

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

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**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-7R $\alpha$  or common cytokine receptor  $\gamma_c$  chain ( $\gamma_c$ ) genes were deleted in thymocytes just before positive selection. We found that  $\gamma_c$  expression was required to signal the differentiation of MHC class I (MHC-I)-specific thymocytes into CD8<sup>+</sup> 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<sup>+</sup> T cells, even into regulatory Foxp3<sup>+</sup>CD4<sup>+</sup> T cells which require  $\gamma_c$  signals for survival. Importantly, IL-7 and IL-15 were identified as the cytokines responsible for CD8<sup>+</sup> cytotoxic T cell lineage specification *in vivo*. Additionally, we found that small numbers of aberrant CD8<sup>+</sup> T cells expressing *Runx3d* could arise without  $\gamma_c$  signaling, but these cells were developmentally arrested before expressing cytotoxic lineage genes. Thus,  $\gamma_c$ -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.**

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Abbreviations used: cKO, conditional KO; DN, double negative; DP, double positive;  $\gamma_c$ , 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<sup>-</sup>CD8<sup>-</sup> double-negative (DN) stage 3 (DN3) thymocytes to differentiate into DN4 thymocytes and, at the second checkpoint, signaling by fully assembled  $\alpha\beta$ -TCR induces CD4<sup>+</sup>CD8<sup>+</sup> 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  $\alpha\beta$  thymocytes is less certain and remains controversial, even though IL-7 signaling is known to be required for  $\gamma\delta$  T cell development (Maki et al., 1996; Candéas et al., 1997). Although it has been a long held perspective that essentially all aspects of  $\alpha\beta$  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).

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Because preselection DP thymocytes do not express IL-7R $\alpha$  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-7R $\alpha$  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<sup>+</sup> T helper lineage cells or MHC-I–selected CD8<sup>+</sup> 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  $\gamma_c$ -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  $\gamma_c$  or IL-7R $\alpha$  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  $\gamma_c$ -dependent cytokine signaling during positive selection, we have now generated conditional KO (cKO) mice in which  $\gamma_c$  or IL-7R $\alpha$  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  $\gamma_c$  or IL-7R $\alpha$  cytokine receptors. By using these novel mice, this study reveals that  $\gamma_c$  expression during positive selection is essential to signal the in vivo differentiation of MHC-I–selected thymocytes into mature CD4 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<sup>+</sup>CD4 T cells which require  $\gamma_c$ -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  $\gamma_c$ -transduced signals are either critical or dispensable for in vivo thymocyte differentiation depending on the MHC specificity of their TCR.

RESULTS

Importance of IL-7R $\alpha$  and  $\gamma_c$  expression during positive selection in the thymus

The IL-7 receptor is composed of two chains, the cytokine receptor common gamma chain ( $\gamma_c$ ; CD132) and the IL-7R $\alpha$  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).  $\gamma_c$  is expressed in varying amounts on all thymocytes at different stages of development, whereas IL-7R $\alpha$  is expressed on early and late stage thymocytes but is extinguished in preselection DP thymocytes (Fig. 1, middle and bottom, black lines). IL-7R $\alpha$  is reexpressed during positive selection on thymocytes at the intermediate (Int; TCR<sup>hi</sup>CD4<sup>+</sup>CD8<sup>lo</sup>) 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.,

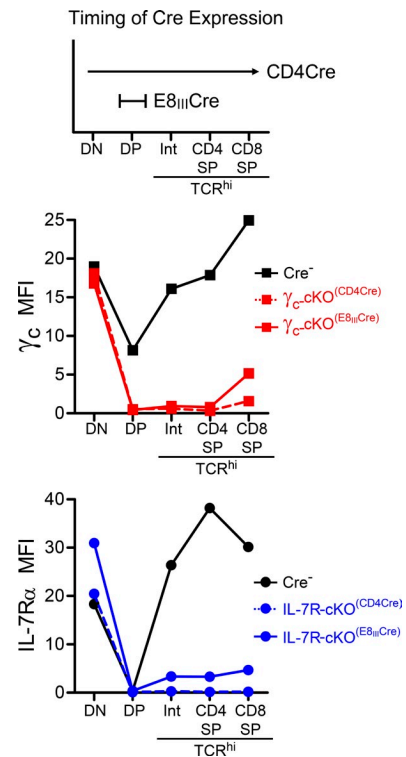
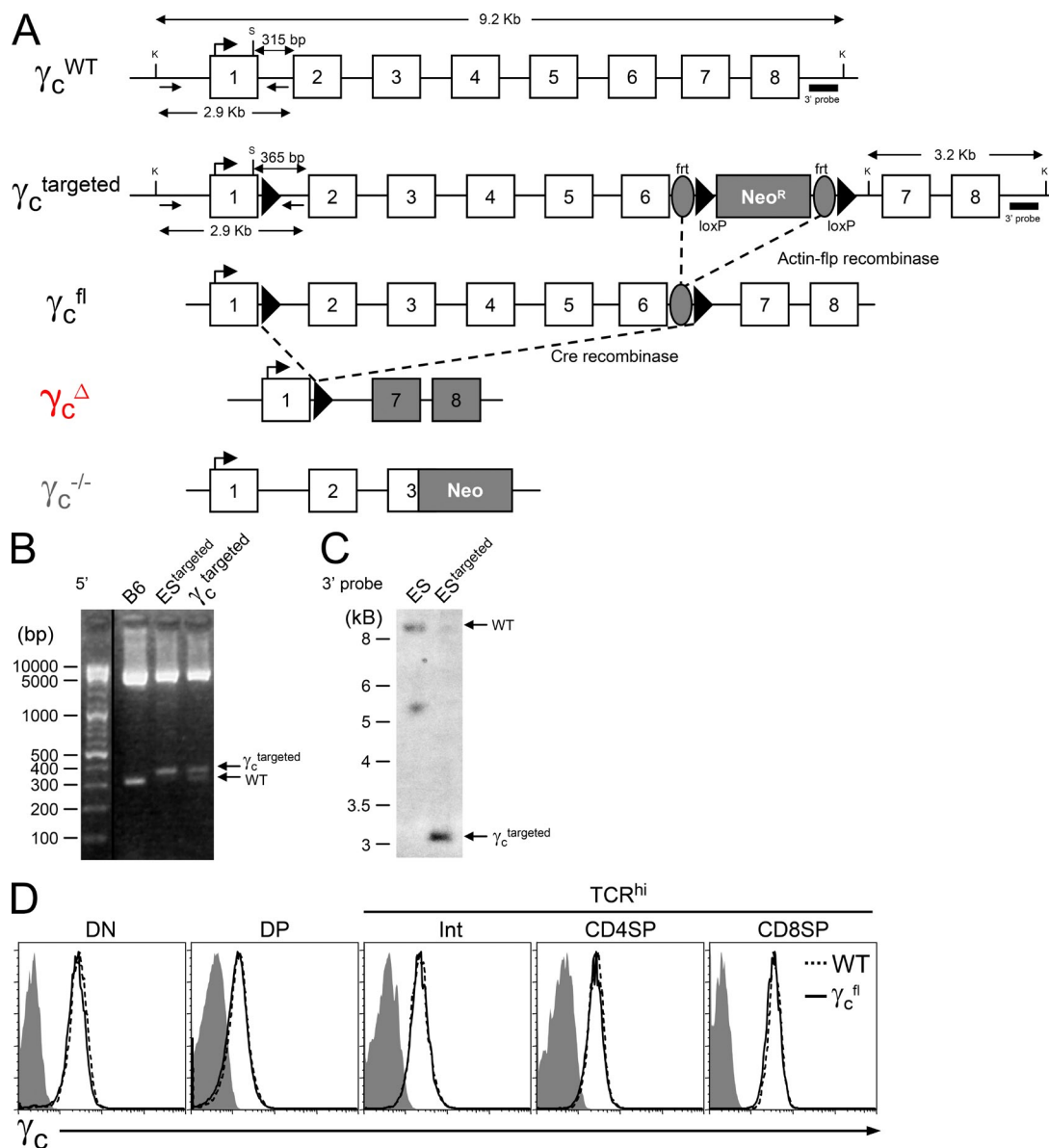


