nature immunology

Suppressor of cytokine signaling 1 is required for the differentiation of CD4+ T cells

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Suppressor of cytokine signaling 1 (Socs1) is critical for the regulation of interferon-γ responses and T cell homeostasis. Although the presentation of the inflammatory disease of Socs1-deficient mice is complex, we have tested here the hypothesis that it originates from inappropriate T cell development and the appearance of autoreactive T cells. Socs1-deficient T cell receptor–transgenic mice showed severely impaired positive selection and a substantial alteration in CD4-CD8 T cell fate specification. These defects were dependent on interferon-γ. Moreover, negative selection was also impaired, suggesting that autoimmunity contributes to the disease observed in *Socs1*$^{-/-}$ mice. We conclude that the constitutive expression of Socs1 in the thymus protects the process of thymic development and selection from the effects of systemic inflammation.

Mice deficient in suppressor of cytokine signaling 1 (Socs1) have a complex phenotype with autoimmune and inflammatory disease characteristics involving many cell types. Socs1 was originally identified as an inhibitor of interleukin 6 (IL-6), a Janus kinase binding and inhibiting protein or as a protein that was cross-reactive with antibody to signal transducers and activators of transcription[1–3]. Socs1-deficient mice have increased elaboration of interferon-γ (IFN-γ)[4] and, in addition, their macrophages and other cell types are hypersensitive to cytokine-mediated signaling[5]. Socs1 is a mediator of classic negative feedback regulation. Cytokine signaling induces the expression of Socs1 that in turn inhibits further signaling. In overexpression studies, Socs1 seems to regulate the response to many cytokines[6]; however, the viability of mice deficient in both Socs1 and IFN-γ suggests that it is essential only for the regulation of IFN-γ[4,5] Additional *in vivo* experiments have indicated involvement of Socs1 in the response to other cytokines, such as prolactin, insulin, IL-4, IL-7, IL-12 and IL-15 (refs. 7–11), although these effects seem to be less severe.

The observation that both gnotobiotic and specific pathogen–free Socs1-deficient mice show equivalent perinatal death seems to eliminate the possibility that normal flora or opportunistic pathogens initiate the disease of *Socs1*$^{-/-}$ mice[5]. Instead, the viability of *Socs1*$^{-/-}$ mice with homozygous deficiency in recombination-activating gene 1 (*Rag1*$^{-/-}$) suggests that T cells are important contributors to the spontaneous IFN-γ production[4]. *Socs1*$^{-/-}$ mice reconstituted with a T cell–specific *Socs1* transgene have a delayed-onset lupus-like autoimmune disease[12], whereas mice with T cell–specific deletion of *Socs1* do not get the disease[8]. Combined with results showing that dendritic cells from Socs1-deficient mice are hypersensitive[12], these results are consistent with involvement of autoreactive T cells and hyperactive dendritic cells in rapid-onset pathology.

Although the main function of Socs1 was thought to be the regulation of cytokine signaling in immune cell homeostasis and in the course of an immune response, Socs1 is most highly expressed in thymus[1,4,8,13]. This expression pattern suggests that the regulation of cytokine signaling could be important for one or more processes that direct T cell development. At present the only known cytokine that affects T cell development is IL-7, and it promotes survival and proliferation of developing thymocytes[14,15]. Mice with homozygous deficiency in IL-7 receptor or in the kinase Jak3 have severely impaired early T cell development[16,17]. However, Socs1 is still expressed in the remaining thymus tissue of each of these mutant mice, suggesting that cytokine signaling is not required for expression of Socs1 in the thymus[4].

Socs1 deficiency alters several aspects of T cell development. For example, Socs1 has been proposed to be important in the negative selection of thymocytes[4]. However, thymocyte deletion in response to superantigen is unaffected in the *Socs1*$^{-/-}$ fetal thymus[4,8]. In addition, a T cell–specific Socs1 deficiency also causes an increase in the number of CD8+ T cells in the thymus and secondary lymphoid organs[8], and this is apparently not diminished in *Socs1*$^{-/-}$ mice with homozygous deficiency in IFN-γ (*Ifng*$^{-/-}$)[18]. Although involvement of cytokines in directing T cells into the CD4 or CD8 fate has been proposed, the mechanistic details of this fate 'decision' are still controversial[19].

To understand the function of Socs1 in thymocyte development, we generated *Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice containing a transgene expressing a T cell receptor (TCR) specific for a fragment of pigeon cytochrome *c* (PCC) and the I-E^{k} molecule (*TcrAND* transgene). Using these mice to examine the function of Socs1 in positive and negative selection of CD4+ T cells, we found that Socs1 deficiency suppressed both positive selection and negative selection and altered the lineage decision of cells

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Published online 29 May 2005; doi:10.1038/ni1211

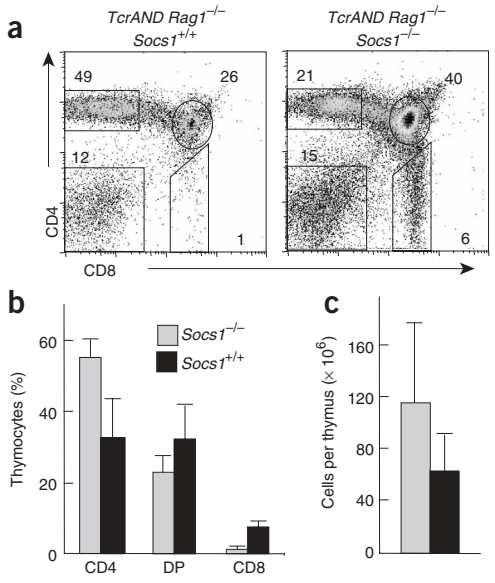

Figure 1 T cell development in *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ thymi is dysregulated. Thymocytes from *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice were stained with monoclonal antibodies to CD8 and CD4. (**a**) Thymic profile determined by flow cytometry. Numbers beside outlined areas indicate percentage of cells in that area. Data are of a representative pair of mice from 20 independent experiments. (**b**) Mean percentages of CD4$^+$ SP, CD8$^+$ SP, and double-positive (DP) thymocytes. Wild-type, $n = 25$; *Socs1*$^{-/-}$, $n = 22$. (**c**) Total number of thymocytes. Wild-type, $n = 23$; *Socs1*$^{-/-}$, $n = 19$.

