Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells

Jung-Hyun Park, Stanley Adoro, Terry Guinter, Batu Erman, Amala S Alag, Marta Catalfamo, Motoko Y Kimura, Yongzhi Cui, Philip J Lucas, Ronald E Gress, Masato Kubo, Lothar Hennighausen, Lionel Feigenbaum & Alfred Singer

Immature CD4⁺CD8⁺ (double-positive (DP)) thymocytes are signaled via T cell antigen receptors (TCRs) to undergo positive selection and become responsive to intrathymic cytokines such as interleukin 7 (IL-7). We report here that cytokine signaling is required for positively selected thymocytes to express the transcription factor Runx3, specify CD8 lineage choice and differentiate into cytotoxic-lineage T cells. In DP thymocytes genetically engineered to be cytokine responsive, IL-7 signaling induced TCR-unsignaled DP thymocytes to express Runx3 and to differentiate into mature CD8⁺ T cells, completely circumventing positive selection. We conclude that TCR-mediated positive selection converts DP cells into cytokine-responsive thymocytes, but it is subsequent signaling by intrathymic cytokines that specifies CD8 lineage choice and promotes differentiation into cytotoxic-lineage T cells.

The fate of T cells developing in the thymus is determined during positive selection by the specificity of their αβ T cell antigen receptors (TCRs)1. Thymocytes at the CD4⁺CD8⁺ (double-positive (DP)) stage of development are signaled by their TCR to undergo positive selection and to differentiate into either CD4⁺ helper T cells or CD8⁺ cytotoxic T cells2. However, most TCRs fail to signal in the thymus because they fail to engage intrathymic ligands, which causes most DP thymocytes to undergo death by neglect3. Consequently, only DP thymocytes that receive a TCR signal successfully complete their differentiation into mature T cells, which has the result that every mature T cell expresses a rigorously screened self-specific TCR.

Before receiving a TCR signal, DP thymocytes are unresponsive to intrathymic cytokines such as interleukin 7 (IL-7; A004205)1,2. Indeed, TCR-unsignaled DP thymocytes do not express IL-7 receptor-α (IL-7Rα; A001267)3 and do have uniquely high expression of suppressor of cytokine signaling 1 (SOCS1), which blocks signal transduction by all common γ-chain (γc) cytokines4. Consequently, despite their expression of IL-4Rα and γc proteins5, TCR-unsignaled DP thymocytes are unresponsive to both IL-7 and IL-4. Moreover, preselection DP thymocytes reside in the thymic cortex, which lacks IL-7-producing cells7, so they may not encounter IL-7 or other γc cytokines unless the cells migrate to other areas of the thymus8,9.

Because TCR signaling in DP thymocytes mediates positive selection and induces the generation of mature CD4⁺ and CD8⁺ T cells, TCR signaling is thought to specify both CD4 and CD8 lineage choices and to drive thymocyte maturation10. Experimentally, DP thymocytes can be induced to differentiate into mature T cells independently of TCR-ligand engagements through the use of agonistic antibodies to TCR11 and pharmacological or genetic mimics of TCR signaling11,12. Although these approaches avoid TCR-ligand engagements, they satisfy the TCR signaling requirement of DP thymocytes. Consequently, TCR-signaled positive selection is generally considered essential for the differentiation of DP thymocytes into mature T cells.

After DP thymocytes are signaled to undergo positive selection, CD4 or CD8 lineage specification is induced by a mechanism that is best explained at present by the kinetic signaling model of T cell development1,10,11. The kinetic signaling model proposes that TCR-mediated positive selection converts cytokine-unresponsive DP thymocytes into cytokine-responsive intermediate thymocytes that are transcriptionally Cd4⁺Cd8⁻ and that lineage specification is then determined in intermediate thymocytes by whether TCR signaling persists or ceases. During positive selection, persistent TCR signaling drives intermediate thymocytes to differentiate into CD4⁺ T cells, whereas cessation of TCR signaling permits intermediate thymocytes to be signaled by

---

Received 4 November 2009; accepted 29 December 2009; published online 31 January 2010; doi:10.1038/ni.1840
intrathymic γc cytokines such as IL-7 and to differentiate into CD8+ T cells. However, key predictions of the kinetic signaling model have not been evaluated in vivo, including the hypothesis that cytokine signaling is required for both CD8 lineage specification and cytotoxic T cell differentiation in the thymus.

The transcription factors that specify CD4 or CD8 lineage choice in positively selected thymocytes have largely been identified14–18. CD4 lineage choice is specified by the zinc-finger transcription factors Th-POK and GATA-3 (refs. 14,17,19), whereas CD8 lineage choice is specified by the Runt-family transcription factor Runx3 (refs. 14,17,20). Th-POK and Runx3 negatively regulate each other’s expression and thus reinforce lineage choices16,17,21–23. In agreement with predictions of the kinetic signaling model, persistent TCR signaling in positively selected intermediate thymocytes can induce Th-POK expression and specify CD4 lineage choice16. However, the intrathymic signal that induces Runx3 expression and specifies CD8 lineage choice has not yet been identified.

