences in the cytokine signaling requirement of MHC-I– and MHC-II–selected thymocytes is a key concept of the kinetic signaling model of T cell development which postulates that cytokine receptor signals specify the lineage fate of MHC-I–selected thymocytes, whereas TCR signals specify the lineage fate of MHC-II–selected thymocytes (Singer et al., 2008). Unfortunately, it has not previously been possible to directly assess the cytokine signaling requirements of positively selected thymocytes in vivo because germline deletion of either γ_c or IL-7Rα impairs T cell development before positive selection at the early DN stage (Cao et al., 1995; Di Santo et al., 1995, 1999).

Consequently, to assess the role of γ_c-dependent cytokine signaling during positive selection, we have now generated conditional KO (cKO) mice in which γ_c or IL-7Rα genes could be deleted after the DN stage in preselection DP thymocytes so that cytokine receptor expression on early thymocytes would be unaffected but positively selected thymocytes would lack either γ_c or IL-7Rα cytokine receptors. By using these novel mice, this study reveals that γ_c expression during positive selection is essential to signal the in vivo differentiation of MHC-I–selected thymocytes into CD8 cytotoxic lineage T cells and into invariant NK T cells (iNKT cells) but is not required to signal in vivo differentiation of MHC-II–selected thymocytes into mature CD4 T cells, even regulatory $Foxp3^+CD4$ T cells which require γ_c-dependent cytokine signals for survival. This study also identifies IL-7 and IL-15 as the cytokines responsible for cytotoxic lineage specification in vivo. Thus, this study documents that γ_c-transduced signals are either critical or dispensable for in vivo thymocyte differentiation depending on the MHC specificity of their TCR.

RESULTS

Importance of IL-7Rα and γ_c expression during positive selection in the thymus

The IL-7 receptor is composed of two chains, the cytokine receptor common gamma chain (γ_c; CD132) and the IL-7Rα chain (CD127), and promotes proliferation, differentiation, and survival of T cell precursor cells in the thymus (Murray et al., 1989; Peschon et al., 1994; Candéias et al., 1997; Tan et al., 2001). γ_c is expressed in varying amounts on all thymocytes at different stages of development, whereas IL-7Rα is expressed on early and late stage thymocytes but is extinguished in preselection DP thymocytes (Fig. 1, middle and bottom, black lines). IL-7Rα is reexpressed during positive selection on thymocytes at the intermediate (Int; $TCR^{hi}CD4^+CD8^{lo}$) stage of development and it is in Int stage thymocytes that CD4 versus CD8 lineage specification occurs (Suzuki et al., 1995; Brugnera et al., 2000; Cibotti et al., 2000; Bosselut et al.,

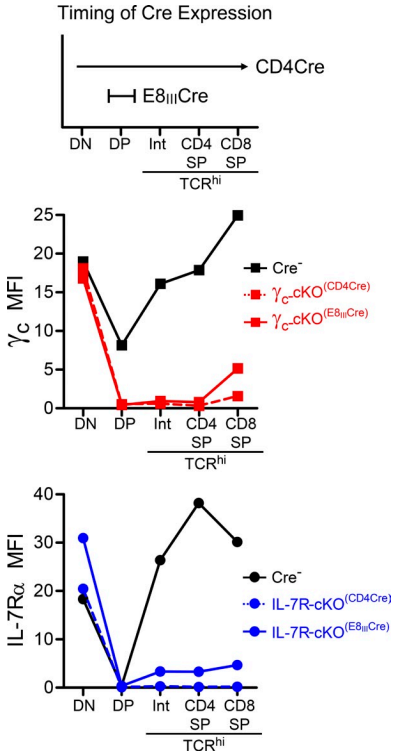

Figure 1. γ_c and IL-7Rα expression on thymocytes from wild-type and cKO mice. (Top) Schematic of CD4Cre and $E8_{III}Cre$ expression in developing thymocytes. Surface expression of γ_c (middle) and IL-7Rα (bottom) was determined on thymocytes from WT (Cre^-) or cKO (Cre^+) mice using CD4Cre (colored dashed lines) or $E8_{III}Cre$ (colored solid lines). Total thymocytes were stained for CD4, CD8α, TCR-β, and γ_c or IL-7Rα and gated on the indicated thymocyte populations. MFI of γ_c and IL-7Rα staining are shown. Data are representative of four independent experiments.

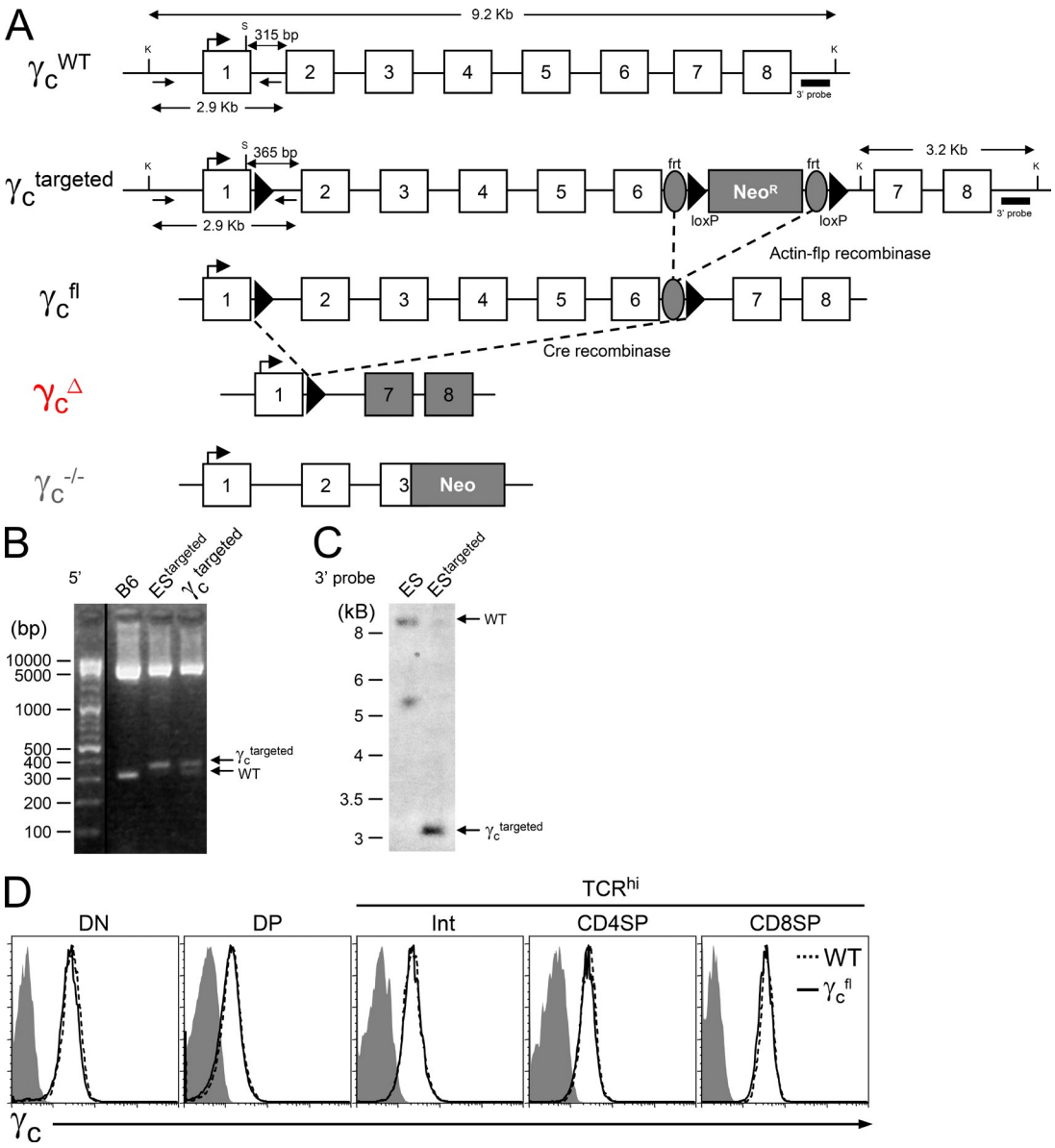

Figure 2. Generation of γ_c-cKO mice. (A) Targeting strategy to generate γ_c-cKO allele. Numbered boxes = exons, gray boxes = out of frame exons as a result of Cre-mediated deletion. Neo^R = neomycin resistance cassette. Black triangles = loxP sites. Gray ovals = frt sites. K, S = Kpn1 and Sac1 restriction sites, respectively. Locations of PCR primers are indicated with facing arrows. Location of 3′ probe for Southern blot is indicated. Configuration of the γ_c germline KO allele used in this study is shown for comparison. (B) PCR confirmation of proper integration of the 5′ end of construct in ES cells and genomic DNA from female knockin offspring. PCR amplification of a 2.9 Kb fragment flanking exon 1 and loxP integration site followed by Sac1 restriction digest reveals a 315 bp WT band and 365 bp targeted band as indicated in A. ES cells have only one targeted band because they are derived from male origin and thus have only one X chromosome. (C) Southern blot confirmation of proper integration of 3′ end of construct. Kpn1 restriction digest results in 9.2 Kb WT fragment and 3.2 Kb targeted fragment as depicted in A. (D) Confirmation that after Actin-flp recombinase-mediated deletion of the Neo^R cassette, expression of the γ_c^{fl} allele is equal to WT γ_c expression in the absence of Cre throughout T cell development.

2003; Singer et al., 2008; Adoro et al., 2012), with IL-7R transduced signals thought to specify the CD8 lineage fate (Yu et al., 2003; Park et al., 2010). In this study, we wished to assess the importance of IL-7Rα and γ_c expression during positive selection for lineage fate specification in vivo. Because germline deletion of either γ_c or IL-7Rα impairs development at the DN stage (Cao et al., 1995; Di Santo et al., 1995, 1999), we constructed γ_c and IL-7Rα floxed alleles which could be conditionally deleted by Cre recombinase in DP thymocytes without reducing their expression in earlier TCR-β$^-$ DN thymocytes (Fig. 2 and Fig. 3). We refer to Cre$^+$ mice with floxed alleles as cKO (γ_c-cKO and IL-7Rα–cKO) mice, and we refer to their deleted alleles, and cells with deleted alleles, as γ_c^{Δ} and IL-7Rα$^{\Delta}$.