normally destined to become CD4$^+$ T cells. A substantial fraction of Socs1-deficient *TcrAND* T cells assumed the CD8 phenotype in a major histocompatibility complex (MHC) class I–independent way. These effects were the result of systemic inflammation and inappropriate production of IFN-γ. We propose that an essential function of Socs1 is to insulate developing thymocytes from systemically produced IFN-γ.

RESULTS

Socs1 regulates the development of CD4$^+$ T cells

Although Socs1 deficiency enhances CD8$^+$ T cell development[4,8,18], the effects of Socs1 deficiency on CD4$^+$ T cell development and T cell selection events have not been investigated. We generated mice with Socs1-deficient T cells specific for PCC by breeding *TcrAND* transgenic mice with *Socs1*$^{+/-}$ mice. *TcrAND Socs1*$^{-/-}$ mice had a disease pathology similar to that of nontransgenic mice, but died with somewhat delayed kinetics (data not shown). This result was probably due to incomplete exclusion of endogenous TCR gene rearrangements.

To generate mice with a truly monospecific T cell population, we further bred *TcrAND Socs1*$^{+/-}$ mice with *Rag1*$^{-/-}$ mice. The resultant *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice were born at the expected mendelian frequency and seemed healthy throughout early life. Thymi from young *Socs1*$^{-/-}$ mice were slightly smaller than those of wild-type mice. In a representative example, the *Socs1*$^{-/-}$ thymus contained about 80% of wild-type total cell numbers (76×10^6 versus 94×10^6, *Socs1*$^{-/-}$ versus wild-type; **Fig. 1a**). In the *Socs1*$^{-/-}$ thymus there was an increased number and frequency of CD4$^+$CD8$^+$ double-positive cells with a concordant decrease in the number and frequency of CD4$^+$CD8$^-$ (CD4$^+$ single-positive (SP)) thymocytes. Most notably, there was a prominent appearance of mature CD4$^-$CD8$^+$ (CD8$^+$ SP) cells (4.3×10^6 versus 0.8×10^6, *Socs1*$^{-/-}$ versus wild-type). The changes in the population frequencies were consistent and significant ($n = 20$; $P < 0.01$, for CD4 SP, CD8 SP, and double-positive, Student's *t*-test; **Fig. 1b**); however, the cellularity was highly variable (**Fig. 1c**). Yet there was no evidence of a systemic stress response. For example, the total number of double-positive thymocytes in *Socs1*$^{-/-}$ mice was actually increased, despite the fact that this population is highly sensitive to the adrenal response brought about by inflammation or the direct administration of corticosteroids[20]. These results suggest that Socs1 has a positive effect on the development of CD4$^+$ T cells and influences the cell fate decision guiding the development of CD4$^+$ and CD8$^+$ T cells.

The requirement for Socs1 in hematopoietic cells

Because *Socs1* is expressed by a wide variety of cell types, we sought to determine whether this positive selection defect was thymocyte intrinsic or the result of Socs1 deficiency in the thymic epithelium or other nonhematopoietic cells. To address this, we lethally irradiated 'allelically marked' wild-type mice and reconstituted them with *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ bone marrow. Then, 4 weeks after reconstitution, we collected thymi and assessed T cell development by analyzing expression of CD4, CD8, CD24, CD69 and $V_\alpha11$ to detect the TCR expressed in *TcrAND* mice. The number of CD4$^+$ SP thymocytes was reduced in mice reconstituted with *Socs1*$^{-/-}$ bone marrow (**Fig. 2a**) and there were fewer mature T cells, as determined by a $V_\alpha11^{hi}$CD24^{lo} phenotype (**Fig. 2b**). The decrease in positive selection in mice reconstituted with *Socs1*$^{-/-}$ bone marrow was also demonstrated by a decrease in the percentage of $V_\alpha11^{hi}$CD69^{hi} thymocytes (**Fig. 2c**). In a representative experiment, CD4$^+$ SP cells constituted 96% of the mature cells ($V_\alpha11^{hi}$CD24^{lo}) in the thymus reconstituted with *Socs1*$^{+/+}$ bone marrow, compared with 71% in the thymus reconstituted with *Socs1*$^{-/-}$ bone marrow (**Fig. 2d**). Most of the remaining cells were CD8$^+$ SP (**Fig. 2d**). The compiled results for subpopulations defined by CD4 and CD8 in total thymocytes (**Fig. 2e**) and mature T cells (**Fig. 2f**) showed that the effect of a *Socs1* deficiency was intrinsic to the bone marrow cells and was not the result of Socs1 deficiency in the thymic epithelium[21].

MHC class II–dependent CD8$^+$ T cell maturation

The fate mis-specification of thymocytes from *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice could result from MHC class II recognition that is improperly 'interpreted' to promote CD8 differentiation, or it could result from MHC class I recognition that is 'enabled' by the loss of Socs1. To distinguish between these two possibilities, we created chimeras by reconstituting irradiated β_2-microglobulin–deficient mice with bone marrow from *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ mice. In such mice, little or no MHC class I is expressed on the epithelial cells of the thymus and CD8$^+$ SP T cells are normally not found[22]. We assessed the thymocyte subpopulations 4 weeks after transfer and, as expected, found that development of CD4$^+$ SP thymocytes was unaltered in β_2-microglobulin–deficient mice reconstituted with *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ bone marrow. In contrast, β_2-microglobulin–deficient mice reconstituted with *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ bone marrow still produced a substantial population of CD8$^+$ SP thymocytes (**Fig. 3a**). Compilation of the data from triplicate recipient mice showed that CD8$^+$ thymocytes consistently developed in irradiated β_2-microglobulin–deficient hosts (**Fig. 3b**).

