Suppressor of Cytokine Signaling-1 Has IFN-γ-Independent Actions in T Cell Homeostasis


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Suppressor of Cytokine Signaling-1 Has IFN-γ-Independent Actions in T Cell Homeostasis


Suppressor of cytokine signaling (SOCS)-1 is a member of a family of proteins that negatively regulate cytokine signaling pathways. We have previously established that SOCS-1 is a key regulator of IFN-γ signaling and that IFN-γ is responsible for the complex inflammatory disease that leads to the death of SOCS-1-deficient mice. In this study, we provide evidence that SOCS-1 is also a critical regulator of IFN-γ-independent immunoregulatory factors. Mice lacking both SOCS-1 and IFN-γ, although outwardly healthy, have clear abnormalities in their immune system, including a reduced ratio of CD4:CD8 T cells in lymphoid tissues and increased expression of T cell activation markers. To examine the contribution of TCR Ag specificity to these immune defects, we have generated two lines of SOCS-1-deficient mice expressing a transgenic TCR specific for an exogenous Ag, OVA (OT-I and OT-II). Although TCR transgenic SOCS-1−/− mice have a longer lifespan than nontransgenic SOCS-1−/− mice, they still die as young adults with inflammatory disease and the TCR transgenic SOCS-1−/− T cells appear activated despite the absence of OVA. This suggests that both Ag-dependent and -independent mechanisms contribute to the disease in SOCS-1-deficient mice. Thus, SOCS-1 is a critical regulator of T cell activation and homeostasis, and its influence extends beyond regulating IFN-γ signaling.


Cytokines control many biological processes including the formation and differentiation of lymphocytes in the immune system. The importance of tight regulation of cytokine action is highlighted by profound perturbations in immune homeostasis seen in mice in which levels of cytokines have been experimentally altered. For example, mice lacking the common γ-chain, a shared component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, have severe combined immunodeficiency (1, 2). IL-6 transgenic mice develop massive plasmacytosis in several organs (3), and mice transgenic for IL-4 exhibit an allergic inflammatory phenotype and abnormal T cell maturation in the thymus (4). Suppressor of cytokine signaling (SOCS)-1 is a key regulator of cytokine signal transduction (5–7). SOCS-1 appears to limit responses to cytokines by inhibiting the activity of Janus kinases (6, 7). Although studies in vitro suggest that SOCS-1 regulates signaling in response to a broad spectrum of cytokines, including IFNα, IFNβ, IFN-γ, IL-2, IL-4, IL-6, IL-7, and TNF (5, 8–13), analyses of SOCS-1-deficient mice have suggested a more specific role in vivo. SOCS-1-deficient mice die before weaning from a complex inflammatory disease characterized by fatty degeneration of the liver and macrophage infiltrates in the lung, pancreas, heart, and skin (14, 15). We have established previously that IFN-γ is a critical mediator of this disease, because mice that are deficient for both SOCS-1 and IFN-γ survive to adulthood and appear in good health (16). Furthermore, lymphocytes have been shown to be necessary for disease progression as mice lacking both SOCS-1 and recombination activating gene-2 (RAG-2) are healthy (17). T lymphocytes, a major source of IFN-γ, appear abnormally activated in SOCS-1−/− mice, and this may contribute to the increased production of IFN-γ that is typical of these mice (17). However, mice lacking both SOCS-1 and IFN-γ eventually develop a variety of inflammatory conditions in later life and have a reduced lifespan, suggesting that SOCS-1 has additional functions in vivo that are independent of IFN-γ (18).

Recent studies have suggested a role for SOCS-1 in T cell development and function. SOCS-1 is strongly expressed in the thymus and to a lesser extent in the spleen (14, 17). SOCS-1 expression is induced in lymphocytes activated in vitro by ligation of the TCR (19). Constitutive expression of SOCS-1 has been shown to inhibit TCR signaling in a reconstituted cell-based system (20). In SOCS-1 transgenic mice, where SOCS-1 expression is driven by a T cell-specific promoter, T cell homeostasis and lymphocyte development are altered (21). The proportion of CD4+ T cells is increased in these mice, with spontaneous activation and apoptosis of T cells evident in the periphery. In addition, overexpression of SOCS-1 in fetal liver-derived hemopoietic progenitors blocks their differentiation during early T cell development (10). Together these data suggest that SOCS-1 may play an important role in T cell regulation in vivo.

To address whether the perturbed T cell homeostasis and T cell activation in SOCS-1−/− mice occurs independently of IFN-γ, or...
whether it is a consequence of the profound disease in these mice, we have analyzed the lymphoid phenotype of mice lacking both SOCS-1 and IFN-γ. T cells from SOCS-1−/− IFN-γ−/− mice appeared activated and T cell subsets were perturbed with a significantly reduced CD4:CD8 ratio. Furthermore, this apparent T cell activation occurred in the absence of reactivity to specific Ag, because it was also a feature of SOCS-1−/− mice expressing a transgenic TCR. TCR transgenic SOCS-1−/− mice have a longer lifespan than SOCS-1−/− mice, indicating that Ag-specific TCR responses contribute to the SOCS-1−/− disease. We conclude that SOCS-1 is a key regulator of T cell homeostasis and activation, and that SOCS-1 regulates other IFN-γ-independent immunoregulatory factors.