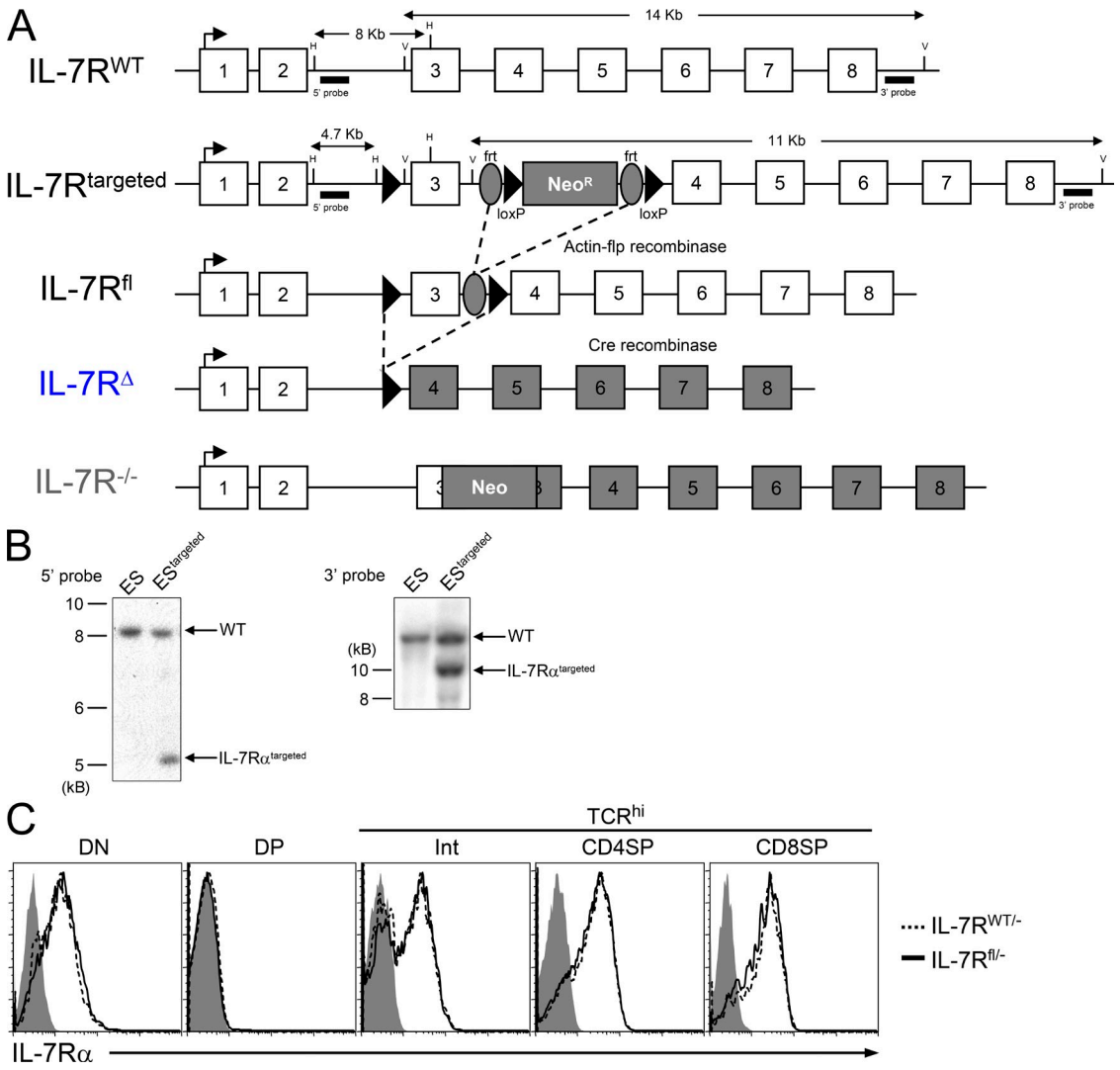

Figure 3. Generation of IL-7Rα-cKO mice. (A) Targeting strategy to generate IL-7Rα-cKO allele. Numbered boxes = exons, gray boxes = out of frame exons as a result of Cre-mediated deletion. Neo^R = neomycin resistance cassette. Black triangles = loxP sites. Gray ovals = frt sites. H, V = HindIII and EcoRV restriction sites, respectively. Location of 5′ and 3′ probes for Southern blot is indicated. Configuration of the IL-7Rα germline KO allele used in this study is shown for comparison sake. (B) Southern blot confirmation of proper integration of 5′ and 3′ ends of targeting construct in ES cells. HindIII restriction digest results in an 8 Kb WT fragment and a 4.7 Kb targeted fragment detected with the 5′ probe. EcoRV restriction digest results in a 14 kB WT fragment and a 11 kB targeted fragment detected with the 3′ probe as depicted in A. (C) Confirmation that after Actin-flp recombinase-mediated deletion of the Neo^R cassette, expression of the IL-7Rα^{fl} allele is equal to WT IL-7Rα expression in the absence of Cre throughout T cell development.

We began our study using two different Cre transgenes, CD4Cre and $E8_{III}$Cre (Lee et al., 2001; Park et al., 2010). The CD4Cre transgene is controlled by CD4 enhancer/promoter elements so Cre expression first begins in late DN thymocytes, persists throughout thymocyte development, and continues in mature T cells (Fig. 1, top). CD4Cre has been commonly used and is known to effectively delete floxed genes, but has the disadvantage that it is continuously expressed in thymocytes and T cells so it is difficult to be certain if Cre-mediated deletions occurred before, during, or after thymocyte positive selection and lineage specification. In contrast, the $E8_{III}$Cre transgene is regulated by $E8_{III}$-CD8α enhancer/promoter elements that limit Cre expression exclusively to preselection DP thymocytes (Fig. 1, top; Park et al., 2010). Because $E8_{III}$Cre expression is limited to preselection DP thymocytes, Cre is present in only a brief, well defined developmental timeframe before lineage specification.

We introduced the CD4Cre and $E8_{III}$Cre transgenes into $\gamma_c^{fl/-}$ and IL-7R$\alpha^{fl/-}$ mice to generate γ_c^{Δ} and IL-7Rα^{Δ} deleted alleles (Fig. 2 and Fig. 3). We found that neither CD4Cre nor $E8_{III}$Cre reduced expression of γ_c or IL-7Rα on TCR-β^- DN thymocytes, but both Cre transgenes induced deletions in DP and postselection Int ($TCR^{hi}CD4^+CD8^{lo}$) and SP thymocytes, with a few more cells escaping deletion with $E8_{III}$Cre than CD4Cre (Fig. 1). Importantly, Cre-mediated deletions did not alter total thymic cellularity (not depicted)