TcrAND Rag1$^{-/-}$*H2*^{d} mice show no positive selection[23], indicating that this TCR does not recognize either H-2A^d or H-2E^d. To determine whether CD8$^+$ SP thymocyte development requires MHC class II

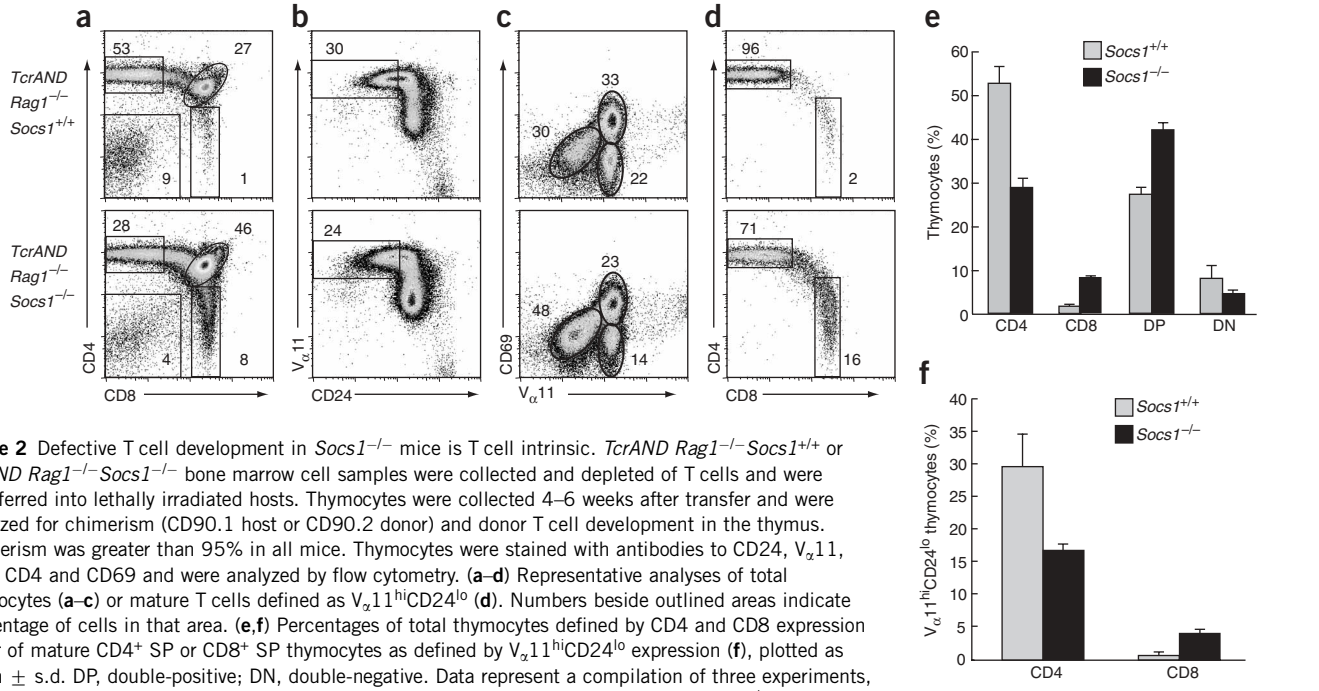

Figure 2 Defective T cell development in *Socs1*$^{-/-}$ mice is T cell intrinsic. *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ bone marrow cell samples were collected and depleted of T cells and were transferred into lethally irradiated hosts. Thymocytes were collected 4–6 weeks after transfer and were analyzed for chimerism (CD90.1 host or CD90.2 donor) and donor T cell development in the thymus. Chimerism was greater than 95% in all mice. Thymocytes were stained with antibodies to CD24, $V_\alpha11$, CD8, CD4 and CD69 and were analyzed by flow cytometry. (**a**–**d**) Representative analyses of total thymocytes (**a**–**c**) or mature T cells defined as $V_\alpha11^{hi}CD24^{lo}$ (**d**). Numbers beside outlined areas indicate percentage of cells in that area. (**e**,**f**) Percentages of total thymocytes defined by CD4 and CD8 expression (**e**) or of mature CD4$^+$ SP or CD8$^+$ SP thymocytes as defined by $V_\alpha11^{hi}CD24^{lo}$ expression (**f**), plotted as mean ± s.d. DP, double-positive; DN, double-negative. Data represent a compilation of three experiments, each with three recipients per genotype. The total number of thymocytes was similar: *Socs1*$^{+/+}$, 47.6 × 10^6 ± 12.3 × 10^6, $n = 9$; *Socs1*$^{-/-}$, 56.7 × 10^6 ± 23.6 × 10^6, $n = 9$.

recognition, we generated *TcrAND Rag1*$^{-/-}$*H2*^{d} mice with or without a Socs1 deficiency and characterized their thymus subpopulations. The analysis showed no CD4$^+$ SP or CD8$^+$ SP thymocytes (**Fig. 3c**). The apparent CD8$^+$ SP cells were immature SP cells with high CD24 expression and low $V_\alpha11$ expression (data not shown). In addition, there was no clearly defined population of mature thymocytes with a $V_\alpha11^{hi}CD24^{lo}$ phenotype (**Fig. 3d**). Even among the few cells in the $V_\alpha11^{hi}CD24^{lo}$ electronic gate, more than 95% did not express either CD4 or CD8 (**Fig. 3e**). We confirmed these results by transferring bone marrow from *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$*H2*^{b} donors into irradiated MHC class II–deficient hosts (*H2-Ab1*$^{-/-}$; data not shown). These results indicated that selection of CD8$^+$ SP *TcrAND* thymocytes in *Socs1*$^{-/-}$ mice occurred through the recognition of MHC class II molecules and that some *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ thymocytes recognized the correct H-2A^b ligand but 'chose' the inappropriate lineage.