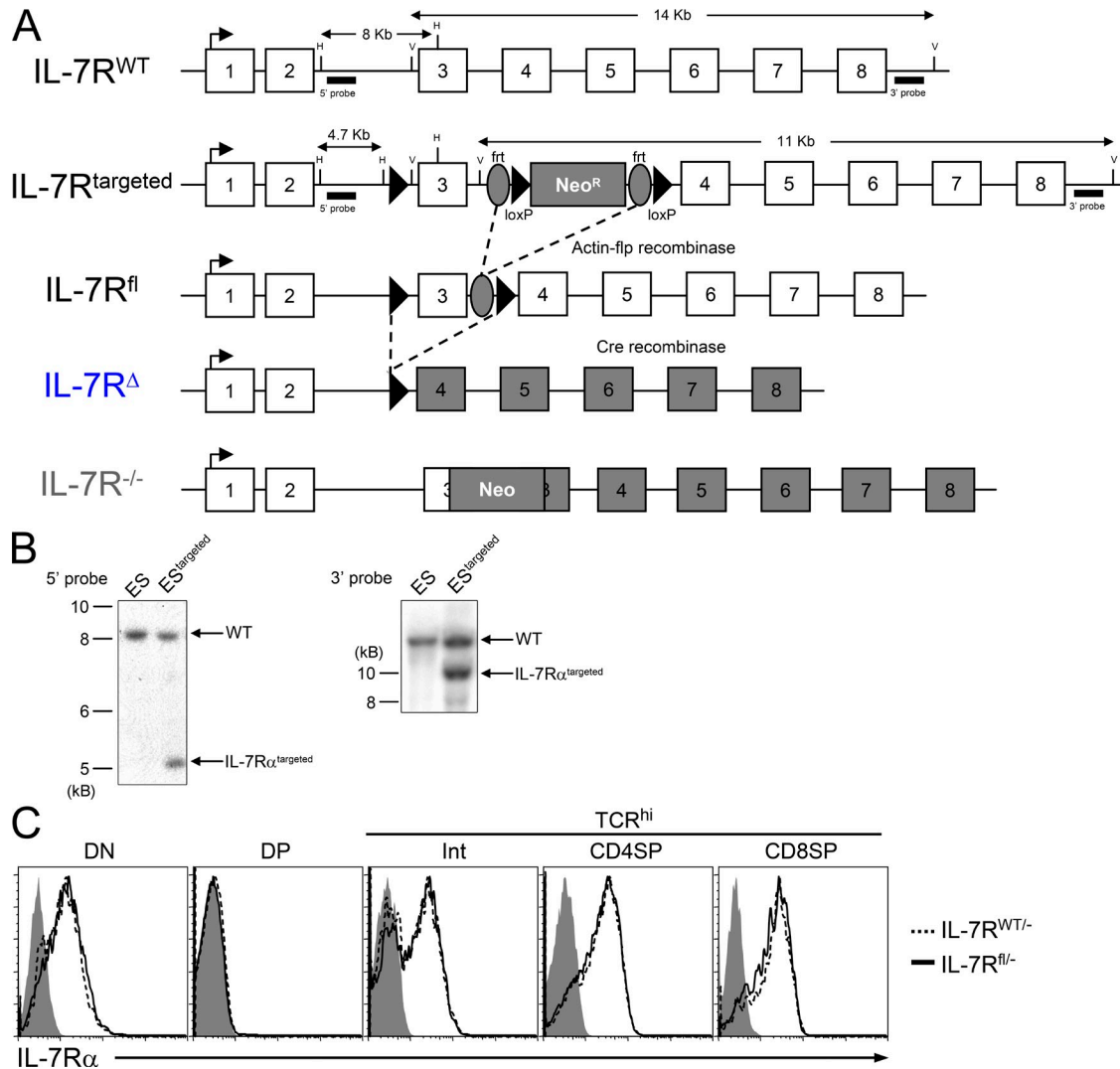
Figure 1.  $\gamma_c$  and IL-7R $\alpha$  expression on thymocytes from wild-type and cKO mice. (Top) Schematic of CD4Cre and E8III-Cre expression in developing thymocytes. Surface expression of  $\gamma_c$  (middle) and IL-7R $\alpha$  (bottom) was determined on thymocytes from WT (Cre<sup>-</sup>) or cKO (Cre<sup>+</sup>) mice using CD4Cre (colored dashed lines) or E8III-Cre (colored solid lines). Total thymocytes were stained for CD4, CD8 $\alpha$ , TCR- $\beta$ , and  $\gamma_c$  or IL-7R $\alpha$  and gated on the indicated thymocyte populations. MFI of  $\gamma_c$  and IL-7R $\alpha$  staining are shown. Data are representative of four independent experiments.



**Figure 2. Generation of  $\gamma_c$ -cKO mice.** (A) Targeting strategy to generate  $\gamma_c$ -cKO allele. Numbered boxes = exons, gray boxes = out of frame exons as a result of Cre-mediated deletion. Neo<sup>R</sup> = 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  $\gamma_c$  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<sup>R</sup> cassette, expression of the  $\gamma_c^{\text{fl}}$  allele is equal to WT  $\gamma_c$  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-7R $\alpha$  and  $\gamma_c$  expression during positive selection for lineage fate specification in vivo. Because germline deletion of either  $\gamma_c$  or IL-7R $\alpha$  impairs development at the DN stage (Cao et al., 1995; Di Santo et al., 1995,

1999), we constructed  $\gamma_c$  and IL-7R $\alpha$  floxed alleles which could be conditionally deleted by Cre recombinase in DP thymocytes without reducing their expression in earlier TCR- $\beta^-$  DN thymocytes (Fig. 2 and Fig. 3). We refer to Cre<sup>+</sup> mice with floxed alleles as cKO ( $\gamma_c$ -cKO and IL-7R $\alpha$ -cKO) mice, and we refer to their deleted alleles, and cells with deleted alleles, as  $\gamma_c^{\Delta}$  and IL-7R $\alpha^{\Delta}$ .



