Identification of lineage-specifying cytokines that signal all CD8⁺-cytotoxic-lineage-fate ‘decisions’ in the thymus

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T cell antigen receptor (TCR) signaling in the thymus initiates positive selection, but the CD8⁺-lineage fate is thought to be induced by cytokines after TCR signaling has ceased, although this remains controversial and unproven. We have identified four cytokines (IL-6, IFN-γ, TSLP and TGF-β) that did not signal via the common γ-chain (γc) receptor but that, like IL-7 and IL-15, induced expression of the lineage-specifying transcription factor Runx3d and signaled the generation of CD8⁺ T cells. Elimination of in vivo signaling by all six of these ‘lineage-specifying cytokines’ during positive selection eliminated Runx3d expression and completely abolished the generation of CD8⁺ single-positive thymocytes. Thus, this study proves that signaling during positive selection by lineage-specifying cytokines is responsible for all CD8⁺-lineage-fate ‘decisions’ in the thymus.

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unlike IL-7 and IL-15, did not substantially upregulate genes encoding pro-survival molecules. We considered the non-γc and γc cytokines that induced Runx3d to be 'lineage-specifying cytokines'. Remarkably, elimination of in vivo signaling by all six lineage-specifying cytokines during positive selection eliminated Runx3d expression and limited Runx1 function to abolish all generation of CD8+ T cells, which proved that signaling by lineage-specifying cytokines was strictly required for CD8+-lineage-fate decisions. We conclude that CD8+-lineage-fate decisions are signaled exclusively by lineage-specifying cytokines during positive selection in the thymus.

**RESULTS**

**Characterization of γc-independent SP8 thymocytes**

We undertook the present study to identify the signals that promote the differentiation of γc-deficient thymocytes into CD8+ T cells and to determine if all CD8+-lineage-fate decisions in the thymus are signaled by cytokines. We began by assessing the generation of SP8 cells in mice in which genes encoding γc (Il2rg) are conditionally deleted in pre-selection DP thymocytes. We used Il2rg<sup>b<sup>+</sup>β<sup>+</sup>C57BL/6-Cre<sup>+</sup> and Il2rg<sup>b<sup>+</sup>β<sup>+</sup>E8III-Cre<sup>+</sup> mice (called γc<sup>KO</sup> here), in which loxP-flanked Il2rg alleles are conditionally deleted just before positive selection (via Cre recombinase, whose transgenic expression is controlled by the E8III Cdb enhancer) and that are devoid of γc proteins during positive selection and thereafter. In γc<sup>KO</sup> mice, the frequency of SP8 cells was only ~30% that in γc-sufficient C57BL/6 (B6) mice (Fig. 1a). To determine if development of the remaining SP8 cells had been signaled by non-γc cytokines, we generated γc<sup>KO</sup> mice expressing a transgene encoding the cytokine-signaling-inhibitor protein SOCS1 (SOCS1<sup>tg</sup>) that binds Jak kinases and specifically inhibits Jak-STAT-mediated signaling. Expression of this transgene further reduced the frequency of SP8 cells in γc<sup>KO</sup> mice to ~10% that in B6 mice (Fig. 1a), which indicated that most SP8 cells in γc-deficient mice were generated by non-γc cytokines that signaled via the Jak-STAT signal-transduction pathway. Notably, SP8 cells in γc<sup>KO</sup> mice upregulated the gene encoding Runx3d (Runx3; called 'Runx3d here) but did not upregulate any genes encoding pro-survival molecules (i.e., Bcl2, Mcl1 or Bcl2l11), unlike SP8 cells in γc-sufficient B6 mice which upregulated both Runx3d and Bcl2 (Fig. 1b). The failure to upregulate genes encoding pro-survival molecules diminished the survival of only the very most mature (CD69<sup>+</sup>Qa2<sup>+</sup>CD24<sup>+</sup>) SP8 thymocytes in γc<sup>KO</sup> mice, which resulted in slightly reduced overall expression of cytotoxicity-related proteins (Supplementary Fig. 1a) and the genes encoding them in SP8 thymocytes.

To identify non-γc cytokines that might signal the generation of SP8 thymocytes, we identified the cytokine receptors expressed on intermediate thymocytes undergoing MHC class I-specific positive selection in mice deficient in MHC class II and the non-classical MHC molecule CD1d, which do not differentiate into unconventional natural killer T cells but instead differentiate only into SP8 cells (Supplementary Fig. 1b–d). MHC class I-specific intermediate thymocytes were phenotypically CD4<sup>+</sup>CD8<sup>+</sup>CD69<sup>+</sup> and expressed components of receptors for five different non-γc cytokines (IL-6, IFN-γ, TSLP, IL-13 and TGF-β) (Supplementary Fig. 1b–d). These five non-γc cytokines became the focus of our study.

Figure 1 The induction of Runx3d by non-γc cytokine signals. (a) Frequency of TCR<sup>hi</sup> SP8 thymocytes in B6, γc<sup>KO</sup>, γc<sup>KO</sup>SOCS1<sup>Tg</sup> and SOCS1<sup>Tg</sup> mice (horizontal axis); numbers in or above bars indicate the frequency of SP8 cells relative to that in B6 mice, set as 100% For γc<sup>KO</sup> mice, SP8 cells that had failed to delete γc were excluded from the analysis. (b) Quantitative PCR analysis of Runx3d, Bcl2, Mcl1 and Bcl2l11 mRNA in pre-selection DP (CD4<sup>+</sup>CD8<sup>+</sup>CD69<sup>+</sup>) and SP8 thymocytes in B6 and γc<sup>KO</sup> mice; results are normalized to those of the control gene Rpl13a, and lines connect results for each type of cell in the same mouse strain. (c,d) Quantitative PCR analysis of Runx3d and Bcl2 mRNA (c) and of Runx1, Runx2 and Cdbb mRNA (d) in B6 pre-selection DP thymocytes cultured in medium alone (far left; red horizontal dashed line) or stimulated with various cytokines during the DP-stimulation assay (Supplementary Fig. 1a); results normalized as in b. (e) Quantitative PCR analysis of Runx3d mRNA in γc<sup>KO</sup> pre-selection DP thymocytes cultured in medium alone or stimulated with various cytokines during the DP-stimulation assay; results normalized as in b. Each symbol (a,c–e) represents an individual mouse (a,c,d) or replicate (e). * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 (two-tailed unpaired t-test). Data are pooled from 3-44 experiments with 5–52 mice per genotype (a), 3–13 experiments (c) or 2–3 experiments (d) as bracketed (a) or versus medium alone (c–e) or are from one of two independent experiments with technical triplicates (b,e) (mean ± s.e.m. throughout).
IL-6, IFN-γ and TSLP signaling induces Runx3d expression