Materials and Methods

Generation and maintenance of mice

SOCS-1−/− and SOCS-1−/− IFN-γ−/− mice were generated as described previously on a mixed 129/Sv and C57BL/6 genetic background (14, 16). IFN-γ−/− mice on an inbred C57BL/6 background (C57BL/6-IFN-γ−/−) were obtained from the Jackson Laboratory (Bar Harbor, ME) via Monash University (Clayton, Victoria, Australia) (22). For studies where mice needed to be on a syngeneic background, SOCS-1−/− mice backcrossed at least 10 generations to C57BL/6 mice were mated with IFN-γ−/− mice to produce mice deficient for both SOCS-1 and IFN-γ. These mice had an identical phenotype to SOCS-1−/− IFN-γ−/− mice generated on a mixed 129/Sv and C57BL/6 genetic background, as described previously (16). The OVA-specific TCR transgenic lines OT-I and OT-II were generated as described (23, 24) and backcrossed to C57BL/6 for at least six generations. RAG-1−/− mice were generated as previously described (25) and kept in microisolators. Mice were routinely housed in clean conventional facilities at The Walter and Eliza Hall Institute (Parkville, Victoria, Australia). To raise mice under germ-free conditions, pups were delivered by Caesarean section and placed with BALB/c foster mothers that had been maintained in germ-free microisolators for several generations. The sterility of this environment was monitored closely with regular testing to confirm the absence of bacterial organisms and a variety of viral pathogens.

Mice were genotyped for SOCS-1 and IFN-γ genes by Southern blot analysis of genomic DNA obtained from tail tips as described (16). OT-I and OT-II TCR transgenic mice and RAG-1−/− mice were identified by flow cytometry of peripheral blood lymphocytes stained with either anti-CD8 and anti-Vα2, anti-CD4 and anti-Vα2 or anti-Thy1 and anti-B220 Abs, respectively.

Flow cytometry

Single-cell suspensions of thymocytes, lymph nodes (inguinal, brachial, axillary, submandibular, and mesenteric), peripheral blood, and splenoocytes were prepared and erythrocytes lysed by incubation in 156 mM ammonium chloride (pH 7.3) at 37°C for 1 min. The cells were stained with mAbs specific for the cell surface markers of interest (CD3, CD4, CD8, CD44; BD PharMingen, San Diego, CA) conjugated to either biotin, FITC, PE, or allophycocyanin followed where necessary by streptavidin-PE or avidin-Texas Red (BD PharMingen) and analyzed by FACS. Dead cells were excluded based on propidium iodide (PI) staining (Sigma-Aldrich, St. Louis, MO). Three-color flow cytometry was performed on a FACSscan (BD Biosciences, Mountain View, CA) using Lysis II or CellQuest software. Lymphocytes were gated based on forward scatter (FSC) and PI profiles. For each analysis, a total of 25–150 × 10⁶ live cells were collected. Analysis was conducted using WEASEL software (F. Battye, The Walter and Eliza Hall Institute of Medical Research).

Adoptive transfer of thymic progenitor T lymphocytes

Thymic T cell progenitors were isolated from SOCS-1−/− and littermate mice (10–14 days of age). Thymocytes were depleted of mature T cells and non-T cell populations by incubation with rat Abs specific for CD3, CD8, B220, Mac1, and Gr-1, followed by removal of Ab-bound cells using Dynabeads conjugated with sheep anti-rat IgG (Dynal Biotech, Oslo, Norway). This depletion was performed twice and the efficiency of depletion was monitored by FACS analysis of the resulting depleted population. Progenitor T cells (1–4 × 10⁵) and syngeneic bone marrow cells (1 × 10⁶) were injected i.v. into gamma-irradiated (11.0 Gy) 8-wk-old wild-type recipient mice. Reconstituted recipients were sacrificed and analyzed by FACS and histology when moribund or at 2 mo postrepopulation. Repopulation efficiency of the spleen varied from 2–10% between experiments; however, no consistent difference in the efficiency was observed between genotypes.

Cell proliferation analysis

Cell proliferation was analyzed by labeling proliferating cells in vivo with the thymidine analog 5-bromo-2′-deoxyuridine (BrDU; Sigma-Aldrich), which was provided continuously for 1 or 3 days in drinking water (0.8 mg/ml plus 2% (w/v) glucose to overcome taste aversion) after an initial i.p. injection of 800 μg in saline. Drinking water bottles were shielded from light and exchanged after 3 days. BrDU incorporation into cellular DNA was detected by staining with a FITC-labeled mAb specific for BrDU (BD PharMingen). Cells were fixed and permeabilized according to manufacturer’s protocols using the BrDU Flow kit (BD PharMingen).

