HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2019 August 14.

Published in final edited form as:

Nat Immunol. 2014 July ; 15(7): 638–645. doi:10.1038/ni.2917.

The transcription factor ThPOK suppresses Runx3 and imposes CD4+ lineage fate by inducing the SOCS suppressors of cytokine signaling

Megan A Luckey[1], **Motoko Y Kimura**[1], **Adam T Waickman**[1], **Lionel Feigenbaum**[2], **Alfred Singer**[1], **Jung-Hyun Park**[1]

[1]Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA.

[2]Leidos Biomedical Research, Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA.

Abstract

Lineage fate in the thymus is determined by mutually exclusive expression of the transcription factors ThPOK and Runx3, with ThPOK imposing the $CD4^+$ lineage fate and Runx3 promoting the $CD8^+$ lineage fate. While it is known that cytokine signals induce thymocytes to express Runx3, it is not known how ThPOK prevents thymocytes from expressing Runx3 and adopting the $CD8^+$ lineage fate, nor is it understood why ThPOK itself imposes the $CD4^+$ lineage fate on thymocytes. We now report that genes encoding members of the SOCS (suppressor of cytokine signaling) family are critical targets of ThPOK and that their induction by ThPOK represses Runx3 expression and promotes the $CD4^+$ lineage fate. Thus, induction of SOCS-encoding genes is the main mechanism by which ThPOK imposes the $CD4^+$ lineage fate in the thymus.

The molecular mechanism that underlies lineage 'choice' in the thymus is a fundamental but still open question in immunology. A competent T cell immune system requires $CD4^+$ helper T cells that recognize major histocompatibility complex (MHC) class II ligands and $CD8^+$ effector T cells that recognize MHC class I ligands[1]. These characteristics are established during thymic selection by the transcription factor ThPOK (encoded by *Zbtb7b*; called '*Thpok*' here), which induces thymocytes that have received MHC class II signaling to become $CD4^+$ T cells[2,3], and by the transcription factor Runx3, which induces cells that have received MHC class I signaling to become $CD8^+$ T cells[4,5]. Unambiguous lineage fate 'decisions' require that individual thymocytes express either ThPOK or Runx3 but not both.

Correspondence should be addressed to J.-H.P. (parkhy@mail.nih.gov).

AUTHOR CONTRIBUTIONS

M.A.L., M.Y.K. and A.T.W. designed and did experiments, and analyzed data; L.F. generated reagents; A.S. designed the study, analyzed data and wrote the manuscript; and J.-H.P. designed and did experiments, analyzed data, provided overall direction and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

How such mutually exclusive expression of lineage-determining factors is achieved remains unresolved.

For Runx3-induced suppression of ThPOK, intrathymic cytokines signal thymocytes to express Runx3 proteins[6] that then bind to a silencer element in the *Thpok* locus to repress its transcription[7,8]. As a result, thymocytes that have received cytokine signaling adopt the $CD8^+$ lineage fate by upregulating Runx3 expression and repressing ThPOK expression[6–8]. In contrast, ThPOK does not bind to the *Runx3* locus to repress its transcription. Consequently, it is not known how ThPOK antagonizes Runx3 and prevents adoption of the $CD8^+$ lineage fate, nor is it known how ThPOK promotes adoption of the $CD4^+$ lineage fate[9,10].

We undertook the present study to identify the mechanism by which ThPOK influences lineage fate determination in developing thymocytes. We found that ThPOK antagonized Runx3 expression by targeting and upregulating genes that encode cytosolic proteins of the SOCS (suppressor of cytokine signaling) family that negatively regulate cytokine signal transduction in T cells[11]. Notably, we observed that in T cells lacking expression of SOCS-encoding genes, ThPOK was functionally inert, as it neither prevented Runx3 expression nor prevented adoption of the $CD8^+$ lineage fate. Reciprocally, in ThPOK-deficient mice, transgenic expression of SOCS1 was sufficient to prevent Runx3 expression and to promote the generation of $CD4^+$ T cells despite the absence of functional ThPOK. We also determined that ThPOK upregulated expression of the coreceptor CD4 during MHC class II–specific positive selection and that that ThPOK function was independent of SOCS proteins. Thus, our study identifies the molecular mechanisms by which ThPOK induces developing T cells to adopt the $CD4^+$ lineage fate during differentiation in the thymus.

RESULTS

ThPOK suppresses Runx3 expression in thymocytes

We began our study by assessing the development of $CD4^+$ T cells in wild-type mice and in helper T cell–deficient (HD) mice ($Thpok^{hd/hd}$ mice)[12,13]. In HD mice, *Thpok* encodes a nonfunctional 'ThPOK^{HD}' protein because of a naturally occurring point mutation in the genomic sequence encoding the second zinc-finger domain of ThPOK[2]. Consequently, the development of $CD4^+$ helper T cells is abolished in HD mice, with the result that thymocytes that have received MHC class II signaling differentiate into $CD8^+$ T cells instead of $CD4^+$ T cells[12–14]. Such a requirement for ThPOK function was illustrated in HD mice expressing a transgene encoding the AND MHC class II–specific T cell antigen receptor (TCR) (Fig. 1a). Unlike AND mice, AND $Thpok^{hd/hd}$ mice failed to generate $CD4^+$ T cells, so that cells positive for the α-chain variable region 11 ($V_\alpha 11$) clonotypic TCR were redirected to the $CD8^+$ lineage (Fig. 1a). To understand the molecular basis of the requirement for functional ThPOK in differentiation into the $CD4^+$ lineage, we examined thymocytes at the $CD69^+CD4^+CD8^{lo}$ intermediate (INT) stage (Fig. 1b, left), because it is in INT thymocytes that lineage fate is determined[1,15,16]. We found that ThPOK mRNA was expressed in INT thymocytes from both AND mice and AND $Thpok^{hd/hd}$ mice (Fig. 1b, right). However, the ThPOK mRNA in AND thymocytes encoded functional ThPOK protein that prevented expression of Runx3 mRNA, whereas the ThPOK mRNA in AND $Thpok^{hd/hd}$

thymocytes encoded the nonfunctional ThPOK^{HD} protein that permitted expression of Runx3 mRNA (Fig. 1b, right). As a result, AND *Thpok*$^{hd/hd}$ thymocytes adopted the CD8$^+$ lineage fate and differentiated into CD4$^-$CD8$^+$ single-positive (CD8SP) thymocytes and CD8$^+$ T cells (Fig. 1a,b). Thus, thymocytes did not simultaneously express functional ThPOK and Runx3.

ThPOK induces expression of SOCS-encoding genes

A transgene encoding SOCS1 that impairs cytokine signaling has been shown to prevent thymocytes that have received MHC class I signaling from expressing Runx3 and differentiating into T cells of the CD8$^+$ lineage[6,17]. The SOCS family of cytosolic proteins suppress cytokine signaling either by interfering with cytokine receptor–associated kinases or by competing with STAT signal transducers for recruitment to activated cytokine receptors[11,18]. At present, there are eight SOCS-encoding genes, of which at least three (*Socs1, Socs3* and *Cish*) are expressed in T cells and encode proteins that impair cytokine signaling. Expression of SOCS-encoding genes is upregulated in cells that have received cytokine signaling to function as a negative feedback mechanism[11,19], but regulation of their expression is incompletely understood. Consequently, we sought to determine if ThPOK might induce expression of one or more of these SOCS-encoding genes in developing thymocytes as the mechanism by which ThPOK antagonized Runx3.

We first compared the expression of SOCS-encoding genes in CD4$^+$CD8$^-$ single-positive (CD4SP) and CD8SP wild-type thymocytes. During thymic selection in wild-type mice, INT thymocytes that have received cytokine signaling express Runx3 and differentiate into CD8SP cells, while INT thymocytes that have received MHC class II signaling express ThPOK and differentiate into CD4SP cells without receiving cytokine signals[1,6,7]. Consequently, signaling by intrathymic cytokines would account for the expression of SOCS-encoding genes in CD8SP thymocytes, but it would not account for such expression in CD4SP thymocytes. Nevertheless, we found that wild-type CD4SP thymocytes did express mRNA from four different SOCS-encoding genes (*Socs1, Socs3, Cish* and *Socs4*), with expression of SOCS1 and Cish mRNA being even higher than that in wild-type CD8SP thymocytes (Fig. 1c). Since these results were not explained by cytokine signaling, they suggested that CD4$^+$ T cells contained an inducer of SOCS-encoding genes, which might be ThPOK.