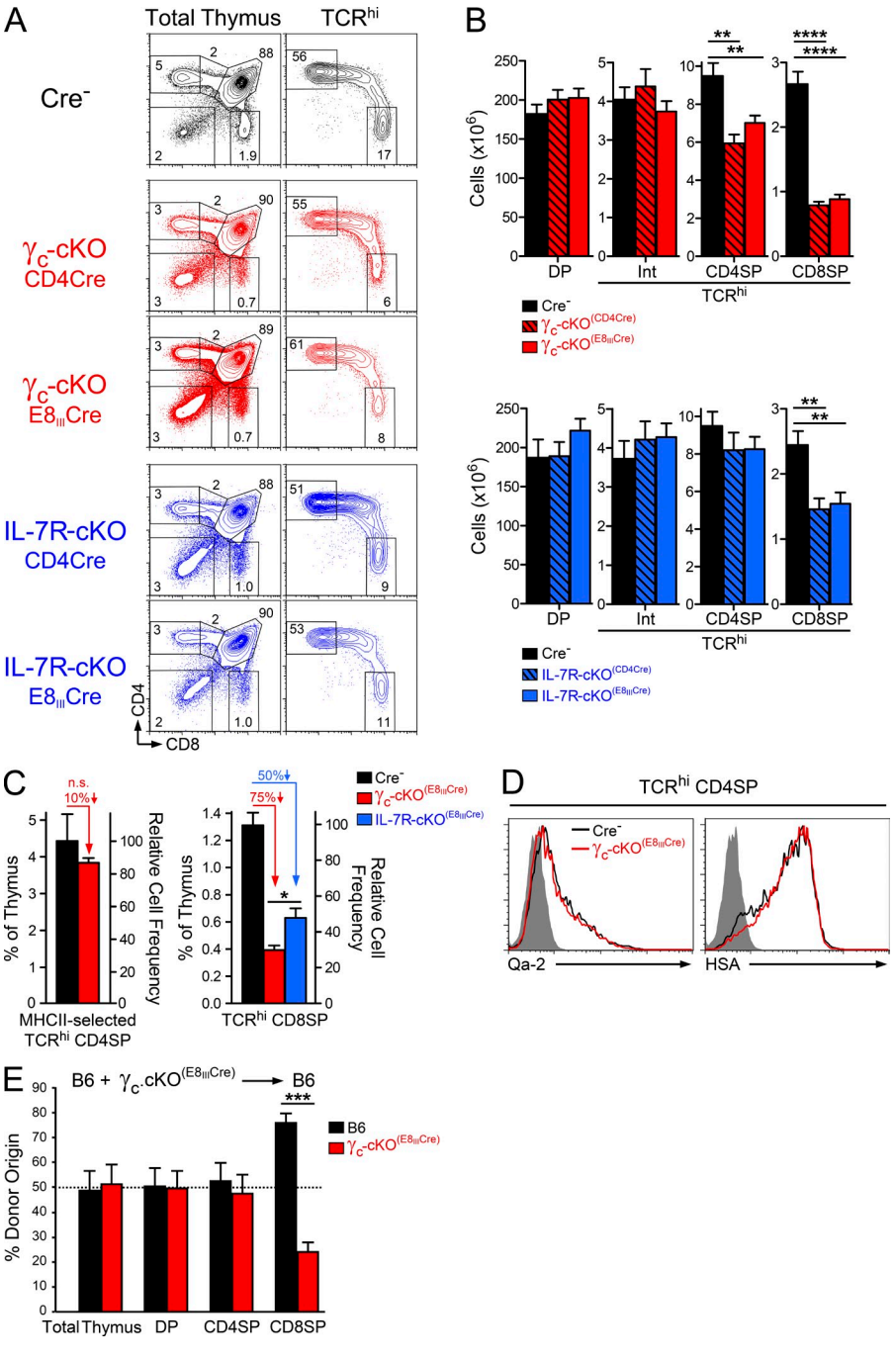

Figure 4. Impact on thymocyte development of targeted deletions of γ_c or IL-7Rα genes. (A) CD4 versus CD8α profiles of total thymus (left) and gated TCR-β^{hi} thymocytes (right) from Cre$^-$ control (black), γ_c-cKO (red), and IL-7Rα–cKO (blue) mice. FACS profiles for both CD4Cre and E8$_{III}$Cre-mediated conditional deletion are shown. Numbers indicate frequencies of cells in gates. Data are representative of four independent experiments. (B, Top) Absolute thymocyte numbers for DP, TCR-β^{hi} Int, TCR-β^{hi} CD4SP, and TCR-β^{hi} CD8SP populations are shown for γ_c-cKO (red) compared with Cre$^-$ control mice (black). Cell numbers for both CD4Cre and E8$_{III}$Cre mice are shown. (B, Bottom) Absolute thymocyte numbers for DP, TCR-β^{hi} Int, TCR-β^{hi} CD4SP, and TCR-β^{hi} CD8SP populations are shown for IL-7Rα–cKO (blue) compared with Cre$^-$ control mice (black). Cell numbers for both CD4Cre and E8$_{III}$Cre mice are shown. Cell numbers were calculated by gating specifically on cells that had deleted γ_c or IL-7Rα. Data represent the mean of 4–13 individual male and female mice aged 5–9 wk from at least four independent experiments. Error bars represent SEM, and all statistically significant changes are marked with asterisks: **, $P < 0.01$; ****, $P < 0.0001$. Any comparisons not marked with asterisks were not significant ($P > 0.05$). (C, Left) Frequency of MHC-II–selected TCR-β^{hi} CD4SP thymocytes from $\beta2m^{-/-}$ γ_c-cKO mice (red) compared with Cre$^-$ control mice (black). γ_c-cKO-derived cells were specifically gated for those that were γ_c-deleted. Data are depicted as frequencies of total thymus on the left y-axis and relative frequencies normalized to Cre$^-$ control on the right y-axis of the histogram plot. Data for E8$_{III}$Cre mice are shown. (C, Right) Frequency of TCR-β^{hi} CD8SP thymocytes from γ_c-cKO mice (red), IL-7Rα–cKO mice (blue), and Cre$^-$ control mice (black) are shown. γ_c-cKO and IL-7 Rα-cKO-derived cells were specifically gated for those that were γ_c- or IL-7Rα–deleted. Data are depicted as frequencies of total thymus on the left y-axis and relative frequencies normalized to Cre$^-$ control on the right y-axis of the histogram plot. Data for E8$_{III}$Cre mice are shown. Means of at least three individual mice are shown, and error bars represent SEM. *, $P < 0.05$; n.s., not significant. (D) Expression of Qa-2 and HSA on TCR-β^{hi} CD4SP thymocytes from γ_c-cKO mice (red; specifically gated on γ_c-deleted cells) and Cre$^-$ control mice (black). Shaded histograms represent negative staining control. Data are representative of five independent experiments. (E) Frequency of donor-derived thymocytes from mixed bone marrow chimeras. Mixed bone marrow chimeras were made by reconstituting lethally irradiated mice with a 1:1 mixture of T cell–depleted bone marrow from wild-type and γ_c-cKO mice. After 8 wk, mice were analyzed for the composition of donor-derived cells within the indicated thymocyte subset using congenic markers to discriminate cells derived from each donor. γ_c-cKO–derived cells were specifically gated for those that were γ_c-deleted. The dashed horizontal line indicates the expected frequency of cells derived from each donor. Means of five individual mice are shown, and error bars represent SEM. ***, $P = 0.0001$.

and did not reduce the number of either DP or TCR^{hi} Int thymocytes (Fig. 4, A and B). However, Cre-mediated deletions significantly reduced the number and frequency of TCR^{hi} thymocytes appearing as CD4SP and CD8SP cells (Fig. 4, A and B). Because both Cre transgenes had similar effects, we decided to exclusively use the E8$_{III}$Cre transgene

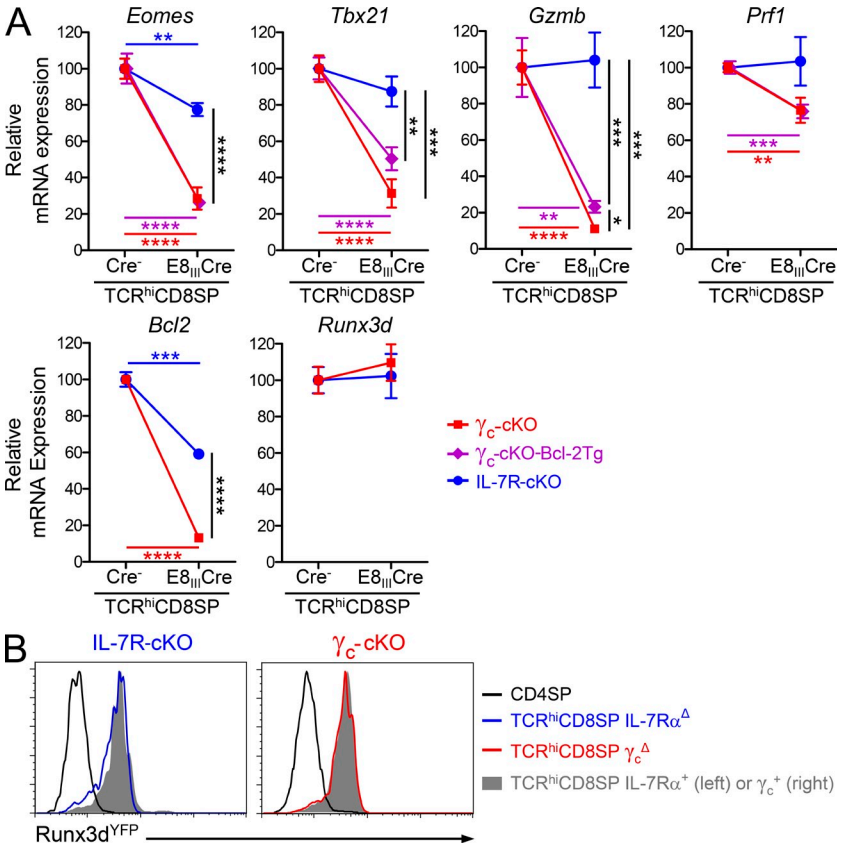

Figure 5. Analysis of CD8 lineage gene expression. (A) Relative mRNA expression of the indicated genes. TCR-β^{hi} CD8SP thymocytes were sorted from Cre$^-$ control mice and TCR-β^{hi} CD8SP thymocytes gated specifically on IL-7Rα–deleted (blue circles) or γ_c-deleted (red squares and purple diamonds) were sorted from $E8_{III}$Cre cKO mice. RNA was isolated and expression of the indicated genes was analyzed by real-time PCR. Purple diamonds indicate γ_c-cKO mice with transgenic expression of Bcl-2. Data are normalized to *Rpl13A* and Cre$^-$ controls. Means of at least three individual mice are shown, and error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (B) Runx3d^{YFP} reporter expression in IL-7Rα–deleted and γ_c-deleted cells. Total thymocytes from IL-7Rα–cKO Runx3d$^{YFP/wt}$ (left) and γ_c-cKO Runx3d $^{YFP/wt}$ (right) were stained for CD4, CD8α, TCR-β and IL-7Rα or γ_c expression. Runx3d^{YFP} reporter expression is compared between TCR-β^{hi} CD4SP cells (black line) and TCR-β^{hi} CD8SP gated on IL-7Rα-deleted (blue line) and IL-7Rα-positive cells (escapees, gray shaded; left), and TCR-β^{hi} CD8SP gated on γ_c-deleted (red line) and γ_c-positive cells (escapees, gray shaded; right). Data are representative of three independent experiments.

for all subsequent experiments so that deletions unequivocally occurred in preselection DP thymocytes before positive selection and lineage specification.

Notably, this analysis necessarily overestimated the impact of γ_c and IL-7Rα deletions on CD4 lineage thymocytes because MHC-I–specific CD8 lineage T cells go through the TCR^{hi}CD4$^+$CD8^{lo} Int stage and are thus unavoidably included in the TCR^{hi} CD4SP gate (Suzuki et al., 1995; Brugnera et al., 2000; Cibotti et al., 2000; Bosselut et al., 2003; Singer et al., 2008; Adoro et al., 2012). To eliminate MHC-I–specific thymocytes from the CD4SP gate, we made γ_c-cKO mice additionally deficient in *B2m* (Fig. 4 C). We observed that deletion of γ_c reduced the number of MHC-II–specific CD4SP thymocytes by only ∼10%, which was not statistically significant, (Fig. 4 C), but it reduced numbers of CD8SP thymocytes by 75% ($P < 0.0001$; Fig. 4 C). Importantly, the undiminished number of CD4SP thymocytes in γ_c-cKO mice was not a result of accumulation of mature thymocytes because of reduced emigration because expression of the maturation markers Qa-2 and HSA (McCaughtry et al., 2007) was unchanged (Fig. 4 D). Moreover, construction of competitive mixed donor bone marrow chimeras revealed no competitive disadvantage of γ_c deficiency by γ_c-cKO CD4SP thymocytes compared with their wild-type counterparts (Fig. 4 E). Thus, deficiency of either γ_c or IL-7Rα during positive selection substantially impaired generation of CD8 lineage thymocytes but only minimally affected generation of CD4 lineage thymocytes.

Impact of IL-7Rα and γ_c on generation of CD8 cytotoxic lineage cells in the thymus

Having determined that IL-7Rα deficiency and γ_c deficiency during positive selection quantitatively reduced generation of CD8SP thymocytes, we then assessed if IL-7Rα and γ_c were important for initiating the cytotoxic lineage program. To do so, we examined expression of cytotoxic lineage-specific genes in purified TCR^{hi} CD8SP thymocytes from IL-7Rα–cKO and γ_c-cKO mice that were electronically sorted to exclude all escapees of Cre-mediated deletion and to include only CD8SP thymocytes with deleted IL-7Rα$^\Delta$ or γ_c^Δ alleles. We specifically focused on expression of Eomesodermin (*Eomes*) and T-bet (*Tbx21*) because these genes encode the defining transcription factors for the cytotoxic lineage program (Pearce et al., 2003; Intlekofer et al., 2005, 2008), and we included expression of Granzyme B (*Gzmb*) and Perforin (*Prf1*) because these genes are associated with cytotoxic capability (Heusel et al., 1994; Kägi et al., 1994; Fig. 5 A, top row).