Because Runx3d specifies the CD8+ cytotoxic-lineage fate, we sought to determine which non-γ-cytokines were able to signal developing thymocytes to express Runx3d. To do this, we developed a two-step in vitro assay, called the 'DP-stimulation assay', in which we electronically sorted pre-selection DP (CD4+CD8+CD69+) thymocytes, transiently stimulated the cells with the phorbol ester PMA plus ionomycin to convert them into cytokine-responsive cells, transferred those cells to a second culture containing either cytokine or medium and then assessed their expression of Runx3d mRNA (Fig. 1c–e and Supplementary Fig. 1e). We confirmed the specificity of the DP-stimulation assay by demonstrating that IL-7 induced Runx3d mRNA only in γc-sufficient B6 cells but not in γc-deficient γcKO cells (Fig. 1c,e) and by demonstrating that IL-7 upregulated Runx3d but not mRNA encoding other Runx proteins or the Runx binding partner protein CBFβ (Fig. 1d). We then tested various non-γ-cytokines in the DP-stimulation assay and identified three non-γ-cytokines (IL-6, IFN-γ and TSLP) that upregulated Runx3d mRNA in both B6 thymocytes and γcKO thymocytes (Fig. 1c,e). Signaling by these three non-γ-cytokines upregulated Runx3d mRNA but not mRNA encoding other Runx proteins or CBFβ (Fig. 1d). Notably, the three non-γ-cytokines (IL-6, IFN-γ and TSLP) stimulated little or no expression of the gene encoding the pro-survival molecule Bcl-2 (Fig. 1c), concordant with our in vivo observation that γc-deficient SP8 cells expressed Runx2, a transcription factor that is expressed in extremely small amounts in thymocytes, where it has no known function13,14 (Fig. 1d).

A complete list of cytokines tested for Runx3d induction in the DP-stimulation assay is provided (Supplementary Fig. 1f). Thus, IL-6, IFN-γ and TSLP were non-γ-cytokines that were able to signal developing thymocytes to express Runx3d.

IL-6, IFN-γ and TSLP generate SP8 cells in FTOC

We assessed the three non-γ-cytokines that upregulated Runx3d expression for their ability to signal thymocytes to differentiate into SP8 cells. For these experiments, we established fetal thymus organ cultures (FTOCs) with thymus lobes at embryonic day 16.5 and cultured them for 5 days, at which point SP8 cells had arisen (Fig. 2a and Supplementary Fig. 2a). Notably, the frequency and number of SP8 cells were 10- to 15-fold higher in B6 FTOCs than in γcKO FTOCs after culture in medium (Fig. 2a–c), with γcKO FTOCs generating fewer than 500 SP8 cells per lobe (Fig. 2c). Also notably, the addition of exogenous IL-6, IFN-γ or TSLP to γcKO FTOCs increased frequency and number of SP8 cells by three- to tenfold (Fig. 2b,c and Supplementary Fig. 2b). The addition of all three non-γ cytokines together (IL-6 plus IFN-γ plus TSLP) increased the number of SP8 cells further, but the increase was not strictly additive (Fig. 2c and Supplementary Fig. 2b); this suggested that they signaled overlapping thymocyte populations. IL-13, which did not induce Runx3d in the DP-stimulation assay, also did not induce SP8 cells in γcKO FTOCs (Supplementary Fig. 2c). Notably, the addition of each non-γ cytokine to B6 FTOCs increased the generation of SP8 cells, as did the addition of IL-7 (Supplementary Fig. 2d); this indicated that γc and non-γ cytokines were limiting in FTOCs, so that the addition of any one CD8+ cell-promoting cytokine quantitatively increased the generation of SP8 cells. The increase in the number of SP8 cells by exogenous non-γ cytokines was not due to the extensive proliferation of a small number of endogenously generated SP8 cells, because SP8 thymocytes (unlike immature TCRβ+ SP thymocytes) incorporated little of the thymidine analog EdU, added during the final 14 h of FTOC (Fig. 2d). We concluded that the non-γ-cytokines IL-6, IFN-γ and TSLP were able to signal developing thymocytes to differentiate into SP8 cells.

Non-γ-cytokines signal the generation of SP8 cells in vivo

To determine if endogenously produced IL-6, IFN-γ and TSLP actually signaled the generation of SP8 cells in the thymus, we
eliminated in vivo signaling by these cytokines either by deleting the gene encoding the cytokine itself (i.e., IL-6) or by deleting a component of their surface receptor (for example, IFN-γR or TSLPR) in γcKO mice (Fig. 3). We found that eliminating signaling by one cytokine had a modest effect on the generation of SP8 cells, as eliminating TSLP signaling had no significant effect, and the effect of eliminating signaling by IL-6 or IFN-γ managed to just achieve significance (Fig. 3a). In contrast, eliminating signaling by any two of these cytokines in γcKO mice (for example, γcKOIL-6KOIFN-γRKO mice) resulted in a highly significant reduction in both the frequency and the number of SP8 cells (Fig. 3b), which suggested that these cytokines had overlapping effects in vivo.