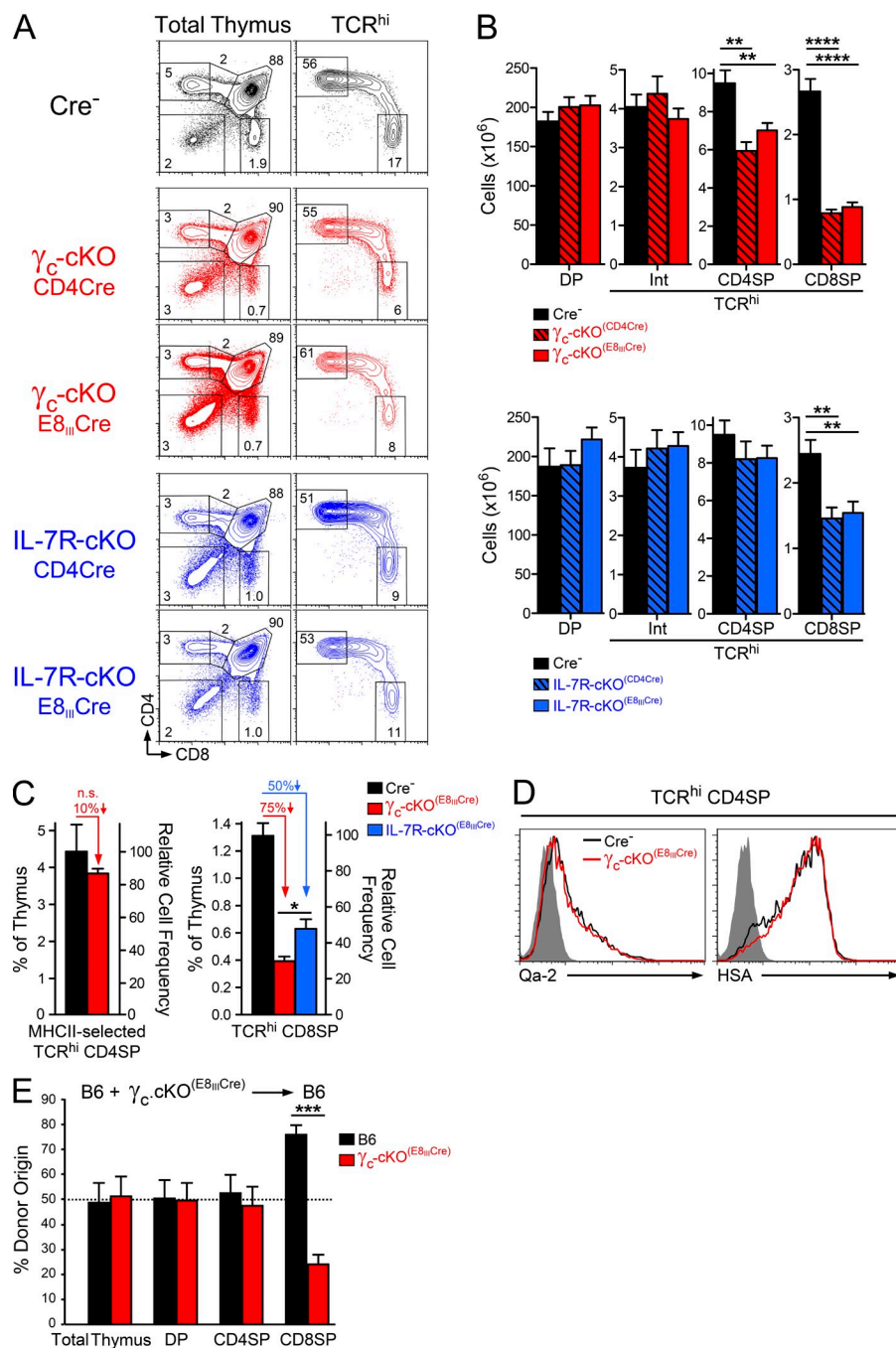
**Figure 3. Generation of IL-7R $\alpha$ -cKO mice.** (A) Targeting strategy to generate IL-7R $\alpha$ -cKO allele. Numbered boxes = exons, gray boxes = out of frame exons as a result of Cre-mediated deletion. Neo<sup>R</sup> = neomycin resistance cassette. Black triangles = loxP sites. Gray ovals = 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-7R $\alpha$  germline KO allele used in this study 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-flip recombinase-mediated deletion of the Neo<sup>R</sup> cassette, expression of the IL-7R $\alpha$ <sup>fl/-</sup> allele is equal to WT IL-7R $\alpha$  expression in the absence of Cre throughout T cell development.

We began our study using two different Cre transgenes, CD4Cre and E8<sub>III</sub>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<sub>III</sub>Cre transgene is regulated by E8<sub>III</sub>-CD8 $\alpha$  enhancer/promoter elements that limit Cre expression exclusively to

preselection DP thymocytes (Fig. 1, top; Park et al., 2010). Because E8<sub>III</sub>Cre expression is limited to preselection DP thymocytes, Cre is present in only a brief, well defined developmental timeframe before lineage specification.