The present study investigates whether CD8 lineage specification and CD8+ T cell differentiation in the thymus requires signaling by intrathymic cytokines. We show that in positively selected thymocytes, γc cytokine activation of intracellular signal transducer and activator of transcription (STAT) molecules was needed to induce Runx3 expression, specify CD8 lineage choice and promote the differentiation of cytotoxic-lineage T cells in vivo. We also show that IL-7 signaling of DP thymocytes genetically engineered to be cytokine responsive circumvented positive selection by inducing preselection DP thymocytes to differentiate into mature CD8+ T cells in the complete absence of TCR signaling. Our observations substantially enhance the understanding of positive selection, lineage specification and T cell differentiation in the thymus.

RESULTS

γc cytokine signaling is required for thymic CD8+ T cell generation

In the present study we assessed whether signals transduced by intrathymic γc cytokines are required to specify CD8 lineage choice and promote the differentiation of cytotoxic-lineage T cells in the thymus (Supplementary Fig. 1). Signaling by IL-7, the main cytokine expressed in the thymus, is transduced by STAT5a and STAT5b molecules, so we examined the effect of deleting Stat5a and Stat5b in positively selected thymocytes24. To avoid interfering with cytokine signal transduction in early CD4+CD8− double-negative (DN) thymocytes, we conditionally deleted Stat5a and Stat5b in thymocytes beyond the DN4 stage of differentiation. We used a Cre transgene construct (E8III-Cre) that uses the E8III enhancer and promoter elements from Cd8a to drive expression of Cre recombinase in preselection immature single-positive and DP thymocytes (Fig. 1a). To confirm the developmental timing of E8III-Cre-mediated deletion, we introduced the E8III-Cre transgene into Rosa26-loxP-STOP-loxP-green fluorescent protein (GFP) reporter mice and observed that most preselection DN thymocytes that were TCRβ+CD4+CD8− were GFP−, whereas DP thymocytes and their post-selection progeny (CD8+ or CD4+ single-positive (SP) thymocytes and TCR+ DN thymocytes) were GFP+ (Supplementary Fig. 2a). We then introduced the E8III-Cre transgene into mice carrying a loxP-flanked allele encoding Stat5 to generate E8III-Cre*Stat5fl/− mice (Stat5-cKO mice) and confirmed that Stat5 expression and IL-7–induced Stat5 phosphorylation were abrogated in DP thymocytes and their post-selection progeny (CD8+ or CD4+ single-positive (SP) thymocytes and TCR+ DN thymocytes) were GFP+ (Supplementary Fig. 2b).

Notably, conditional deletion of Stat5a and Stat5b by E8III-Cre in preselection DP thymocytes had no effect on overall thymocyte cellularity or on the generation of CD4+ T cells (Fig. 1c). In contrast, conditional deletion of Stat5a and Stat5b in preselection DP thymocytes resulted in a 50% lower frequency of CD8 SP (CD8SP) thymocytes in Stat5-cKO mice than that in wild-type mice (P < 0.005; Fig. 1c), which revealed that expression of Stat5a and Stat5b in DP thymocytes was important for their differentiation into CD8+ T cells.

Nevertheless, substantial numbers of CD8+ T cells were still present among Stat5-cKO thymocytes. One explanation could be that other cytokines, such as IL-4, might activate STAT proteins such as STAT6 to induce differentiation of CD8+ T cells in Stat5-cKO mice. Alternatively, IL-7 might signal through other STAT molecules in Stat5-deficient T cells, even though IL-7 signals are transduced...
impairs γc cytokine signal transduction by binding to γc-associated Jak3 kinase molecules and preventing their phosphorylation. As determined by intracellular staining for Myc, nearly all thymocytes in SOCS1-Tg mice expressed the Socs1 transgene. Mature TCRb thymocytes were unable to phosphorylate STAT5 in response to IL-7 stimulation in SOCS1-Tg mice, in contrast to results obtained with wild-type cells (Fig. 2c). Developmentally, SOCS1-Tg mice were specifically devoid of CD8SP thymocytes (Fig. 2b and Supplementary Fig. 3), which confirmed that cytokine signal transduction was required for CD8+ T cell generation in the thymus. The absence of CD8SP thymocytes in SOCS1-Tg mice was caused by a block in CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.

γc cytokine signaling induces Runx3 expression

We next investigated whether cytokine signaling induced expression of Runx3, the transcription factor thought to specify CD8 lineage choice. CD8 lineage choice occurs in intermediate thymocytes that are transcriptionally Cd4+ Cd8– and that are the immediate progeny of TCR–signaled DP thymocytes (Supplementary Fig. 1). We used a well-established in vitro model of positive selection in which DP thymocytes were induced to differentiate into intermediate thymocytes through the use of phorbol 12-myristate 13-acetate and ionomycin and were subsequently stimulated with IL-7 to induce differentiation into CD8SP T cells. CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.