Because of the potential for cytokine redundancy, we thought that full appreciation of the contribution of non-γc cytokine signaling to the generation of SP8 cells might require the elimination of in vivo signaling by all three non-γc cytokines (IL-6, IFN-γ and TSLP). Accordingly, we generated mice in which signaling by γc and the three non-γc cytokines was eliminated (γcKOIL-6KOIFN-γRKO TSLPRKO; called ‘CytoQuad’ here). Notably, CytoQuad thymocytes differed from γcKOIL-6KOIFN-γRKO thymocytes in that TSLP signaling would be eliminated only in CytoQuad thymocytes. Comparison of these two mouse strains demonstrated that the frequency of SP8 cells and the number of SP8 cells were both significantly lower in CytoQuad mice than in γcKOIL-6KOIFN-γRKO mice (Fig. 3b), which revealed the contribution of TSLP signaling to the generation of SP8 cells. Thus, signaling by TSLP, as well as by IL-6 and IFN-γ, contributed to the generation of SP8 cells, but the contribution of each was obscured by cytokine redundancy.

Notably, comparison of the thymus profiles of γcKO mice with that of CytoQuad mice revealed that elimination of signaling by all three non-γc cytokines (IL-6, IFN-γ and TSLP) affected the generation of SP8 cells but not that of other thymocyte subsets (Fig. 3c). Quantifying the effect of non-γc cytokines on the generation of SP8 cells revealed that the frequency of SP8 cells and the number of SP8 cells were both significantly lower in CytoQuad mice than in γcKO mice (Fig. 3b) and also revealed that the CytoQuad thymus contained only 8% of the SP8 cells present in the B6 thymus (Fig. 3d). Notably, the SP8 cells that were generated in the thymus of both γcKO mice and CytoQuad mice had low or absent expression of Bcl-2 and failed to survive in the lymphoid periphery (Supplementary Fig. 3). We concluded that in vivo signaling by the non-γc cytokines IL-6, IFN-γ and TSLP promoted the generation of SP8 cells and that in vivo signaling by these three cytokines accounted for most, but not quite all, of the SP8 thymocytes generated in γc-deficient mice.
\( \gamma_c \) and non-\( \gamma_c \) cytokines signal overlapping SP8 subsets

Having documented that non-\( \gamma_c \) cytokines (IL-6, IFN-\( \gamma \) and TSLP) induced Runx3d and signaled the generation of SP8 cells in \( \gamma_c \)-deficient mice, we considered these cytokines to be lineage-specifying cytokines. We then sought to determine if \( \gamma_c \) and non-\( \gamma_c \) lineage-specifying cytokines signaled generation of the same or different SP8 cells, such that some were generated by \( \gamma_c \) cytokines and others were generated by non-\( \gamma_c \) cytokines. Three experimental observations suggested that \( \gamma_c \) lineage-specifying cytokines and non-\( \gamma_c \) lineage-specifying cytokines generated overlapping, not distinct, SP8 cells. First, non-\( \gamma_c \) cytokine signaling did not generate additional SP8 cells in \( \gamma_c \)-sufficient mice, as the frequency of SP8 cells was no higher in B6 mice than in IL-6\(^{KO}\)IFN-\( \gamma \)R\(^{KO}\)TSLPR\(^{KO} \) mice (Fig. 4a). Second, \( \gamma_c \) cytokines and non-\( \gamma_c \) cytokines generated SP8 cells with a similar repertoire of TCR \( \beta \)-chain variable (\( V_\beta \)) regions, as the pattern of TCR \( V_\beta \) use was similar in \( \gamma_c \)-sufficient (B6) mice and \( \gamma_c \)-deficient (\( \gamma_c^{KO} \)) mice (Fig. 4b). Third, \( \gamma_c \) cytokines and non-\( \gamma_c \) cytokines did not ‘preferentially’ generate high-affinity SP8 cells relative to their generation of low-affinity SP8 cells, as assessed by mouse strains with transgenic expression of TCRs (OT-I or HY): the number of SP8 thymocytes with high-affinity OT-I TCRs was essentially equal to those with low-affinity HY TCRs in both \( \gamma_c \)-sufficient mice and \( \gamma_c \)-deficient mice (Fig. 4c). We concluded that in \( \gamma_c \)-sufficient mice, \( \gamma_c \) cytokine signals and non-\( \gamma_c \) cytokine signals did not generate distinct SP8 populations, so the contribution of non-\( \gamma_c \) cytokines to the generation of SP8 cells would be best appreciated in mice lacking \( \gamma_c \) cytokine signals (Supplementary Fig. 4).

Identification of TGF-\( \beta \) as a lineage-specifying cytokine

Analysis of CytoQuad mice revealed that \( \gamma_c \) and non-\( \gamma_c \) lineage-specifying cytokines together accounted for ~92% of the SP8 cells generated in the B6 thymus (Fig. 3d). Notably, each of these lineage-specifying cytokines utilizes Jak-STAT proteins to transduce intracellular signals. Consequently, the few SP8 thymocytes that arose in CytoQuad mice, like those in \( \gamma_c^{KO} \)SOCS1\(^{KO} \) mice, appeared to be generated by an as-yet-unidentified lineage-specifying cytokine that signaled independently of Jak-STAT proteins\(^{23,31} \). To identify such a cytokine, we performed computational analyses to search for binding motifs for cytokine-signal-transducing molecules in Runx3d regulatory sequences conserved in humans and mice (Supplementary Fig. 5a).

As expected from our current observations, we found multiple STAT-binding motifs in Runx3d conserved sequences (Supplementary Fig. 5a). In addition, we found multiple binding motifs for the signal transducer SMAD (Supplementary Fig. 5a), which suggested that TGF-\( \beta \) signaling might also contribute to the regulation of Runx3d.