To assess possible induction of the expression of SOCS-encoding genes by ThPOK, we constructed two new mouse lines, L1 and L6, with transgenic expression of ThPOK (ThPOK-Tg); their expression of ThPOK was different from that of the preexisting C8 ThPOK-Tg mouse line[3]. Together with wild-type (C57BL/6 (B6)) mice, the three ThPOK-Tg mouse lines formed an ascending hierarchy of ThPOK expression in CD4$^+$ T cells *in vivo* (wild-type < L1 < C8 < L6) (Fig. 1d, left). All three ThPOK-encoding transgenes were driven by regulatory elements of the human gene encoding CD2 that forced ThPOK expression in all T lineage cells and caused nearly all thymocytes to adopt the CD4$^+$ lineage fate (Fig. 1d, right). In addition to having higher expression of ThPOK than that of wild-type CD4$^+$ T cells, ThPOK-Tg CD4$^+$ T cells had higher expression of SOCS1, SOCS3 and Cish mRNA (Fig. 1e and Supplementary Fig. 1a). In fact, the expression of each of those mRNAs

was directly and quantitatively related to the expression of ThPOK mRNA in $CD4^+$ T cells (Fig. 1e). Such a direct quantitative relationship with ThPOK expression revealed that ThPOK was responsible, either directly or indirectly, for upregulating expression of those SOCS-encoding genes. The inductive effect of ThPOK on expression of SOCS-encoding genes was cell intrinsic (Supplementary Fig. 1b) and was specific mainly for *Socs1, Socs3* and *Cish,* with little effect on *Socs4* (Fig. 1e). These results identified *Socs1, Socs3* and *Cish* as direct or indirect downstream targets of ThPOK.

ThPOK is a transcriptional activator of SOCS-encoding genes

To demonstrate that the higher expression of SOCS-encoding genes was a direct effect of ThPOK expression and was not dependent on signaling via the TCR, we generated MHC class II–deficient mice with transgenic expression of ThPOK. In these mice, MHC class I–selected T cells are redirected into the $CD4^+$ lineage and express mismatched CD4 coreceptors so that TCR signaling is absent. Notably, in these mice, the expression of SOCS-encoding genes increased with increasing expression of ThPOK (Fig. 2a), which suggested that the inductive effect of ThPOK was not dependent on TCR signaling.

Next, to determine if ThPOK affected the transcription of SOCS-encoding genes, we introduced a *Socs1* reporter gene (in which sequence encoding a nonfunctional human CD4 protein was inserted into the endogenous *Socs1* locus to report *Socs1* transcriptional activity[20]) into wild-type and ThPOK-Tg mice. We observed that sur face expression of the human CD4 reporter protein was directly and quantitatively related to the ThPOK mRNA content in $CD4^+$ T cells (Fig. 2b), which suggested that the inductive effect of ThPOK on expression of SOCS-encoding genes was transcriptional. Analysis of the *5′* upstream regulatory region of two such genes (*Socs1* and *Cish*) revealed conserved noncoding sequences with two potential ThPOK-binding sites in each that corresponded to their promoter regions (Supplementary Fig. 2). We cloned those promoter elements into a promoterless luciferase reporter vector to assess the effect of ThPOK on the promoters' activity. We found that the transcriptional activity of both the *Socs1* promoter and the *Cish* promoter was increased in a dose-dependent manner by cotransfection of 293T human embryonic kidney cells with cDNA encoding functional ThPOK protein but was unaffected by cotransfection of cDNA encoding nonfunctional mutant ThPOK protein (Fig. 2c). We concluded that ThPOK was able to specifically target the promoters of *Socs1* and *Cish* to increase transcription.

ThPOK needs SOCS-encoding genes to suppress Runx3

We then assessed the possibility that induction of SOCS-encoding genes was the mechanism by which ThPOK suppressed Runx3 expression. We considered that ThPOK upregulated expression of at least three different SOCS-encoding genes (*Socs1, Socs3* and *Cish;* Fig. 1e), each of which could suppress cytokine signaling and prevent Runx3 expression[11,18]. Because *Socs1* had the highest expression among these genes in thymocytes, we examined the specific effect of germline deletion of *Socs1* (*Socs1*$^{-/-}$) on ThPOK-induced suppression of Runx3 expression. We ensured that all *Socs1*$^{-/-}$ mice were also deficient in interferon-γ (IFN-γ; *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice) to avoid any possibility of IFN-γ-mediated inflammation, which might secondarily affect lineage fate 'decisions' in the thymus[21].

Germline deletion of *Socs1* did in fact influence lineage fate 'decisions', as *Socs1*$^{-/-}$ mice had a much higher frequency of CD8SP thymocytes and a lower ratio of CD4^{+} cells to CD8^{+} cells[21,22] (Fig. 3a). To directly assess the effect of SOCS1 deficiency on ThPOK function, we generated ThPOK-Tg *Socs1*$^{-/-}$ mice in which all *Socs1*$^{-/-}$ thymocytes expressed ThPOK. Whereas SOCS1-sufficient thymocytes from ThPOK-Tg mice and ThPOK-Tg *Ifng*$^{-/-}$ mice were devoid of CD8SP cells, SOCS1-deficient thymocytes from ThPOK-Tg *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice contained substantial numbers of mature (TCRβ^{hi}) CD8SP cells (Fig. 3b,c and Supplementary Fig. 3). The generation of those mature CD8SP cells was not due to variegated ThPOK-Tg expression, because CD8SP thymocytes from ThPOK-Tg *Socs1*$^{-/-}$ mice had the same expression of ThPOK mRNA as that of their CD4SP counterparts (Fig. 3d). Unexpectedly, those CD8SP thymocytes not only expressed ThPOK mRNA but also expressed Runx3 mRNA (Fig. 3d). The Runx3 expressed was functional despite ThPOK expression, as revealed by expression of the Runx3 target gene *Cd103* (ref. 23) (Fig. 3d) and by the appearance of mature CD8^{+} T cells in the periphery (Fig. 3e). Thus, in the absence of SOCS1, transgenically expressed ThPOK failed to suppress Runx3 expression and failed to prevent developing thymocytes from adopting the CD8^{+} lineage fate, which resulted in unusual ThPOK-expressing CD8^{+} T cells.

To demonstrate that the effect of SOCS1 deficiency on the generation of CD8SP thymocytes in ThPOK-Tg mice was cell intrinsic and was not secondary to peripheral inflammation induced by SOCS1-deficient T cells, we performed two different types of experiments. First, we assessed the generation of mature CD8SP thymocytes in 9-day-old (neonatal) ThPOK-Tg *Socs1*$^{-/-}$ mice that were IFN-γ deficient and were too young to have sufficient numbers of peripheral T cells to induce peripheral inflammation (Fig. 3f). Nevertheless, we observed that mature (TCRβ^{hi}) CD8SP thymocytes were still generated in ThPOK-Tg *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonates (Fig. 3f). Second, we assessed the generation of mature CD8SP thymocytes in immunodeficient (*Rag2*$^{-/-}$) chimeras 8–10 weeks after reconstitution with a mixture of equal numbers of bone marrow stem cells from ThPOK-Tg *Socs1*$^{+/+}$ donor mice and ThPOK-Tg *Socs1*$^{-/-}$ donor mice. In such chimeras, SOCS1-sufficient ThPOK-Tg thymocytes (identified by a *Socs1*$^{+}$ allele) and SOCS1-deficient ThPOK-Tg thymocytes (identified by the absence of a *Socs1*$^{+}$ allele and the presence of *Socs1*$^{-/-}$ alleles) would both be equally subjected to peripheral inflammation induced by SOCS1-deficient T cells. Nevertheless, chimeric CD8SP thymocytes were entirely and exclusively of *Socs1*$^{-/-}$ origin, unlike chimeric CD4SP thymocytes, which were of dual origin (Fig. 3g). Thus, the inability of the transgenically expressed ThPOK to prevent SOCS1-deficient thymocytes from adopting the CD8^{+} lineage fate was cell intrinsic and was not secondary to peripheral inflammation.