γc cytokine signaling induces Runx3 expression

We next investigated whether cytokine signaling induced expression of Runx3, the transcription factor thought to specify CD8 lineage choice. CD8 lineage choice occurs in intermediate thymocytes that are transcriptionally Cd4+ Cd8– and that are the immediate progeny of TCR–signaled DP thymocytes (Supplementary Fig. 1). We used a well-established in vitro model of positive selection in which DP thymocytes were induced to differentiate into intermediate thymocytes through the use of phorbol 12-myristate 13-acetate and ionomycin and were subsequently stimulated with IL-7 to induce differentiation into CD8SP T cells. CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.

γc cytokine signaling induces Runx3 expression

We next investigated whether cytokine signaling induced expression of Runx3, the transcription factor thought to specify CD8 lineage choice. CD8 lineage choice occurs in intermediate thymocytes that are transcriptionally Cd4+ Cd8– and that are the immediate progeny of TCR–signaled DP thymocytes (Supplementary Fig. 1). We used a well-established in vitro model of positive selection in which DP thymocytes were induced to differentiate into intermediate thymocytes through the use of phorbol 12-myristate 13-acetate and ionomycin and were subsequently stimulated with IL-7 to induce differentiation into CD8SP T cells. CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.

γc cytokine signaling induces Runx3 expression

We next investigated whether cytokine signaling induced expression of Runx3, the transcription factor thought to specify CD8 lineage choice. CD8 lineage choice occurs in intermediate thymocytes that are transcriptionally Cd4+ Cd8– and that are the immediate progeny of TCR–signaled DP thymocytes (Supplementary Fig. 1). We used a well-established in vitro model of positive selection in which DP thymocytes were induced to differentiate into intermediate thymocytes through the use of phorbol 12-myristate 13-acetate and ionomycin and were subsequently stimulated with IL-7 to induce differentiation into CD8SP T cells. CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.

γc cytokine signaling induces Runx3 expression

We next investigated whether cytokine signaling induced expression of Runx3, the transcription factor thought to specify CD8 lineage choice. CD8 lineage choice occurs in intermediate thymocytes that are transcriptionally Cd4+ Cd8– and that are the immediate progeny of TCR–signaled DP thymocytes (Supplementary Fig. 1). We used a well-established in vitro model of positive selection in which DP thymocytes were induced to differentiate into intermediate thymocytes through the use of phorbol 12-myristate 13-acetate and ionomycin and were subsequently stimulated with IL-7 to induce differentiation into CD8SP T cells. CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.

γc cytokine signaling induces Runx3 expression

We next investigated whether cytokine signaling induced expression of Runx3, the transcription factor thought to specify CD8 lineage choice. CD8 lineage choice occurs in intermediate thymocytes that are transcriptionally Cd4+ Cd8– and that are the immediate progeny of TCR–signaled DP thymocytes (Supplementary Fig. 1). We used a well-established in vitro model of positive selection in which DP thymocytes were induced to differentiate into intermediate thymocytes through the use of phorbol 12-myristate 13-acetate and ionomycin and were subsequently stimulated with IL-7 to induce differentiation into CD8SP T cells. CD8 lineage development and was not due to redirected differentiation of major histocompatibility complex (MHC) class I–selected thymocytes into the CD4 lineage, because MHC class II–deficient SOCS1-Tg mice were additionally devoid of mature CD4 lineage thymocytes (Supplementary Fig. 3), although they did contain the phenotypically CD4+CD8+ intermediate cells that are precursors of both CD4-lineage and CD8-lineage mature T cells. Together these results demonstrate that STAT-mediated signaling by intrathymic γc cytokines is specifically required for CD8+ T cell generation in the thymus.
addition of either IL-7 or IL-4 to overnight cultures (Fig. 3b), which demonstrated that signaling by IL-7 and other γ cytokines induces Runx3 gene expression in mature CD8+ T cells as well as positively selected thymocytes.

Because Runx3 expression was downstream of cytokine signaling, we tested if transgenic Runx3 expression was able to specify CD8 lineage choice in the absence of γ cytokine signals. To test this possibility, we generated mice that transgenically expressed Runx3 (Runx3-Tg mice) using human CD2 (CD2) control elements that are expressed in essentially all thymocytes. Consistent with the role of Runx3 in CD8 lineage specification, we found that Runx3-Tg mice contained mature TCRβ+CD8+ T cells (Fig. 3c, Supplementary Fig. 3a). Thymic profiles of SOCS1-Tg, SOCS1-Tg–Bcl-2-Tg and wild-type mice (Supplementary Fig. 3b) showed similar percent DP thymocytes sorted from SOCS1-Tg–Bcl-2-Tg and wild-type mice. Numbers above bracketed lines (top, c) indicate percent TCRhi cells (c) or Vα11hi cells (variable α-region 11; d); numbers in outlined areas (bottom, c,d; top, e) indicate frequency of cells in each subset. Data are representative of six (a), three (b,d) or two (c,e) experiments.