To assess whether the few SP8 cells in CytoQuad mice might have been signaled by TGF-\( \beta \), we carefully analyzed the molecular profile of CytoQuad SP8 cells and found that it resembled that of B6 SP8 cells, except that the expression of Runx2 mRNA was significantly higher in CytoQuad SP8 cells than in B6 SP8 cells (Fig. 5a). Even though Runx2 exerts no known function in the normal thymus, TGF-\( \beta \) upregulates Runx2 in bone\(^{24,25} \), and TGF-\( \beta \) signaling upregulated Runx2 in the DP-stimulation assay (Fig. 1d). TGF-\( \beta \) also upregulates expression of the integrin CD103 (\( \alpha_\delta \)) on peripheral CD8\(^{+} \) T cells\(^{25} \), and we found that CytoQuad SP8 thymocytes were CD103\(^{hi} \) (Fig. 5a). Consequently, these observations, together with published reports that TGF-\( \beta \) signals precursors of intraepithelial lymphocytes to express the co-receptor CD8\( \alpha_2 \), regulates expression of the cytokine receptor IL-7R \( \alpha \) on SP8 cells\(^{27} \) and signals B cells to express Runx3 (ref. 28), led us to further assess the possibility that TGF-\( \beta \) was the remaining Jak-STAT-independent lineage-specifying cytokine.

To do that, we first assessed the ability of TGF-\( \beta \) to signal the generation of SP8 cells in \( \gamma_c^{KO} \) FTOCs. Exogenous TGF-\( \beta \) significantly increased both the frequency and the number of SP8 cells (Fig. 5b), and the molecular profile of these SP8 cells bore a striking resemblance to that of SP8 thymocytes in adult CytoQuad mice, including upregulated (but still very low) expression of Runx2 mRNA and CD103\(^{hi} \) surface expression (Fig. 5b). Thus, TGF-\( \beta \) signaling did upregulate Runx3d and generate SP8 cells in \( \gamma_c^{KO} \) FTOCs, even though it had not upregulated Runx3d in the DP-stimulation assay.

As an explanation, we think that SMAD-mediated transduction of TGF-\( \beta \) signaling might require more time to upregulate Runx3d than was available in the \textit{in vitro} DP-stimulation assay.

Having determined that TGF-\( \beta \) signaling generated SP8 cells in FTOC, we wanted to then assess the effect on SP8 cells of eliminating \textit{in vivo} TGF-\( \beta \) signaling during positive selection. However, elimination of \textit{in vivo} TGF-\( \beta \) signaling triggers a devastating lymphoproliferative disorder that is usually fatal\(^{29,30} \). Fortunately, we found that \( \gamma_c \) deficiency prevented disease and that \( \gamma_c^{KO} \) mice with deletion of the gene encoding TGF-\( \beta \)-R1 in pre-selection DP thymocytes (\( \gamma_c^{KO} \)TGF-\( \beta \)-R1\(^{KO} \))...
mice) remained healthy and disease free (data not shown). As confirmation that in vivo TGF-β signaling had been eliminated in these healthy mice, SP8 cells in γcKO TGF-βR1KO mice were CD103lo, whereas SP8 cells from related mice with intact TGF-β signaling were all CD103hi (Fig. 5c and Supplementary Fig. 5b). Thus, these results identified TGF-β as a fourth non-γc lineage-specifying cytokine.

Elimination of all lineage-specifying cytokine signals
To confirm that TGF-β was the final and Jak-STAT-independent cytokine responsible for the generation of SP8 cells, we eliminated in vivo TGF-β signaling during positive selection by generating γcKO SOCS1TGF-βR1KO mice. Notably, γcKO SOCS1TGF-βR1KO mice expressed EIIIII-Cre, so the only difference between them was the potential for TGF-β signaling during positive selection. Remarkably, whereas γcKO SOCS1TGF-βR1KO mice generated a few SP8 thymocytes (~12 × 10⁴), γcKO SOCS1TGF-βR1KO mice appeared to be devoid of SP8 cells (Fig. 6). Careful quantification revealed ~6 × 10⁴ total SP8 cells in the γcKO SOCS1TGF-βR1KO mouse, of which 2 × 10³ were documented escapes from Cre-mediated deletion of the gene encoding TGF-β1 (as revealed by high expression of CD103) or from the failure to express SOCS1 (as revealed by absent expression of the Myc epitope tag that was on the transgenically expressed SOCS1 protein). In fact, the very few remaining SP8 cells in γcKO SOCS1TGF-βR1KO mice represented a remarkable 99.9% depletion relative to the ~3.2 × 10⁴ SP8 cells in B6 mice, and we think these few SP8 cells arose only because they were signaled by cytokines before their surface cytokine receptors were completely deleted. In comparison, thymocyte populations with conditional deficiency in TGF-β1 mice were essentially devoid of SP8 cells (Fig. 6). We concluded that TGF-β was a Jak-STAT-independent lineage-specifying cytokine that promoted the generation of SP8 cells. More notably, we concluded that all CD8⁺-lineage-fate decisions in the thymus required cytokine signaling.

The generation of SP8 cells by Runx1 requires cytokine signaling
Because the generation of SP8 cells can be mediated by either Runx3d or Runx1 (ref. 31), we were surprised that SP8 cells were not generated by Runx1 in the absence of cytokine-signdaled Runx3d expression (Fig. 6). To understand why this was the case, we first confirmed that Runx1 and Runx3d could each promote the generation of SP8 cells in mice deficient in the other, in that SP8 cells were generated in mice with conditional deficiency in Runx1 induced by CD4-Cre (Runx1cKO) and mice deficient in Runx3d (via expression of yellow fluorescent protein (YFP) from both Runx3d alleles: Runx3dTg(YFP/YFP)) but not in mice deficient in both (Runx1cKO Runx3dTg(YFP/YFP)) (Fig. 7a). These results confirmed that Runx1 and Runx3d were each able to promote the generation of SP8 cells and they excluded the possibility of any substantial contribution by Runx2, since the doubly deficient mice were essentially devoid of SP8 cells (Fig. 7a).