While ThPOK-Tg *Socs1*$^{-/-}$ thymocyte populations included mature CD8^{+} T cells, it was evident that most thymocytes in ThPOK-Tg *Socs1*$^{-/-}$ mice did not express Runx3 and did not adopt the CD8^{+} lineage fate (Fig. 3b–e). To understand why this was true, we considered that ThPOK upregulated the expression of *Socs3* and *Cish,* in addition to upregulating the expression of *Socs1.* Consequently, we hypothesized that ThPOK-Tg *Socs1*$^{-/-}$ cells that adopted the CD8^{+} lineage fate might not have upregulated expression of *Socs3* or *Cish* in amounts sufficient to prevent Runx3 expression. This was precisely the case, as ThPOK-Tg *Socs1*$^{-/-}$ CD8^{+} T cells had only low expression of SOCS3 and Cish mRNA, whereas

ThPOK-Tg *Socs1*$^{-/-}$ CD4^{+} T cells had high expression of both (Fig. 4a). Notably, such CD8^{+} T cells were truly of the CD8^{+} T cell lineage because after being stimulated by antibody to the TCR and antibody to the costimulatory receptor CD28, they differentiated into cytotoxic effector cells with high expression of Runx3 and granzyme B (Fig. 4b,c). Thus, ThPOK-Tg *Socs1*$^{-/-}$ cells that did not upregulate expression of either *Socs3* or *Cish* adopted the CD8^{+} lineage fate, whereas cells that upregulated expression of one or both adopted the CD4^{+} lineage fate. To potentially further increase the frequency of cells adopting the CD8^{+} lineage fate, we attempted to generate ThPOK-Tg mice deficient in multiple SOCS-encoding genes. However, we were unable to obtain viable offspring with such multiple deficiencies. We concluded that repression of Runx3 expression and prevention of the CD8^{+} lineage fate by ThPOK strictly required the induction of *Socs1, Socs3* or *Cish.* Without upregulated expression of at least one of those genes, ThPOK itself neither repressed Runx3 expression nor influenced T cell lineage 'choice' (Supplementary Fig. 4).

Transgenic expression of SOCS1 can replace ThPOK

Having discovered that ThPOK repressed Runx3 expression and the CD8^{+} lineage fate by inducing the expression of genes encoding members of the SOCS family, we sought to determine if expression of a transgene encoding a SOCS protein could overcome the requirement for ThPOK in CD4^{+} lineage 'choice' To investigate this possibility, we introduced into HD mice a transgene encoding SOCS1 driven by proximal promoter-enhancer elements of *Lck*[24] (*Thpok*$^{hd/hd}$ SOCS1-Tg mice) and assessed expression of ThPOK and Runx3 mRNA. The transgene encoding SOCS1 did not alter expression of ThPOK mRNA in INT thymocytes from *Thpok*$^{hd/hd}$ SOCS1-Tg mice but it resulted in much lower expression of Runx3 mRNA (Fig. 5a). Thus, INT thymocytes in *Thpok*$^{hd/hd}$ SOCS1-Tg mice lacked both of the lineage-specifying transcription factors ThPOK and Runx3. Nonetheless, these cells gave rise to mature (CD24^{lo}TCRβ^{hi}) CD4^{+} T cells in both the thymus and periphery (Fig. 5b), with 14 × 10^{6} CD4^{+} T cells in *Thpok*$^{hd/hd}$ SOCS1-Tg lymph nodes (Fig. 5c). To exclude the possibility that these findings resulted from unappreciated functioning of ThPOK^{HD} protein, we also introduced the transgene encoding SOCS1 into *Thpok*$^{-/-}$ mice that were completely devoid of ThPOK protein[25]. Analysis of *Thpok*$^{-/-}$ SOCS1-Tg mice again revealed the presence of mature CD4^{+} T cells in both the thymus and periphery (Fig. 6a), with 15 × 10^{6} CD4^{+} T cells in the lymph nodes (Fig. 6b). Thus, expression of the transgene encoding SOCS1 in ThPOK-deficient mice resulted in the generation of ThPOK-deficient CD4^{+} T cells.

ThPOK upregulates CD4 expression during positive selection

To further understand the contribution of ThPOK to differentiation into the CD4^{+} lineage, we further characterized the ThPOK-independent CD4^{+} T cells that arose in *Thpok*$^{hd/hd}$ SOCS1-Tg mice. These cells were specific for MHC class II, as deficiency in MHC class II eliminated their generation (Fig. 5b). As further evidence of their MHC class II specificity, ThPOK-independent CD4^{+} T cells were selected by the AND TCR, as shown by the presence of clonotypic $V_{\alpha}11^{hi}$ CD4^{+} T cells in both the thymus and periphery of AND *Thpok*$^{hd/hd}$ SOCS1-Tg mice (Fig. 7a). The ThPOK-independent CD4^{+} T cells from AND *Thpok*$^{hd/hd}$ SOCS1-Tg mice were functional cells of the CD4^{+} lineage, as they produced

interleukin 4 but not IFN-γ under T helper type 2-polarizing conditions (Fig. 7b) and had helper function, as revealed by upregulation of the expression of the ligand for the costimulatory receptor CD40 in response to stimulation with antibody to the TCR (Fig. 7c).

Notably, we observed that ThPOK-independent $CD4^+$ T cells differed from conventional $CD4^+$ T cells in surface expression of CD4 (Figs. 5b, 6a and 7a). Quantification of CD4 expression revealed that both the expression of CD4 mRNA and surface expression of CD4 protein were significantly lower in ThPOK-independent $CD4^+$ T cells than in conventional wild-type $CD4^+$ T cells (Fig. 7d). The basis for this reduction was revealed by quantification of the surface expression of CD4 during positive selection in the thymus (Fig. 7e). While CD4 expression was upregulated during the differentiation of INT thymocytes into mature $CD4^+$ T cells in ThPOK-intact mice (i.e., wild-type mice and SOCS1-Tg mice), CD4 expression was not upregulated during differentiation in ThPOK-deficient mice (i.e., *Thpok*$^{hd/hd}$ SOCS1-Tg mice and *Thpok*$^{-/-}$SOCS1-Tg mice) (Fig. 7e). As a result, CD4 expression was significantly lower on ThPOK-deficient CD4SP thymocytes than on ThPOK-sufficient CD4SP thymocytes (Fig. 7e). Thus, these results identified ThPOK as being responsible for the upregulation of CD4 expression during MHC class II–specific positive selection in the thymus.

Because upregulation of CD4 expression prevents TCR signaling from being prematurely disrupted during MHC class II–specific positive selection and increasing the potential for lineage errors[26], the failure of ThPOK-deficient thymocytes to upregulate CD4 expression would result in increased lineage-specification errors. Consistent with this expectation, many MHC class II–selected thymocytes in AND *Thpok*$^{hd/hd}$ SOCS1-Tg mice did not differentiate into $CD4^+$ T cells (Fig. 7a). Thus, upregulation of CD4 expression during MHC class II–specific positive selection was a function of ThPOK that was not mediated by SOCS proteins.

DISCUSSION

ThPOK is considered the master regulator of $CD4^+$ lineage fate because it directs positively selected thymocytes to differentiate into T cells of the $CD4^+$ lineage regardless of the MHC specificity of their TCR[2,3,14]. However, the mechanism by which ThPOK imposes $CD4^+$ lineage fate on developing T cells has remained enigmatic. Our study has now identified SOCS-encoding genes as direct targets of ThPOK and has documented that the induction of these genes by ThPOK prevented Runx3 expression and was responsible for $CD4^+$ lineage–fate determination. Thus, ThPOK imposed the $CD4^+$ helper T cell lineage fate on developing T cells by inducing the expression of SOCS-encoding genes to prevent Runx3 expression and block $CD8^+$ lineage ‘choice’.

We found that ThPOK targeted at least three genes (*Socs1, Socs3* and *Cish*) encoding SOCS proteins that suppressed cytokine signaling in T cells and prevented the expression of Runx3. In cells lacking expression of SOCS-encoding genes, transgenically expressed ThPOK was observed to be functionally inert in that it permitted developing T cells to express Runx3 and to adopt the $CD8^+$ cytotoxic T cell lineage fate. The resulting SOCS-deficient $CD8^+$ cytotoxic T cells were unique among T cells in their simultaneous

expression of both ThPOK and Runx3. Despite simultaneously expressing both of these lineage-specifying factors, the SOCS-deficient T cells that were Runx3$^+$ displayed no ambiguity in their lineage fate 'decision', as they differentiated into the CD8$^+$ cytotoxic T cell lineage. Indeed, Runx3 seemed to be fully functional in SOCS-deficient CD8$^+$ T cells despite the presence of ThPOK, as these cells expressed the cytotoxic-lineage molecules granzyme B and perforin. These unique T cells demonstrate that ThPOK must induce the expression of SOCS-encoding genes to prevent Runx3 expression and to impose the CD4$^+$ lineage fate.