CFSE labeling of T cells

CFSE labeling was performed as previously described (26). Isolated lymph node T cells from either SOCS-1−/− IFN-γ−/− or IFN-γ−/− mice were resuspended in PBS containing 0.1% (w/v) BSA (Sigma-Aldrich) and 5 μM CFSE ( Molecular Probes, Eugene, OR) at 1 × 10⁶ cells/ml for 10 min at 37°C. Cells were then washed and 2 × 10⁶ CFSE-labeled T cells were injected i.v. into either SOCS-1−/− IFN-γ−/− or IFN-γ−/− mice. Six days after injection the mice were sacrificed and 2.5 × 10⁶ CFSE-labeled cells from spleen and lymph node were analyzed by FACS.

Fetal thymic organ culture (FTOC)

Fetal thymic lobes were obtained at embryonic day 15 from plug-timed pregnant SOCS-1−/− IFN-γ−/− or IFN-γ−/− mice. FTOC was performed in culture media consisting of 15 mM HEPES-buffered RPMI, 10% (v/v) FCS, and 50 μM 2-ME. Cultures were placed on filters placed on media-soaked Gelfoam in groups of two per well of a 24-well plate for 12 days, with a media change every 6 days of culture as described previously (27). At the end of culture, thymocytes were harvested from lobes, counted, and analyzed by FACS.

Cell survival analysis

Lymph node cells were stained with the mAb CD44, CD4, and CD8 as described above and T cell subsets were isolated after sorting by FACS. Cells were plated into 96-well plates at 5 × 10⁵ cells/well in RPMI supplemented with 10% (v/v) heat-inactivated FCS and 50 μM 2-ME but no additional cytokines. At various time periods afterward cells were harvested, stained with PI, and the percentage of live cells determined by FACS.

Statistical analyses

Statistical analyses were performed using ANOVA tables with Bonferroni adjustments for multiple testing.

Results

Lymphoid abnormalities in SOCS-1−/− IFN-γ−/− mice

Lymphoid compartments of healthy SOCS-1−/− IFN-γ−/− mice were compared with those of control IFN-γ−/− mice to determine whether T cell homeostasis and activation was altered in SOCS-1−/− mice independently of IFN-γ and disease. SOCS-1−/− IFN-γ−/− mice were shown to have enlarged lymph nodes, which was reflected by an increase in the total number of lymph node cells (Table I). The spleen was also slightly larger, although the total number of lymphocytes present was decreased (Table I). There was no significant change in thymic cellularity or size (Table I).

T lymphocyte subsets in the thymus, spleen, lymph node, and peripheral blood of age- and sex-matched mice were examined by FACS. The ratio of mature (CD3+CD4−CD8−) single positive (SP) CD4:CD8 cells in the thymus was decreased in SOCS-1−/− IFN-γ−/− mice, although no overall difference was observed in the ratio of total SP CD4:CD8 cells. This difference was also seen in peripheral lymphoid organs (Table I and Fig. 1, A and B). The ratio of CD4:CD8 T cells was decreased by 30–35% in the spleen, lymph node, and peripheral blood (Table I). Interestingly, the total number of both CD4+ and CD8+ T cells from the thymus and lymph node was increased in SOCS-1−/− IFN-γ−/− mice while splenic numbers were slightly decreased (Table I). However, the total pool of mature T cells, combining thymic, lymph node, and splenic CD4+...
and CD8<sup>+</sup> T cells appeared to be increased in SOCS-1<sup>−/−</sup> IFN-γ<sup>−/−</sup> mice (188 ± 67 × 10<sup>6</sup> T cells) compared with IFN-γ<sup>−/−</sup> mice (126 ± 44 × 10<sup>6</sup> T cells; n = 6). Although not all T cells are accounted for in this analysis (T cells in the blood and other tissues were not included), the increased T cell numbers in SOCS-1<sup>−/−</sup> IFN-γ<sup>−/−</sup> mice suggests a general role for SOCS-1 in T cell homeostasis influencing overall T cell numbers, as well as maintaining the balance of CD4<sup>+</sup> to CD8<sup>+</sup> T cells. The reduction in ratio of CD4<sup>+</sup>:CD8<sup>+</sup> T cells was consistent in SOCS-1<sup>−/−</sup> IFN-γ<sup>−/−</sup> mice aged from 14 days to 3 mo (data not shown). A similar perturbation of T cell subset was seen in the peripheral lymphoid organs of 10-day-old SOCS-1<sup>−/−</sup> mice, and was more pronounced in the thymus of SOCS-1<sup>−/−</sup> mice relative to SOCS-1<sup>−/−</sup> IFN-γ<sup>−/−</sup> mice (Fig. 1, D and E). As previously described, no difference in the CD4:CD8 ratio between wild-type animals and IFN-γ<sup>−/−</sup> mice was observed (Ref. 22 and Table I). All other lymphoid subsets were present in normal proportions in SOCS-1<sup>−/−</sup> IFN-γ<sup>−/−</sup> mice, including NK and NKT cells from the thymus, lymph node, and spleen (data not shown).