To elucidate why Runx1 did not promote the generation of SP8 cells in thymocytes that had not received cytokine signaling, we sought to determine if Runx1 might require cytokine signaling to generate SP8 cells. To address this possibility, we determined the effect of eliminating γc cytokine signaling in Runx3d-deficient mice. Indeed, γc deficiency reduced the Runx1-mediated generation of SP8 cells in γcKO Runx3dTg(YFP/YFP) mice by three- to fivefold (Fig. 7b), which indicated that signaling by γc cytokines was in fact required for the Runx1-mediated generation of SP8 cells. The simplest explanation was that γc cytokine signaling upregulated expression of Runx1 (or possibly Cbfβ), but this was not the case, as γc-deficient SP8 cells and γc-deficient SP8 cells in Runx3dTg(YFP/YFP) mice contained the same

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**Figure 5** TGF-β contributes to generation of γc-independent CD8⁺ T cells. (a) Quantitative PCR analysis of Runx3d, Runx1 and Runx2 mRNA in pre-selection DP and SP8 thymocytes from B6 and CytoQuad mice (left), and flow-cytometry analysis of CD103 expression on DP and SP8 thymocytes from CytoQuad mice (right); mRNA results are normalized to those of Rp113a, and lines connect results for each type of cell in the same mouse strain. (b) Flow cytometry (left) of TCRβhi thymocytes from γcKO mice, assessed on day 5 of FTTC with medium or TGF-β, and quantification of SP8 cells generated in FTTC (middle left); right, quantitative PCR analysis of Runx3d, Runx1 and Runx2 mRNA in (middle right) and flow-cytometry analysis of CD103 on (right) DP and SP8 thymocytes from γcKO mice, assessed after FTTC with TGF-β (analyzed and presented as in a). Numbers in outlined areas (left) indicate percent SP8 cells among TCRβhi thymocytes. (c) Expression of CD103 on SP8 thymocytes from γcKO, γcKO TGF-βR1KO, CytoQuad and SOCS1KO mice, presented as mean fluorescence intensity (MFI). Each symbol (b (middle left), c) represents an individual thymic lobe (b (middle left)) or mouse (c). *P < 0.05 and ****P < 0.0001, as bracketed or versus γcKO (two-tailed unpaired t-test). Data are from one experiment representative of two experiments with technical triplicates from one to six mice (pooled results) or two experiments with eight to twelve lobes (b: mean ± s.e.m.) or two experiments with four mice per genotype (c: mean ± s.e.m.).
Cytokine signaling of MHC class II–selected SP8 cells

Finally, we sought to determine if cytokine signaling was also required for the misdirected differentiation of MHC class II–selected thymocytes into SP8 cells. Misdirected differentiation occurs in ThPOK-deficient mice, such that MHC class II–selected thymocytes incorrectly differentiate into SP8 cells, and this was especially evident in mice deficient in ThPOK and B2-microglobulin (ThPOK<sup>−/−</sup>B2m<sup>−/−</sup> mice) (Fig. 7e and Supplementary Fig. 6b). To assess the requirement for cytokine signaling, we compared γ<sup>−/−</sup>-sufficient ThPOK<sup>−/−</sup>B2m<sup>−/−</sup> mice with γ<sup>−/−</sup>-deficient ThPOK<sup>−/−</sup>B2m<sup>−/−</sup> mice. We found that in this context, γ<sup>−</sup> deficiency resulted in two- to threefold fewer misdirected SP8 thymocytes (Fig. 7e and Supplementary Fig. 6b); this demonstrated that cytokine signaling was important even for the generation of SP8 cells by developmental misdirection. We concluded that signaling by lineage-specifying cytokines was required for all CD8<sup>+</sup>-lineage-fate decisions in the thymus (Supplementary Fig. 7).

DISCUSSION

Our present study has proven that signaling by lineage-specifying cytokines during positive selection is responsible for all CD8<sup>+</sup>-lineage-fate decisions in the thymus. Lineage-specifying cytokines are a diverse group of cytokines that consist of two γ cytokines (previously identified as IL-7 and IL-15) and four non-γ cytokines (IL-6, IFN-γ, TSLP and TGF-β) with disparate immunological functions in the periphery<sup>35–39</sup>. However, in the thymus, all of these cytokines induced Runx3d and signaled the generation of SP8 cells, whether they induced genes encoding pro-survival molecules or not. Most notably, elimination of signaling by all six lineage-specifying cytokines during positive selection abolished the generation of SP8 cells, which revealed that the cytokine requirement for determination of the CD8<sup>+</sup> lineage could not be circumvented by other signals in the thymus, including TCR signals.

Determination of the CD4<sup>+</sup>-lineage fate versus the CD8<sup>+</sup>-lineage fate in the thymus is currently best described by the kinetic signaling model<sup>4,6,13,15</sup>. A unique requirement of this model is that the CD8<sup>+</sup>-lineage fate must be signaled by cytokines and not by TCRs, which signal the CD4<sup>+</sup>-lineage fate. While many predictions of the kinetic signaling model have been fulfilled<sup>13,18,40</sup>, this key precept has remained controversial and unproven. In the present study, abolishing the generation of SP8 cells via the elimination of cytokine signaling documented that CD8<sup>+</sup>-lineage-fate decisions required cytokine signaling during positive selection and that the requirement for cytokine signaling was not circumvented by signaling from other receptors in the thymus, including TCRs. In fact, cytokine signaling was required even for the generation of SP8 cells during MHC class II–specific positive selection by misdirected differentiation in ThPOK-deficient mice.