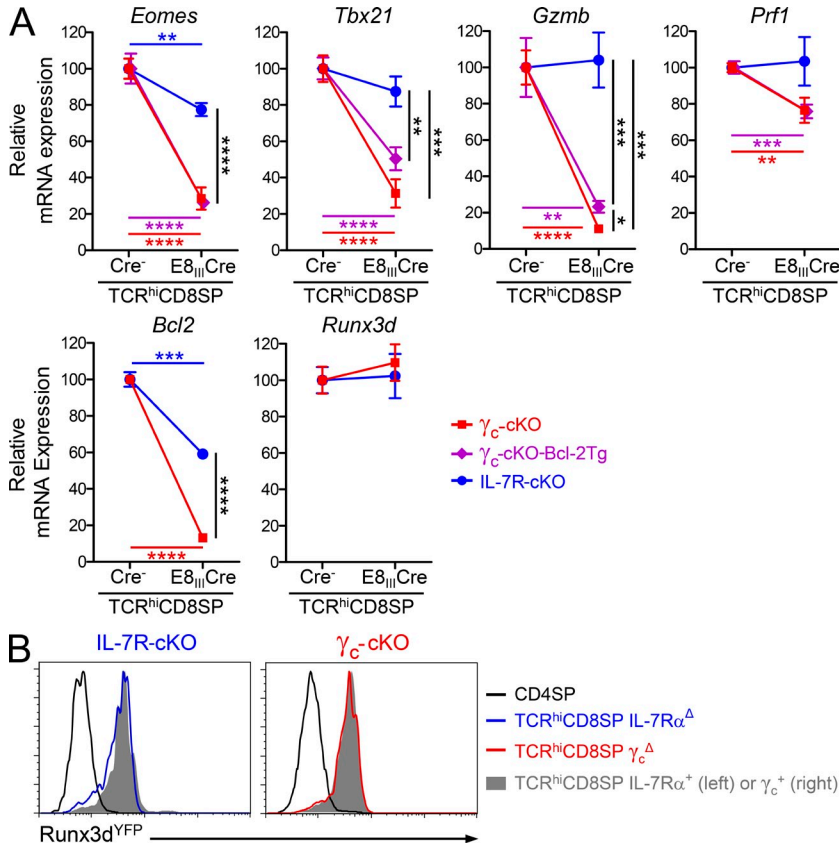
We introduced the CD4Cre and E8<sub>III</sub>Cre transgenes into  $\gamma_c^{fl/-}$  and IL-7R $\alpha^{fl/-}$  mice to generate  $\gamma_c^{\Delta}$  and IL-7R $\alpha^{\Delta}$  deleted alleles (Fig. 2 and Fig. 3). We found that neither CD4Cre nor E8<sub>III</sub>Cre reduced expression of  $\gamma_c$  or IL-7R $\alpha$  on TCR- $\beta^{-}$  DN thymocytes, but both Cre transgenes induced deletions in DP and postselection Int (TCR<sup>hi</sup>CD4<sup>+</sup>CD8<sup>lo</sup>) and SP thymocytes, with a few more cells escaping deletion with E8<sub>III</sub>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  $\gamma_c$  or IL-7R $\alpha$  genes.** (A) CD4 versus CD8 $\alpha$  profiles of total thymus (left) and gated TCR- $\beta$ <sup>hi</sup> thymocytes (right) from Cre<sup>-</sup> control (black),  $\gamma_c$ -cKO (red), and IL-7R $\alpha$ -cKO (blue) mice. FACS profiles for both CD4Cre and E8<sub>III</sub>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- $\beta$ <sup>hi</sup> Int, TCR- $\beta$ <sup>hi</sup> CD4SP, and TCR- $\beta$ <sup>hi</sup> CD8SP populations are shown for  $\gamma_c$ -cKO (red) compared with Cre<sup>-</sup> control mice (black). Cell numbers for both CD4Cre and E8<sub>III</sub>Cre mice are shown. (B, Bottom) Absolute thymocyte numbers for DP, TCR- $\beta$ <sup>hi</sup> Int, TCR- $\beta$ <sup>hi</sup> CD4SP, and TCR- $\beta$ <sup>hi</sup> CD8SP populations are shown for IL-7R $\alpha$ -cKO (blue) compared with Cre<sup>-</sup> control mice (black). Cell numbers for both CD4Cre and E8<sub>III</sub>Cre mice are shown. Cell numbers were calculated by gating specifically on cells that had deleted  $\gamma_c$  or IL-7R $\alpha$ . 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 MHCII-selected TCR- $\beta$ <sup>hi</sup> CD4SP thymocytes from  $\beta 2m^{-/-}$   $\gamma_c$ -cKO mice (red) compared with Cre<sup>-</sup> control mice (black).  $\gamma_c$ -cKO-derived cells were specifically gated for those that were  $\gamma_c$ -deleted. Data are depicted as frequencies of total thymus on the left y-axis and relative frequencies normalized to Cre<sup>-</sup> control on the right y-axis of the histogram plot. Data for E8<sub>III</sub>Cre mice are shown. (C, Right) Frequency of TCR- $\beta$ <sup>hi</sup> CD8SP thymocytes from  $\gamma_c$ -cKO mice (red), IL-7R $\alpha$ -cKO mice (blue), and Cre<sup>-</sup> control mice (black) are shown.  $\gamma_c$ -cKO and IL-7 R $\alpha$ -cKO-derived cells were specifically gated for those that were  $\gamma_c$ - or IL-7 R $\alpha$ -deleted. Data are depicted as frequencies of total thymus on the left y-axis and relative frequencies normalized to Cre<sup>-</sup> control on the right y-axis of the histogram plot. Data for E8<sub>III</sub>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- $\beta$ <sup>hi</sup> CD4SP thymocytes from  $\gamma_c$ -cKO mice (red; specifically gated on  $\gamma_c$ -deleted cells) and Cre<sup>-</sup> 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  $\gamma_c$ -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.  $\gamma_c$ -cKO-derived cells were specifically gated for those that were  $\gamma_c$ -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<sup>hi</sup> Int thymocytes (Fig. 4, A and B). However, Cre-mediated deletions significantly reduced the number and frequency of TCR<sup>hi</sup> 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<sub>III</sub>Cre transgene



**Figure 5. Analysis of CD8 lineage gene expression.** (A) Relative mRNA expression of the indicated genes. TCR-β<sup>hi</sup> CD8SP thymocytes were sorted from Cre<sup>-</sup> control mice and TCR-β<sup>hi</sup> CD8SP thymocytes gated specifically on IL-7Rα-deleted (blue circles) or γ<sub>c</sub>-deleted (red squares and purple diamonds) were sorted from E8<sup>III</sup>Cre cKO mice. RNA was isolated and expression of the indicated genes was analyzed by real-time PCR. Purple diamonds indicate γ<sub>c</sub>-cKO mice with transgenic expression of Bcl-2. Data are normalized to *Rpl13A* and Cre<sup>-</sup> 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<sup>YFP</sup> reporter expression in IL-7Rα-deleted and γ<sub>c</sub>-deleted cells. Total thymocytes from IL-7Rα-cKO Runx3d<sup>YFP/wt</sup> (left) and γ<sub>c</sub>-cKO Runx3d<sup>YFP/wt</sup> (right) were stained for CD4, CD8α, TCR-β and IL-7Rα or γ<sub>c</sub> expression. Runx3d<sup>YFP</sup> reporter expression is compared between TCR-β<sup>hi</sup> CD4SP cells (black line) and TCR-β<sup>hi</sup> CD8SP gated on IL-7Rα-deleted (blue line) and IL-7Rα-positive cells (escapes, gray shaded; left), and TCR-β<sup>hi</sup> CD8SP gated on γ<sub>c</sub>-deleted (red line) and γ<sub>c</sub>-positive cells (escapes, gray shaded; right). Data are representative of three independent experiments.

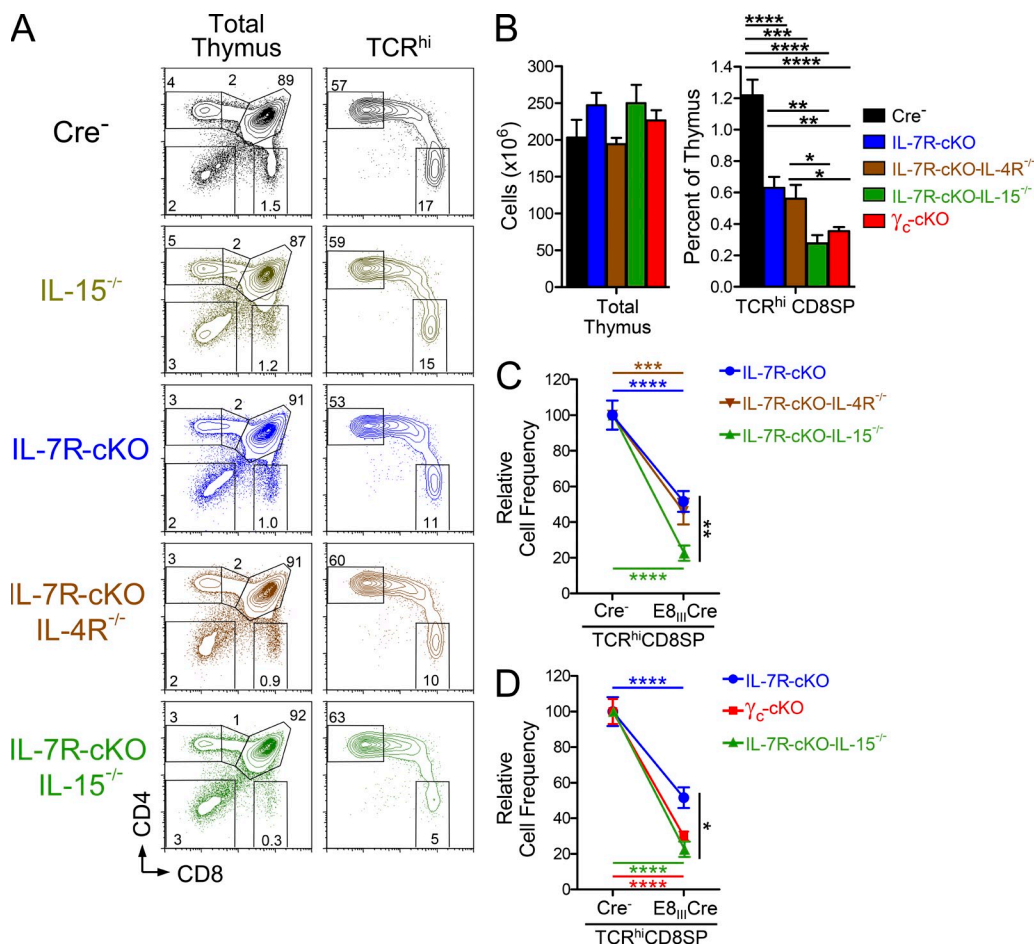
for all subsequent experiments so that deletions unequivocally occurred in preselection DP thymocytes before positive selection and lineage specification.

