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-7Rα or common cytokine receptor γ chain (γc) genes were deleted in thymocytes just before positive selection. We found that γc 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 γc 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 γc signaling, but these cells were developmentally arrested before expressing cytotoxic lineage genes. Thus, γc-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.
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 γc–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 γc 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 γc–dependent cytokine signaling during positive selection, we now have generated conditional KO (cKO) mice in which γc 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 γc or IL-7Rα cytokine receptors. By using these novel mice, this study reveals that γc 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 γc–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 γc–transduced signals are either critical or dispensable for in vivo thymocyte 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 γc expression during positive selection in the thymus

The IL-7 receptor is composed of two chains, the cytokine receptor common gamma chain (γc; 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). γc 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; TCRhiCD4+CD8lo) 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., 2005; Sun et al., 2005; Egawa et al., 2007; Egawa and Littman, 2008; Wang et al., 2008). Total thymocytes were stained for CD4, CD8, IL-7Rα, and γc expression on thymocytes from wild-type (Cre−) or cKO (Cre+) mice using CD4Cre (colored dashed lines) or E8IIICre (colored solid lines).

Figure 1. γc and IL-7Rα expression on thymocytes from wild-type and cKO mice. [Top] Schematic of CD4Cre and E8iiICre expression in developing thymocytes. Surface expression of γc (middle) and IL-7Rα (bottom) was determined on thymocytes from WT (Cre−) or cKO (Cre+) mice using CD4Cre (colored dashed lines) or E8iiICre (colored solid lines). Total thymocytes were stained for CD4, CD8α, TCR-β, and γc or IL-7Rα and gated on the indicated thymocyte populations. MFI of γc and IL-7Rα staining are shown. Data are representative of four independent experiments.
we constructed $\gamma_c^c$ and IL-7R $\alpha^c$ 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$^+$ mice with floxed alleles as cKO ($\gamma_c^c$-cKO and IL-7R $\alpha^c$-cKO) mice, and we refer to their deleted alleles, and cells with deleted alleles, as $\gamma_c^c$ and IL-7R $\alpha^c$.

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 $\alpha^c$ and $\gamma_c^c$ expression during positive selection for lineage fate specification in vivo. Because germline deletion of either $\gamma_c$ or IL-7R $\alpha^c$ impairs development at the DN stage (Cao et al., 1995; Di Santo et al., 1995, 1999), we constructed $\gamma_c$ and IL-7R $\alpha^c$ 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$^+$ mice with floxed alleles as cKO ($\gamma_c^c$-cKO and IL-7R $\alpha^c$-cKO) mice, and we refer to their deleted alleles, and cells with deleted alleles, as $\gamma_c^c$ and IL-7R $\alpha^c$. 

Figure 2. Generation of $\gamma_c^c$-cKO mice. (A) Targeting strategy to generate $\gamma_c^c$-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 $\gamma_c$ 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$^R$ cassette, expression of the $\gamma_c^c$ allele is equal to WT $\gamma_c$ expression in the absence of Cre throughout T cell development.
We began our study using two different Cre transgenes, CD4Cre and E8IIICre (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 E8IIICre transgene is regulated by E8III-CD8α enhancer/promoter elements that limit Cre expression exclusively to preselection DP thymocytes (Fig. 1, top; Park et al., 2010). Because E8IIICre 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 E8IIICre transgenes into γcΔ− and IL-7RαΔ− mice to generate γcΔ and IL-7RαΔ deleted alleles (Fig. 2 and Fig. 3). We found that neither CD4Cre nor E8IIICre reduced expression of γc or IL-7Rα on TCR-β− DN thymocytes, but both Cre transgenes induced deletions in DP and postselection Int (TCRhiCD4+CD8lo) and SP thymocytes, with a few more cells escaping deletion with E8IIICre than CD4Cre (Fig. 1). Importantly, Cre-mediated deletions did not alter total thymic cellularity (not depicted).
TCRhi 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 E8IIICre transgene and did not reduce the number of either DP or TCRβhi Int thymocytes (Fig. 4, A and B). However, Cre-mediated deletions significantly reduced the number and frequency of TCRβhi thymocytes as CD4SP and CD8SP cells.
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 γc and IL-7Rα deletions on CD4 lineage thymocytes because MHC-I–specific CD8 lineage T cells go through the TCRβ−CD4+CD8lo Int stage and are thus unavoidably included in the TCRβ−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 γc-cKO mice additionally deficient in B2m (Fig. 4 C). We observed that deletion of γc reduced the number of MHC-II–specific CD8SP 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 γc-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 chimera revealed no competitive disadvantage of γc-deficient CD8SP thymocytes compared with their wild-type counterparts (Fig. 4 E). Thus, deficiency of either γc 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 γc on generation of CD8 cytotoxic lineage cells in the thymus