Before our present study, it was difficult to distinguish the effect of cytokine signaling on CD8<sup>+</sup>-lineage-fate decisions from an effect of cytokine signaling on thymocyte survival. However, the non-γ lineage-specifying cytokines identified here (IL-6, IFN-γ, TSLP and TGF-β) lacked pro-survival function but signaled the generation of SP8 cells anyway, which effectively excluded any possibility that cytokine signaling simply promoted the survival of otherwise CD8<sup>+</sup>-lineage-committed cells. These non-γ cytokines display diverse functions in the periphery, as IL-6, IFN-γ and TSLP are pro-inflammatory<sup>35–37</sup> and TGF-β is anti-inflammatory<sup>41</sup>. In the thymus, IFN-γ is produced by the NKT1 subset of natural killer T cells<sup>42,43</sup>, TSLP is produced by medullary thymic epithelial cells<sup>44</sup>, IL-6 is produced by epithelial cells and fibroblasts<sup>35</sup>, and TGF-β is produced by thymic epithelial cells<sup>45</sup> and macrophages<sup>46</sup>. It is also conceivable that non-γ cytokines produced in the periphery can enter the thymus and contribute to CD8<sup>+</sup>-lineage determination. For example, the misdirected differentiation of MHC class II–selected cells with transgenic
expression of the AND TCR into SP8 cells that is observed in SOCS1-deficient mice is thought to be due to elevated serum concentrations of the pro-inflammatory cytokines IFN-γ and IL-6 as a consequence of SOCS1 deficiency.47, Also, we observed during this study that the number of SP8 thymocytes in neonatal IL-6−/− mice was greater in those from an Il6−/− mother than in those from an Il6+/− mother (data not shown), which suggested that peripheral IL-6 from the mother was able to augment the generation of SP8 thymocytes. 

Our present study has delineated the cytokine-signaling requirements for the differentiation of SP8 cells in the thymus from the cytokine-signaling requirements for the survival of SP8 cells in the periphery. Six different lineage-specifying cytokines (IL-7, IL-15, IL-6, IFN-γ, TSLP and TGF-β) were found to signal the generation of SP8 thymocytes, but the maintenance of naive CD8+ T cells in the periphery requires IL-7 signaling.48 As a result, γc−/−CD8+ cells cannot survive in the periphery, but this does not mean that non-γc cytokines do not normally contribute to the generation of peripheral CD8+ T cells. In fact, we think that individual SP8 cells might normally be signaled by several cytokines (for example, γc cytokines and non-γc cytokines) during their differentiation in the thymus. As an example, B6 SP8 thymocytes were generated mostly by γc cytokines, but they were also CD103hi, which indicated that they were signaled by TGF-β as well.

Our study has also provided new insights into the effect of cytokine signaling on the Runx1 and Runx3d-mediated generation of SP8 cells. Cytokine signaling was needed to induce Runx3d expression and was also required for Runx1’s function in developing SP8 cells. However, the molecular basis for the Runx1 cytokine requirement remains uncertain, but one possibility is that γc cytokine signaling is necessary to induce the expression of molecular co-factors such as AP4 (ref. 49), which contributes to silencing of the gene encoding the co-receptor CD4 (ref. 50). In any event, Runx1 and Runx3d are thought to be redundant for the generation of SP8 cells, because each generates SP8 cells in mice genetically deficient in the other, and it has been presumed that both Runx factors participate in the generation of SP8 cells in ‘normal’ (i.e., Runx-sufficient) mice. However, the fact that cytokine-induced Runx3d inhibits Runx1 expression makes it difficult for both Runx factors to participate in the generation of SP8 cells in ‘normal’ (i.e., Runx-sufficient) thymocytes. Instead, we think that Runx3d is the CD8+–lineage-specifying transcription factor and Runx1 is not, and that Runx1 mediates the generation of SP8 cells only in Runx3d-deficient mice, because cytokine signaling of Runx3d-deficient thymocytes cannot result in the Runx3d-mediated inhibition of Runx1 expression.

Finally, redundancy among lineage-specifying cytokines in the thymus caused difficulty in appreciating the contribution of individual non-γc cytokines to the generation of SP8 cells. However, we think that another result of cytokine redundancy is that the number of SP8 thymocytes is determined by the total amount, rather than the identity, of CD8+-lineage-specifying cytokines available to positively selected thymocytes in vivo. Consequently, the exogenous addition of any one lineage-specifying cytokine would be expected to increase the total number of SP8 thymocytes in vivo, as we observed in FTOCs.

In conclusion, our present study has identified a diverse group of lineage-specifying cytokines whose signaling was strictly required during positive selection for all CD8+-lineage-fate decisions in the thymus. In addition, this study has identified an interplay between
Differential requirements for Runx proteins in CD4 repression

Targeted disruption of the mouse transforming growth factor β receptor 1 (TGFβR1) in thymocytes; and A.S. designed and supervised the study, analyzed data and provided helpful discussions; A.A. and B.E. generated and performed experiments, supported by the Intramural Research Program of the US National Institutes of Health, the National Cancer Institute, the Center for Cancer Research and the US National Institutes of Health (R01AI097244-01A1 to T.E.).