Notably, this analysis necessarily overestimated the impact of γ<sub>c</sub> and IL-7Rα deletions on CD4 lineage thymocytes because MHC-I-specific CD8 lineage T cells go through the TCR<sup>hi</sup>CD4<sup>+</sup>CD8<sup>lo</sup> Int stage and are thus unavoidably included in the TCR<sup>hi</sup> 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<sub>c</sub>-cKO mice additionally deficient in *B2m* (Fig. 4 C). We observed that deletion of γ<sub>c</sub> 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<sub>c</sub>-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 (McCaughy et al., 2007) was unchanged (Fig. 4 D). Moreover, construction of competitive mixed donor bone marrow chimeras revealed no competitive disadvantage of γ<sub>c</sub> deficiency by γ<sub>c</sub>-cKO CD4SP thymocytes compared with their wild-type counterparts (Fig. 4 E). Thus, deficiency of either γ<sub>c</sub> 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<sub>c</sub> on generation of CD8 cytotoxic lineage cells in the thymus**

Having determined that IL-7Rα deficiency and γ<sub>c</sub> deficiency during positive selection quantitatively reduced generation of CD8SP thymocytes, we then assessed if IL-7Rα and γ<sub>c</sub> were important for initiating the cytotoxic lineage program. To do so, we examined expression of cytotoxic lineage-specific genes in purified TCR<sup>hi</sup> CD8SP thymocytes from IL-7Rα-cKO and γ<sub>c</sub>-cKO mice that were electronically sorted to exclude all escapes of Cre-mediated deletion and to include only CD8SP thymocytes with deleted IL-7Rα<sup>Δ</sup> or γ<sub>c</sub><sup>Δ</sup> 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<sup>III</sup>Cre<sup>-</sup> and E8<sup>III</sup>Cre<sup>+</sup> 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<sup>III</sup>Cre<sup>-</sup> 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<sub>c</sub>-deficient CD8SP thymocytes from γ<sub>c</sub>-cKO mice were



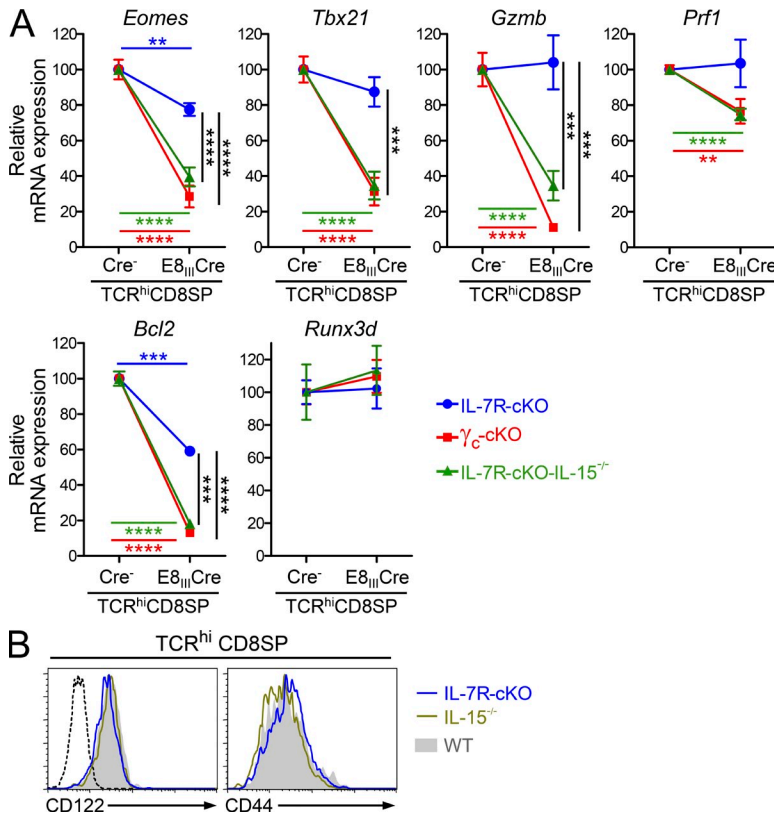
**Figure 6. Contribution of IL-15 to generation of CD8 lineage cells.** (A) Thymocyte profiles resulting from E8<sub>III</sub>Cre-mediated deletion of IL-7Rα alone or in combination with IL-4Rα or IL-15 germline deficiency. Total thymocytes were analyzed for expression of CD8α, CD4, and TCR-β from Cre<sup>-</sup> control (black), germline IL-15<sup>-/-</sup> (olive green), E8<sub>III</sub>Cre IL-7R-cKO (blue), IL-7R-cKO combined with germline IL-4R<sup>-/-</sup> (brown) or IL-15<sup>-/-</sup> (bright green) mice. Data are representative of at least three independent experiments. (B) Total thymocyte number and frequency of TCR-β<sup>hi</sup> CD8SP thymocytes from mice in A shown in comparison to γ<sub>c</sub>-cKO mice (red). γ<sub>c</sub>-cKO- and IL-7Rα-cKO-derived cells were specifically gated for those that were γ<sub>c</sub>- 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<sup>hi</sup> CD8SP thymocytes after E8<sub>III</sub>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<sup>hi</sup> CD8SP thymocyte frequencies were analyzed from total thymocytes. Data are shown as frequencies normalized to Cre<sup>-</sup> 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<sup>hi</sup> CD8SP thymocytes resulting from deletion of IL-7Rα alone (blue line) or in combination with IL-15 (green line) compared with frequency of γ<sub>c</sub>-deleted TCR-β<sup>hi</sup> CD8SP thymocytes from γ<sub>c</sub>-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<sub>c</sub>-deficient CD8SP thymocytes had very low expression of *Eomes*, *Tbx21*, or *Gzmb* and contained reduced levels of *Pf1* mRNA (Fig. 5 A, top row). In addition, γ<sub>c</sub>-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<sub>c</sub>-deficient CD8SP thymocytes from γ<sub>c</sub>-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<sub>c</sub> 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<sub>c</sub>-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-7R $\alpha$ -cKO thymocytes.** (A) Relative mRNA expression of the indicated genes. IL-7R $\alpha$ -cKO and  $\gamma_c$ -cKO data from Fig. 5 A are shown compared with TCR- $\beta^{hi}$  CD8SP thymocytes sorted from IL-7R $\alpha$ -cKO-IL-15<sup>-/-</sup> mice by gating specifically for IL-7R $\alpha$ -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-7R $\alpha$ -deleted TCR- $\beta^{hi}$  CD8SP thymocytes from IL-7R $\alpha$ -cKO (blue line) and IL-7R $\alpha$ -cKO-IL-15<sup>-/-</sup> (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,  $\gamma_c$ -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  $\gamma_c$ -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-7R $\alpha$ -cKO and  $\gamma_c$ -cKO mice and found that both IL-7R $\alpha$ -deficient and  $\gamma_c$ -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)  $\gamma_c$  expression is essential for cytotoxic lineage specification; (2) a small minority of aberrant CD8SP thymocytes can express *Runx3d* in the absence of  $\gamma_c$ -transduced signals, but such CD8 cells are arrested in their development before expression of cytotoxic lineage genes; (3) in the absence of  $\gamma_c$ , *Runx3d* fails to induce cytotoxic lineage gene expression; and (4) there exists a  $\gamma_c$ -dependent cytokine that can signal generation of CD8 cytotoxic lineage thymocytes independently of IL-7R $\alpha$ .