Effector functions of Socs1-deficient T cells

Although the CD8$^+$ SP cells in the thymus of *Socs1*$^{-/-}$ mice seem mature, they may not be committed to the CD8 cytotoxic lineage. Thus, we tested the functional maturity of these cells by stimulating

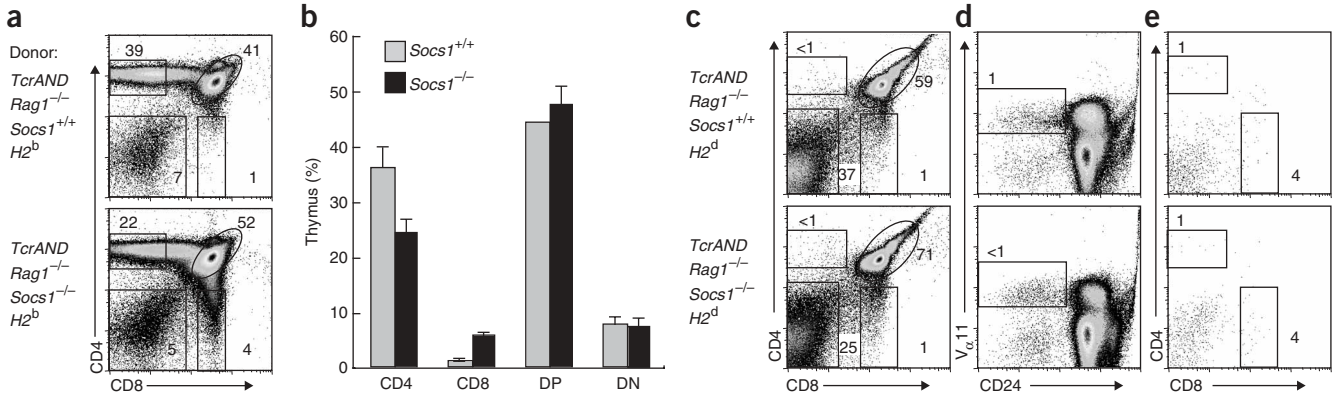

Figure 3 *TcrAND Socs1*$^{-/-}$ CD8$^+$ T cells are selected on MHC class II. (**a**,**b**) *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ bone marrow cells were collected and transferred into lethally irradiated β_2 microglobulin–deficient CD90.1 hosts. At 4–6 weeks after transfer, thymocytes were analyzed for chimerisim (CD90.1 host or CD90.2 donor) and the percentage of donor thymic subsets was defined by CD4 and CD8 expression. (**a**) Flow cytometry. (**b**) Mean values with the standard deviation for the triplicate recipients for one representative experiment. Data are representative of two independent experiments with a total of six recipients for each donor genotype. (**c**–**e**) Total thymocytes (**c**,**d**) or mature T cells (**e**) from *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$*H2*^{d} or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$*H2*^{d} mice were stained with monoclonal antibodies to CD24, $V_\alpha11$, CD8 and CD4 and were analyzed by flow cytometry as described in **Figure 2**. Numbers beside outlined areas (**a**,**c**–**e**) indicate percentage of cells in that area. Data are a representative pair of mice from three experiments.

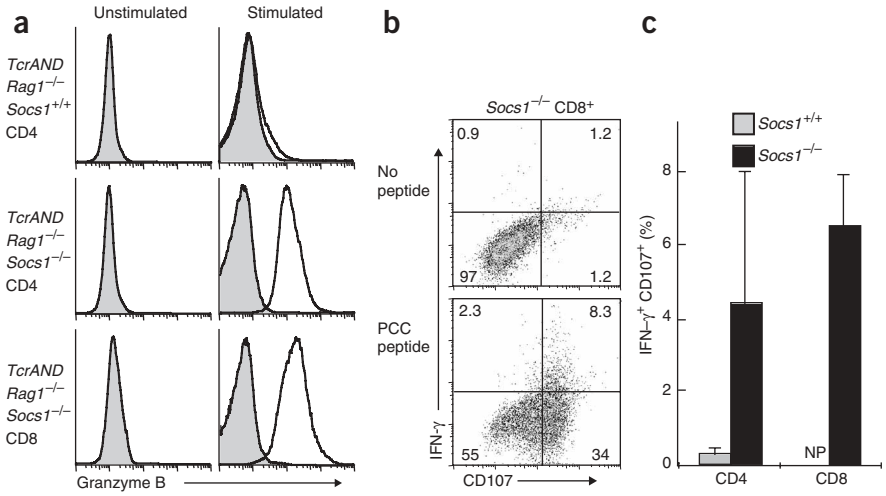

Figure 4 CD8$^+$ SP *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ thymocytes acquire CD8$^+$ T cell effector functions. Thymocytes were activated for 3 d with PCC peptide and T cell–depleted B10.A splenocyte samples. (**a**) Unstimulated or stimulated cells were then stained with isotype control (filled histograms) or antibody to granzyme B (open histograms). Data are one representative experiment of three. (**b**,**c**) Cells activated for 3 d were restimulated for 2 h with peptide and T cell–depleted splenocyte samples in the presence of antibodies to CD107a and CD107b. (**b**) Cells were then collected and stained for IFN-γ production after 6 h. Numbers in quadrants indicate percentage of cells in that area. (**c**) Average frequency of thymocytes staining with both IFN-γ and CD107 from four mice in three experiments (with s.d.). CD8$^+$ SP *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ cells are not present (NP).

wild-type or *Socs1*$^{-/-}$ thymocytes with T cell–depleted splenocyte samples and PCC peptide for 3 d. We stained naive and activated cells for intracellular granzyme B expression and analyzed the cells by flow cytometry. As expected, activated CD4$^+$ SP thymocytes from *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ mice expressed no detectable granzyme B. In contrast, the *Socs1*$^{-/-}$ CD8$^+$ SP and CD4$^+$ SP thymocytes were both uniformly positive for granzyme B after activation (**Fig. 4a**). The induction was at least as high as that measured for ovalbumin-specific effector CD8$^+$ T cells (data not shown).