Consequently, we asked if transgenic expression of Bcl2 would permit the generation of CD8+ T cells in the absence of γ cytokine signaling among Socs1-transgenic thymocytes. Introduction of the Bcl2 transgene in SOCS1-Tg mice resulted in a higher CD4SP thymocyte frequency and, more importantly, restored the generation of CD8SP thymocytes (Fig. 3e). Notably, Bcl2 transgene expression is known to generate unconventional CD8SP thymocytes that arise independently of MHC class I recognition, fail to attain functional competence, and may be the direct progeny of negatively selected DN thymocytes rather than positively selected DP thymocytes (data not shown). Notably, analysis of CD8SP thymocytes from Bcl-2-Tg–SOCS1-Tg mice revealed that they did not express either Runx3 mRNA or Runx1 mRNA (both of which encode Runx proteins with similar DNA-binding sites and similar downstream target genes; Fig. 3e). These results demonstrate that positively selected thymocytes do not express Runx3 in the absence of γ cytokine signaling, despite the appearance of unconventional CD8SP thymocytes induced by transgenic Bcl2 expression.

### CD8 lineage specification in the absence of TCR signaling

Next we investigated whether cytokine signaling could induce CD8 lineage specification in the absence of TCR-mediated positive selection signals. By genetic manipulation, we generated mice in which preselection DP thymocytes were responsive to IL-7 signaling (Fig. 4a and Supplementary Fig. 4). First, we introduced an Il7r transgene into Socs1−/− mice to generate IL-7Rα−/−–Socs1−/−KO mice. Then, to eliminate TCR signaling, we bred IL-7Rα−/−–Socs1−/−KO mice with mice deficient in the tyrosine kinase Zap70 (Zap70−/−) mice to generate IL-7Rα−/−–Socs1−/−–Zap70−/−KO mice (7SZ mice). Consistent with the absence of Zap70 expression, 7SZ thymocytes were arrested at the DP stage and lacked positively selected SP T cells (Fig. 4a). But unlike normal DP thymocytes, 7SZ DP thymocytes were IL-7 responsive, as exogenous IL-7 strongly induced STAT5 phosphorylation in vitro (Fig. 4a).
Because 7SZ DP thymocytes phosphorylated STAT5 in response to IL-7 stimulation, we assessed whether IL-7 signaling would induce Runx3 expression. Purified fresh 7SZ DP thymocytes did not have detectable expression of Runx3 mRNA, but culture of purified 7SZ DP thymocytes with IL-7 markedly upregulated their expression of Runx3 mRNA, which eventually reached an amount equivalent to that in peripheral CD8⁺ lymph node T cells (Fig. 4b). These results demonstrate that cytokines such as IL-7 can induce Runx3 expression even in TCR-unsignaled DP thymocytes, as long as the DP thymocytes are capable of transducing cytokine signals.

To assess the consequences of cytokine signaling in TCR-unsignaled DP thymocytes, we assessed the developmental progression of 7SZ DP thymocytes in in vitro cultures supplemented with IL-7 or IL-4 (Fig. 4c). Cell recovery of 7SZ DP thymocytes was essentially 100% after 5 d in culture with IL-7 but was much lower with IL-4 and even lower in medium-alone conditions (Fig. 4c). Notably, we observed that after 5 d in IL-7-supplemented cultures, almost all (≥95%) 7SZ DP thymocytes had terminated CD4 expression and most had differentiated into CD8SP T cells (Fig. 4c), both events concordant with their Runx3 expression (Fig. 4b). As expected, cytokine-unresponsive DP thymocytes from wild-type and Zap70-KO mice remained DP (Fig. 4c, Supplementary Fig. 6), which confirmed that cytokine signaling is a necessary component for DP thymocyte differentiation.

IL-7-induced CD8SP T cells were mature CD8⁺ T cells by phenotypic criteria, including expression of TCR, Qa-2, granzyme B and perforin (Fig. 4d). Most IL-7-induced 7SZ CD8⁺ T cells were TCRβ⁺ and resembled in vivo–generated mature CD8⁺ T cells from wild-type mice. However, about 30% of IL-7-induced 7SZ CD8⁺ T cells were surface TCRβ⁻, which indicated that they lacked TCRαβ proteins, consistent with their TCR-independent differentiation (Fig. 4d). The Qa-2 marker is expressed on mature T cells that have acquired functional competence and, all IL-7-induced 7SZ CD8⁺ T cells, including those that were TCRβ⁺, were Qa-2⁺ and also contained granzyme B and perforin proteins (Fig. 4d), which revealed that they had differentiated into functional cytotoxic-lineage T cells. However, because 7SZ T cells lack Zap70 expression, we could not use cytotoxic assays to assess the cytotoxic function of these CD8⁺ 7SZ T cells. The kinase Syk could potentially transduce TCR signals in the absence of Zap70 expression, but neither DP nor SP 7SZ thymocytes expressed Syk (Supplementary Fig. 6). We conclude that in vitro IL-7 signaling in cytokine-responsive preselection DP thymocytes induces Runx3 expression, specifies CD8 lineage choice and promotes their differentiation into phenotypically mature cytotoxic-lineage T cells, despite the absence of TCR-mediated positive selection signals.