Thymocyte development was investigated in greater detail. No differences were observed in the CD44/CD25 defined subsets of CD4<sup>+</sup> CD8<sup>+</sup> thymocytes (data not shown). However, a decrease was observed in the percentage of CD4<sup>+</sup>CD8<sup>+</sup> (double positive; DP) thymocytes present (87 ± 2% for IFN-γ<sup>−/−</sup> compared with 81 ± 3% for SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup>, n = 5). Interestingly, this reduction was mainly due to a decrease in the DP thymocytes expressing the TCR (4.6 ± 1.8 × 10<sup>7</sup> CD3<sup>+</sup>DP in IFN-γ<sup>−/−</sup> mice compared with 3.3 ± 1.2 × 10<sup>7</sup> CD3<sup>+</sup>DP in SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup>; n = 5) (Fig. 1A). Therefore, although overall numbers of thymocytes were unchanged, DP numbers decreased while SP numbers increased. These differences could reflect enhanced positive or impaired negative selection, or hypersensitivity to immunoregulatory factors impacting at this point of thymocyte development. These possibilities are currently under investigation.

To determine whether the CD4:CD8 imbalance originated in the thymus and reflected increased production of CD8<sup>+</sup> relative to CD4<sup>+</sup> T cells, FTOC were prepared from embryonic day 15 SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup> and control IFN-γ<sup>−/−</sup> embryos. Again, a reduction in the ratio of CD4:CD8 T cells was observed in SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup> samples (Fig. 1C). There was an increase in the number of CD8<sup>+</sup> T cells generated in the FTOC, whereas fewer CD4<sup>+</sup> T cells were generated compared with IFN-γ<sup>−/−</sup> samples. No change in the number of DP T cells or overall thymic cell number was observed (data not shown). A similar decrease in the ratio of CD4:CD8 T cells was observed in FTOC of SOCS-1<sup>−/−</sup> embryos compared with wild-type or SOCS-1<sup>−/−</sup> littermate embryos, with an increase in the number of CD8<sup>+</sup> cells, although the number of CD4<sup>+</sup> cells was unchanged (Fig. 1F and data not shown).

### Table I. Perturbed lymphoid subsets in SOCS-1<sup>−/−</sup> IFN-γ<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Organ</th>
<th>Nos. of Cells (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Weight (mg)</th>
<th>Percentage of CD4&lt;sup&gt;+&lt;/sup&gt; DP SP Cells (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Percentage of CD4&lt;sup&gt;+&lt;/sup&gt; SP Cells (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Percentage of CD8&lt;sup&gt;+&lt;/sup&gt; DP SP Cells (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Percentage of CD8&lt;sup&gt;+&lt;/sup&gt; SP Cells (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Ratio of CD4:CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Thymus</td>
<td>220 ± 40</td>
<td>68 ± 3</td>
<td>60 ± 2</td>
<td>12 ± 2</td>
<td>9 ± 1</td>
<td>2 ± 0</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td>230 ± 90</td>
<td>52 ± 14</td>
<td>65 ± 4</td>
<td>11 ± 4</td>
<td>13 ± 2</td>
<td>3 ± 1</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td>SOCS-1&lt;sup&gt;−/−&lt;/sup&gt;IFN-γ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Spleen</td>
<td>190 ± 70</td>
<td>44 ± 12</td>
<td>63 ± 1</td>
<td>21 ± 6*</td>
<td>23 ± 1*</td>
<td>8 ± 2*</td>
<td>2.8 ± 0.1*</td>
</tr>
<tr>
<td>Wild type</td>
<td>Lymph node</td>
<td>53 ± 16</td>
<td>49 ± 4</td>
<td>45 ± 4</td>
<td>24 ± 7</td>
<td>27 ± 2</td>
<td>14 ± 4</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td>69 ± 26</td>
<td>44 ± 5</td>
<td>40 ± 4</td>
<td>28 ± 10</td>
<td>26 ± 4</td>
<td>18 ± 6</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>SOCS-1&lt;sup&gt;−/−&lt;/sup&gt;IFN-γ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Blood</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td>180 ± 54*</td>
<td>81 ± 10*</td>
<td>33 ± 2*</td>
<td>60 ± 21*</td>
<td>32 ± 3*</td>
<td>58 ± 20*</td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>SOCS-1&lt;sup&gt;−/−&lt;/sup&gt;IFN-γ&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.7 ± 0.2*</td>
</tr>
</tbody>
</table>

* Cells from wild-type, IFN-γ<sup>−/−</sup> and SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup> tissues were analyzed for the total cell number in each organ and expression of CD4 and CD8 by FACS. Thymocytes were gated on CD3<sup>+</sup> cells representing mature T cells for CD4 and CD8 analysis. Data are mean ± SD from at least six age-matched mice.

* ND. Not determined.

* p < 0.007.