Exocytosis of cytolytic granules, which correlates with cytolytic activity, exposes CD107a and CD107b (also called LAMP-1 and LAMP-2, respectively), and this can be demonstrated by flow cytometry[23]. To further characterize the phenotype of CD8$^+$ SP T cells in *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice, we stimulated thymocytes with PCC and T cell–depleted splenocyte samples in the presence of antibodies to CD107a and CD107b and then stained the cells for intracellular IFN-γ (representative data for *Socs1*$^{-/-}$ CD8$^+$ SP cells, **Fig. 4b**). Over the period of induction, a substantial proportion of either CD4$^+$ SP or CD8$^+$ SP thymocytes from *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ expressed both CD107 and IFN-γ, whereas almost none of the CD4$^+$ SP thymocytes from *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ mice showed this characteristic (**Fig. 4c**). *TcrAND Socs1*$^{-/-}$ thymocytes did not spontaneously produce IFN-γ or expose surface CD107a and CD107b in the absence of antigen (data not shown). Thus, the absence of Socs1 results in the differentiation of MHC class II–specific CD4$^+$ and CD8$^+$ T cells that acquire mature CD8$^+$ T cell effector functions.

Negative selection of *Socs1*$^{-/-}$ T cells

Because the block in positive selection of *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ T cells and selection of the CD8$^+$ T cell fate are consistent with a partial block in TCR signaling, we considered the possibility that negative selection might also be altered. *TcrAND* transgenic thymocytes are negatively selected at the transition from double-positive to SP in the presence of H-2A^s, and this accurately reflects the timing and anatomy of the deletion of self-reactive thymocytes[24,25]. To determine whether Socs1 is required for this process, we reconstituted irradiated, 'allelically marked' *H2*$^{b/s}$ mice with *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ bone marrow and analyzed them 4 weeks later. In these mice, the *TcrAND* thymocytes were positively selected by H-2A^b and were negatively selected by H-2A^s. Consistent with published results[25], the proportion of CD4$^+$ SP cells was substantially reduced by the inclusion of *H2A*^{s} (**Fig. 2a**, top, versus **Fig. 5a**, top). Moreover, as assessed by the proportion of $V_\alpha11^{hi}CD24^{lo}$ cells, there were almost no mature thymocytes present in these mice (**Fig. 2b**, top, versus **Fig. 5b**, top). From the small and indistinct population of cells in this electronic gate, there was a continuum of CD4 and CD8 expression (**Fig. 5c**, top). This result perhaps reflects a small number of immature or atypical T cells, but in any case, the presence of *H2A*^{s} caused potent

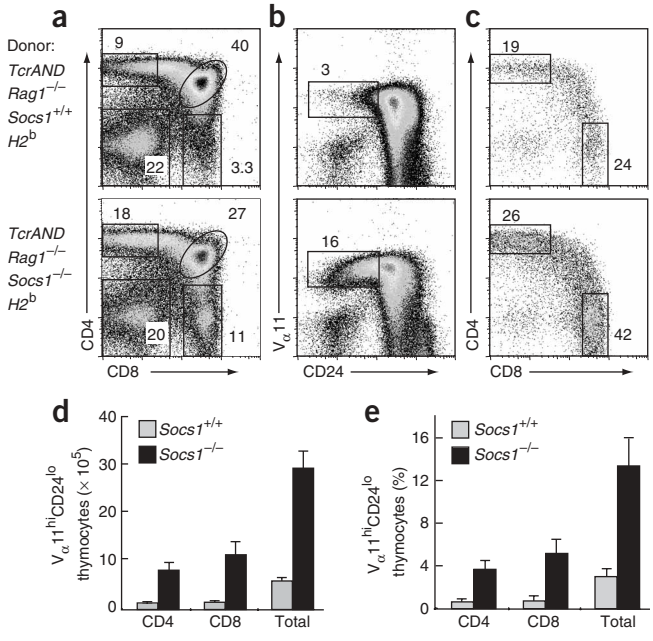

Figure 5 Negative selection of *Socs1*$^{-/-}$ thymocytes is defective. T cell–depleted *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$*H2*^{b} or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ *H2*^{b} bone marrow was collected and was used to reconstitute lethally irradiated *H2*$^{b/s}$ hosts. Cells were stained with monoclonal antibodies to CD90.1 (host), CD90.2 (donor), CD24, $V_\alpha11$, CD8 and CD4 and were analyzed by flow cytometry for chimerism and T cell development. (**a**–**c**) Representative profiles of total donor thymus (**a**,**b**) or mature donor thymocytes (**c**) as described in **Figure 2**. (**d**) Mean cell number ± s.d. ($n = 3$) of the $V_\alpha11^{hi}CD24^{lo}$, mature T cell population for each genotype. (**e**) Mean frequency ± s.d. ($n = 3$) of the $V_\alpha11^{hi}CD24^{lo}$, mature T cell population for each genotype. The experiment was done three times with similar results.