Exogenous IL-7 signaling of DP thymocytes in vivo

The 7SZ thymus contained cytokine-responsive DP thymocytes but no CD8SP cells, which raised the possibility that 7SZ DP thymocytes do not have access to endogenous γc cytokine in sufficient amounts to be signaled in the thymic cortex. Consequently, we tested if exogenous IL-7 could induce cytokine-responsive 7SZ DP thymocytes to differentiate into CD8⁺ T cells in vivo. Systemic IL-7 administration by a subcutaneously implanted osmotic pump did induce the differentiation of modest numbers of TCRβ⁺, CD24⁺, Qa-2⁻ and CD4⁻ mature CD8⁺ T cells in 7SZ mice (Fig. 5). Thus, exogenously administered IL-7 induces cytokine-responsive DP thymocytes to differentiate in vivo into phenotypically mature CD8⁺ T cells in the absence of TCR-signaled positive selection signals.

Because Runx3 is a downstream target of IL-7, we also asked if transgenic Runx3 expression was itself sufficient to induce TCR-unsignaled DP thymocytes to differentiate into mature CD8⁺ T cells in vivo. However, the thymus of Runx3-Tg–Zap70-KO mice lacked mature CD8⁺ T cells (Fig. 5), which confirmed that Runx3 transgene expression does not fully replace cytokine signaling in generating CD8⁺ T cells.

Given the modest effect of systemic IL-7 administration on CD8⁺ T cell generation in 7SZ mice, we next used a mouse IÎ² transgene driven by the proximal Lck promoter as an intrathymic source of IL-7. We introduced the IÎ² transgene into 7SZ mice with cytokine-responsive DP thymocytes and into IL-7Rα⁻–Tg–Zap70-KO mice (7Z mice) with...
DISCUSSION

Our present study fundamentally changes the understanding of TCR-mediated positive selection signals have been classically thought to specify CD4-CD8 lineage choice and to drive the differentiation of DP thymocytes into mature T cells. However, our present study has documented that TCR-mediated positive selection signals do not specify CD8 lineage choice and are not sufficient to drive the differentiation of DP thymocytes into mature CD8+ T cells.
Instead, TCR-mediated positive selection signals make it possible for DP thymocytes and/or their immediate progeny to be signaled by intrathymic cytokines, which is accomplished by TCR-mediated termination of Cd8a and Socs1 expression and by TCR-mediated induction of Ifnγ and Ccr7 expression. Termination of Cd8a and Socs1 expression in TCR-signaled DP thymocytes disrupts MHC class I–specific TCR signaling and permits γc cytokine signaling, whereas persistent TCR signaling (such as that mediated by MHC class II–specific TCRs) prevents the transduction of γc cytokine signals in both developing CD4+ thymocytes and mature T cells. Induction of Ifnγ and Ccr7 expression permits TCR-signaled DP thymocytes to migrate into cytokine-rich areas of the thymus (such as the corticomedullary junction and thymic medulla) and to bind IL-7.

CD8 lineage specification is thought to be mediated in positively selected thymocytes by the transcription factor Runx3 (refs. 14, 17, 20). In DP thymocytes signaled by their TCR to undergo positive selection, Runx3 is not expressed until DP thymocytes have converted transcriptionally into Cd4+Cd8− intermediate thymocytes and have received STAT-mediated cytokine signals. Runx3 may be a direct STAT target, as sequence analysis has revealed three potential STAT-binding sites in the Runx3 distal promoter (data not shown). When expressed in IL-7–signaled intermediate thymocytes, Runx3 mediates the transcriptional events referred to as ‘coreceptor reversal,’ activating the Cd4 silencer to extinguish Cd4 expression and activating the E8, Cd8a transcriptional enhancer to reactivate Cd8a expression, which converts intermediate thymocytes into Cd4+ Cd8+ (that is, CD8 lineage) thymocytes. Notably, in addition to mediating coreceptor reversal, Runx3 silences expression of Zbtb7b, the gene that encodes Th-POK, a transcription factor important for CD4 lineage specification. Thus, STAT-mediated induction of Runx3 results in CD8 lineage specification because Runx3 converts positively selected thymocytes into Cd4+ Cd8+ (that is, CD8 lineage) cells and suppresses Th-POK to extinguish CD4 lineage potential.

Our findings provide an additional explanation for why it is critical to prevent DP thymocytes from being signaled by intrathymic cytokines. It is now believed that IL-7 signaling would interfere with preselection DP thymocytes undergoing death by neglect. Our present study has additionally suggested that IL-7 signaling in preselection DP thymocytes can circumvent positive selection and drive DP thymocytes to differentiate into CD8 lineage T cells expressing unscreened TCRs.