A cell intrinsic mechanism drives the altered ratio of CD4:CD8 T cells in SOCS-1<sup>−/−</sup> mice

To determine whether the altered ratio of CD4:CD8 T cells is cell intrinsic or is due to an altered environment in SOCS-1<sup>−/−</sup> mice, such as altered cytokine levels, thymic progenitor T cells from SOCS-1<sup>−/−</sup> or control mice (both Ly5.2<sup>+</sup>) were adoptively transferred to irradiated Ly5.1<sup>+</sup> congenic wild-type recipients, in combination with syngeneic bone marrow. Donor cells were identified from recipient tissue at 2 wk, 1 mo, and 2 mo after reconstitution by staining cells with Abs specific for the donor Ly5 isomorph (Fig. 1G). At all time points measured, a decreased ratio of CD4:CD8 T cells was generated from the donor SOCS-1<sup>−/−</sup> progenitors compared with those cells generated from control wild-type progenitor T cells; however, there was no detectable difference in the ratio of CD4:CD8 T cells in the total cell populations harvested from each lymphoid organ (Fig. 1, G and H). The donor T cells were mature as defined by CD3 expression. Similar changes in the ratio of CD4:CD8 T cells were observed in the spleen and lymph node of lethally irradiated IFN-γ<sup>−/−</sup> mice transplanted with SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup> bone marrow (data not shown). Hence, this perturbation of T cell populations could be generated in the context of a wild-type environment, suggesting that in the absence of SOCS-1, T cells may be either responding abnormally to steady-state cytokine levels or producing altered amounts or types of cytokine themselves.

T cell activation in SOCS-1<sup>−/−</sup> mice occurs in the absence of IFN-γ

Previous studies have shown that T cells from SOCS-1<sup>−/−</sup> mice display features of activated T cells, such as an increase in cell size and expression of the activation markers CD44, CD69, and CD25 (17, 28, 29). These features were also typical of T cells from SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup> mice. An increase in the percentage of cells expressing high levels of CD44 (CD44<sup>hi</sup>) was observed in all lymphoid tissues examined from SOCS-1<sup>−/−</sup>IFN-γ<sup>−/−</sup> mice aged 14 days to 3 mo, compared with IFN-γ<sup>−/−</sup> control mice (Fig. 2, A and B and data not shown). The most profound change was the increase of CD44 expression by peripheral CD8<sup>+</sup> cells (Fig. 2B).
Small increases were seen in the proportion of cells in the periphery expressing CD69 or CD25, early markers of activation (data not shown). In addition, SOCS-1⁻/⁻ IFN-γ⁻/⁻ T cells were substantially larger, with a large shift in FSC, consistent with an activated/memory phenotype (Fig. 2B). This may account for some of the increase in peripheral lymphoid organ weight (Table I). The level of expression of CD3 by SOCS-1⁻/⁻ IFN-γ⁻/⁻ peripheral T cells was decreased relative to IFN-γ⁻/⁺ cells, but the expression of CD62L was unchanged (data not shown). In addition, T cells from SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice expressed only basal levels of the IL-12R β1 subunit, which is highly expressed on activated T cells (30, 31).

FTOCs were performed to examine whether the increase in apparent activation of T cells originated in the thymus. There was no increase in the proportion of CD44⁺ cells in FTOCs from SOCS-1⁻/⁻ IFN-γ⁻/⁻ compared with control IFN-γ⁻/⁺ embryos (Fig. 2C), suggesting that the increase in the proportion of CD44⁺ cells was not intrinsic to the thymus and was dependent on extrathyMIC factors.

In addition to the expression of activation markers, effector T cells exhibit a number of properties such as increased proliferation. T cells from SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice were examined to see if the increases in activation marker expression reflected true effector function. T cell proliferation was measured in vivo via BrdU uptake. BrdU incorporation was marginally greater in the lymph node and spleen but unchanged in the thymus of SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice compared with IFN-γ⁻/⁺ mice, suggesting more active cell division in both the CD4⁺ and CD8⁺ subpopulations in the periphery (Fig. 3A). The difference in proliferation was greater for the CD8⁺ T cells compared with the CD4⁺ T cells, and this may contribute to the decreased CD4⁻/CD8⁻ T cell ratio.

To examine whether this increase in proliferation was intrinsic to the T cells or caused by environmental factors, lymph node T cells were labeled with CFSE and transplanted into recipient mice. As the cells proliferate, intracellular CFSE levels decrease in proportion to the number of cell divisions, and this can be measured by FACS. CD8⁻ cells from SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice were found to proliferate more rapidly than IFN-γ⁻/⁺ cells when transferred into either SOCS-1⁻/⁻ IFN-γ⁻/⁻ or IFN-γ⁻/⁺ recipients (Fig. 3B). To determine whether T cells from IFN-γ⁻/⁺ mice were capable of proliferation, CFSE-labeled lymph node T cells were transplanted into RAG1⁻/⁻ mice to stimulate homeostatic proliferation. In this lymphopenic environment, both the SOCS-1⁻/⁻ IFN-γ⁻/⁻ and IFN-γ⁻/⁺ T cells proliferated; however, T cells from SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice underwent a greater number of divisions than those from IFN-γ⁻/⁺ mice (Fig. 3C).