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

Although deficiency of either  $\gamma_c$  or IL-7R $\alpha$  during positive selection impaired the generation of CD8SP thymocytes, it was evident that  $\gamma_c$  deficiency had a greater quantitative effect

than IL-7R $\alpha$  deficiency. Indeed, CD8SP thymocyte numbers were reduced 75% by  $\gamma_c$  deficiency but were only reduced 50% by IL-7R $\alpha$  deficiency (Fig. 4 C). These findings supported the concept that, in addition to IL-7, the thymus must contain a  $\gamma_c$ -dependent cytokine that induces CD8 cytotoxic lineage specification independently of IL-7R $\alpha$ .

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

We next examined the possibility that IL-15 was the unknown  $\gamma_c$ -dependent cytokine we were seeking by breeding IL-7R $\alpha$ -cKO mice with IL-15<sup>-/-</sup> 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-7R $\alpha$ /IL-15 double deficiency significantly reduced both the frequency and number of CD8SP thymocytes below that of IL-7R $\alpha$  deficiency alone (Fig. 6, A–D). Indeed, impairment of CD8SP thymocyte generation as a result of IL-7R $\alpha$ /IL-15 double deficiency was indistinguishable from that of  $\gamma_c$  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-7R $\alpha$ /IL-15 double-deficient mice were not cytotoxic lineage cells because they displayed very low expression of *Eomes*, *Tbx21*, *Gzmb*, and *Pf1* mRNA (Fig. 7 A). Thus, IL-7R $\alpha$ /IL-15 double deficiency replicated  $\gamma_c$  deficiency, identifying IL-15 as the  $\gamma_c$ -dependent cytokine that induced the generation CD8 cytotoxic lineage T cells independently of IL-7R $\alpha$ .

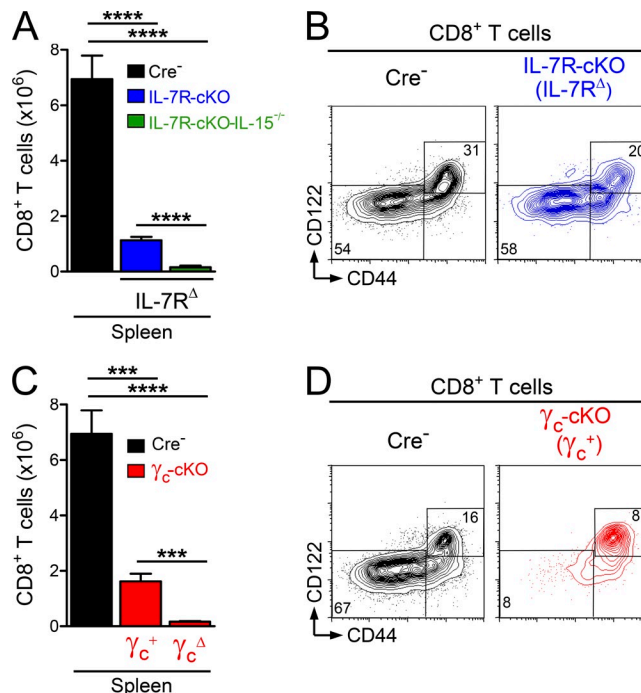
IL-15 is known to signal CD8 T cells that display a CD-44<sup>hi</sup>CD122<sup>hi</sup> memory phenotype (Dubois et al., 2006). Consequently, we wondered if the CD8SP thymocytes that were induced by IL-15 during positive selection in IL-7R $\alpha$ -cKO mice also displayed a memory phenotype. Contrary to this possibility, examination of CD8SP thymocytes induced by IL-15 in IL-7R $\alpha$ -cKO mice revealed that they were CD-44<sup>lo</sup>CD122<sup>lo</sup> 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-7R $\alpha$  and IL-15 during positive selection induces generation of naive cytotoxic lineage CD8 T cells.

#### Impact of IL-7R $\alpha$ and $\gamma_c$ on peripheral CD8 T cells

We next wished to determine if CD8SP thymocytes generated by IL-15 in IL-7R $\alpha$ -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-7R $\alpha$ -cKO and IL-7R $\alpha$ -cKO-IL-15<sup>-/-</sup> mice. We found that IL-7R $\alpha$ -deficient CD8 T cells were present in the periphery of IL-7R $\alpha$ -cKO mice, albeit in reduced numbers, and these cells were IL-15 dependent, as they essentially disappeared in IL-7R $\alpha$ /IL15 double-deficient mice (Fig. 8 A). Interestingly, IL-15-dependent IL-7R $\alpha$ -deficient CD8 T cells that displayed a naive phenotype in the thymus also displayed a CD-122<sup>lo</sup>CD44<sup>lo</sup> naive phenotype in the periphery (Fig. 8 B), indicating that IL-7R $\alpha$ -deficient CD8 T cells did not acquire a memory phenotype despite peripheral lymphopenia. Therefore, although IL-15 supported the survival of IL-7R $\alpha$ -deficient cells, IL-15 did not drive the homeostatic expansion of CD8 T cells in the absence of IL-7R $\alpha$  signaling. Unlike the naive CD8 T cells populating the periphery of IL-7R $\alpha$ -cKO mice, the periphery of  $\gamma_c$ -cKO mice contained CD8 T cells that were overwhelmingly the progeny of  $\gamma_c$ <sup>+</sup> thymocytes that had escaped  $\gamma_c$  deletion and had undergone lymphopenia-induced homeostatic expansion as revealed by their CD-122<sup>hi</sup>CD44<sup>hi</sup> memory phenotype (Fig. 8, C and D).