Notably, conditional deletion of the genes encoding the E protein transcription factors HEB and E2A results in intrathymic generation of mature CD8+ T cells even in the absence of TCR signaling, similar to the intrathymic generation of mature CD8+ T cells in IL-7-Tg 7SZ mice reported here. However, the effects of deficiency in HEB and E2A on thymocyte development were ascribed to a proposed ‘gatekeeper’ function for HEB and E2A in preventing DP thymocyte differentiation until a functional βTcR was produced and to a proposed ‘default pathway’ for CD8+ T cell differentiation for thymocytes not instructed to become CD4+ T cells. Our present study offers an alternative explanation for the phenotype observed in HEB-E2A–deficient thymocytes. We suggest that in the absence of HEB and E2A transcription factors, preselection DP thymocytes are responsive to intrathymic cytokines such as IL-7 and express Ccr7 despite absent TCR signaling, which causes HEB-E2A–deficient preselection thymocytes to migrate to cytokine-rich thymic areas, where they are signaled by cytokines to express Runx3 and to differentiate into phenotypically mature CD8+ T cells. In fact, in support of this perspective, HEB-E2A–deficient preselection DP thymocytes are IL-7Rα−Ccr7+ (ref. 44). Thus, we propose that HEB and E2A contribute to the cytokine-unresponsive phenotype of preselection DP thymocytes in wild-type mice by downregulating expression of IL-7Rα and Ccr7 and by upregulating expression of Socs1 in these cells.

Although our present study has documented that CD8 lineage choice and CD8+ T cell differentiation require cytokine signaling, it has also documented that CD4 lineage choice and CD4+ T cell differentiation are γc cytokine independent and are unaffected by the ability of positively selected thymocytes to transduce γc cytokine signals. Because IL-7 is produced by thymic stromal cells and is the most prevalent intrathymic cytokine, we expected that conditional deletion of Stat5a and Stat5b in preselection thymocytes would be sufficient to abrogate CD8+ T cell generation. However, conditional deletion of Stat5a and Stat5b in preselection DP thymocytes diminished but did not abrogate CD8+ T cell generation. One possibility was that other cytokines such as IL-4 contribute to CD8+ T cell generation. However, IL-4 was much less effective than IL-7 in supporting thymocyte survival and differentiation, which suggested that IL-7 signaling might be mediated by other STAT molecules, such as STAT6, in STAT5-deficient cells. Although STAT5 deficiency does affect the activity of other STAT molecules in nonlymphoid cells, STAT redundancy, as observed in our present study, has not been documented before in T cells, to our knowledge. We think the mechanism underlying our observation is that STAT6 and other STAT proteins can be recruited to phosphorylated Tyr449 in the IL-7Rα cytosolic tail and can then be phosphorylated. In wild-type cells, other STAT proteins are probably outcompeted by STAT5, which presumably binds to phosphorylated Tyr449 with the highest affinity. Indeed, mice deficient in both Stat5 and Stat6 contained considerably fewer CD8+ T cells in the thymus than did either wild-type mice or mice deficient in only one of the other STAT protein, whereas CD4+ T cell numbers and overall thymocyte numbers were equivalent, which demonstrates that CD8+ T cells require STAT-mediated cytokine signals for their generation in the thymus. Blocking γc cytokine signaling in developing thymocytes using a Socs1 transgene confirmed the critical importance of cytokine signaling for the generation of CD8+ T cells but not CD4+ T cells.

Cytokine signaling is not strictly indispensable for the appearance of CD8SP cells in the thymus, as we detected such cells in SOCS1-Tg–Bcl-2-Tg mice despite the absence of γc cytokine signal transduction. Unconventional CD8SP thymocytes have been detected previously in Bcl-2-Tg mice and are highly unusual because they arise independently of MHC class I expression and fail to achieve functional maturity. Our present study has further documented that these unconventional CD8SP thymocytes arose independently of γc cytokine signaling and, when they did not receive γc cytokine signals, did not express Runx3 (or Runx1). We think such unconventional CD8 thymocytes may not be the immediate progeny of DP thymocytes but instead may derive from DN thymocytes that, in the presence of the Bcl2 transgene, are signaled by their βTcR directly to upregulate CD8 expression. Thus, we think that unconventional thymocytes, despite their CD8SP phenotype, do not require and have not undergone CD8 lineage specification because they do not arise from bipotent precursors (for example, DP thymocytes).

In conclusion, signaling by intrathymic cytokines is necessary for CD8 lineage specification and CD8+ T cell development. Because preselection DP thymocytes reside in a cytokine-insufficient region of the thymic cortex and are cytokine unresponsive, we propose that TCR signaling converts DP thymocytes into cytokine-responsive cells that migrate to cytokine-rich regions of the thymus and are signaled by endogenous cytokines. Thus, thymic generation of
CD8\(^+\) T cells requires a carefully choreographed sequence initiated by TCR signaling and executed by cytokine signaling.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.


**Note:** Supplementary information is available on the Nature Immunology website.

**ACKNOWLEDGMENTS**

We thank R. Bosoretii, N. El Kassar, R. Hodes, D. Singer and N. Taylor for critical reading of the manuscript; S. Habu (Tokai University) for mouse Runx3 CDNA; A. Weiss (University of California San Francisco) for fluorescein isothiocyanate–conjugated monoclonal antibody to Syk (5F5); J. Ihle (St. Jude Children’s Research Hospital) for Sox11+ Tg/− mice; and S. Sharro, A. Adams and L. Granger for flow cytometry. Supported by the Intramural Research Program of the US National Institutes of Health, the National Cancer Institute and the Center for Cancer Research.