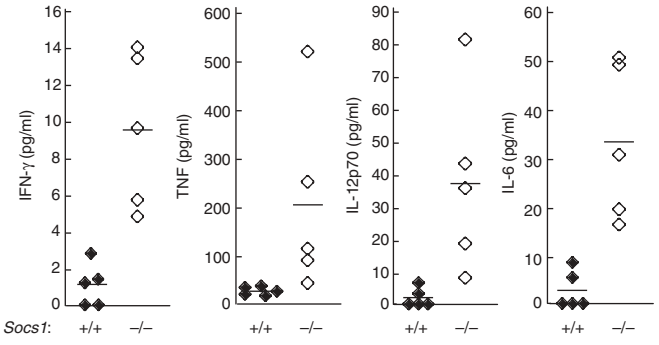

Figure 6 Inflammatory cytokines are increased in *TcrAND Rag1*$^{-/-}$ *Socs1*$^{-/-}$ serum. Serum samples were analyzed by inflammatory cytokine bead array for IFN-γ, TNF, the p70 subunit of IL-12 (IL-12p70) and IL-6 in *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/+}$ or *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice. Each data point is an individual mouse; horizontal bars indicate the average of five mice. Filled symbols, *Socs1*$^{+/+}$; open symbols, *Socs1*$^{-/-}$.

negative selection. In recipients of bone marrow from *TcrAND Rag1*$^{-/-}$ *Socs1*$^{-/-}$ mice, this loss of mature T cells was substantially 'rescued' (**Fig. 5a–c**). As assessed by the total number (**Fig. 5d**) or proportion (**Fig. 5e**) of cells, the absence of Socs1 considerably diminished the process of negative selection. We obtained similar data with mice bred to be *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$*H2*$^{b/s}$ (data not shown).

IFN-γ requirements for *Socs1*$^{-/-}$ developmental defects

To assess the production of proinflammatory cytokines in *Socs1*$^{-/-}$ mice, we measured the concentrations of IFN-γ, tumor necrosis factor (TNF), IL-12 and IL-6 in the serum of *TcrAND Rag1*$^{-/-}$ mice with or without *Socs1*. Whereas the serum cytokine concentrations in *Socs1*$^{+/+}$ mice were near background, *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice had high and variable concentrations of all four cytokines (**Fig. 6**). Thus, even *Socs1*$^{-/-}$ mice with 'monospecific', non-autoreactive T cells produce inflammatory cytokines.

Because the chronic exposure to inflammatory cytokines such as IFN-γ and TNF can depress TCR-mediated signaling[26], we assessed whether IFN-γ was required for the changes in T cell development. To determine the contribution of IFN-γ to the thymic phenotype, we generated *TcrAND Ifng*$^{-/-}$*Socs1*$^{-/-}$ mice. These mice completely lost the *TcrAND* CD8$^+$ SP cells present in *TcrAND Ifng*$^{+/+}$*Socs1*$^{-/-}$ mice (**Fig. 7a–c**). The defect in positive selection was lost, as determined by the production of CD4$^+$ SP and double-positive cells (**Fig. 7d**). This correlated with a reduction in the concentrations of all cytokines to near background. TNF was increased in *TcrAND Ifng*$^{-/-}$*Socs1*$^{-/-}$ mice (32 ± 7.3 pg/ml, $n = 5$) compared with that of *Socs1*$^{+/+}$ controls (19 ± 4.3 pg/ml, $n = 4$), and IL-6 was slightly increased as well (6.9 ± 5.5 pg/ml, $n = 5$, versus 2.8 ± 4.9 pg/ml, $n = 4$).

If systemic inflammation affects the development of Socs1-deficient thymocytes, it might be predicted that neonates would have less inflammation and no defects in T cell development. Thymi from mice 5 d postpartum had decreased numbers of total thymocytes and mature CD4$^+$ cells, but no appearance of mature CD8$^+$ T cells. These mice had slight increases in serum TNF and IFN-γ concentrations, reinforcing the hypothesis that the alteration in lineage commitment results from systemic inflammation (data not shown). Thus, *Socs1*$^{-/-}$ T cell developmental defects are highly dependent on IFN-γ but may also reflect the contribution of other cytokines such as TNF.

DISCUSSION

The data presented here build on studies showing that Socs1 is profoundly important for many aspects of immune homeostasis[6]. In this study we have shown that Socs1 deficiency dysregulates thymic selection. Compared with *TcrAND Rag1*$^{-/-}$ control mice, Socs1-deficient mice had reduced numbers of mature thymocytes and a fate determination that was not in accord with the specificity of the TCR. *TcrANDRag1*$^{-/-}$ thymocytes normally differentiate to become CD4$^+$ T cells with almost complete fidelity, yet in the absence of Socs1 there was a substantial population that assumed a CD8 phenotype. This abnormal differentiation was further shown to require MHC class II recognition but not MHC class I recognition. Moreover, by the criterion of granzyme b expression in activated cells, even the resulting CD4$^+$ T cells seem to have an incompletely resolved phenotype. A caveat to this observation is the possibility that these thymocytes are differentiated T helper type 1 cells. Activated human T helper type 1 cell lines have been shown by gene array experiments to express more granzyme b mRNA than do T helper type 2 cells[27]. We maintain that CD4$^+$ SP thymocytes from *Socs1*$^{-/-}$ cells are unlikely to be T helper type 1 cells, because after 3 d of antigen-mediated stimulation, less than 10% were able to produce IFN-γ. In contrast, all of the CD4$^+$ thymocytes were positive for granzyme b. This inappropriate expression of a CD8 effector–specific gene may contribute to the disease progression in *Socs1*$^{-/-}$ mice.