T cells expressing dual lineage factors arose in ThPOK-Tg mice that were also deficient in SOCS1. Because ThPOK induced the expression of *Socs3* and *Cish* in addition to that of *Socs1,* it was unexpected that deficiency in only *Socs1* affected ThPOK function. However, SOCS1-deficient T cells in ThPOK-Tg mice that adopted the CD8$^+$ lineage fate additionally lacked expression of both *Socs3* and *Cish,* whereas T cells in the same mice that adopted the CD4$^+$ lineage fate had high expression of *Socs3* or *Cish* or both. We suggest that ThPOK is expressed in amounts that are insufficient to ensure binding to all or multiple ThPOK targets in every T cell so that ThPOK may bind randomly to different SOCS-expressing targets in individual T cells. If ThPOK binds only to *Socs1* in some T cells, then ThPOK in those T cells in SOCS1-deficient mice will not induce expression of any SOCS protein, rendering transgenic ThPOK functionally inert and allowing those T cells to express Runx3 and to differentiate into the CD8$^+$ cytotoxic T cell lineage, as we observed.

In addition to demonstrating that the expression of SOCS-encoding genes was required for ThPOK to promote CD4$^+$ lineage specification, our study has also demonstrated the reciprocal result, in that enforced SOCS1 expression largely replaced ThPOK in the generation of CD4$^+$ T cells. Specifically, transgenic expression of SOCS1 in ThPOK-deficient mice directed many MHC class II–selected cells to adopt the CD4$^+$ lineage fate, which confirmed that the expression of SOCS-encoding genes was the mechanism by which ThPOK promoted the CD4$^+$ lineage fate. The observation that enforced SOCS1 expression obviated the need for ThPOK extends the understanding of CD4$^+$ lineage specification and integrates together three disparate findings about lineage fate determination in the thymus. The first such finding is our demonstration here that ThPOK induced the expression of SOCS-encoding genes. The second is the fact that suppression of cytokine signaling in developing T cells prevents Runx3 expression and prevents CD8$^+$ lineage specification[6]. Third is a report showing that CD4 T cells arise 'by default' in mice lacking both the ThPOK and Runx3 lineage-specifying transcription factors[27]. On the basis of those three findings, our observation here that enforced SOCS1 expression permitted the generation of CD4$^+$ T cells in ThPOK-deficient mice can be explained as the differentiation of T cells that expressed neither ThPOK nor Runx3. Thus, this perspective suggests that CD4$^+$ lineage 'choice' may require only the absence of Runx3 expression and that ThPOK during normal T cell development upregulates expression of genes encoding SOCS proteins that suppress Runx3 expression.

However, SOCS1 produced by transgenically enforced expression did not completely replace ThPOK in ThPOK-deficient mice, as some MHC class II–selected cells in *Thpok*$^{-/-}$ SOCS1-Tg mice differentiated into CD8$^+$ T cells with mismatched TCRs and coreceptors.

The appearance of T cells with such mismatch usually results from errors in lineage ‘choice’ during positive selection. We propose that the appearance of $CD8^+$ T cells with such mismatch in *Thpok*$^{-/-}$ SOCS1-Tg mice could also be ascribed to erroneous lineage ‘choices’ that were the result of the failure of ThPOK-deficient thymocytes to upregulate CD4 expression during MHC class II–specific positive selection[26]. The upregulation of CD4 expression during MHC class II–specific positive selection is necessary to ensure that TCR signaling persists throughout selection[26], but the factors responsible for this have not been previously identified, to our knowledge. Our study has now identified ThPOK as inducing the upregulation of CD4 expression during MHC class II–specific positive selection and has identified such upregulation as a function of ThPOK that cannot be achieved by SOCS1.

The previously unknown functions of ThPOK that we have identified in this study (induction of the expression of SOCS-encoding genes and upregulation of CD4 expression) provide new insights into the mechanism by which lineage ‘choice’ occurs during positive selection. As described in the kinetic signaling model[1,16], positive selecting TCR signals transiently inhibit cytokine signaling and induce the differentiation of $CD4^+CD8^+$ double-positive cells into INT thymocytes that are transcriptionally *Cd4*$^+$*Cd8*$^-$ and phenotypically $CD69^+CD4^+CD8^{lo}$. Because INT thymocytes maintain CD4 expression, MHC class II −specific positive selection signals persist to induce ThPOK expression[7]. We suggest that ThPOK then upregulates CD4 expression to maintain MHC class II signaling and upregulates genes encoding SOCS proteins to suppress cytokine signaling and prevent Runx3 expression, with cells then differentiating into mature $CD4^+$ T cells. Conversely, because INT thymocytes lose CD8 expression, MHC class I–specific positive-selection signals cease, which allows intrathymic cytokines to induce Runx3 expression[6]. Runx3 then silences *Thpok,* silences *Cd4* and reactivates *Cd8* and thus causes INT thymocytes to undergo ‘coreceptor reversal’ and become transcriptionally *Cd4*$^-$ *Cd8*$^+$ cells that differentiate into mature $CD8^+$ T cells[1,28]. In conclusion, our study has demonstrated that the expression of SOCS-encoding genes is the mechanism by which ThPOK represses Runx3 expression and imposes the $CD4^+$ lineage fate during positive selection. Thus, SOCS-encoding genes have a critical role in lineage-fate determination during positive selection in the thymus.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

Mice

C57BL/6, AND, and MHC class II–deficient mice were obtained from The Jackson Laboratory. SOCS1-Tg mice, which express cDNA encoding mouse SOCS1 under control of the proximal *Lck* promoter, have been described and were provided by M. Kubo[24]. *Thpok*$^{hd/hd}$ mice were provided by D. Kappes[12]. *Thpok*$^{-/-}$ and ThPOK-Tg mice (line C8) were provided by R. Bosselut[3,25]. Mice with germline deficiency in *Socs1* and *Ifng* were provided by James Ihle[29]. Human CD4–*Socs1* reporter mice have been described[20] and were provided by T. Kay. Mice with transgenic expression of ThPOK were generated by cloning of cDNA encoding mouse ThPOK under the control of enhancer-promoter elements of the

gene encoding human CD2, followed by injection into fertilized oocytes. Mice of both sexes between 4 and 16 weeks of age were used for analyses. Animal experiments were approved by the National Cancer Institute Animal Care and Use Committee, and all mice were cared for in accordance with guidelines of the US National Institutes of Health.

Flow cytometry

Lymphocytes were isolated from organs and were electronically sorted or were analyzed on a LSR II or FACSAria II. Lymph node cells were collected from the submandibular, inguinal, axillary and mesenteric lymph nodes. Dead cells were excluded by gating on forward light scatter and staining with propidium iodide. Flow cytometry data were analyzed with software designed by the Division of Computer Research and Technology at the US National Institutes of Health. The following antibodies were used for staining: antibody to TCRβ (anti-TCRβ; H57–957), anti-$V_{\alpha}11$ (RR8–1), anti-CD4 (GK1.5) and anti-CD8a (53–6-7; all from BD Biosciences); anti-Qa-2 (69H1–9-9), anti-CD24 (M1/69), antibody to the α-chain of the receptor for interleukin 7 (anti-IL-7Rα; A7R34), antibody to the CD40 ligand (MR1), anti-CD69 (H1.2F3) and anti-CD103 (2EF; all from eBioscience); and anti-IL-4 (11B11) and anti-IFN-γ (XMg1.2; both from BioLegend).

Construction of mixed-bone marrow chimeras

Bone marrow chimeras were generated by reconstitution of irradiated (600 rads) *Rag2*$^{-/-}$ recipient mice with donor bone marrow depleted of T cells and mixed at ratio of 1:1 (ThPOK-Tg.*Socs1*$^{+/+}$ to ThPOK-Tg.*Socs1*$^{-/-}$; a total of 15×10^6 cells). Chimeric mice were analyzed 8–10 weeks after reconstitution.

In vitro T helper type 2 differentiation of CD4$^+$ T cells

CD4$^+$ and CD8$^+$ T cells were sorted electronically from lymph nodes and were stimulated for 5 d with plate-bound anti-CD3 (145.2C11; BioLegend) and anti-CD28 (37.51; BioLegend), each at a concentration of 1 μg/ml, in the presence of recombinant mouse IL-4 (20 ng/ml; Peprotech) and anti-mouse IFN-γ (10 μg/ml; BioLegend). For intracellular staining of IL-4 and IFN-γ (both antibodies identified above), cells were restimulated for 3 h with PMA (phorbol 12-myristate 13-acetate) and ionomycin (both from Sigma) with the addition of brefeldin A, then were fixed and permeabilized with IC fixation buffer (eBioscience).