**AUTHOR CONTRIBUTIONS**

J.-H.P. did experiments, analyzed data and contributed to the writing of the manuscript; S.A., T.G., M.C., M.Y.K. and P.J.L. did experiments and analyzed data; B.E. and A.S.A. constructed some of the experimental mice; Y.C., R.E.G., M.K. and A.S. conceptualized the research, directed the study, analyzed data and wrote the manuscript.

**COMPETING INTERESTS STATEMENT**

The authors declare no competing financial interests.

Published online at http://www.nature.com/natureimmunology/. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.
**ONLINE METHODS**

**Animals.** E8III-Cre-Tg mice, which express cDNA encoding Cre recombinase under the control of the mouse E8III-Cd8a enhancer-promoter elements, and Runx3−/− mice, which express cDNA encoding hemagglutinin-tagged mouse Runx3 driven by the human CD2 promoter, were newly generated for this study. Other transgenic mice models used in this study were as follows: Bcl-2−/− mice, which express cDNA encoding human Bcl-2 driven by the Lck proximal promoter; SOCS1−/− mice, which express cDNA encoding Myc-tagged mouse SOCS1 driven by the Lck proximal promoter; IL-7−/− mice, which express cDNA encoding mouse IL-7 driven by the Lck proximal promoter; IL-7Rα−/− mice, which express cDNA encoding mouse IL-7Rα driven by the human CD2 promoter; and AND TCR−/− transgenic mice.

Mice whose Stat5a and Stat5b loci are flanked with loxP sites have been reported. Stat6−/− mice were from Taconic; Socs1−/−Tfng−/− mice were provided by J. Ihle and were bred to generate Socs1−/−Tfng−/− mice; Zap70−/−, MHC class II−/− and MHC-KO mice were bred in our own colony. C57BL/6 (wild-type) mice were from Charles River. Notably, all Socs1−/− mice were maintained as Socs1−/−Tfng−/− to avoid systemic inflammation. 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.

**Cell culture and cell purification.** Single-cell suspensions were prepared from thymus and lymph nodes by gentle teasing with forceps and were analyzed freshly or after overnight in vitro culture (5 × 10⁶ cells per ml) in a 7.5% CO₂ atmosphere in RPMI-1640 medium supplemented with 10% (vol/vol) FCS. Where indicated, cells were stimulated with mouse recombinant IL-7 (10 ng/ml) or IL-4 (40 ng/ml; Peprotech). CD4⁺ and CD8⁺ lymph node T cells were purified by depletion of immunoglobulin-positive cells plus either CD8⁺ or CD4⁺ cells, respectively, with antibody-mediated magnetic cell sorting.

**Flow cytometry.** Antibodies with the following specificities were used for staining: CD4 (GK1.5 and RM4.5), CD8α (53-6-7), CD8β (53-5.8), TCRβ (H57-597), IL-4Rx (M1), phosphorylated STAT5 (47), p-STAT6 (J71-773; all from BD Pharmingen); CD24 (30-F1), IL-7Rx (A7R34), Qa-2 (69H1-9-9; all from eBioscience); STAT5 (9363) and phosphorylated STAT6 (9361; polyclonal antibodies; both from Cell Signaling Technology); and fluorescein isothiocyanate–conjugated anti-Myc (9E10; Sigma). Donkey secondary antibodies to rabbit IgG (711-096-152) were from Jackson ImmunoResearch Laboratories. Intracellular staining was done as described. Fluorescein isothiocyanate–conjugated anti-Syk (5F5) was a gift from A. Weiss.

For cell surface immunofluorescence analysis, cells were analyzed on a FACSQuant Vantage SEM (Becton Dickinson) with four-decade logarithmic amplification. Dead cells were excluded by forward light-scatter gating and propidium iodide staining. Data were analyzed using software designed by the Division of Computer Research and Technology, US National Institutes of Health.

**ALZET osmotic pump installation.** Infusion of recombinant mouse IL-7 (PeproTech) was achieved by subcutaneous implantation of ALZET osmotic pumps (Durect), which released 10 µg IL-7 per day for 2 weeks.

**RNA hybridization and RT-PCR.** Total RNA were isolated with TriZol and reverse transcribed into cDNA. cDNA samples were amplified using specific primers in an RT-PCR system. Primers for murine Bcl-2 were as follows: sense, 5′-CATCTAAGGGCATCACAGACCTG-3′; antisense, 5′-GGTGAGCCTCGTTCATTCAT-3′. Primers for Runx3 were as follows: sense, 5′-GGTCAGACCCACTTGGTTGG-3′; antisense, 5′-GCTGAGCTCTGTTCACTCAT-3′. PCR products were analyzed by electrophoresis on agarose gels. RT-qPCR primers were designed using the Primer3 software.

**Statistical analyses.** Data were expressed as mean ± SD. Student’s two-tailed t-tests with P values of less than 0.05 were considered significant.