In addition to proliferation, cell survival is an important factor in activation and T cell homeostasis (reviewed by Ref. 32). Survival of T cells was assessed by annexin V staining of cells taken directly from the mouse (data not shown), or by culturing cells in cytokine-deficient media. No significant differences were observed in the survival of SOCS-1⁻/⁻ IFN-γ⁻/⁻ T cells compared with IFN-γ⁻/⁺ T cells (Fig. 3D). This contrasts with results obtained from SOCS-1⁻/⁻ mice where increased apoptosis occurs, presumably due to the severe disease state of these mice (15).

An increase in CTL activity is another measure of effector function of CD8⁺ T cells. CTL assays were performed by anti-CD3 redirected lysis of lymphocyte-labeled P815 cells from freshly isolated T cells from either SOCS-1⁻/⁻ IFN-γ⁻/⁻ or IFN-γ⁻/⁺ mice. This showed no increase in the CTL activity of T cells from SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice (data not shown).

**FIGURE 1.** The ratio of CD4⁻/CD8⁻ T cells is decreased in SOCS-1⁻/⁻ IFN-γ⁻/⁻ mice. CD³⁻ mature thymocytes and lymph node cells from 70- to 80-day-old IFN-γ⁻/⁺ and SOCS-1⁻/⁻ IFN-γ⁻/⁻ (A and B) and 10-day-old SOCS-1⁻/⁻,⁻/⁻ and SOCS-1⁻/⁻ (D and E) mice were analyzed by FACS for the expression of CD4 and CD8 and the ratio of CD4⁻/CD8⁻ T cells was calculated. *, p < 0.001. Data are representative of at least six age-matched pairs of mice. CD3⁻ T cell subsets generated in FTOC from IFN-γ⁻/⁻ and SOCS-1⁻/⁻ IFN-γ⁻/⁻ embryos (C and SOCS-1⁻/⁻,⁻/⁻ and SOCS-1⁻/⁻ embryos (F). G and H, Lethally irradiated Ly5.1 congenic wild-type mice were repopulated with thymic progenitor T cells from either SOCS-1⁻/⁻ or SOCS-1⁻/⁻ mice and splenocytes from recipient mice analyzed by FACS 2 mo later. G, Donor cells were identified in the spleen by staining with an anti-Ly5.2 Ab. H, Splenocytes stained with Abs specific for CD4 and CD8. Left panel, CD4/CD8 profile of total splenocytes; right panel, CD4/CD8 profile of donor cells (gated for Ly5.2⁻).
Ag-specific TCR activation contributes to the SOCS-1−/− phenotype

T cells have been proposed to be critical mediators of the inflammatory disease caused by loss of SOCS-1−/− (17), and as such, abnormal activation of T cells is likely to drive downstream events. A CD44high phenotype is characteristic of memory and activated T cells that have interacted with specific Ag via the TCR (33), but is also characteristic of cells undergoing homeostatic proliferation that is not dependent on high-affinity Ag interaction (34–36). To determine whether T cell activation in SOCS-1−/− mice occurs in an Ag-independent manner or is driven by specific Ag, we generated TCR-transgenic SOCS-1−/− mice. Because CD8+ cells are commonly activated in SOCS-1−/− IFN-γ−/− mice, we crossed SOCS-1−/− mice with an MHC class I-restricted TCR-transgenic mouse line, OT-I, that expresses an OVA-specific TCR on all CD8+ T cells (23). In the absence of exogenous OVA, all T cells should be naive.

In contrast to CD8+ cells from OT-I SOCS-1−/− controls, which appeared naive (CD44low/low), an increased proportion of CD8+ T cells in OT-I SOCS-1−/− mice were CD44high (Fig. 4, A–D). Similar to SOCS-1−/− IFN-γ−/− mice, this activation was profound in all peripheral lymphoid tissues, and was also seen to a lesser extent in the thymus (Fig. 4, B and D and data not shown). Peripheral CD8+ cells from OT-I SOCS-1−/− mice were slightly larger than those from control OT-I mice, and expressed lower levels of CD3 (Fig. 4E), features typical of activated T cells. Serum IFN-γ measured by ELISA was detectable in 33% of OT-I SOCS-1−/− but only 5% of OT-I SOCS-1+/+ mice, compared with 80% of SOCS-1−/− mice (n ≥ 15 for each genotype; data not shown).