Luciferase reporter assay

The Matinspector software tool (Genomatix) and the rVista comparative tool (version 2.0) were used for analysis of evolutionarily conserved regions and potential ThPOK-binding sites in *Socs1* and *Cish*. *5′* upstream regions of the transcriptional initiation sites corresponding to the *Socs1* or *Cish* promoter were cloned into the promoterless pGL3-enhancer firefly luciferase reporter vector (Promega). cDNA encoding wild-type ThPOK or a mutant form of ThPOK whose BTB-POZ domain was destroyed was cloned into the pMACS LNGFR-IRES expression vector (Miltenyi Biotec). Luciferase reporter and ThPOK expression vectors were transfected together with pRL-CMV renilla luciferase vectors for normalization into 293T cells (American Type Culture Collection). Cell lysates were

assayed with the Dual-Luciferase Reporter Assay System (Promega) and results were 'read' in a luminometer.

Quantitative RT-PCR

Total RNA was isolated from sorted cells with TRIzol (Invitrogen) or with an RNeasy Mini kit (Qiagen). RNA was reverse-transcribed into cDNA by oligo(dT) priming with the QuantiTect Reverse Transcription kit (Qiagen). An ABI PRISM 7900HT Sequence Detection System and the QuantiTect SYBR Green detection system (Qiagen) were used for quantitative RT-PCR. Primers sequences are as follows. *Socs1* (forward, 5′-CCGCTCCCACTCCGATTA-3′, and reverse, 5′-GCACCAAGAAG GTGCCCA-3′), *Socs3* (forward, 5′-TTTCGCTTCGGGACTAGCTC-3′, and reverse, 5′-TTGCTGTGGGTGACCATGG-3′), *Cish* (forward, 5′-ACCTTCGGGAATCTGGGTG-3′, and reverse, 5′-GGGAAGGCCAGGATTCGA-3′), *Socs4* (forward, 5′-CGGAGAGATCCGTCCAGAAA-3′, and reverse, 5′ CTGTCAGCACTTCGACTCCG-3′), *Cd4* (forward, 5′-TCCAACCTAAGGGTTCAGGACAG-3′, and reverse, 5′-AGGTCTTTGGTGGACTTTTGTACG-3′), *Hprt* (forward, 5′-GCGATGATGAACCAGGTTATGA-3′, and reverse, 5′-ACAATGTGATGGCCTCCCAT-3′), *Rpl13* (forward, 5′-CGAGGCATGCTGCCCCACAA-3′, and reverse, 5′-AGCAGGGACCACCATCCGCT-3′), *Runx3* (forward, 5′-GCGACATGGCTTCCAACAGC-3′, and reverse, 5′-CTTAGCGCGCCGCTGTTCTCGC-3′), ThPOK *(Zbtb7b;* called *'Thpok'* here) (forward, 5′-GGTCTTGAGTACCGCACCCACA-3′, and reverse, 5′-CC CCTGTTCCCATCACGGTT-3′) and granzyme B *(Gzmb)* (forward, 5′-CC TCCAGGACAAAGGCAGGGGA-3′, and reverse, 5′-CCCACATATCGCCT CAGGCTGC-3′).

Statistical analysis

Prism (GraphPad) was used for statistical analyses. Statistical significance was determined with Student's *t*-test. A P value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank D. Singer and R. Hodes for critical review of this manuscript; R. Bosselut (National Cancer Institute) for *Thpok*$^{-/-}$ and ThPOK-Tg (C8) mice; J. Ihle (St. Jude Children's Research Hospital) for mice with germline deficiency in *Socs1* and *Ifng;* D. Kappes (Fox Chase Cancer Center) for *Thpok*$^{hd/hd}$ mice; T. Kay (St. Vincent's Institute of Medical Research) for human CD4–*Socs1* reporter mice; and M. Kubo (RIKEN) for SOCS1-Tg mice. Supported by the Intramural Research Program of the US National Institutes of Health, National Cancer Institute, Center for Cancer Research.

References

1. Singer A, Adoro S & Park JH Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat. Rev. Immunol 8, 788–801 (2008). [PubMed: 18802443]

2. He X et al. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature 433, 826–833 (2005). [PubMed: 15729333]
3. Sun G et al. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat. Immunol 6, 373–381 (2005). [PubMed: 15750595]
4. Sato T et al. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. Immunity 22, 317–328 (2005). [PubMed: 15780989]
5. Taniuchi I et al. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell 111, 621–633 (2002). [PubMed: 12464175]
6. Park JH et al. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. Nat. Immunol 11, 257–264 (2010). [PubMed: 20118929]
7. He X et al. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. Immunity 28, 346–358 (2008). [PubMed: 18342007]
8. Setoguchi R et al. Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. Science 319, 822–825 (2008). [PubMed: 18258917]
9. Taniuchi I & Ellmeier W Transcriptional and epigenetic regulation of CD4/CD8 lineage choice. Adv. Immunol 110, 71–110 (2011). [PubMed: 21762816]
10. Xiong Y & Bosselut R The enigma of CD4-lineage specification. Eur. J. Immunol 41, 568–574 (2011). [PubMed: 21341258]
11. Yoshimura A, Naka T & Kubo M SOCS proteins, cytokine signalling and immune regulation. Nat. Rev. Immunol 7, 454–465 (2007). [PubMed: 17525754]
12. Dave VP, Allman D, Keefe R, Hardy RR & Kappes DJ HD mice: a novel mouse mutant with a specific defect in the generation of $CD4^+$ T cells. Proc. Natl. Acad. Sci. USA 95, 8187–8192 (1998). [PubMed: 9653162]
13. Keefe R, Dave V, Allman D, Wiest D & Kappes DJ Regulation of lineage commitment distinct from positive selection. Science 286, 1149–1153 (1999). [PubMed: 10550051]
14. He X, Park K & Kappes DJ The role of ThPOK in control of CD4/CD8 lineage commitment. Annu. Rev. Immunol 28, 295–320 (2010). [PubMed: 20307210]
15. Bosselut R, Guinter TI, Sharrow SO & Singer A Unraveling a revealing paradox: Why major histocompatibility complex I-signaled thymocytes “paradoxically” appear as CD4+8lo transitional cells during positive selection of CD8+ T cells. J. Exp. Med 197, 1709–1719 (2003). [PubMed: 12810689]
16. Brugnera E et al. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. Immunity 13, 59–71 (2000). [PubMed: 10933395]
17. Adoro S et al. Coreceptor gene imprinting governs thymocyte lineage fate. EMBO J. 31, 366–377 (2012). [PubMed: 22036949]
18. Croker BA, Kiu H & Nicholson SE SOCS regulation of the JAK/STAT signalling pathway. Semin. Cell Dev. Biol 19, 414–422 (2008). [PubMed: 18708154]
19. Palmer DC & Restifo NP Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. Trends Immunol. 30, 592–602 (2009). [PubMed: 19879803]
20. Chong MM et al. Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent $CD8^+$ T cell differentiation. Immunity 18, 475–487 (2003). [PubMed: 12705851]
21. Catlett IM & Hedrick SM Suppressor of cytokine signaling 1 is required for the differentiation of $CD4^+$ T cells. Nat. Immunol 6, 715–721 (2005). [PubMed: 15924143]
22. Yu Q et al. Cytokine signal transduction is suppressed in preselection doublepositive thymocytes and restored by positive selection. J. Exp. Med 203, 165–175 (2006). [PubMed: 16390939]
23. Grueter B et al. Runx3 regulates integrin alpha E/CD103 and CD4 expression during development of $CD4^-/CD8^+$ T cells. J. Immunol 175, 1694–1705 (2005). [PubMed: 16034110]
24. Hanada T et al. A mutant form of JAB/SOCS1 augments the cytokine-induced JAK/STAT pathway by accelerating degradation of wild-type JAB/CIS family proteins through the SOCS-box. J. Biol. Chem 276, 40746–40754 (2001). [PubMed: 11522790]

25. Wang L et al. The zinc finger transcription factor Zbtb7b represses CD8-lineage gene expression in peripheral CD4+ T cells. Immunity 29, 876–887 (2008). [PubMed: 19062319]
26. Sarafova SD et al. Upregulation of CD4 expression during MHC class II-specific positive selection is essential for error-free lineage choice. Immunity 31, 480–490 (2009). [PubMed: 19747858]
27. Egawa T & Littman DR ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. Nat. Immunol 9, 1131–1139 (2008). [PubMed: 18776905]
28. Singer A New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. Curr. Opin. Immunol 14, 207–215 (2002). [PubMed: 11869894]
29. Marine JC et al. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. Cell 98, 609–616 (1999). [PubMed: 10490100]