We identified a second defect in Socs1-deficient mice in the process of thymocyte negative selection. One study reported no effect of Socs1 in the streptococcal enterotoxin B model of negative selection in mice conditionally deficient in Socs1 (ref. 8). However, we found negative selection in the *TcrAND* H-2A$^{b/s}$ model was compromised by loss of *Socs1*. A likely explanation is that streptococcal enterotoxin B is a stronger stimulus of negative selection. Whereas streptococcal

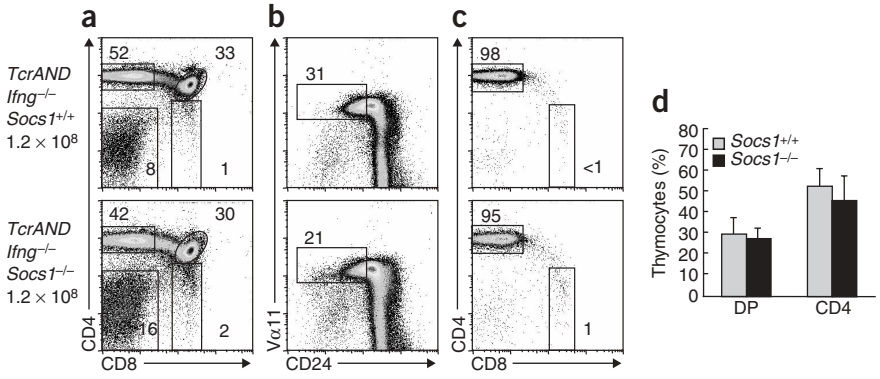

Figure 7 IFN-γ is an important mediator of the *Socs1*$^{-/-}$ T cell developmental defects. (**a–c**) Whole thymi (**a**,**b**) or mature T cells (**c**) from *TcrAND Ifng*$^{-/-}$*Socs1*$^{+/+}$ or *TcrAND Ifng*$^{-/-}$*Socs1*$^{-/-}$ mice were stained for CD24, $V_\alpha11$, CD8 and CD4 and were gated on $V_\alpha11^{hi}$CD24^{lo}. Numbers beside outlined areas indicate percentage of cells in that area. (**d**) Frequency of double-positive (DP) and CD4$^+$ populations, with s.d. Data are representative of four *Socs1*$^{+/+}$ and five *Socs1*$^{-/-}$ mice.

enterotoxin B elicits both proliferation and cytokine production of mature T cells, antigen-presenting cells bearing H-2A$^{b/s}$ were incapable of stimulating naive *TcrAND* T cells to divide (data not shown). The failure of negative selection in *Socs1*$^{-/-}$ mice suggests that autoimmunity may be an inciting factor in the disease of *Socs1*$^{-/-}$ mice.

Socs1 deficiency manifests compound effects. Socs1-deficient T cells themselves produce or stimulate the high production of inflammatory cytokines, including IFN-γ. At the same time, Socs1-deficient thymocytes are hypersensitive to cytokine stimulation[8]. Is one of these effects responsible for changes in T cell development or does one effect 'multiply' the other? The presence of chronic inflammation is not itself sufficient to alter T cell development, as motheaten mice, which are deficient in the tyrosine phosphatase SHP-1, also show evidence of chronic inflammation, and yet they do not show the changes in T cell development noted for Socs1-deficient mice[28,29]. Conversely, thymocytes from Socs1-deficient mice, lacking circulating cytokines, do not demonstrate lineage mis-specification. Neither *TcrAND Ifng*$^{-/-}$*Socs1*$^{-/-}$ nor neonatal *TcrAND Rag1*$^{-/-}$*Socs1*$^{-/-}$ mice produce mature CD8$^+$ SP thymocytes. We conclude that Socs1 is essential to insulate the ongoing process of thymic development from episodic inflammatory cytokine exposure that would otherwise cause the emergence of mis-specified or overtly autoreactive T cells.

Deficiency in Socs1 might also be expected to dysregulate the cytokine signaling that occurs during development, and a primary candidate for this is IL-7. Indeed, Socs1 negatively regulates CD8 development in an IL-7-dependent way[8], and OT-I TCR–transgenic *Socs1*$^{-/-}$ mice have increased numbers of CD8$^+$ T cells[18]. This is consistent with an *in vitro* model of T cell development in which cytokines are required for the completion of CD8$^+$ thymocyte differentiation[19,30]. In this model, positive selection uniformly results in an initial downmodulation of CD8 from the cell surface and, lacking a continued signal through CD8, MHC class I–specific thymocytes require cytokines such as IL-7, IL-4 or IFN-γ to complete maturation. MHC class II–specific cells mature in response to continued TCR- and CD4-mediated signals[19,30]. Although such a cell-intrinsic hypersensitivity to cytokine signaling could be a contributing factor to the phenotypes noted here, for the reasons stated above, we do not favor it as a primary explanation for the alteration in lineage commitment or the loss of negative selection.

The second model proposed to explain CD4 and CD8 lineage commitment has been called the 'strength of signal' model. The proposal for this model is that the strength of the signal emanating from the TCR determines the propensity to assume either a CD4 ('strong') or CD8 ('weak') phenotype[31–35]. Concordant with an alteration in lineage commitment, the absence of Socs1 caused a partial loss of positive and negative selection, and superficially this could be seen as evidence that Socs1 is a positive regulator of thymocyte signaling. However, Socs1 may directly inhibit TCR-mediated signaling in nonlymphoid transfected cells[36], and chronic stimulation of T cells by proinflammatory cytokines results in decreased responsiveness to TCR ligation[37–39]. The simplest explanation is that the loss of Socs1 mainly affects T cell signaling indirectly through the attenuating effects of inflammatory cytokines. Although there may be a cell-intrinsic effect of Socs1 on T cell development, it may not be sufficient to alter lineage commitment. However, we also do not favor the idea that simple signaling attenuation is responsible for the changes noted in T cell development. Instead, it is possible that hypersensitive thymocytes stimulated by cytokines have an altered signaling network that responds abnormally to TCR-mediated signals. This signaling aberration is not universal, as we found no changes in two of the essential signaling pathways important for positive selection; neither the TCR-mediated release of free calcium nor the phosphorylation of the kinases Erk1 and Erk2 seemed to be altered in the double-positive thymocytes of *Socs1*$^{-/-}$ mice (data not shown).