OT-I SOCS-1−/− mice survived longer than nontransgenic SOCS-1−/− mice (mean survival of 27 ± 6.1 days, compared with 12 ± 3.4 days for nontransgenic SOCS-1−/− mice), suggesting a role for Ag-driven TCR activation in the generation of the SOCS-1−/− disease (Fig. 5). Histological analyses showed that most dying OT-I SOCS-1−/− mice exhibited similar pathological changes to dying SOCS-1−/− IFN-γ−/− mice (28). The OT-I SOCS-1−/− mice did not exhibit the liver degeneration of SOCS-1−/− mice although small hemoipoietic foci were present in the organ (Fig. 6). The major lesions in 70–80% of the OT-I SOCS-1−/− mice, as in SOCS-1−/− IFN-γ−/− mice, were invasion of the lung, pancreas, muscle, heart, and cornea by inflammatory cells (Fig. 6). However, the number of eosinophils in the infiltrates was lower than in SOCS-1−/− IFN-γ−/− mice, as was the extent of the organ infiltrates (Fig. 6).

Delayed and less extensive disease development in OT-I SOCS-1−/− mice suggests that Ag activation of TCR is required for rapid disease development. Although TCR transgenes in general inhibit rearrangement of endogenous TCR genes, a caveat to these experiments is that a small subset of T cells is selected on endogenously rearranged TCR, and abnormal behavior by these cells may induce disease development. To overcome this complication, OT-I SOCS-1−/− mice were derived on a RAG-1-deficient background. Initially, these mice were generated in conventional mouse facilities, and had a slightly increased lifespan compared with OT-I SOCS-1−/− mice, surviving an average of 43 ± 19 days (Fig. 5). IFN-γ was not detectable in the serum of OT-I SOCS-1−/− RAG-1−/− mice (n = 12; data not shown).

Because RAG-1−/− mice are immunocompromised and several OT-I SOCS-1−/− RAG-1−/− controls also became sick, we rederived this line in a strictly germ-free animal facility. These mice survived longer than those in conventional facilities (mean survival = 75 ± 24 days) but then succumbed to a similar disease to that described for OT-I SOCS-1−/− mice raised in conventional mouse facilities (Fig. 5). Interestingly, CD8+ cells in the periphery of these mice appeared to be activated. Compared with corresponding cells from littermate controls, OT-I SOCS-1−/− RAG-1−/− CD8+ cells were larger, expressed lower levels of the TCR, and an increased proportion of cells had a CD44high phenotype (data not shown). Hence, this apparent activation of CD8+ cells was occurring in a sterile environment and in the strict absence of stimulation by exogenous Ag. This suggests that while Ag stimulation of TCR contributes to rapid disease development in SOCS-1−/− mice, other factors drive the activation of T cells and disease that develops in young adult mice lacking SOCS-1. Furthermore,
in contrast to the morbidity of OT-1 SOCS-1<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/RAG-1<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice, SOCS-1<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/RAG-1<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice generated in a germ-free environment remained healthy and did not die prematurely (Fig. 5).

Shown are the means ± SD of 3–6 pairs of mice in each group. B, CFSE-labeled T cells from either IFN-γ<sup>−/−</sup> or SOCS-1<sup>−/−</sup>/IFN-γ<sup>−/−</sup> lymph nodes were analyzed 6 days after transfer into either IFN-γ<sup>−/−</sup> or SOCS-1<sup>−/−</sup>/IFN-γ<sup>−/−</sup> recipients. C, CFSE-labeled T cells from either IFN-γ<sup>−/−</sup> or SOCS-1<sup>−/−</sup>/IFN-γ<sup>−/−</sup> lymph nodes were analyzed 6 days after injection into RAG-1<sup>−/−</sup> mice. D, T cells from SOCS-1<sup>−/−</sup>/IFN-γ<sup>−/−</sup> mice do not show increases in cell death compared with T cells from IFN-γ<sup>−/−</sup> mice. T cells were sorted by FACS into the following fractions: CD4<sup>+</sup>/H<sup>11001</sup>CD44<sup>low</sup>, CD4<sup>+</sup>/H<sup>11001</sup>CD44<sup>high</sup>, CD8<sup>+</sup>/H<sup>11001</sup>CD44<sup>low</sup>, CD8<sup>+</sup>/H<sup>11001</sup>CD44<sup>high</sup>, and plated into cytokine-deficient media. Cell survival was analyzed by FACS at various time points by PI exclusion.

FIGURE 3. Increased proliferation of SOCS-1<sup>−/−</sup>/IFN-γ<sup>−/−</sup> T cells in vivo. A, CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from IFN-γ<sup>−/−</sup> (○) and SOCS-1<sup>−/−</sup>/IFN-γ<sup>−/−</sup> (●) mice were analyzed by FACS for BrdU incorporation.