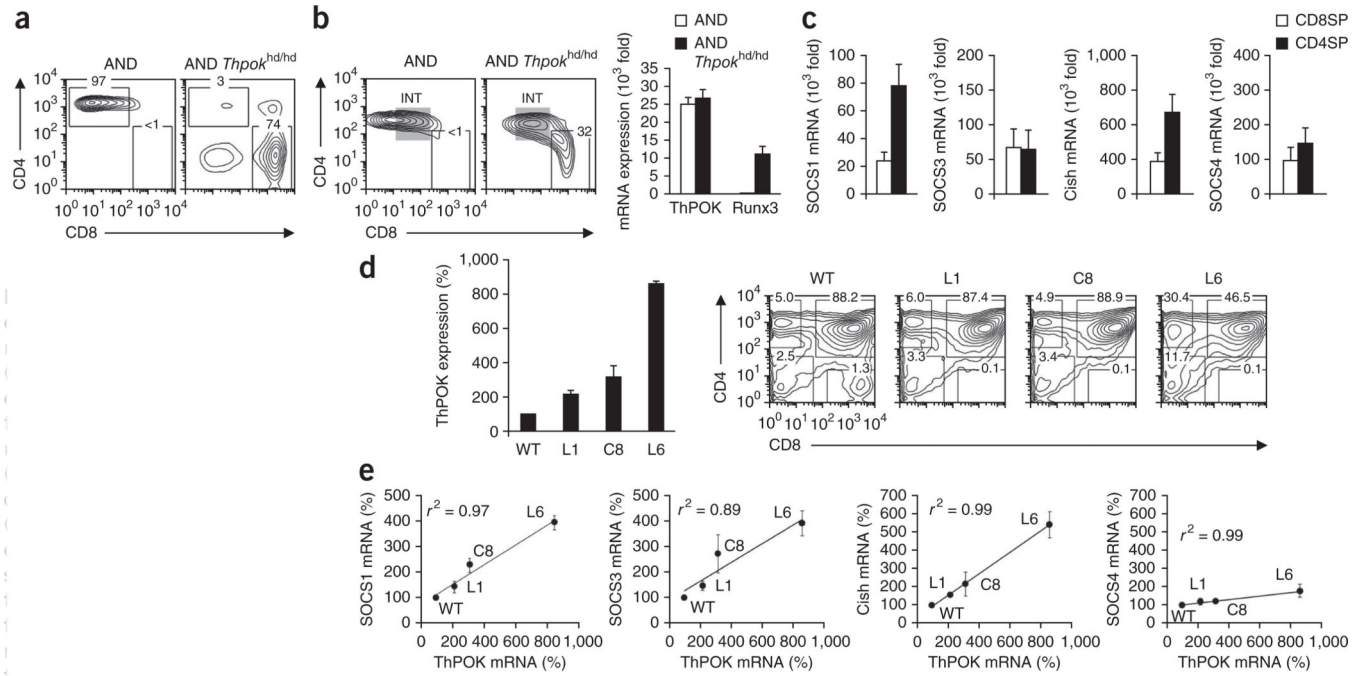

Figure 1.

ThPOK induces the expression of genes encoding members of the SOCS family. (**a**) CD4-versus-CD8 profiles of clonotypic $V_\alpha 11^+$ $CD24^{lo}$ T cells from the lymph nodes of AND mice and AND *Thpok*$^{hd/hd}$ mice. (**b**) Quantitative RT-PCR analysis of ThPOK and Runx3 mRNA (right) in $CD69^+CD4^+CD8^{lo}$ INT cells (shaded area, left plots) sorted electronically from AND thymocytes and AND *Thpok*$^{hd/hd}$ thymocytes (left); mRNA results are presented relative to those of control HPRT mRNA (encoding hypoxanthine guanine phosphoribosyl transferase). (**c**) Quantitative RT-PCR analysis of SOCS1, SOCS3, Cish and SOCS4 mRNA in mature CD4SP and CD8SP thymocytes sorted from wild-type B6 mice (presented as in **b**, right). (**d**) Expression of ThPOK mRNA in $CD4^+$ T cells from the lymph nodes of wild-type mice (WT) and L1, C8 and L6 ThPOK-Tg mice (left), and CD4-versus-CD8 profiles of total thymocytes from those mice (right); mRNA results presented relative to those of wild-type cells, set as 100%. (**e**) Quantitative RT-PCR analysis of SOCS1, SOCS3, Cish and SOCS4 mRNA in purified $CD4^+$ T cells from lymph nodes of wild-type, L1, C8 and L6 mice, relative to results for HPRT mRNA, plotted against ThPOK mRNA in the same cells and normalized to results for wild-type cells, set as 100%. Numbers adjacent to outlined areas (**a,b,d**, right) indicate percent cells in each. Data are representative of more than five experiments (**a**) or three (**b,d,e**) or four (**c**) independent experiments (mean and s.e.m. for PCR results).

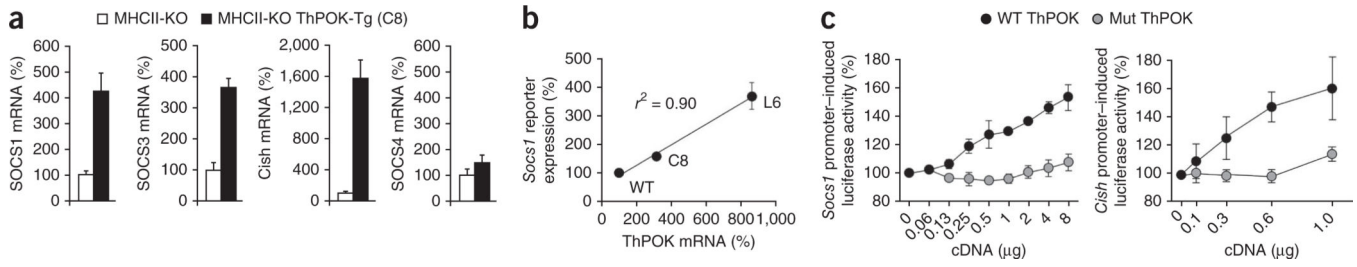

Figure 2.

ThPOK is a transcriptional activator of SOCS-encoding genes. (**a**) Quantitative RT-PCR analysis of SOCS1, SOCS3, Cish and SOCS4 mRNA in MHC class I–selected T cells purified from lymph nodes of MHC class II–deficient mice (MHCII-KO) and MHC class II–deficient ThPOK-Tg mice (ThPOK-Tg MHCII-KO); results are presented relative to those of MHC class II–deficient cells, set as 100%. (**b**) Surface expression of the *Socs1* reporter protein on T cells from lymph nodes of wild-type mice and C8 or L6 ThPOK-Tg mice, plotted against ThPOK mRNA expression and presented relative to expression in wild-type cells, set as 100%. (**c**) Firefly luciferase activity in 293T cells transfected with a promoterless firefly luciferase reporter vector containing a genomic DNA fragment corresponding to the mouse *Socs1* or *Cish* promoter plus increasing amounts (horizontal axis) of cDNA encoding wild-type ThPOK (WT ThPOK) or mutant nonfunctional ThPOK with a defect in its BTB-POZ domain (Mut ThPOK); results were normalized to those of cotransfected renilla luciferase and are presented relative to those of untransfected cells, set as 100%. Data are representative of two independent experiments with two mice per group (**a**; mean and s.e.m.), three independent experiments (**b**; mean and s.e.m.) or three to five independent experiments (**c**; mean ± s.e.m. of triplicate assays).