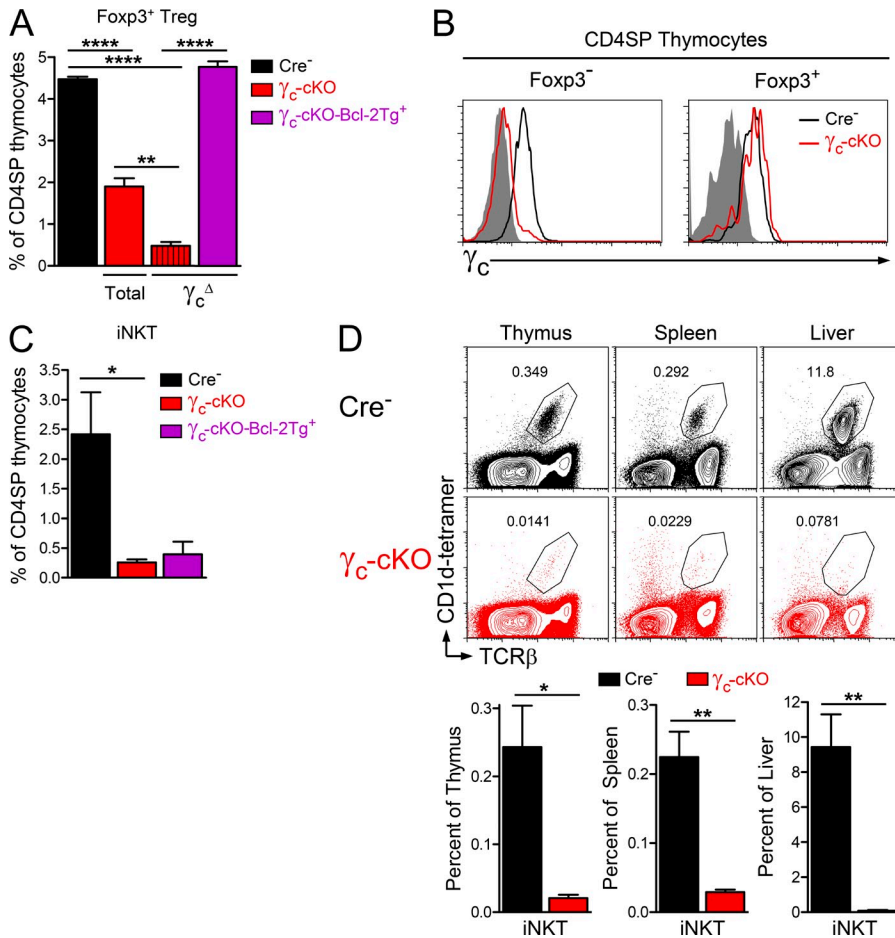
#### Impact of $\gamma_c$ on generation of CD4 regulatory and iNKT cells

Finally, we wished to understand the small but distinct effect that  $\gamma_c$  deletion had on the generation of CD4 lineage thymocytes. We considered that  $\gamma_c$  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  $\gamma_c$  deletion on induction of regulatory and iNKT cell subpopulations in  $\gamma_c$ -cKO mice.



**Figure 8. Analysis of peripheral CD8 T cells from IL-7R $\alpha$ -cKO and  $\gamma_c$ -cKO mice.** (A) Absolute cell number of splenic CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cell numbers were determined from splenocytes for Cre<sup>-</sup> control (black bar), IL-7R $\alpha$ -cKO (blue bar), and IL-7R $\alpha$ -cKO-IL-15<sup>-/-</sup> (green bar) mice. CD8<sup>+</sup> T cells from IL-7R $\alpha$ -cKO and IL-7R $\alpha$ -cKO-IL-15<sup>-/-</sup> mice were specifically gated on IL-7R $\alpha$ -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- $\beta$ <sup>+</sup> CD8<sup>+</sup> splenocytes were analyzed for frequency of naive and CD44<sup>hi</sup>CD122<sup>hi</sup> memory phenotype cells among IL-7R $\alpha$ -deleted cells (blue) compared with Cre<sup>-</sup> controls (black). Data are representative of three independent experiments. (C) Absolute CD8<sup>+</sup> T cell numbers were determined from splenocytes from Cre<sup>-</sup> control (black bar), and  $\gamma_c$ -cKO (red bars) mice. CD8<sup>+</sup> T cells from  $\gamma_c$ -cKO mice were specifically gated on  $\gamma_c$ -undeleted (labeled  $\gamma_c$ <sup>+</sup>) or  $\gamma_c$ -deleted (labeled  $\gamma_c$  $\Delta$ ) 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- $\beta$ <sup>+</sup> CD8<sup>+</sup> splenocytes were analyzed for frequency of naive and CD44<sup>hi</sup>CD122<sup>hi</sup> memory phenotype cells among  $\gamma_c$ <sup>+</sup> (escapes) cells from  $\gamma_c$ -cKO mice (red) compared with Cre<sup>-</sup> controls (black). Data are representative of three independent experiments.

Analysis of Foxp3 expression revealed that  $\gamma_c$ -deficient CD4SP thymocytes in  $\gamma_c$ -cKO mice were nearly devoid of Foxp3<sup>+</sup> cells and that the Foxp3<sup>+</sup> CD4SP cells that were present were nearly all escapees of Cre-mediated  $\gamma_c$  deletion (Fig. 9, A and B). Enrichment among Foxp3<sup>+</sup> thymocytes of cells that had escaped Cre-mediated  $\gamma_c$  deletion suggested that  $\gamma_c$  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  $\gamma_c$ -cKO mice to replace the survival benefit lost by  $\gamma_c$  deficiency. Interestingly, we found that transgenic overexpression of the prosurvival protein Bcl-2 fully restored Foxp3<sup>+</sup>



**Figure 9. Impact of  $\gamma_c$  deletion on CD4<sup>+</sup> regulatory T cells and iNKT cells.** (A) TCR- $\beta^{\text{hi}}$  CD4SP thymocytes were analyzed for Foxp3<sup>+</sup> cells from Cre<sup>-</sup> control mice (black bar) and for  $\gamma_c$ -deleted Foxp3<sup>+</sup> cells from  $\gamma_c$ -cKO (red bars) and  $\gamma_c$ -cKO-Bcl2 transgenic (purple bar) mice. Total Foxp3<sup>+</sup> CD4SP thymocytes from  $\gamma_c$ -cKO mice are shown (plain red) or after specifically gating on  $\gamma_c$ -deleted Foxp3<sup>+</sup> CD4SP thymocytes (red with black). Frequencies of Foxp3<sup>+</sup> 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)  $\gamma_c$  expression on Foxp3<sup>-</sup> (non-T reg cells) and Foxp3<sup>+</sup> CD4SP thymocytes. CD4SP thymocytes were gated on Foxp3<sup>-</sup> and Foxp3<sup>+</sup> subsets and analyzed for expression of  $\gamma_c$  from  $\gamma_c$ -cKO (red) compared with Cre<sup>-</sup> 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- $\beta^+$  CD1d-tetramer<sup>+</sup> iNKT cells from Cre<sup>-</sup> control (black bar),  $\gamma_c$ -cKO (red bar), and  $\gamma_c$ -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  $\gamma_c$ -cKO (red) or Cre<sup>-</sup> control (black) mice were analyzed for expression of TCR- $\beta$  and binding of CD1d-tetramer. Data are representative of at least three individual mice. (D, Bottom) Quantitation of pooled data from Cre<sup>-</sup> control (black) or  $\gamma_c$ -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  $\gamma_c$ -deficient CD4SP thymocytes in  $\gamma_c$ -cKO mice (Fig. 9 A), revealing that  $\gamma_c$  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  $\gamma_c$ -deficient CD4SP thymocytes (Fig. 9 C), which was also observed in the spleen and liver of  $\gamma_c$ -cKO mice (Fig. 9 D). However, the appearance of iNKT cells among  $\gamma_c$ -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  $\gamma_c$  eliminated all iNKT cells, including DN iNKT cells. Thus,  $\gamma_c$  signaling was necessary for differentiation, as well as for survival, of iNKT cells in the thymus. We conclude that expression of  $\gamma_c$  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  $\gamma_c$  and IL-7R $\alpha$  expression for signaling the differentiation of positively selected thymocytes into functionally distinct T cell subsets. The results of this study revealed that  $\gamma_c$  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<sup>+</sup>CD4<sup>+</sup> T cells. Moreover, the present results identified IL-7 and IL-15 as the cytokines responsible for CD8 cytotoxic lineage specification in vivo. Unexpectedly,  $\gamma_c$  was not required for induction of *Runx3d* expression, and *Runx3d* was not sufficient to induce cytotoxic lineage gene expression in the absence of  $\gamma_c$  cytokine signaling. Thus, the present study documents that  $\gamma_c$  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<sup>hi</sup>CD4<sup>+</sup>CD8<sup>lo</sup> 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<sup>+</sup>CD8<sup>lo</sup> 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<sup>+</sup>CD8<sup>lo</sup> Int into CD8SP thymocytes. The present study provides compelling in vivo support for this perspective by demonstrating that  $\gamma_c$  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<sub>III</sub>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  $\gamma_c$  independent because  $\gamma_c$  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-7R $\alpha$  expression eliminated two-thirds of  $\gamma_c$ -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  $\gamma_c$ -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  $\gamma_c$ -dependent cytokine as IL-15 and not IL-4. The CD8 T cells induced by IL-15 in the thymus of IL-7R $\alpha$ -cKO mice unexpectedly displayed a naive CD44<sup>lo</sup>CD122<sup>lo</sup> 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-7R $\alpha$ -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-7R $\alpha$  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-7R $\alpha$  expression. Regarding IL-7R $\alpha$ , it should be noted that IL-7R $\alpha$  cannot only pair with  $\gamma_c$  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-7R $\alpha$ /IL-15 double deficiency on CD8SP thymocytes