Having determined that IL-7Rα deficiency and γc deficiency during positive selection quantitatively reduced generation of CD8SP thymocytes, we then assessed if IL-7Rα and γc were important for initiating the cytotoxic lineage program. To do so, we examined expression of cytotoxic lineage–specific genes in purified TCRβhi CD8SP thymocytes from IL-7Rα−cKO and γc-cKO mice that were electronically sorted to exclude all escapees of Cre-mediated deletion and to include only CD8SP thymocytes with deleted IL-7Rα or γc alleles. We specifically focused on expression of Eomesoderm (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 E8IIICre− and E8IIICre+ 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 E8IIICre− mice (Fig. 5 A, top row), documenting that CD8 cytotoxic lineage thymocytes could be generated in vivo in the absence of IL-7Rα-transduced signals. In marked contrast, γc-deficient CD8SP thymocytes from γc-cKO mice were
during positive selection was essential for generating cytotoxic lineage CD8 T cells, but expression of IL-7R 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-7R–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, γc-deficient CD8SP thymocytes also contained undiminished levels of Runx3d mRNA (Fig. 5 A, bottom row), but, in this case, Runx3d mRNA expression was markedly not cytotoxic lineage cells because they failed to express most cytotoxic lineage genes (Fig. 5 A, top row). Indeed, γc-deficient CD8SP thymocytes had very low expression of Eomes, Thx21, or Gzmb and contained reduced levels of Pyp1 mRNA (Fig. 5 A, top row). In addition, γc-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 γc-deficient CD8SP thymocytes from γc-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 γc during positive selection was essential for generating cytotoxic lineage CD8 T cells, but expression of IL-7Rγc 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-7Rγc–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, γc-deficient CD8SP thymocytes also contained undiminished levels of Runx3d mRNA (Fig. 5 A, bottom row), but, in this case, Runx3d mRNA expression was markedly...
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Discordant with absent expression of cytotoxic lineage-specific genes. That is, γc-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 γc-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 γc-cKO mice and found that both IL-7Rα-deficient and γc-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) γc expression is essential for cytotoxic lineage specification; (2) a small minority of aberrant CD8SP thymocytes can express Runx3d in the absence of γc-transduced signals, but such CD8 cells are arrested in their development before expression of cytotoxic lineage genes; (3) in the absence of γc, Runx3d fails to induce cytotoxic lineage gene expression; and (4) there exists a γc-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 γc or IL-7Rα during positive selection impaired the generation of CD8SP thymocytes, it was evident that γc deficiency had a greater quantitative effect than IL-7Rα deficiency. Indeed, CD8SP thymocyte numbers were reduced 75% by γc 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 γc-dependent cytokine that induces CD8 cytotoxic lineage specification independently of IL-7Rα.

We first examined the possibility that IL-4 was the unknown γc-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 γc-dependent cytokine that signaled CD8SP thymocytes independently of IL-7Rα.

We next examined the possibility that IL-15 was the unknown γc-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 γc 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*, *Thx21*, *Gzmh*, and *Ppf1* mRNA (Fig. 7 A). Thus, IL-7Rα/IL-15 double deficiency replicated γc deficiency, identifying IL-15 as the γc-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 CD44hiCD122hi 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 CD-44hiCD122hi 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 γc 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 those cells were IL-15 dependent, as they essentially disappeared in IL-7Rα/IL-15 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 CD-122hiCD44hi 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 γc–KO mice contained CD8 T cells that were overwhelmingly the progeny of γc+ thymocytes that had escaped γc deletion and had undergone lymphopenia-induced homeostatic expansion as revealed by their CD-122hiCD44hi memory phenotype (Fig. 8, C and D).