Comparison of cytotoxic lineage gene expression in CD8SP thymocytes from $E8_{III}$Cre$^-$ and $E8_{III}$Cre$^+$ mice revealed that IL-7Rα–deficient CD8SP thymocytes from IL-7Rα–cKO mice expressed cytotoxic lineage genes at comparable levels to wild-type CD8SP thymocytes from $E8_{III}$Cre$^-$ mice (Fig. 5 A, top row), documenting that CD8 cytotoxic lineage thymocytes could be generated in vivo in the absence of IL-7R–transduced signals. In marked contrast, γ_c-deficient CD8SP thymocytes from γ_c-cKO mice were

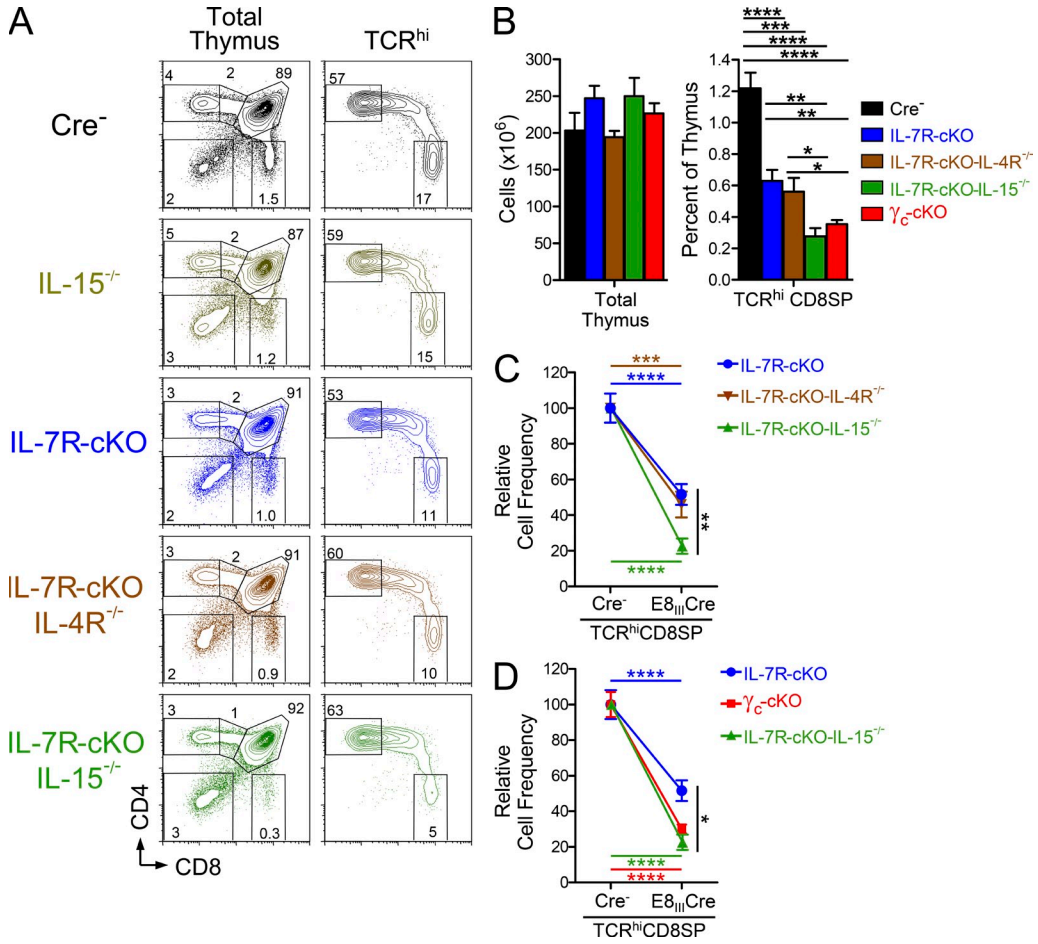

Figure 6. Contribution of IL-15 to generation of CD8 lineage cells. (A) Thymocyte profiles resulting from $E8_{III}$Cre-mediated deletion of IL-7Rα alone or in combination with IL-4Rα or IL-15 germline deficiency. Total thymocytes were analyzed for expression of CD8α, CD4, and TCR-β from Cre^- control (black), germline $IL\text{-}15^{-/-}$ (olive green), $E8_{III}$Cre IL-7R-cKO (blue), IL-7R-cKO combined with germline $IL\text{-}4R^{-/-}$ (brown) or $IL\text{-}15^{-/-}$ (bright green) mice. Data are representative of at least three independent experiments. (B) Total thymocyte number and frequency of TCR-β^{hi} CD8SP thymocytes from mice in A shown in comparison to γ_c-cKO mice (red). γ_c-cKO– and IL-7Rα–cKO-derived cells were specifically gated for those that were γ_c- or IL-7Rα–deleted. Data represent the means after pooling at least six individual mice from at least three independent experiments. (C) Relative frequency of TCR-β^{hi} CD8SP thymocytes after $E8_{III}$Cre-mediated deletion of IL-7Rα alone (blue line) or in combination with germline deletion of IL-4Rα (brown line) or IL-15 (green line). IL-7Rα–deleted TCR-β^{hi} CD8SP thymocyte frequencies were analyzed from total thymocytes. Data are shown as frequencies normalized to Cre^- controls. Data represent the mean after pooling at least eight individual mice from at least two independent experiments. (D) Relative frequency of IL-7Rα–deleted TCR-β^{hi} CD8SP thymocytes resulting from deletion of IL-7Rα alone (blue line) or in combination with IL-15 (green line) compared with frequency of γ_c-deleted TCR-β^{hi} CD8SP thymocytes from γ_c-cKO mice (red line). Data were obtained as for Fig. 6 C. Data represent the mean of at least six individual mice from at least three independent experiments. (B–D) Error bars represent SEM. All statistically significant changes are marked with asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Any comparisons not marked with asterisks were not significant.

not cytotoxic lineage cells because they failed to express most cytotoxic lineage genes (Fig. 5 A, top row). Indeed, γ_c-deficient CD8SP thymocytes had very low expression of *Eomes*, *Tbx21*, or *Gzmb* and contained reduced levels of *Prf1* mRNA (Fig. 5 A, top row). In addition, γ_c-deficient CD8SP thymocytes contained little, if any, *Bcl-2* mRNA, so it was possible that these cells died before they could express cytotoxic lineage genes (Fig. 5 A, bottom row). However, this was not the case because γ_c-deficient CD8SP thymocytes from γ_c-cKO–Bcl-2 transgenic (Bcl-2Tg) mice that overexpress the human prosurvival protein Bcl-2 still failed to express cytotoxic lineage genes (Fig. 5 A, top row). Thus, expression of γ_c during positive selection was essential for generating cytotoxic lineage CD8 T cells, but expression of IL-7Rα was not.

We then examined expression of *Runx3d*, which is considered to be a master regulator of CD8 lineage T cells (Taniuchi et al., 2002; Ehlers et al., 2003; Woolf et al., 2003; Egawa et al., 2007; Egawa and Littman, 2008). IL-7Rα–deficient CD8SP thymocytes expressed *Runx3d* mRNA at undiminished levels (Fig. 5 A, bottom row), which was concordant with their expression of cytotoxic lineage-specific mRNAs. Much to our surprise, however, γ_c-deficient CD8SP thymocytes also contained undiminished levels of *Runx3d* mRNA (Fig. 5 A, bottom row), but, in this case, *Runx3d* mRNA expression was markedly

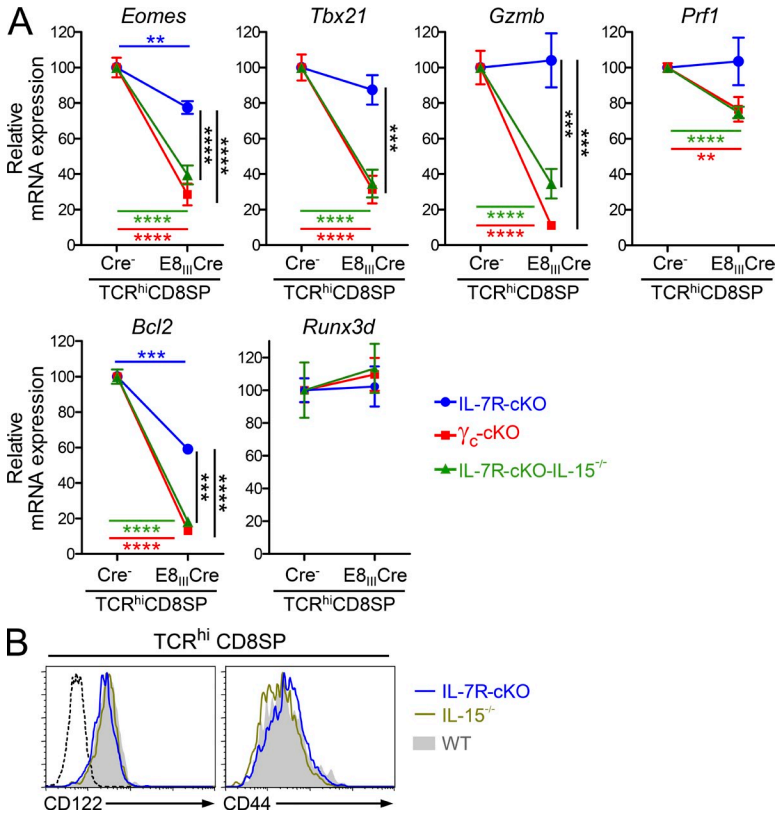

Figure 7. IL-15 is required for induction of cytotoxic lineage genes in IL-7Rα–cKO thymocytes. (A) Relative mRNA expression of the indicated genes. IL-7Rα–cKO and γ_c-cKO data from Fig. 5 A are shown compared with TCR-β^{hi} CD8SP thymocytes sorted from IL-7Rα–cKO–IL-15$^{-/-}$ mice by gating specifically for IL-7Rα–deleted thymocytes. Data are normalized to *Rpl13A* and Cre$^-$ controls. Means of at least three mice are shown, and error bars represent SEM. All statistically significant changes are marked with asterisks: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Any comparisons not marked with asterisks were not significant. (B) Expression of CD122 and CD44 on IL-7Rα–deleted TCR-β^{hi} CD8SP thymocytes from IL-7Rα–cKO (blue line) and IL-7Rα–cKO–IL-15$^{-/-}$ (olive green line) mice compared with WT (shaded histograms). Dotted line represents negative staining control. Data are representative of three independent experiments.

discordant with absent expression of cytotoxic lineage-specific genes. That is, γ_c-deficient CD8SP thymocytes did not become cytotoxic lineage cells even though they expressed *Runx3d*.

Because the disconnect between expression of *Runx3d* and cytotoxic lineage genes in γ_c-deficient CD8SP thymocytes was unexpected, we independently examined *Runx3d* expression using the Runx3d-YFP reporter (Egawa and Littman, 2008). We bred the Runx3d-YFP reporter allele into both IL-7Rα–cKO and γ_c-cKO mice and found that both IL-7Rα–deficient and γ_c-deficient CD8SP thymocytes expressed Runx3d-YFP at similar levels to normal CD8SP thymocytes (Fig. 5 B), confirming that CD8SP thymocytes could express Runx3d without becoming cytotoxic lineage cells.

Based on these results, we conclude that: (1) γ_c expression is essential for cytotoxic lineage specification; (2) a small minority of aberrant CD8SP thymocytes can express Runx3d in the absence of γ_c-transduced signals, but such CD8 cells are arrested in their development before expression of cytotoxic lineage genes; (3) in the absence of γ_c, Runx3d fails to induce cytotoxic lineage gene expression; and (4) there exists a γ_c-dependent cytokine that can signal generation of CD8 cytotoxic lineage thymocytes independently of IL-7Rα.

Contribution of IL-15 to generation of CD8 cytotoxic lineage cells

Although deficiency of either γ_c or IL-7Rα during positive selection impaired the generation of CD8SP thymocytes, it was evident that γ_c deficiency had a greater quantitative effect than IL-7Rα deficiency. Indeed, CD8SP thymocyte numbers were reduced 75% by γ_c deficiency but were only reduced 50% by IL-7Rα deficiency (Fig. 4 C). These findings supported the concept that, in addition to IL-7, the thymus must contain a γ_c-dependent cytokine that induces CD8 cytotoxic lineage specification independently of IL-7Rα.