was identical to but not greater than that of  $\gamma_c$  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  $\gamma_c$ -dependent cytokine receptors as opposed to other cytokine receptors, it was nevertheless surprising to find that conditional deletion of  $\gamma_c$  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  $\gamma_c$  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<sup>+</sup>CD8<sup>lo</sup> Int thymocytes into CD8SP thymocytes. However, the failure of  $\gamma_c$ -deficient CD8SP thymocytes to express cytotoxic lineage genes revealed that *Runx3d* was insufficient, in the absence of  $\gamma_c$ , to specify the cytotoxic lineage fate, indicating that cytotoxic lineage specification required CD8SP thymocytes to express both  $\gamma_c$  and *Runx3d*.

One question raised by the generation of small numbers of  $\gamma_c$ -deficient CD8SP thymocytes in  $\gamma_c$ -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  $\gamma_c$ -dependent cytokine signals could induce *Runx3d* expression in developing thymocytes (Park et al., 2010). Although the present study indicates that intrathymic signals other than  $\gamma_c$  can also induce *Runx3d* expression to generate small numbers of CD8SP thymocytes, we think the unidentified intrathymic signal is most likely transduced by a  $\gamma_c$ -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  $\gamma_c$  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,  $\gamma_c$ -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- $\beta$  can up-regulate CD8 $\alpha$  expression (Konkel et al., 2011), and we have found that several  $\gamma_c$ -independent cytokines, including IL-6 and IFN- $\gamma$ , signal CD8 T cells to up-regulate *Runx3d* but not Bcl-2 (unpublished data), resembling  $\gamma_c$ -deficient CD8SP thymocytes.

A second question raised by the generation of small numbers of  $\gamma_c$ -deficient CD8SP thymocytes in  $\gamma_c$ -cKO mice was why these  $\gamma_c^-$  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  $\gamma_c$



signaling, which does not occur in normal mice but only occurs in  $\gamma_c$ -deficient mice. In fact,  $\gamma_c$ -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  $\gamma_c$ -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  $\gamma_c^{-/-}$  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  $\gamma_c$ -deficient CD8SP thymocytes from germline  $\gamma_c^{-/-}$  mice and  $\gamma_c$ -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  $\gamma_c$ , we were surprised to find that  $\gamma_c$  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  $\gamma_c$  expression was the CD1d-restricted iNKT cell subset. Generation of iNKT cells was eliminated by deletion of  $\gamma_c$  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  $\gamma_c$  and IL-7R $\alpha$ , the present study documents that  $\gamma_c$ -dependent cytokine receptors exclusively signal MHC-I-selected thymocytes to differentiate into functional T cells and that the requirement for  $\gamma_c$ -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<sub>III</sub>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  $\gamma_c$  and IL-7R $\alpha$  cKO mice.** To generate  $\gamma_c$ -cKO mice, a targeting construct was generated to replace the endogenous *Il2fg* gene, which resulted in the insertion of a loxP site after exon 1 and an frt/loxP-flanked neomycin resistance (Neo<sup>R</sup>) cassette after exon 6, referred to as the

$\gamma_c^{\text{targeted}}$  allele. Proper integration of the 5' and 3' ends of the construct were verified by PCR and Southern blot analysis.

IL-7R $\alpha$ -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<sup>R</sup> cassette in intron 3. The resulting allele is referred to as IL-7R $\alpha^{\text{targeted}}$ . Proper integration of the 5' and 3' ends were confirmed by Southern blot analysis.

ES cells with proper integration of the  $\gamma_c^{\text{targeted}}$  and IL-7R $\alpha^{\text{targeted}}$  alleles were injected into blastocysts, which were then implanted into pseudo-pregnant female recipients. Resulting chimeric mice containing the  $\gamma_c^{\text{targeted}}$  and IL-7R $\alpha^{\text{targeted}}$  knockin alleles were bred to Actin-flp transgenic female mice to induce germline deletion of the Neo<sup>R</sup> selection cassette, giving rise to the  $\gamma_c^{\text{fl}}$  and IL-7R $\alpha^{\text{fl}}$  alleles. After confirming germline transmission of the knockin alleles,  $\gamma_c^{\text{fl}}$  and IL-7R $\alpha^{\text{fl}}$  mice were then backcrossed and maintained on a B6 background. For the majority of experiments,  $\gamma_c^{\text{fl}}$  and IL-7R $\alpha^{\text{fl}}$  mice were bred to  $\gamma_c^{-/-}$  and IL-7R $\alpha^{-/-}$  germline KO mice, respectively, to generate hemizygous  $\gamma_c^{\text{fl}/-}$  females or  $\gamma_c^{\text{fl}/Y}$  males, and IL-7R $\alpha^{\text{fl}/-}$  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(TM $\beta$ 1), CD4 (RM4-5), CD8 (53-6.7), TCR- $\beta$  (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<sub>3</sub> 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  $\times$  10<sup>6</sup> cells from a 1:1 mixture of B6 and  $\gamma_c$ -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- $\beta^{\text{hi}}$  CD8SP  $\gamma_c$ -deleted or IL-7R $\alpha$ -deleted thymocytes were obtained by depletion of CD4<sup>+</sup> cells using anti-CD4 microbeads (GK1.5; Miltenyi Biotec) followed by staining with anti-TCR- $\beta$ , anti-CD8 $\alpha$ , anti-CD4 (RM4-4), and anti-CD132 or anti-CD127. For Cre<sup>+</sup> mice, TCR- $\beta^{\text{hi}}$  CD8SP cells were specifically sorted to be negative for either  $\gamma_c$  or IL-7R $\alpha$  to exclude cells that escaped Cre-mediated deletion. For Cre<sup>-</sup> control mice, CD8SP TCR<sup>hi</sup> 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*, *Pf1*, *Bcl2*, and SYBR green (QIAGEN) for distal *Runx3d* (Table S1). Relative expression levels were calculated using the  $\Delta\Delta\text{Ct}$  method using *Rpl13A* as the housekeeping gene and values from Cre<sup>+</sup> samples were normalized to the values from Cre<sup>-</sup> 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>.

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