**Impact of γc on generation of CD4 regulatory and iNKT cells**

Finally, we wished to understand the small but distinct effect that γc deletion had on the generation of CD4 lineage thymocytes. We considered that γc 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 γc deletion on induction of regulatory and iNKT cell subpopulations in γc–KO mice.
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+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, \( \gamma_c \) 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⁎CD4⁺CD8lo T 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⁺CD8lo 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⁺CD8lo Int into CD8SP thymocytes. The present study provides compelling in vivo support for this perspective by demonstrating that γc 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 E8IIICre-expressing cKO mice so that Cre recombinase was expressed in preselection DP thymocytes and then terminated. As a result, we can be confident that MHC-II differentiation was really γc independent because γc 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 γc–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 γc–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 γc–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 CD44loCD122lo 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 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 γc deficiency, our current results are concordant with studies indicating that TSLP signaling does not discernibly affect 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 γc–dependent cytokine receptors as opposed to other cytokine receptors, it was nevertheless surprising to find that conditional deletion of γc 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 γc 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⁺CD8lo Int thymocytes into CD8SP thymocytes. However, the failure of γc–deficient CD8SP thymocytes to express cytotoxic lineage genes revealed that Runx3d was insufficient, in the absence of γc, to specify the cytotoxic lineage fate, indicating that cytotoxic lineage specification required CD8SP thymocytes to express both γc and Runx3d.

One question raised by the generation of small numbers of γc–deficient CD8SP thymocytes in γc–cKO mice concerned the intrathymic signal that induced their expression of Runx3d. Runx3d has been proposed as a master regulator required for cytotoxic lineage gene expression (Woolf et al., 2003; Egawa et al., 2007; Park et al., 2010) and we previously documented that γc–dependent cytokine signals could induce Runx3d expression in developing thymocytes (Park et al., 2010). Although the present study indicates that intrathymic signals other than γc can also induce Runx3d expression to generate small numbers of CD8SP thymocytes, we think the unidentified intrathymic signal is most likely transduced by a γc–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 γc, 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, γc–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 γc–independent cytokines, including IL-6 and IFN-γ, signal CD8 T cells to up-regulate Runx3d but not Bcl-2 (unpublished data), resembling γc–deficient CD8SP thymocytes.

A second question raised by the generation of small numbers of γc–deficient CD8SP thymocytes in γc–cKO mice was why these γc– 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 γc.
signaling, which does not occur in normal mice but only occurs in γδ-deficient mice. In fact, γδ-deficient CD8SP thymocytes and T cells have been found in germline-deleted γδ−/− mice (Cao et al., 1995; Di Santo et al., 1995, 1999). However, it was recently reported that γδ-deficient CD8SP thymocytes from germline γδ−/− 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 γδ− 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 γδ-deficient CD8SP thymocytes from germline γδ−/− mice and γδ-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 γc, we were surprised to find that γc signaling (probably induced by IL-2; Vang et al., 2008) was only required for the survival of newly generated Treg cells and was not required to either their differentiation or expression of Foxp3. In fact, the only CD4 T cell subset whose development required γc expression was the CD1d-restricted iNKT cell subset. Generation of iNKT cells was eliminated by deletion of γc, 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. Because iNKT cells are a unique subset of CD4 T cells, it was recently reported that IL-15 transgene. Consequently, our results are consistent with a re-

**Flow cytometry.** Monoclonal antibodies with the following specificities were obtained from BD and ebioscience: CD132 (4G3), CD127 (A7R34), CD122(TMB1), CD4 (RM4-5), CD8 (S3-6.7), TCRI-B (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.

**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 γδ-cKO-derived T cell–depleted bone marrow cells 6 h after irradiation. Chimeric mice were analyzed 8 wk after reconstitution.

**Cell sorting for qPCR.** TCRI-β6 CD8SP γδ−/δ− or IL-7Rα−/− thymocytes were obtained by depletion of CD4+ cells using anti-CD4 microbeads (GK1.5; Miltenyi Biotec) followed by staining with anti-TCR-β, anti-CD8a, anti-CD4 (RM4-4), and anti-CD132 or anti-CD127. For Cre+ mice, TCRI-β6 CD8SP cells were specifically sorted to be negative for either γc or IL-7Rα to exclude cells that escaped Cre-mediated deletion. For Cre− control mice, CD8SP TCRI-β6 cells were sorted. Cells were electronically sorted to >95% purity using a FACSAria (BD).

**qPCR gene expression analysis.** Total RNA was isolated 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, Thx21, Gzmb, Prf1, Bcl2, and SYBR green (QJAGEN) for distal Rnuc3d (Table S1). Relative expression levels were calculated using the ΔΔct 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.

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IL-7 and IL-15 specify CD8 cytotoxic lineage fate | McCaughtry et al.