Socs1 apparently has multiple functions in the immune system. It is a primary mediator of negative feedback control in IFN-γ-mediated cellular activation. It is induced in response to IFN-γ stimulation[1], and IFN-γ stimulated macrophages[5], hepatocytes[40] and dendritic cells[12] from *Socs1*$^{-/-}$ mice show a high and persistent state of activation. In contrast, Socs1 is constitutively expressed in developing thymocytes[4] and apparently functions not as negative feedback but as a means of dampening the effects of cytokine stimulation in otherwise cytokine-responsive cells. What is the advantage of expressing all the components required for cytokine responsiveness, only to block the response with a constitutive inhibitor? One possibility is that positive selection and lineage commitment are physiologically responsive to cytokine stimulation, but the natural process only tolerates a weak signal. This response might be a part of a paracrine cytokine pathway that guides thymic development, but may also reflect a natural feedback mechanism for the regulated production of T cells. Just as inflammatory responses mobilize bone marrow neutrophils as a means of augmenting an immune response, perhaps the rate or outcome of thymic maturation is responsive to the onset of a viral infection. Socs1 could function to modulate this activity, allowing subtle changes in the number of cytotoxic T cells produced, but not allowing inflammatory cytokines to severely distort the process. Whether this is a physiological component of thymus development, *Socs1*$^{-/-}$ mice afford a means of looking at the global changes in gene expression associated with aberrant lineage commitment.

METHODS

Mice. *Socs1*$^{+/-}$ mice on a mixed F1 C57BL/6:129 background were obtained from J. Ihle (St. Jude Children's Research Hospital, Memphis, Tennessee). The mice used in these studies were backcrossed (N5) to C57BL/6 (Jackson Labs). *Socs1*$^{+/-}$ mice were bred with *Tg(TcrAND)53Hed Rag1*$^{-/-}$ mice, which carry the *AND* TCR transgene (*TcrAND*); the resulting *TcrAND Rag1*$^{+/-}$*Socs1*$^{+/-}$ mice were bred to generate *TcrAND Rag1*$^{-/-}$*Socs1*$^{+/-}$ mice. The presence of the *TcrAND* transgene was determined by PCR. Genotyping for *Socs1* was done by PCR with three primers: P3 (5′-GCCTCCTTGACGAGTTCTTCTG-3′), P4 (5′-GCTAGCTAGTCCTAGCCTGTGGG-3′) and P5 (5′-ACATTGTCAGCCAGG CACCCACTCC-3′). Amplification with P3 and P5 produced a 185–base pair band indicating a mutant allele; P4 and P5 produced a band of 427 base pairs indicating a wild-type allele. Reactions were done with 10% glycerol. *Ifng*$^{-/-}$, C57BL/6J-Thy-1.1, B10.S(9R) and B10.A mice were from Jackson Labs. Mice with homozygous deficiency in the gene encoding β_2-microglobulin and in *H2-Ab1* were from C. Surh (The Scripps Research Institute, La Jolla, California) or Jackson Labs[22,41]. Mice were housed in specific pathogen–free vivaria. Experiments used mice 3–6 weeks of age. All mice were treated in accordance with institutional guidelines and protocols approved by the University of California San Diego Institutional Animal Care and Use Committee.

Flow cytometry. Antibodies to CD4, CD24, CD69, CD90.1, CD90.2, TCRβ and IFN-γ were from Ebiosciences. Antibodies to V$_\alpha$11, CD8, H-2D^b, H-2A^b, CD107a and CD107b were from BD Pharmingen. Antibody to granzyme B was from Caltag. The inflammatory cytokine bead array was from BD Pharmingen. Intracellular staining was done with Cytofix/cytoperm from BD Pharmingen. Samples were analyzed on a Becton Dickenson FACSCalibur.

Bone marrow chimeras. Mice were irradiated at the Moores UCSD Cancer Center. A lethal dose of 1,000 rads was administered. Bone marrow samples were depleted of T cells and equal numbers were transferred into three recipient mice for each genotype. The mice then received bone marrow by intravenous injection through the tail vein. At 4 weeks after transfer, mice were assessed for chimerism by expression of CD90.1 and CD90.2; chimerism was more than 95% in all mice. Data are presented as the mean and s.d. of the three mice per group.

Thymocyte activation. Thymocytes were collected and placed into culture with T cell–depleted B10A splenocyte samples at a ratio of 1:1 with 30 μM PCC peptide (Genemed Synthesis). Cells were stained for $V_{\alpha}11$, CD4, CD8 and intracellular granzyme B either immediately *ex vivo* or after 3 d of activation. Expression of granzyme B was determined by flow cytometry. Alternatively, cells were restimulated with antigen-presenting cells with or without peptide, first for 2 h in the presence of antibodies to CD107a and CD107b. At 2 h, brefeldin A and monensin were added to inhibit cytokine secretion. After 6 h of culture, cells were collected and stained for CD4 and CD8 followed by intracellular staining for IFN-γ or an isotype control.

Negative selection. B6.PL mice were bred with B10.S(9R) mice. The resulting mice are H-$2^{b/s}$, and were allelically distinguishable from the donor mice: CD90.1^{+} versus CD90.1^{-}, respectively. Irradiation and reconstitution were done as described above.

Data analysis. The Microsoft Excel program was used to calculate means and standard deviations.

ACKNOWLEDGMENTS

We thank V. Lieuw for help with strain construction; and M. McGargill for discussions. Supported by National Institutes of Health (AI21372-20 and AI37988-09 to S.M.H. and Hematology Training Grant 5T32DK07233 to I.M.C.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 23 November 2004; accepted 14 April 2005
Published online at http://www.nature.com/natureimmunology/

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