We first examined the possibility that IL-4 was the unknown γ_c-dependent cytokine that signaled CD8SP thymocytes independently of IL-7Rα. Because all thymocytes express surface IL-4Rα (Yu et al., 2006), we bred IL-7Rα–cKO mice with IL-4Rα$^{-/-}$ mice to generate mice whose positively selected thymocytes were deficient in both IL-7Rα and IL-4Rα expression. However, IL-4Rα deficiency did not further reduce CD8SP thymocytes below that induced by IL-7Rα deficiency alone (Fig. 6, A–C). Thus, IL-4 was not the γ_c-dependent cytokine that signaled CD8SP thymocytes independently of IL-7Rα

We next examined the possibility that IL-15 was the unknown γ_c-dependent cytokine we were seeking by breeding IL-7Rα–cKO mice with IL-15$^{-/-}$ mice. As previously reported, deficiency of IL-15 alone did not reduce the generation of conventional CD8SP thymocytes (Dubois et al., 2006). Impressively, however, IL-7Rα/IL-15 double deficiency significantly reduced both the frequency and number of CD8SP thymocytes below that of IL-7Rα deficiency alone (Fig. 6, A–D). Indeed, impairment of CD8SP thymocyte generation as a result of IL-7Rα/IL-15 double deficiency was indistinguishable from that of γ_c deficiency and

resulted in a 75% reduction in both frequency and number of CD8SP thymocytes (Fig. 6, A–D). Moreover, the CD8SP thymocytes that were generated in IL-7Rα/IL-15 double-deficient mice were not cytotoxic lineage cells because they displayed very low expression of *Eomes*, *Tbx21*, *Gzmb*, and *Prf1* mRNA (Fig. 7 A). Thus, IL-7Rα/IL-15 double deficiency replicated γ_c deficiency, identifying IL-15 as the γ_c-dependent cytokine that induced the generation CD8 cytotoxic lineage T cells independently of IL-7Rα.

IL-15 is known to signal CD8 T cells that display a CD44^{hi}CD122^{hi} memory phenotype (Dubois et al., 2006). Consequently, we wondered if the CD8SP thymocytes that were induced by IL-15 during positive selection in IL-7Rα–cKO mice also displayed a memory phenotype. Contrary to this possibility, examination of CD8SP thymocytes induced by IL-15 in IL-7Rα–cKO mice revealed that they were CD44^{lo}CD122^{lo} phenotypically naive, cytotoxic lineage cells (Fig. 7 B). Thus, IL-15 signals during positive selection induce the generation of naive, not memory, CD8SP thymocytes. We conclude that signaling by IL-7Rα and IL-15 during positive selection induces generation of naive cytotoxic lineage CD8 T cells.

Impact of IL-7Rα and γ_c on peripheral CD8 T cells

We next wished to determine if CD8SP thymocytes generated by IL-15 in IL-7Rα–cKO mice emigrated to the periphery and were maintained by IL-15 (Surh and Sprent, 2005). To do so, we examined peripheral CD8 T cells from IL-7Rα–cKO and IL-7Rα–cKO–IL-15$^{-/-}$ mice. We found that IL-7Rα–deficient CD8 T cells were present in the periphery of IL-7Rα–cKO mice, albeit in reduced numbers, and these cells were IL-15 dependent, as they essentially disappeared in IL-7Rα/IL15 double-deficient mice (Fig. 8 A). Interestingly, IL-15–dependent IL-7Rα–deficient CD8 T cells that displayed a naive phenotype in the thymus also displayed a CD122^{lo}CD44^{lo} naive phenotype in the periphery (Fig. 8 B), indicating that IL-7Rα–deficient CD8 T cells did not acquire a memory phenotype despite peripheral lymphopenia. Therefore, although IL-15 supported the survival of IL-7Rα–deficient cells, IL-15 did not drive the homeostatic expansion of CD8 T cells in the absence of IL-7Rα signaling. Unlike the naive CD8 T cells populating the periphery of IL-7Rα–cKO mice, the periphery of γ_c-cKO mice contained CD8 T cells that were overwhelmingly the progeny of γ_c^+ thymocytes that had escaped γ_c deletion and had undergone lymphopenia-induced homeostatic expansion as revealed by their CD122^{hi}CD44^{hi} memory phenotype (Fig. 8, C and D).

Impact of γ_c on generation of CD4 regulatory and iNKT cells

Finally, we wished to understand the small but distinct effect that γ_c deletion had on the generation of CD4 lineage thymocytes. We considered that γ_c expression, although dispensable for generation of most CD4SP thymocytes, might be important for generation of specific CD4 lineage subsets. Consequently we assessed the effect of γ_c deletion on induction of regulatory and iNKT cell subpopulations in γ_c-cKO mice.

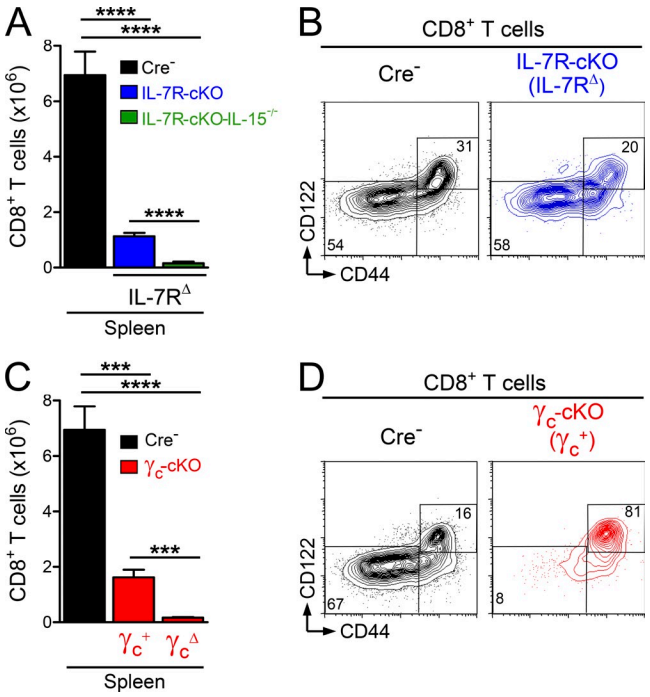

Figure 8. Analysis of peripheral CD8 T cells from IL-7Rα–cKO and γ_c-cKO mice. (A) Absolute cell number of splenic CD8$^+$ T cells. CD8$^+$ T cell numbers were determined from splenocytes for Cre$^-$ control (black bar), IL-7Rα–cKO (blue bar), and IL-7Rα–cKO–IL-15$^{-/-}$ (green bar) mice. CD8$^+$ T cells from IL-7Rα–cKO and IL-7Rα–cKO–IL-15$^{-/-}$ mice were specifically gated on IL-7Rα–deleted cells. Data represent the mean of at least seven individual mice from at least four independent experiments, and error bars represent SEM. ****, $P < 0.0001$. (B) TCR-β$^+$ CD8$^+$ splenocytes were analyzed for frequency of naive and CD44^{hi}CD122^{hi} memory phenotype cells among IL-7Rα–deleted cells (blue) compared with Cre$^-$ controls (black). Data are representative of three independent experiments. (C) Absolute CD8$^+$ T cell numbers were determined from splenocytes from Cre$^-$ control (black bar), and γ_c-cKO (red bars) mice. CD8$^+$ T cells from γ_c-cKO mice were specifically gated on γ_c-undeleted (labeled γ_c^+) or γ_c-deleted (labeled γ_c^Δ) cells. Data represent the mean of at least seven individual mice from at least three independent experiments, and error bars indicate SEM. ***, $P < 0.001$; ****, $P < 0.0001$. (D) TCR-β$^+$ CD8$^+$ splenocytes were analyzed for frequency of naive and CD44^{hi}CD122^{hi} memory phenotype cells among γ_c^+ (escapees) cells from γ_c-cKO mice (red) compared with Cre$^-$ controls (black). Data are representative of three independent experiments.

Analysis of Foxp3 expression revealed that γ_c-deficient CD4SP thymocytes in γ_c-cKO mice were nearly devoid of Foxp3$^+$ cells and that the Foxp3$^+$ CD4SP cells that were present were nearly all escapees of Cre-mediated γ_c deletion (Fig. 9, A and B). Enrichment among Foxp3$^+$ thymocytes of cells that had escaped Cre-mediated γ_c deletion suggested that γ_c signaling either provided a significant survival benefit or was required to induce Foxp3 expression (Burchill et al., 2007). To clarify this issue, we introduced the Bcl-2Tg into γ_c-cKO mice to replace the survival benefit lost by γ_c deficiency. Interestingly, we found that transgenic overexpression of the prosurvival protein Bcl-2 fully restored Foxp3$^+$