**Supplementary Figure 1.** Schematic presentation of the kinetic signaling model of CD4–CD8 lineage choice.
Supplementary Figure 2. E8III-Cre transgene is expressed in DP thymocytes and terminates STAT5 activity in CD8SP and CD4SP thymocytes from Stat5fl/– mice. (a) Introduction of the E8III-Cre transgene into Rosa26-loxP-STOP-loxP-GFP reporter mice reveals that the transgene induces Cre expression in DP thymocytes, as DP thymocytes and their developmental progeny (CD8SP and CD4SP thymocytes) are GFP+. (b) IL-7 fails to induce p-STAT5 in mature thymocytes from STAT5-cKO mice. Freshly isolated thymocytes from WT (E8III-Cre Stat5+/+) and STAT5-cKO (E8III-Cre Stat5fl/–) mice were stimulated with IL-7 (shaded curve) or medium (open curve) for 30 min and assayed for intracellular p-STAT5 content (left). Surface IL-4Rα expression upon overnight IL-7 stimulation was determined on TCRβhi-gated thymocytes (right).
Supplementary Figure 3. SOCS1-Tg blocks generation of CD8⁺ and MHC-I selected thymocytes. Thymocytes from WT, SOCS1-Tg, and MHC-II-KO-SOCS1-Tg mice were assessed for CD4 versus CD8 expression. Data are representative of 3 independent experiments.
**Supplemental Figure 4.** Developmental implications of cytokine-unresponsive versus cytokine-responsive DP thymocytes. Pre-selection DP thymocytes are normally cytokine-unresponsive because they express high levels of SOCS1 and express little or no IL-7Rα (left panel). Consequently, TCR-mediated positive selection signaling is required to convert DP thymocytes into IL-7-responsive Cd4+Cd8⁻ intermediate thymocytes by terminating SOCS1 and inducing IL-7Rα expression, so that the intermediate cells can then be signaled by IL-7 to differentiate into mature CD8⁺ T cells (left). However, pre-selection DP thymocytes from IL-7Rα-Tg·SOCS1-KO mice are IL-7-responsive even in the absence of TCR signaling (right panel). To ensure that pre-selection DP thymocytes from IL-7Rα-Tg·SOCS1-KO mice had not been TCR-signaled, we additionally made them ZAP70-deficient, so that mice were IL-7Rα-Tg·SOCS1-KO·ZAP70-KO (simply referred to as 7SZ mice). IL-7 signaling of cytokine-responsive pre-selection 7SZ DP thymocytes would be predicted to induce their differentiation into mature CD8⁺ T cells, completely circumventing TCR-mediated positive selection (right panel).
Supplementary Figure 5. *In vitro* IL-7 has no effect on DP thymocytes from ZAP70-KO or MHC-KO mice. Purified DP thymocytes from ZAP70-KO (a) or MHC-KO mice (b) were cultured with either IL-7 or medium for 5 days and then assessed for surface CD4 and CD8 expression.
Supplementary Figure 6. SYK kinase is not expressed in IL-7 responsive DP or IL-7-induced CD8SP thymocytes from 7SZ mice. Intracellular (i.c.) SYK kinase expression was determined in freshly isolated 7SZ DP cells and in IL-7-induced 7SZ CD8^+ SP cells using anti-SYK monoclonal antibodies. LN B cells from B6 mice were used as positive control for SYK staining (right).
**Supplementary Figure 7.** CD8+ T cells in LN of IL-7 transgenic 7SZ mice. Mature CD8+ T cells were identified in lymph nodes of IL-7-Tg·7SZ mice. Mature CD8+ T cells were identified as TCRβ<sup>hi</sup>CD24−Qa-2+ cells. Boxes identify mature CD8+ T cells in each plot, and the frequency of cells in each box is shown.
Supplementary Figure 8. CD8+ T cell generation in the thymus requires sequential signaling by TCR and γc-cytokines. (a) A graphic summary of our results with cytokine-responsive DP thymocytes from 7SZ mice, revealing that IL-7 signaling is needed to induce CD8 lineage-specification and differentiation of TCR-unsignaled pre-selection DP thymocytes into mature CD8+ T cells. (b) Synthesizing previous studies13,34 with our current results reveals a new understanding of CD8+ T cell differentiation and indicates that sequential signaling by TCR and IL-7 is required for DP thymocytes to differentiate into mature CD8+ T cells. TCR signaling converts cytokine-unresponsive DP thymocytes into IL-7-responsive intermediate thymocytes that are transcriptionally Cd4+8– and Ccr7+. Because they are transcriptionally Cd4+8–, intermediate thymocytes specifically lose surface CD8 coreceptor expression, causing MHC-I-specific TCR signaling to cease and the cells to regain their ability to transduce cytokine signals. Cytokine-responsive intermediate thymocytes then encounter IL-7 by migrating to IL-7 richer areas of the thymus (cortico-medullary junction and medulla), which is facilitated by e.g. CCR7 expression. After encountering IL-7, STAT-mediated IL-7 signaling induces Runx3 expression which specifies CD8-lineage choice by mediating coreceptor reversal, i.e. the transcriptional conversion of Cd4+8– intermediate thymocytes into Cd4–8+ cells, and by providing the survival signals required for differentiation of CD8-committed thymocytes into mature CD8+ cytotoxic-lineage T cells.