AUTHOR CONTRIBUTIONS
R.E. designed the study, performed experiments, analyzed data and contributed to the writing of the manuscript; T.K., X.T., T.I.G. and T.E. performed experiments, analyzed data and provided helpful discussions; A.A. and R.E. generated experimental mice; and A.S. designed and supervised the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Mice with IoxP-flanked alleles encoding γc were bred to E8III-Cre to obtain γcKO mice as previously described. SOCS1JOmice were provided by M. Kubo; Tslpr+/-; (TSLPRKO)mice were provided by W. Leonard, Tgbr1Flmice were provided by W. Chen. Runx3Δp/Δp; Tgbr1Flmice were provided by D. Littman; and Zbtb7a-/- (ThPko) mice were synthesized by R. Bosselut. Tgbr1Flmice were bred to E8III-Cre mice to obtain TGF-βR1ΔKO mice, while CbfbKO and Runx1ΔFOXK1 (from The Jackson Laboratory) were bred to CD4+Cre mice to obtain CBFRKO and Runx1ΔKO mice. Il6−/-, Il6r−/-, Il1r−/- (IL-4Rα−/−), B2mKO, HY RAGKO, OT-I RAGKO and H2-Ab−/+Cd1d−/− (MICHIKOCD1ΔKO) mice were bred in our own animal colony at the National Cancer Institute. C57BL/6 (B6) mice were obtained from the Frederick National Laboratory for Cancer Research. All animal experiments were approved by the National Cancer Institute Animal Care and Use Committee, and mice were cared for in accordance with National Institutes of Health guidelines.

Flow cytometry. Monoclonal antibodies with the following specificities were used: CD131 (4G3), CD103 (M290), CD126 (D7715A7), CD24 (M1/69), CD25 (7D4), CD5 (53-7.3), CD122 (TM-Beta 1), T-bet (94-46) and CD4 (RM4-4), all from Becton Dickinson; CD119 (2E2), CD127 (A7R34), CD197 (4B12), CD4 (7D4), CD5 (53-7.3), CD122 (TM-Beta 1), T-bet (04-46) and CD4 (RM4-4), all from eBioscience; CD69 (H1.2F3), CD124 (I015F8), granzyme B (GB11), CD8 (53-7.3), Eomes (Dan11mag), perforin (OMAK-D) and TCR γδ (2.4G2, custom made by Harlan), followed by staining with fluorochrome-conjugated antibodies (identified above). For intracellular staining of cells, surface staining was performed first (antibodies identified above), followed by fixation and permeabilization with either the Fixation/Permeabilization Solution Kit (BD) or the Transcription Factor Staining Buffer Set (eBioscience), followed by intracellular staining (antibodies identified above). Cells were acquired using an LSRII or LSRFortessa (Becton Dickinson). Doublet and dead cells were excluded from analysis by forward light-scatter and propidium iodide gating. Data were analyzed using EIB-Flow Control software developed at the Institute of Health guidelines.

FTOC. Thymic lymphocytes were stained with anti-CD4 microbeads on a FACSAria II. To obtain pre-selection DP thymocytes, whole thymocytes were stained for CD4, CD8, and CD69 (antibodies identified above) and electronically sorted to obtain purified CD4+CD8+CD69+ cells. To obtain SP8 thymocytes, whole thymocytes were depleted of CD4+ cells with anti-CD4 microbeads on MACS columns (Miltenyi Biotec); then stained for CD4, CD8, TCRβ and CD132 (antibodies identified above) and then electronically sorted to obtain either purified CD4+CD8+TCRβ+ cells (B6) or purified γc+CD4+8+TCRβ+ cells (γcΔKO).

Quantitative real time PCR. Total RNA was isolated using RNeasy Mini or the RNAasy Micro kit (Qiagen), genomic DNA was removed from samples using TURBO DNA-free Kit (Applied Biosystems) and CDNA was synthesized using SuperScript III with oligo(dT) primers (Invitrogen). TaqMan primers and probes (Applied Biosystems) or Quant iTect SYBR Green detection system (Qiagen) reagents were used for real time PCR, and samples were analyzed on an ABI PRISM 7900HT Sequence Detection System or QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The following TaqMan assays were used: Bcl2 Mm00477631_m1, Mcl-1 Mm00725832_s1, Bcl2 Mm00437783_m1, Runx1 Mm01213405_m1 and Rpl13a Mm01612986_g1. The following primers were used for SYBR green assays: Runx1 F: 5'-CGGCAATGTTGGAATGTTGTT-3', R: 5'-CCCGGATGAGTGCTTCTGTT-3', Runx2 F: 5'-CCGATCGACTGTGAGGTGACT-3', R: 5'-AGGATGCGAGGAGGATCTTTG-3', R: 5'-CGGGCATTACGAGGAACCTACG-3', R: 5'-CTTACGGCGCCGTCGTCTCCG-3', CBFB F: TGGTGTACAAAGGACCAACACTG-3', R: 5'-TGTGGCAGTCGCTTCCCTGC-3' and Rpl13a F: 5'-CGAGGCGATCTGTCGCCACAAA-3', R: 5'-AGCAGGGACACACCCACTGG-3'. All mRNA values were determined by quantitative PCR and results are presented relative to those of Rpl13a.

All mRNA values were determined by quantitative PCR and results are presented relative to those of Rpl13a.

DP-stimulation assay. Pre-selection DP thymocytes (CD4+CD8+ΔKO) were electronically sorted and cultured with 0.3 ng/ml PMA (Sigma) and 0.3 µg/ml ionomycin (Sigma) for 16 h, then were washed and cultured in medium alone for 10 h. Cells were then co-cultured with cytokines for 16–20 h, harvested and assayed for mRNA expression. All mRNA values were determined by quantitative PCR and are presented relative to those of Rpl13a.

The following cytokines were used: IL-7 (10 ng/ml, Peprotech), IL-6 (45 ng/ml, eBioscience), IFN-γ (25 ng/ml, Peprotech), TSLP (25 ng/ml, eBioscience), TGF-β (10 ng/ml, eBioscience), IL-13 (25 ng/ml, eBioscience), IL-4 (40 ng/ml, Peprotech), IFN-α (50 ng/ml, eBioscience) and IFN-β (50 ng/ml, Peprotech).