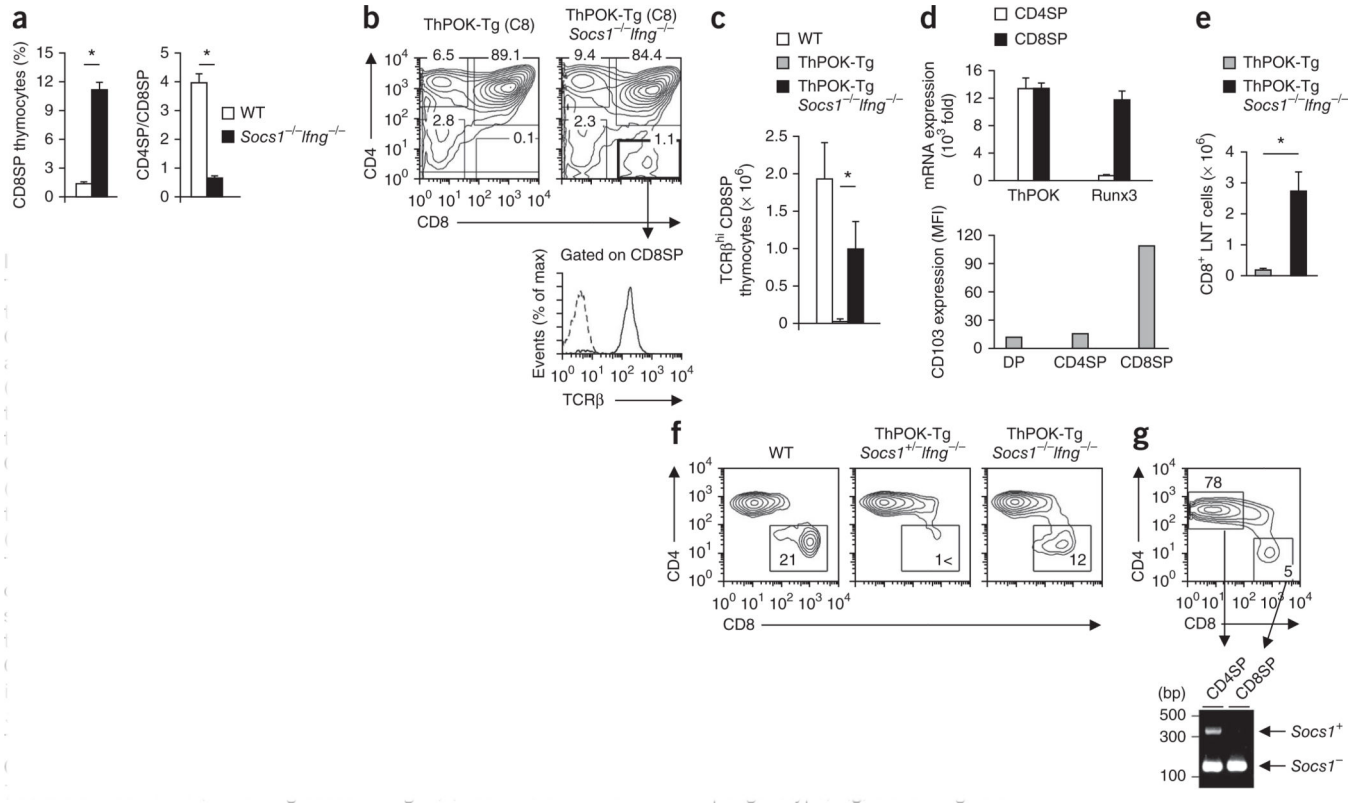

Figure 3.

Imposition of the CD4$^+$ lineage fate by ThPOK requires SOCS1. (**a**) Frequency of CD8SP thymocytes (left) and ratio of CD4SP cells to CD8SP cells (CD4SP/CD8SP; right) in wild-type and SOCS1-deficient *(Socs1$^{-/-}$Ifng$^{-/-}$)* mice. (**b**) Surface expression of CD4 and CD8 on ThPOK-Tg thymocytes and C8 ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* thymocytes (top), and TCRβ expression (solid line) on the CD8SP ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* thymocytes gated above (bottom right; dashed line, control antibody). Numbers adjacent to outlined areas (top) indicate percent cells in each. (**c**) Quantification of TCRβ^{hi} CD8SP thymocytes in wild-type, ThPOK-Tg and ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* mice. (**d**) Expression of ThPOK and Runx3 mRNA in CD4SP and CD8SP thymocytes sorted from ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* mice, presented relative to that of HPRT mRNA (top), and surface expression of CD103 on CD4$^+$CD8$^+$ double-positive (DP), CD4SP and CD8SP cells from the same mice (bottom). MFI, mean fluorescence intensity. (**e**) Quantification of CD8$^+$ T cells in lymph nodes (CD8$^+$ LNT cells) from ThPOK-Tg and ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* mice. (**f**) CD4-versus-CD8 profiles of CD24^{lo}TCRβ^{hi} thymocytes from neonatal (9-day-old) wild-type, ThPOK-Tg *Socs1$^{+/-}$Ifng$^{-/-}$* and ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* mice. Numbers in outlined areas indicate percent mature CD8SP cells, quantified as 1,300 ± 414 × 10^3 cells (wild-type), 65 ± 7 × 10^3 cells (ThPOK-Tg *Socs1$^{+/-}$*) and 793 ± 38 × 10^3 cells (ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$*) (mean ± s.e.m. of three mice per genotype). (**g**) Donor origins of TCRβ^{hi} mature CD4SP and CD8SP cells (top) In an Irradiated *Rag2$^{-/-}$* host given a mixture of equal numbers of ThPOK-Tg *Socs1$^{+/+}$* and ThPOK-Tg *Socs1$^{-/-}$Ifng$^{-/-}$* bone marrow stem cells, determined by genotyplng of sorted CD4SP and CD8SP thymocytes by PCR of genomic DNA (bottom). Numbers adjacent to outlined areas (top) indicate percent CD4SP cells (top left) or CD8SP

cells (bottom right). * $P < 0.001$ (Student's *t*-test). Data are representative of five independent experiments (**a–c,e**; mean and s.e.m. of one mouse per group), two independent experiments with two mice per genotype (**d**; error bars, s.e.m.), three independent experiments (**f**) or four independent experiments (**g**).

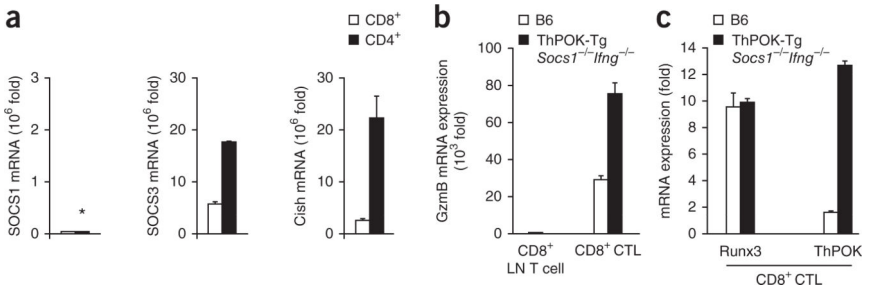

Figure 4.

ThPOK-Tg *Socs1*$^{-/-}$ thymocytes that adopt the CD8^{+} cytotoxic T cell fate have minimal expression of any SOCS-encoding gene. (**a**) Quantitative RT-PCR analysis of SOCS1, SOCS3 and Cish mRNA in CD8^{+} or CD4^{+} T cells sorted from ThPOK-Tg *Socs1*$^{-/-}$*Ifng*$^{-/-}$ lymph nodes, presented relative to that of HPRT mRNA. *, SOCS1 mRNA in CD4^{+} T cells from wild-type lymph nodes. (**b**) Quantitative RT-PCR analysis of granzyme B (GzmB) mRNA in resting lymph node CD8^{+} T cells (CD8^{+} LN T cells) and CD8^{+} cytotoxic T lymphocytes generated *in vitro* (CD8^{+} CTL), purified from B6 and ThPOK-Tg *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice; results are presented relative to those of HPRT mRNA. (**c**) Quantitative RT-PCR analysis of ThPOK and Runx3 mRNA in B6 and ThPOK-Tg *Socs1*$^{-/-}$*Ifng*$^{-/-}$ CD8^{+} cytotoxic T lymphocytes, presented relative to that of mRNA encoding ribosomal protein L13. Data are representative of two independent experiments (mean and s.e.m. of three mice per group).

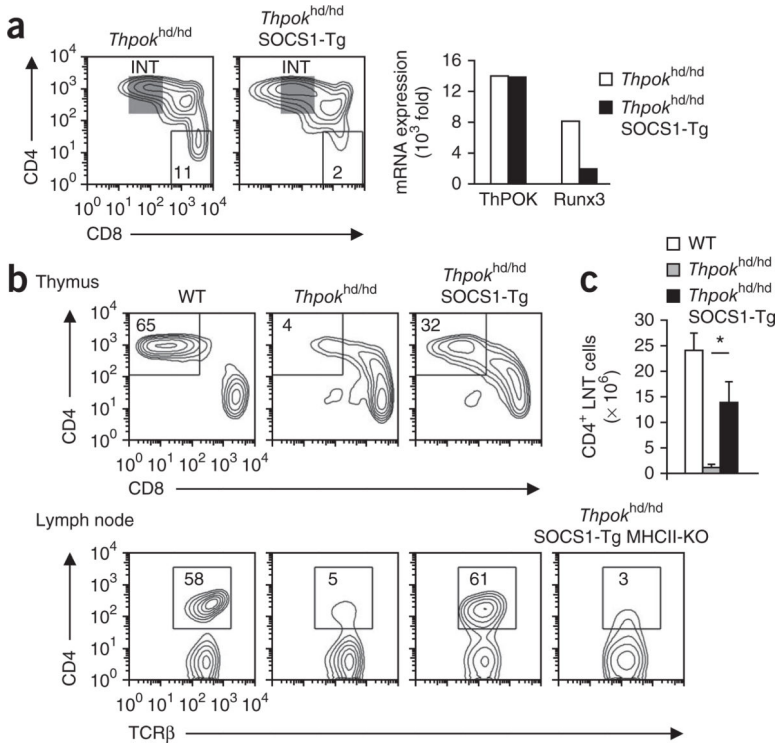

Figure 5.