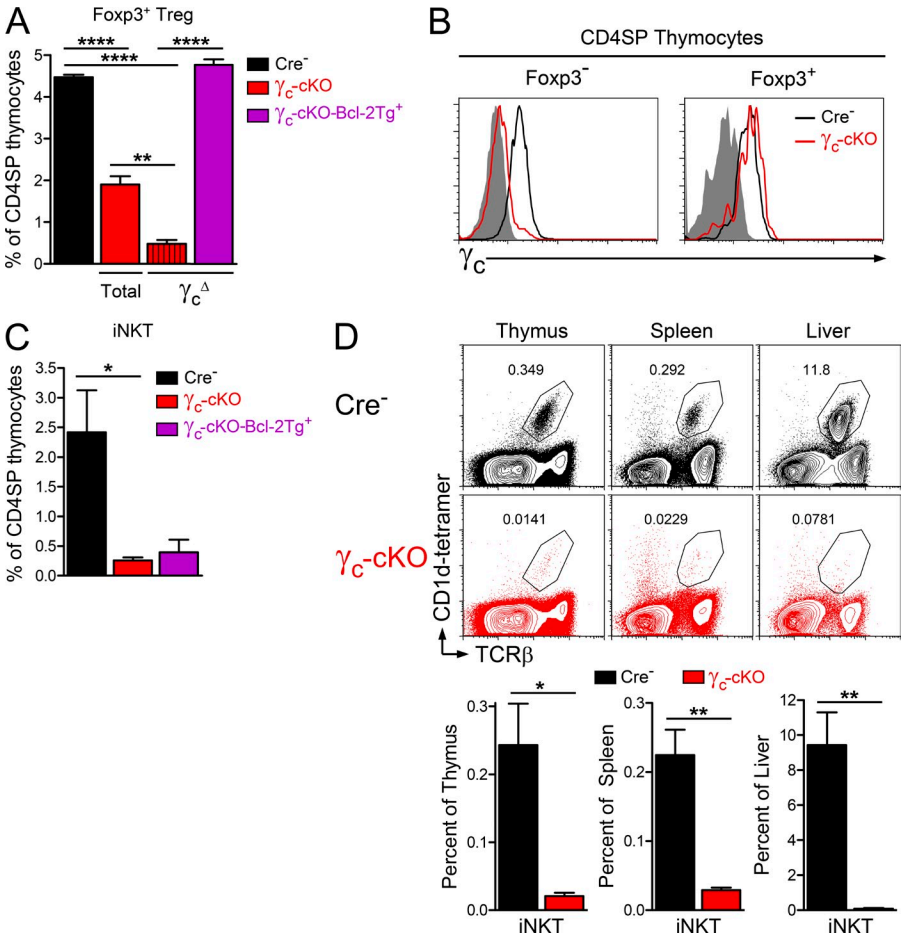

Figure 9. Impact of γ_c deletion on CD4+ regulatory T cells and iNKT cells. (A) TCR-β^{hi} CD4SP thymocytes were analyzed for Foxp3+ cells from Cre− control mice (black bar) and for γ_c-deleted Foxp3+ cells from γ_c-cKO (red bars) and γ_c-cKO-Bcl2 transgenic (purple bar) mice. Total Foxp3+ CD4SP thymocytes from γ_c-cKO mice are shown (plain red) or after specifically gating on γ_c-deleted Foxp3+ CD4SP thymocytes (red with black). Frequencies of Foxp3+ cells among CD4SP cells are shown. Means of at least three mice are shown, and error bars represent SEM. **, $P < 0.01$; ****, $P < 0.0001$. (B) γ_c expression on Foxp3− (non–T reg cells) and Foxp3+ CD4SP thymocytes. CD4SP thymocytes were gated on Foxp3− and Foxp3+ subsets and analyzed for expression of γ_c from γ_c-cKO (red) compared with Cre− control (black) mice. Gray histograms represent negative staining controls. Data are representative of at least three individual mice. (C) CD4SP thymocytes were analyzed for TCR-β+ CD1d-tetramer+ iNKT cells from Cre− control (black bar), γ_c-cKO (red bar), and γ_c-cKO-Bcl2 transgenic (purple bar) mice. Frequencies of iNKT cells among CD4SP cells are shown. Means of at least three mice are shown, and error bars indicate SEM. *, $P < 0.05$. (D, Top) The frequency of iNKT cells in various tissues. Total thymocytes, splenocytes, or lymphocytes prepared from the liver of γ_c-cKO (red) or Cre− control (black) mice were analyzed for expression of TCR-β and binding of CD1d-tetramer. Data are representative of at least three individual mice. (D, Bottom) Quantitation of pooled data from Cre− control (black) or γ_c-cKO (red) mice. Means of at least three mice are shown, and error bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$.

T reg cell numbers among γ_c-deficient CD4SP thymocytes in γ_c-cKO mice (Fig. 9 A), revealing that γ_c expression was only necessary for survival of newly arising T reg cells but was not necessary to induce Foxp3 expression.

Analysis of CD1d-tetramer binding cells revealed a striking reduction in iNKT cells among γ_c-deficient CD4SP thymocytes (Fig. 9 C), which was also observed in the spleen and liver of γ_c-cKO mice (Fig. 9 D). However, the appearance of iNKT cells among γ_c-deficient CD4SP thymocytes was not restored by the Bcl-2Tg (Fig. 9 C) even though we confirmed that the Bcl-2Tg was expressed in thymic iNKT cells (not depicted). Indeed, deletion of γ_c eliminated all iNKT cells, including DN iNKT cells. Thus, γ_c signaling was necessary for differentiation, as well as for survival, of iNKT cells in the thymus. We conclude that expression of γ_c during positive selection of CD4 lineage thymocytes is required for the differentiation of iNKT cells, but it is only required for the survival of newly arising T reg cells in the thymus.

DISCUSSION

The present study has assessed the importance of γ_c and IL-7Rα expression for signaling the differentiation of positively selected thymocytes into functionally distinct T cell subsets. The results of this study revealed that γ_c expression was necessary to signal differentiation of MHC-I–selected thymocytes into either CD8 cytotoxic lineage T cells or iNKT cells but was not necessary to signal differentiation of conventional MHC-II–selected thymocytes into CD4 T cells, even regulatory Foxp3+CD4+ T cells. Moreover, the present results identified IL-7 and IL-15 as the cytokines responsible for CD8 cytotoxic lineage specification in vivo. Unexpectedly, γ_c was not required for induction of *Runx3d* expression, and *Runx3d* was not sufficient to induce cytotoxic lineage gene expression in the absence of γ_c cytokine signaling. Thus, the present study documents that γ_c expression is either critical or dispensable for signaling in vivo thymocyte differentiation and specifying lineage choice, depending on the MHC specificity of their TCR.

Lineage choice in the thymus is currently thought to be best described by the kinetic signaling model which posits that lineage choice occurs in $TCR^{hi}CD4^{+}CD8^{lo}$ Int thymocytes by whether Int thymocytes continue to be signaled by their TCR or are instead signaled by their cytokine receptors, depending on the MHC specificity of their TCR (Singer et al., 2008). In the kinetic signaling model, cytokine receptors signal $CD4^{+}CD8^{lo}$ Int thymocytes to specifically differentiate into CD8SP cells by inducing expression of Runx proteins which both silence *Cd4* and reactivate *Cd8* gene expression (Taniuchi et al., 2002; Sato et al., 2005; Egawa et al., 2007; Egawa and Littman, 2008), resulting in the phenotypic conversion of $CD4^{+}CD8^{lo}$ Int into CD8SP thymocytes. The present study provides compelling in vivo support for this perspective by demonstrating that γ_c cytokine receptor expression during positive selection was only required by MHC-I–selected thymocytes and was not required by MHC-II–selected thymocytes. Note that we specifically used $E8_{III}$Cre-expressing cKO mice so that Cre recombinase was only expressed in preselection DP thymocytes and then terminated. As a result, we can be confident that MHC-II differentiation was really γ_c independent because γ_c deletions occurred before MHC-II–specific DP thymocytes underwent positive selection and not after they had differentiated into CD4SP T cells.

It has been thought that IL-7 was the only cytokine or a major cytokine that signaled MHC-I–specific thymocytes to differentiate into CD8 cytotoxic lineage T cells (Yu et al., 2003; Park et al., 2010). In fact, the present study revealed that conditional deletion of IL-7Rα expression eliminated two-thirds of γ_c-dependent CD8 cytotoxic lineage T cells, verifying that IL-7 is the predominant cytokine signaling MHC-I–specific thymocyte differentiation. However, the present results also revealed that another γ_c-dependent cytokine, other than IL-7, also contributed to the in vivo generation of CD8 cytotoxic lineage T cells and we were surprised to identify the other γ_c-dependent cytokine as IL-15 and not IL-4. The CD8 T cells induced by IL-15 in the thymus of IL-7Rα–cKO mice unexpectedly displayed a naive $CD44^{lo}CD122^{lo}$ phenotype, which was curious because IL-15 is the cytokine generally associated with maintenance of memory CD8 T cells (Surh and Sprent, 2005). Intriguingly, we also found that IL-15–generated CD8 T cells did not homeostatically expand or acquire memory markers in the periphery of IL-7Rα–cKO mice, even though these mice were lymphopenic. Although the impact of IL-15 signaling on CD8 T cell differentiation in the absence of IL-7Rα proteins will be the subject of additional study, our present findings document that IL-15 induces the differentiation and supports the survival of naive CD8 T cells that are deficient in IL-7Rα expression. Regarding IL-7Rα, it should be noted that IL-7Rα cannot only pair with γ_c to form the IL-7 receptor but it can also pair with the TSLP receptor chain to comprise the receptor for thymic stromal lymphopoietin (TSLP; Pandey et al., 2000; Park et al., 2000). However, because the effect of IL-7Rα/IL-15 double deficiency on CD8SP thymocytes was identical to but not greater than that of γ_c deficiency, our current results are concordant with studies indicating that TSLP signaling does not discernibly effect CD8 T cell development (Al-Shami et al., 2004).

Although there was no reason to consider CD8 cytotoxic lineage fate to be a unique consequence of signaling by γ_c-dependent cytokine receptors as opposed to other cytokine receptors, it was nevertheless surprising to find that conditional deletion of γ_c abrogated the generation of most (75%), but not all, CD8SP thymocytes. Notably, the small number of CD8SP thymocytes that were generated in the absence of γ_c did express *Runx3d*, but they did not express the hallmark genes of the cytotoxic lineage: *Eomes*, *Tbx21*, and *Gzmb*. Their expression of *Runx3d* was consistent with *Runx3d* expression being necessary for the phenotypic conversion of $CD4^{+}CD8^{lo}$ Int thymocytes into CD8SP thymocytes. However, the failure of γ_c-deficient CD8SP thymocytes to express cytotoxic lineage genes revealed that *Runx3d* was insufficient, in the absence of γ_c, to specify the cytotoxic lineage fate, indicating that cytotoxic lineage specification required CD8SP thymocytes to express both γ_c and *Runx3d*.

One question raised by the generation of small numbers of γ_c-deficient CD8SP thymocytes in γ_c-cKO mice concerned the intrathymic signal that induced their expression of *Runx3d*. Runx3 has been proposed as a master regulator required for cytotoxic lineage gene expression (Woolf et al., 2003; Egawa et al., 2007; Cruz-Guilloty et al., 2009) and we previously documented that γ_c-dependent cytokine signals could induce *Runx3d* expression in developing thymocytes (Park et al., 2010). Although the present study indicates that intrathymic signals other than γ_c can also induce *Runx3d* expression to generate small numbers of CD8SP thymocytes, we think the unidentified intrathymic signal is most likely transduced by a γ_c-independent cytokine receptor and not by the TCR. Previously, we have shown that overexpression of SOCS1, which inhibits signaling by multiple cytokines in addition to γ_c cytokines (Starr et al., 1997; Metcalf, 1999), blocked induction of *Runx3d*, arguing for a role for cytokine signaling in *Runx3d* induction (Park et al., 2010). In addition, γ_c-deficient CD8SP thymocytes did not express Bcl-2, whereas TCR signaling up-regulates Bcl-2 expression, arguing against a role for TCR signals inducing *Runx3d*. In contrast, it has been suggested that TGF-β can up-regulate CD8α expression (Konkel et al., 2011), and we have found that several γ_c-independent cytokines, including IL-6 and IFN-γ, signal CD8 T cells to up-regulate *Runx3d* but not Bcl-2 (unpublished data), resembling γ_c-deficient CD8SP thymocytes.