Alignment of Runx3d distal promoter region. VISTA (http://genome.lbl.gov/vista/index.shtml) was used for analysis of conservation between human and mouse genomic Runx3d regulatory elements. The Genomatix MatInspector software package was used to predict nuclear factor binding motifs in these conserved regions.

Statistical analysis. Statistical analysis was performed with GraphPad Prism 7 software using the two-tailed unpaired t-test. P values of 0.05 or less were considered significant.

A Life Sciences Reporting Summary for this paper is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.
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Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample size was determined empirically, at least two independent experiments were conducted.

2. Data exclusions
   Describe any data exclusions.
   For gc-cKO mice, animals with more than 20% gc+ escapee thymocytes were excluded.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   The experimental findings were reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Animals were allocated to groups based on their genotype.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   No blinding was used.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   □ Confirmed
   □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □ A statement indicating how many times each experiment was replicated
   □ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Flow Control version 4.2.0.4
FlowJo version 10
GraphPad Prism 7.01

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

BD: CD132 (clone 4G3, Cat # 554457, Lot # 6348614 and 7096903), CD103 (clone M290, Cat # 557494, Lot # 4128518, Cat # 557495, Lot # 84906, Cat # 562772, Lot # 5288786), CD126 (clone D7715A7, Cat # 554462, Lot # 24780), CD69 (clone H1.2F3, Cat # 553237, Lot # 6291860, Cat # 553236, Lot # 4111613) CD4 (clone RM-4-5, Cat # 557681, Lot # 6053873), TCRb (clone HS7-597, Cat # 553169, Lot # 3290527), CD25 (clone 7D4, Cat # 553070, Lot # 00504), CD5 (clone 53-7.3, Cat # 553020), T-bet (clone O4-46, Cat # 561267, Lot # 4234512), CD24 (clone M1/96, Cat # 553260), anti-mouse TCR-Vb screening panel (Cat #; 557004)

eBioscience: CD119 (clone 2E2, Cat # 13-1191-82, Lot # E16529-103), CD127 (clone A7R34, Cat # 13-1271-82, Lot # E02715-1630, Cat # 12-1271-83, Lot # E01471-1630), CD197 (clone 4B12, Cat # 13-1971-85 Lot # E02799-1632, Cat # 12-1971-83, Lot # E01589-1632, Cat # 17-1971-82, Lot # 4314325), CD4 (clone RM4-4, Cat # 11-0043-85, Lot # E00087-1630 and E00087-1633) CD4 (clone RM4-5, Cat # 25-0042-82, Lot # 4304293), CD130 (clone KGP130, Cat # 17-1302-82, Lot # E12886-102), CD213a1 (clone 13MOKA, Cat # 12-2130-80, Lot # E12988-107), CD122 (clone TM-Beta 1, Cat # 13-1222-82), Qa2 (clone 69H1-9-9, Cat # 11-5996-82), CD28 (clone 37.51, Cat # 13-0281-85, Lot # E028247), Perforin (clone OMAK-D, Cat # 17-9392-80, Lot # 4339809), Eomes (clone Dan11mag, Cat # S0-4875-82, Lot # E15619-102);

BioLegend: CD124 (clone I015F8, Cat # 144804, Lot # B193122) and IFNgRb (clone MOB-47, Cat # 113604, Lot # B180126), TCRb (clone HS7-597, Cat # 109218, Lot # B206104), C4D (clone RM4-5, Cat # 100508, Lot # B192084), CDBb (clone YT5156.7.7, Cat # 126612, Lot # B156364), Granzyme B (clone GB11, Cat # 515405, Lot # B213985);

Invitrogen: CD8a (clone 5H10, Cat #MCD0828, Lot # 1741798 and 1832959);

R&D Systems: c-Myc (clone 9E10, Cat # IC3696R, Lot # AEID0116201); Polyclonal antibodies specific for TSLPR (Cat # FAB5461P, Lot # AAG00414091) and TGFbRII (Cat # FAB532P, Lot # AACQ0715051).

Antibodies were chosen based on the validation statements for species (mouse) and application (flow cytometry) on the manufacturer’s website.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No cell lines were used.

b. Describe the method of cell line authentication used.

No cell lines were used.

c. Report whether the cell lines were tested for mycoplasma contamination.

No cell lines were used.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No cell lines were used.

Nature Immunology: doi:10.1038/ni.3847
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

All mice were on a C57BL/6 background. Both male and female mice were used. Any Tgfbr1.cKO mice (and controls in experiments that contained Tgfbr1.cKO mice) were analyzed at age 3-5 weeks, for fetal thymic organ cultures day 16.5 embryos were used, all other mice were 5-10 weeks old.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.
Flow Cytometry Reporting Summary

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Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.
   Thymus and lymph node single cell suspensions were prepared by gently tweezing the organs with forceps in cold HBSS supplemented with 0.5% BSA and 0.5% NaN3.

6. Identify the instrument used for data collection.
   BD LSRII Special Order Research Product (5 lasers: 355nm, 403nm, 488nm, 594nm, 640nm)
   BD FACSAria II Cell Sorter

7. Describe the software used to collect and analyze the flow cytometry data.
   Data collection: BD FACSDIVA
   Data analysis: Flow Control version 4.2.0.4 and FlowJo version 10

8. Describe the abundance of the relevant cell populations within post-sort fractions.
   Pre-selection DP thymocytes: 99-100%
   Mature SP8 thymocytes: 97-99%

9. Describe the gating strategy used.
   Doublets were excluded using forward light-scatter gating followed by gating on lymphocytes based on FSC-SSC. Dead cells were excluded by gating on propidium iodide negative cells. TCRbhi gates were set to only include the TCRb+ mature cells but not TCRb- immature single positive cells within the CD8SP population. CD4SP and CD8SP gates were set to contain cells that are CD4+CD8- or CD4-CD8+, respectively. gc- gates were set based on gc-cells from gc-cKO mice.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