SOCS1 can replace ThPOK function during commitment to the $CD4^+$ lineage in the thymus. (**a**) Quantitative RT-PCR analysis of ThPOK and Runx3 mRNA (right) in $CD69^+CD4^+CD8^{lo}$ INT thymocytes sorted electronically from *Thpok*$^{hd/hd}$ and *Thpok*$^{hd/hd}$ SOCS1-Tg mice (left), presented relative to that of HPRT mRNA. (**b**) Expression of CD4 and CD8 on $CD24^{lo}TCR\beta^{hi}$ thymocytes from *Thpok*$^{hd/hd}$ and *Thpok*$^{hd/hd}$ SOCS1-Tg mice (top), and expression of CD4 and TCRβ on $TCR\beta^+$ lymph node cells from those mice and a *Thpok*$^{hd/hd}$ SOCS1-Tg MHC class II–deficient mouse (bottom). Numbers in outlined areas indicate percent CD4SP cells (top) or percent peripheral $TCR\beta^+$ $CD4^+$ T cells (bottom). (**c**) Quantification of $CD4^+$ lymph node T cells ($CD4^+$ LNT cells) from wild-type, *Thpok*$^{hd/hd}$ and *Thpok*$^{hd/hd}$ SOCS1-Tg mice. $*P < 0.001$ (Student's f-test). Data are representative of two independent experiments (**a**) or five independent experiments (**b,c**; mean and s.e.m. in **c**).

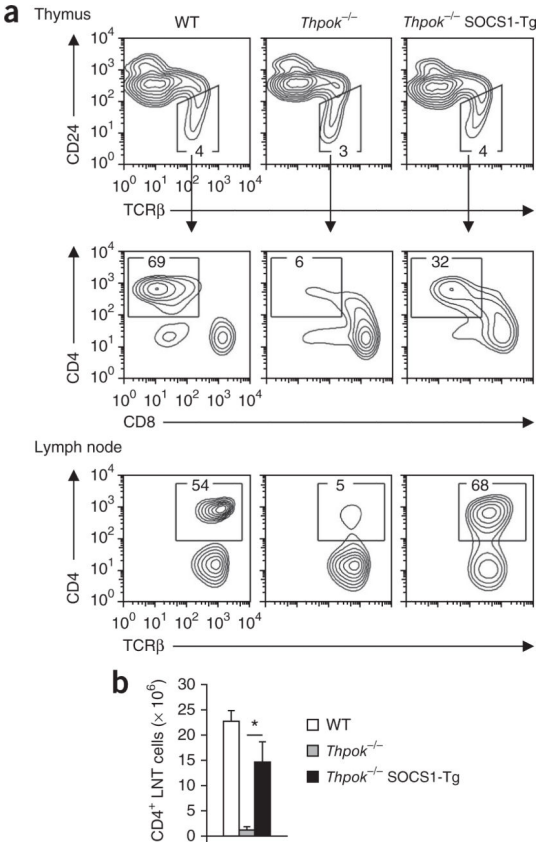

Figure 6.

Transgenically expressed SOCS1 can replace ThPOK in the generation of $CD4^+$ T cells. (**a**) Expression of CD24 and TCRβ by whole thymocytes from wild-type, *Thpok*$^{-/-}$ and *Thpok*$^{-/-}$ SOCS1-Tg mice (top) and expression of CD4 and CD8 on the $CD24^{lo}TCR\beta^{hi}$ thymocytes gated above (middle), as well as expression of CD4 and TCRβ on $TCR\beta^+$ lymph node cells (bottom). Numbers in outlined areas indicate percent $CD24^{lo}TCR\beta^{hi}$ thymocytes (top) or CD4SP thymocytes (middle) or peripheral $TCR\beta^+$ $CD4^+$ T cells (bottom). (**b**) Quantification of $CD4^+$ lymph node T cells from wild-type, *Thpok*$^{-/-}$ and *Thpok*$^{-/-}$ SOCS1-Tg mice. $*P < 0.001$ (Student's *t*-test). Data are representative of seven independent experiments (mean and s.e.m. of seven mice per genotype in **b**).

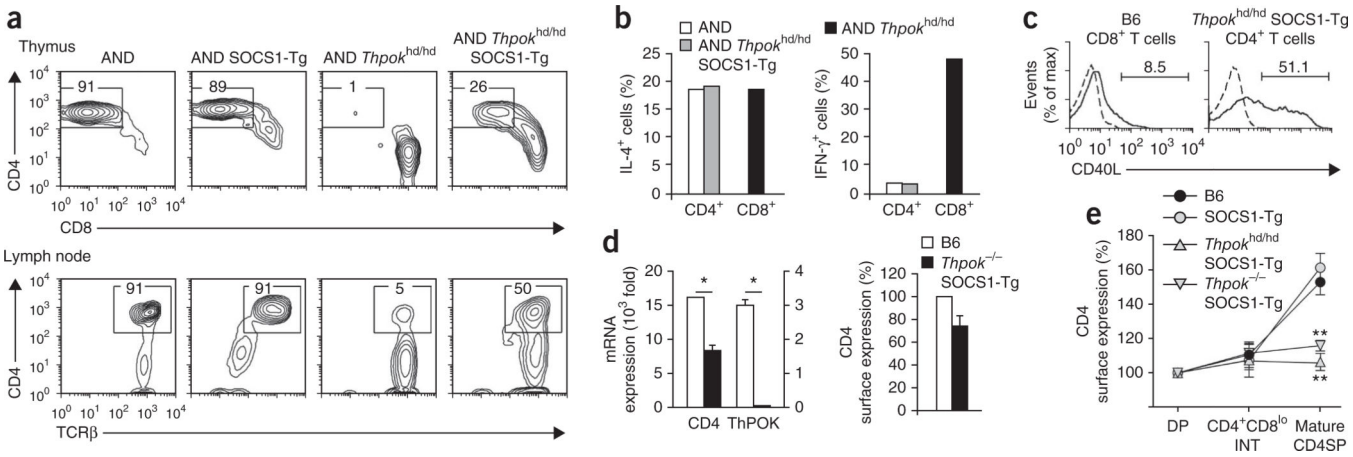

Figure 7.

ThPOK-independent and ThPOK-dependent generation of CD4$^+$ T cells. (**a**) Expression of CD4 and CD8 on CD24^{lo}V$_\alpha$11^{hi} thymocytes from mice of various genotypes (top) and expression of CD4 and TCRβ by clonotypic V$_\alpha$11$^+$ lymph node cells (bottom). Numbers in outlined areas indicate percent CD4SP thymocytes (top) or peripheral TCRβ$^+$ CD4$^+$ T cells (bottom). (**b**) Frequency of cells expressing interleukin 4 (IL-4) and IFN-γ among purified AND CD4$^+$ T cells, AND *Thpok*$^{hd/hd}$ SOCS1-Tg CD4$^+$ T cells and AND *Thpok*$^{hd/hd}$ CD8$^+$ T cells exposed for 5 d to T helper type 2-polarizing conditions. (**c**) Surface expression of the CD40 ligand (CD40L; solid lines) on T cells purified from lymph nodes of B6 and *Thpok*$^{hd/hd}$ SOCS1-Tg mice and then stimulated overnight with plate-bound antibody to the TCR (5 μg/ml); dashed lines, control antibody. Numbers above bracketed lines indicate percent CD40 ligand-expressing cells. (**d**) Quantitative RT-PCR analysis of CD4 and ThPOK mRNA (left) and surface expression of CD4 (assessed by flow cytometry; right) by CD4$^+$ T cells sorted electronically from lymph nodes of B6 and *Thpok*$^{-/-}$*SOCS1-Tg* mice, presented relative to results for HPRT mRNA (left) or wild-type cells, set as 100% (right). (**e**) Surface expression of CD4 on CD4$^+$CD8$^+$ double-positive, INT and CD4SP thymocytes of various genotypes (key), normalized to that of CD4$^+$CD8$^+$ double-positive cells, set as 100%. *$P < 0.01$ and **$P < 0.001$ (Student's *t*-test). Data are representative of three independent experiments (**a,d,e**; mean and s.e.m. of three mice per group in **d,e**) or two independent experiments (**b,c**).