A second question raised by the generation of small numbers of γ_c-deficient CD8SP thymocytes in γ_c-cKO mice was why these γ_c^{-} CD8SP thymocytes did not express cytotoxic lineage genes. Importantly, it was not because of shortened survival as a result of absent Bcl-2 expression because forced overexpression of transgenic Bcl-2 still did not allow their expression of cytotoxic lineage genes. Instead, we think these thymocytes are an aberrant population of CD8SP thymocytes that are developmentally arrested as a result of absent γ_c

signaling, which does not occur in normal mice but only occurs in γ_c-deficient mice. In fact, γ_c-deficient CD8SP thymocytes and T cells have been found in germline-deleted $\gamma_c^{-/-}$ mice (Cao et al., 1995; Di Santo et al., 1995, 1999). However, it was recently reported that γ_c-deficient CD8SP thymocytes from germline $\gamma_c^{-/-}$ mice expressing a Bcl-2 transgene could generate antiviral cytotoxic T cells in response to infection (Decaluwe et al., 2010), which would appear to conflict with our present findings that such CD8 T cells were developmentally arrested before their expression of cytotoxic lineage genes. We would like to suggest that a potentially interesting explanation for this apparent discrepancy is that the viral infection provoked the host to produce inflammatory cytokines that signaled γ_c^- CD8SP thymocytes and/or T cells to up-regulate expression of cytotoxic lineage genes. Alternatively, it might be speculated that mature thymocytes are programmed to use the cytokines they were exposed to as early DN thymocytes, which would predict that γ_c-deficient CD8SP thymocytes from germline $\gamma_c^{-/-}$ mice and γ_c-cKO mice are responsive to different cytokines. Future experiments will attempt to resolve this issue.

Finally, although most CD4 lineage thymocytes were unaffected by deletion of γ_c, we were surprised to find that γ_c signaling (probably induced by IL-2; Vang et al., 2008) was only required for the survival of newly generated T reg cells and was not required to induce either their differentiation or expression of Foxp3. In fact, the only CD4 T cell subset whose development required γ_c expression was the CD1d-restricted iNKT cell subset. Generation of iNKT cells was eliminated by deletion of γ_c but was not restored by transgenic overexpression of Bcl-2. Because iNKT cells are a unique subset of CD4 T cells, we specifically verified that CD4 iNKT cells expressed the Bcl-2 transgene. Consequently, our results are consistent with a required role of IL-15 both for differentiation and survival of iNKT cells (Gordy et al., 2011). In conclusion, by generating and examining novel mice with conditional deletion of γ_c and IL-7Rα, the present study documents that γ_c-dependent cytokine receptors exclusively signal MHC-I–selected thymocytes to differentiate into functional T cells and that the requirement for γ_c-dependent cytokine signaling strikingly differs with the MHC specificity of the $\alpha\beta$-TCR.

MATERIALS AND METHODS

Animals. C57BL/6 (B6) and B6.CD45.1 mice were obtained from Frederick Cancer Research and Development Center. IL-7R$\alpha^{-/-}$, IL-4R$\alpha^{-/-}$, and $\gamma_c^{-/-}$ mice were purchased from The Jackson Laboratory. Human Bcl-2 transgenic (Sentman et al., 1991) and $E8_{III}$Cre mice (Park et al., 2010) have been previously reported. IL-15$^{-/-}$ mice were provided by T. Waldmann under license from Taconic. Actin-flp, CD4Cre, and Runx3d-YFP mice were provided by L. Tessarollo, R. Bosselut, and D. Littman, respectively. *B2m*$^{-/-}$ mice were maintained in our own facility. Animal experiments were approved by the National Cancer Institute Animal Care and Use Committee, and all mice were cared for in accordance with US National Institutes of Health guidelines.

Generation of γ_c and IL-7Rα cKO mice. To generate γ_c-cKO mice, a targeting construct was generated to replace the endogenous *Il2rg* gene, which resulted in the insertion of a loxP site after exon 1 and an frt/loxP-flanked neomycin resistance (Neo^R) cassette after exon 6, referred to as the $\gamma_c^{targeted}$ allele. Proper integration of the 5′ and 3′ ends of the construct were verified by PCR and Southern blot analysis.

IL-7Rα–cKO mice were generated by creating a targeting construct to replace exon 3 of the *Il7ra* gene and insert a loxP site in intron 2 and an frt/loxp-flanked Neo^R cassette in intron 3. The resulting allele is referred to as IL-7R$\alpha^{targeted}$. Proper integration of the 5′ and 3′ ends were confirmed by Southern blot analysis.

ES cells with proper integration of the $\gamma_c^{targeted}$ and IL-7R$\alpha^{targeted}$ alleles were injected into blastocysts, which were then implanted into pseudo-pregnant female recipients. Resulting chimeric mice containing the $\gamma_c^{targeted}$ and IL-7R$\alpha^{targeted}$ knockin alleles were bred to Actin-flp transgenic female mice to induce germline deletion of the Neo^R selection cassette, giving rise to the γ_c^{fl} and IL-7Rα^{fl} alleles. After confirming germline transmission of the knockin alleles, γ_c^{fl}, and IL-7Rα^{fl} mice were then backcrossed and maintained on a B6 background. For the majority of experiments, γ_c^{fl} and IL-7Rα^{fl} mice were bred to $\gamma_c^{-/-}$ and IL-7R$\alpha^{-/-}$ germline KO mice, respectively, to generate hemizygous $\gamma_c^{fl/-}$ females or $\gamma_c^{fl/Y}$ males, and IL-7R$\alpha^{fl/-}$ mice, which resulted in more efficient Cre-mediated deletion. Cre-negative littermates were used as controls for all experiments. Mice were used between 5 and 9 wk of age.

Flow cytometry. Monoclonal antibodies with the following specificities were obtained from BD and eBioscience: CD132 (4G3), CD127 (A7R34), CD122(TMβ1), CD4 (RM4-5), CD8 (53–6.7), TCR-β (H57–597), Qa-2 (1–1-2), HSA (M1/69), CD45.2 (104), CD44 (IM7), and Foxp3 (FJK-16s using eBioscience Foxp3 staining kit). PE-conjugated CD1d tetramer was obtained from the National Institutes of Health tetramer facility.

Single-cell suspension of thymus and spleen were obtained by gentle tweezing of the organs with forceps. For iNKT cell isolation, livers were perfused with PBS followed by mashing through a fine mesh to obtain single cell suspensions. Lymphocytes were then isolated by Percoll gradient centrifugation. Cells were resuspended in 40% Percoll and layered over 70% Percoll. After centrifugation for 20 min at 900 *g*, lymphocytes at the interface were collected. Cells were stained in HBSS + 0.5% BSA + 0.5% NaN_3 at 4°C. Cells were analyzed on an LSRII or LSRFortessa (BD). Dead cells and doublets were excluded by forward light scatter and propidium-iodide staining. Data were analyzed using FlowJo software (Tree Star, Inc.).

Mixed bone marrow chimeras. Mixed radiation bone marrow chimeras were generated by reconstituting lethally irradiated (950R) recipient mice with a total of 10–15 × 10^6 cells from a 1:1 mixture of B6 and γ_c-cKO–derived T cell–depleted bone marrow cells 6 h after irradiation. Chimeric mice were analyzed 8 wk after reconstitution.

Cell sorting for qPCR. TCR-β^{hi} CD8SP γ_c-deleted or IL-7Rα–deleted thymocytes were obtained by depletion of $CD4^+$ cells using anti-CD4 microbeads (GK1.5; Miltenyi Biotec) followed by staining with anti–TCR-β, anti-CD8α, anti-CD4 (RM4-4), and anti-CD132 or anti-CD127. For Cre^+ mice, TCR-β^{hi} CD8SP cells were specifically sorted to be negative for either γ_c or IL-7Rα to exclude cells that escaped Cre-mediated deletion. For Cre^- control mice, CD8SP TCR^{hi} cells were sorted. Cells were electronically sorted to >95% purity using a FACSAria (BD).

qPCR gene expression analysis. Total RNA was isolated by using TRIzol (Invitrogen) and cDNA was synthesized using the SuperScript III kit (Invitrogen) with oligo(dT) priming. Genomic DNA was removed using DNA-free kit (Ambion), and amplification of gene-specific products was achieved using TaqMan probes (Applied Biosystems) for *Eomes*, *Tbx21*, *Gzmb*, *Prf1*, *Bcl2*, and SYBR green (QIAGEN) for distal *Runx3d* (Table S1). Relative expression levels were calculated using the $\Delta\Delta$Ct method using *Rpl13A* as the housekeeping gene and values from Cre^+ samples were normalized to the values from Cre^- controls.

Statistical methods. SEM and p-values were determined using Prism software (GraphPad Software, Inc.). P-values were calculated using a two-tailed unpaired Student's *t* test with 95% confidence interval.

Online supplemental material. PCR primer sequences are available in Table S1. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20121505/DC1.

We would like to thank Richard Hodes, Leonid Pobezinsky, François Van Laethem, and Heather Cohen for critical review of the manuscript, and Susan Sharrow, Larry Granger, and Tony Adams for flow cytometry expertise.

This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

The authors have no conflicting financial interests.

Submitted: 10 July 2012
Accepted: 2 October 2012

REFERENCES

Adoro, S., T. McCaughtry, B. Erman, A. Alag, F. Van Laethem, J.H. Park, X. Tai, M. Kimura, L. Wang, A. Grinberg, et al. 2012. Coreceptor gene imprinting governs thymocyte lineage fate. *EMBO J.* 31:366–377. http://dx.doi.org/10.1038/emboj.2011.388

Al-Shami, A., R. Spolski, J. Kelly, T. Fry, P.L. Schwartzberg, A. Pandey, C.L. Mackall, and W.J. Leonard. 2004. A role for thymic stromal lymphopoietin in $CD4^+$ T cell development. *J. Exp. Med.* 200:159–168. http://dx.doi.org/10.1084/jem.20031975

Bosselut, R., T.I. Guinter, S.O. Sharrow, and A. Singer. 2003. Unraveling a revealing paradox: Why major histocompatibility complex I-signaled thymocytes "paradoxically" appear as $CD4^+8^{lo}$ transitional cells during positive selection of $CD8^+$ T cells. *J. Exp. Med.* 197:1709–1719. http://dx.doi.org/10.1084/jem.20030170

Brugnera, E., A. Bhandoola, R. Cibotti, Q. Yu, T.I. Guinter, Y. Yamashita, S.O. Sharrow, and A. Singer. 2000. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity.* 13:59–71. http://dx.doi.org/10.1016/S1074-7613(00)00008-X

Burchill, M.A., J. Yang, C. Vogtenhuber, B.R. Blazar, and M.A. Farrar. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J. Immunol.* 178:280–290.

Candéias, S., K. Muegge, and S.K. Durum. 1997. IL-7 receptor and VDJ recombination: trophic versus mechanistic actions. *Immunity.* 6:501–508. http://dx.doi.org/10.1016/S1074-7613(00)80338-6

Cao, X., E.W. Shores, J. Hu-Li, M.R. Anver, B.L. Kelsall, S.M. Russell, J. Drago, M. Noguchi, A. Grinberg, E.T. Bloom, et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity.* 2:223–238. http://dx.doi.org/10.1016/1074-7613(95)90047-0

Chong, M.M., A.L. Cornish, R. Darwiche, E.G. Stanley, J.F. Purton, D.I. Godfrey, D.J. Hilton, R. Starr, W.S. Alexander, and T.W. Kay. 2003. Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent CD8+ T cell differentiation. *Immunity.* 18:475–487. http://dx.doi.org/10.1016/S1074-7613(03)00078-5

Cibotti, R., A. Bhandoola, T.I. Guinter, S.O. Sharrow, and A. Singer. 2000. CD8 coreceptor extinction in signaled CD4(+)CD8(+) thymocytes: coordinate roles for both transcriptional and posttranscriptional regulatory mechanisms in developing thymocytes. *Mol. Cell. Biol.* 20:3852–3859. http://dx.doi.org/10.1128/MCB.20.11.3852-3859.2000

Cruz-Guilloty, F., M.E. Pipkin, I.M. Djuretic, D. Levanon, J. Lotem, M.G. Lichtenheld, Y. Groner, and A. Rao. 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J. Exp. Med.* 206:51–59. http://dx.doi.org/10.1084/jem.20081242

Decaluwe, H., M. Taillardet, E. Corcuff, I. Munitic, H.K. Law, B. Rocha, Y. Rivière, and J.P. Di Santo. 2010. Gamma(c) deficiency precludes CD8+ T cell memory despite formation of potent T cell effectors. *Proc. Natl. Acad. Sci. USA.* 107:9311–9316. http://dx.doi.org/10.1073/pnas.0913729107

Di Santo, J.P., I. Aifantis, E. Rosmaraki, C. Garcia, J. Feinberg, H.J. Fehling, A. Fischer, H. von Boehmer, and B. Rocha. 1999. The common cytokine receptor gamma chain and the pre-T cell receptor provide independent but critically overlapping signals in early α/β T cell development. *J. Exp. Med.* 189:563–574. http://dx.doi.org/10.1084/jem.189.3.563

Di Santo, J.P., W. Müller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc. Natl. Acad. Sci. USA.* 92:377–381. http://dx.doi.org/10.1073/pnas.92.2.377

Dubois, S., T.A. Waldmann, and J.R. Müller. 2006. ITK and IL-15 support two distinct subsets of CD8+ T cells. *Proc. Natl. Acad. Sci. USA.* 103:12075–12080. http://dx.doi.org/10.1073/pnas.0605212103

Egawa, T., and D.R. Littman. 2008. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nat. Immunol.* 9:1131–1139. http://dx.doi.org/10.1038/ni.1652

Egawa, T., R.E. Tillman, Y. Naoe, I. Taniuchi, and D.R. Littman. 2007. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J. Exp. Med.* 204:1945–1957. http://dx.doi.org/10.1084/jem.20070133

Ehlers, M., K. Laule-Kilian, M. Petter, C.J. Aldrian, B. Grueter, A. Würch, N. Yoshida, T. Watanabe, M. Satake, and V. Steimle. 2003. Morpholino antisense oligonucleotide-mediated gene knockdown during thymocyte development reveals role for Runx3 transcription factor in CD4 silencing during development of CD4-/CD8+ thymocytes. *J. Immunol.* 171:3594–3604.

Gordy, L.E., J.S. Bezbradica, A.I. Flyak, C.T. Spencer, A. Dunkle, J. Sun, A.K. Stanic, M.R. Boothby, Y.W. He, Z. Zhao, et al. 2011. IL-15 regulates homeostasis and terminal maturation of NKT cells. *J. Immunol.* 187:6335–6345. http://dx.doi.org/10.4049/jimmunol.1003965

He, X., X. He, V.P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B.A. Roe, and D.J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature.* 433:826–833. http://dx.doi.org/10.1038/nature03338

Heusel, J.W., R.L. Wesselschmidt, S. Shresta, J.H. Russell, and T.J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell.* 76:977–987. http://dx.doi.org/10.1016/0092-8674(94)90376-X

Intlekofer, A.M., N. Takemoto, E.J. Wherry, S.A. Longworth, J.T. Northrup, V.R. Palanivel, A.C. Mullen, C.R. Gasink, S.M. Kaech, J.D. Miller, et al. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* 6:1236–1244. http://dx.doi.org/10.1038/ni1268

Intlekofer, A.M., A. Banerjee, N. Takemoto, S.M. Gordon, C.S. Dejong, H. Shin, C.A. Hunter, E.J. Wherry, T. Lindsten, and S.L. Reiner. 2008. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science.* 321:408–411. http://dx.doi.org/10.1126/science.1159806

Kägi, D., B. Ledermann, K. Bürki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature.* 369:31–37. http://dx.doi.org/10.1038/369031a0

Konkel, J.E., T. Maruyama, A.C. Carpenter, Y. Xiong, B.F. Zamarron, B.E. Hall, A.B. Kulkarni, P. Zhang, R. Bosselut, and W. Chen. 2011. Control of the development of $CD8\alpha\alpha$+ intestinal intraepithelial lymphocytes by TGF-β. *Nat. Immunol.* 12:312–319. http://dx.doi.org/10.1038/ni.1997

Lee, P.P., D.R. Fitzpatrick, C. Beard, H.K. Jessup, S. Lehar, K.W. Makar, M. Pérez-Melgosa, M.T. Sweetser, M.S. Schlissel, S. Nguyen, et al. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity.* 15:763–774. http://dx.doi.org/10.1016/S1074-7613(01)00227-8

Maki, K., S. Sunaga, and K. Ikuta. 1996. The V-J recombination of T cell receptor-gamma genes is blocked in interleukin-7 receptor-deficient mice. *J. Exp. Med.* 184:2423–2427. http://dx.doi.org/10.1084/jem.184.6.2423

McCaughtry, T.M., M.S. Wilken, and K.A. Hogquist. 2007. Thymic emigration revisited. *J. Exp. Med.* 204:2513–2520. http://dx.doi.org/10.1084/jem.20070601

Metcalf, D. 1999. The SOCS-1 story. *Exp. Hematol.* 27:1715–1723. http://dx.doi.org/10.1016/S0301-472X(99)00120-4

Murray, R., T. Suda, N. Wrighton, F. Lee, and A. Zlotnik. 1989. IL-7 is a growth and maintenance factor for mature and immature thymocyte

subsets. *Int. Immunol.* 1:526–531. http://dx.doi.org/10.1093/intimm/1.5.526

Pandey, A., K. Ozaki, H. Baumann, S.D. Levin, A. Puel, A.G. Farr, S.F. Ziegler, W.J. Leonard, and H.F. Lodish. 2000. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat. Immunol.* 1:59–64.

Park, J.H., S. Adoro, T. Guinter, B. Erman, A.S. Alag, M. Catalfamo, M.Y. Kimura, Y. Cui, P.J. Lucas, R.E. Gress, et al. 2010. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. *Nat. Immunol.* 11:257–264. http://dx.doi.org/10.1038/ni.1840

Park, L.S., U. Martin, K. Garka, B. Gliniak, J.P. Di Santo, W. Muller, D.A. Largaespada, N.G. Copeland, N.A. Jenkins, A.G. Farr, et al. 2000. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: formation of a functional heteromeric complex requires interleukin 7 receptor. *J. Exp. Med.* 192:659–670. http://dx.doi.org/10.1084/jem.192.5.659

Pearce, E.L., A.C. Mullen, G.A. Martins, C.M. Krawczyk, A.S. Hutchins, V.P. Zediak, M. Banica, C.B. DiCioccio, D.A. Gross, C.A. Mao, et al. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science.* 302:1041–1043. http://dx.doi.org/10.1126/science.1090148

Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180:1955–1960. http://dx.doi.org/10.1084/jem.180.5.1955

Sato, T., S. Ohno, T. Hayashi, C. Sato, K. Kohu, M. Satake, and S. Habu. 2005. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity.* 22:317–328. http://dx.doi.org/10.1016/j.immuni.2005.01.012

Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell.* 67:879–888. http://dx.doi.org/10.1016/0092-8674(91)90361-2

Singer, A., S. Adoro, and J.H. Park. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat. Rev. Immunol.* 8:788–801. http://dx.doi.org/10.1038/nri2416

Starr, R., T.A. Willson, E.M. Viney, L.J. Murray, J.R. Rayner, B.J. Jenkins, T.J. Gonda, W.S. Alexander, D. Metcalf, N.A. Nicola, and D.J. Hilton. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature.* 387:917–921. http://dx.doi.org/10.1038/43206

Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annu. Rev. Immunol.* 21:139–176. http://dx.doi.org/10.1146/annurev.immunol.21.120601.141107

Sun, G., X. Liu, P. Mercado, S.R. Jenkinson, M. Kypriotou, L. Feigenbaum, P. Galéra, and R. Bosselut. 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nat. Immunol.* 6:373–381. http://dx.doi.org/10.1038/ni1183

Surh, C.D., and J. Sprent. 2005. Regulation of mature T cell homeostasis. *Semin. Immunol.* 17:183–191. http://dx.doi.org/10.1016/j.smim.2005.02.007

Suzuki, H., J.A. Punt, L.G. Granger, and A. Singer. 1995. Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. *Immunity.* 2:413–425. http://dx.doi.org/10.1016/1074-7613(95)90149-3

Tan, J.T., E. Dudl, E. LeRoy, R. Murray, J. Sprent, K.I. Weinberg, and C.D. Surh. 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. USA.* 98:8732–8737. http://dx.doi.org/10.1073/pnas.161126098

Taniuchi, I., M. Osato, T. Egawa, M.J. Sunshine, S.C. Bae, T. Komori, Y. Ito, and D.R. Littman. 2002. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell.* 111:621–633. http://dx.doi.org/10.1016/S0092-8674(02)01111-X

Vang, K.B., J. Yang, S.A. Mahmud, M.A. Burchill, A.L. Vegoe, and M.A. Farrar. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J. Immunol.* 181:3285–3290.

Wang, L., K.F. Wildt, J. Zhu, X. Zhang, L. Feigenbaum, L. Tessarollo, W.E. Paul, B.J. Fowlkes, and R. Bosselut. 2008. Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. *Nat. Immunol.* 9:1122–1130. http://dx.doi.org/10.1038/ni.1647

Woolf, E., C. Xiao, O. Fainaru, J. Lotem, D. Rosen, V. Negreanu, Y. Bernstein, D. Goldenberg, O. Brenner, G. Berke, et al. 2003. Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. *Proc. Natl. Acad. Sci. USA.* 100:7731–7736. http://dx.doi.org/10.1073/pnas.1232420100

Yu, Q., B. Erman, A. Bhandoola, S.O. Sharrow, and A. Singer. 2003. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature $CD8^+$ T cells. *J. Exp. Med.* 197:475–487. http://dx.doi.org/10.1084/jem.20021765

Yu, Q., J.H. Park, L.L. Doan, B. Erman, L. Feigenbaum, and A. Singer. 2006. Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. *J. Exp. Med.* 203:165–175. http://dx.doi.org/10.1084/jem.20051836