FIGURE 4. Apparent T cell activation in OT-I SOCS-1<sup>−/−</sup> mice in the absence of OVA. Thymus (A) and lymph node (C) cells were analyzed by FACS for the expression of CD4 and CD8. CD8<sup>+</sup> cells were gated and analyzed for CD44 expression in the thymus (B) and lymph node (D). E, CD3 expression and FSC of CD8<sup>+</sup> lymph node cells from OT-I SOCS-1<sup>−/−</sup> (open histograms) and OT-I SOCS-1<sup>−/−</sup> (filled histograms).
Although CD8\(^+\) T cells appeared to be more dramatically affected by the disruption of SOCS-1, similar but less marked increases in proliferation and CD44 expression were observed for CD4\(^+\) T cells. To examine the effect of specific Ag stimulation of CD4\(^+\) T cells, SOCS-1\(^-/-\) mice were crossed to a MHC class II-restricted TCR-transgenic mouse line, OT-II, that expresses an OVA-specific TCR on all CD4\(^+\) T cells. OT-II SOCS-1\(^-/-\) mice had a similar phenotype and lifespan to OT-I SOCS-1\(^-/-\) mice (Fig. 5 and data not shown). This suggests that SOCS-1 has a role in both CD4\(^+\) and CD8\(^+\) T cells but the effect of SOCS-1 deficiency in CD8\(^+\) T cells is more profound.

Discussion
Previous studies have shown SOCS-1 to be a critical regulator of IFN-\(\gamma\) both in vitro and in vivo (16, 37). The neonatal mortality of SOCS-1\(^-/-\) mice appears to result from a combination of increased production of IFN-\(\gamma\) and hypersensitivity to this cytokine (17, 37). IFN-\(\gamma\) is mainly produced by activated T lymphocytes, NK, and NKT cells (38, 39). Recent studies have shown that lymphocytes are required for the manifestation of the profound SOCS-1\(^-/-\) phenotype, and that SOCS-1\(^-/-\) NKT cells can induce the liver disease characteristic of these mice (17, 40). Furthermore, T cells appear abnormally activated in SOCS-1\(^-/-\) mice, suggesting the possibility that these activated cells contribute to the increased IFN-\(\gamma\) production (17). In this study, we have demonstrated that SOCS-1 has additional physiological roles that are distinct from regulation of IFN-\(\gamma\) signaling.

First, we established that perturbations in T cell development occur in mice lacking both SOCS-1 and IFN-\(\gamma\), indicating that SOCS-1 has other functions in vivo that are independent of IFN-\(\gamma\). Overall T cell numbers were increased in SOCS-1\(^-/-\) IFN-\(\gamma\)\(^-/-\) mice, although decreased T cell numbers were observed in the spleen. Our data suggest that the increase in lymphocyte numbers is due to increased proliferation, but it is unclear why cellularity in the spleen is decreased. However, we have established that this abnormality is unlikely to be due to increased cell death or decreased proliferation. Conceivably, a defect in T cell migration could result in this phenotype. Altered cytokine levels have previously shown to result in differences in T cell homing. For example, IL-15R-deficient mice have a decreased number of T cells.
in the lymph node with no change in the spleen (41). We have investigated cytokine production by various organs in SOCS-1−/− IFN-γ−/− mice compared with IFN-γ−/− controls and have found no differences (Ref. 42 and data not shown). Future studies will address more directly the involvement of T cell migration in this defect.

A feature common to SOCS-1−/− and SOCS-1−/−IFN-γ−/− mice is a decreased ratio of CD4+CD8+ T cells in lymphoid tissues relative to control mice. A decreased ratio of CD4+CD8+ T cells was also evident in FTOCs of SOCS-1−/− and SOCS-1−/−IFN-γ−/− embryos, suggesting that this perturbation may originate in the thymus and may involve responses to cytokines secreted by the thymic stroma. We have examined both proliferation and survival of T cells in SOCS-1−/−IFN-γ−/− and IFN-γ−/− mice. There was no difference in the survival of T cells lacking SOCS-1 although proliferation of CD8+ T cells was increased compared with CD4+ T cells. Therefore, increased proliferation of CD8+ SOCS-1-deficient T cells may be driving the altered ratio of CD4+CD8+ T cells. Although no difference was observed in cell survival after cytokine withdrawal, cytokine effects on survival are being investigated.

Previous studies have shown that cytokines can influence the generation and expansion of different T cell subsets. For instance, IL-12 promotes thymic CD8+ differentiation (27), IL-7-transgenic mice have a decreased ratio of CD4+CD8+ T cells in the periphery, but not in the thymus (43), whereas IL-15R-deficient mice show an increase in the ratio of CD4+CD8+ T cells (41). In the absence of SOCS-1, sustained signaling in response to these or other cytokines may contribute to the altered ratio of CD4+CD8+ T cell ratio and T cell homeostasis. Consistent with this, an increase in the ratio of CD4+CD8+ T cells is apparent in both the thymus and periphery of SOCS-1-transgenic mice, which exhibit reduced responses to cytokines including IL-6 and IL-7 (21).

Adoptive transfer of SOCS-1−/− progenitor T cells to wild-type recipients resulted in a similar perturbation in the CD4:CD8 ratio of donor-derived cells when generated in the context of a wild-type environment. Therefore, this altered T cell ratio does not appear to result from changes in the environment in SOCS-1−/− mice, such as increased cytokine levels, but is more likely to reflect hyper-responsivity to cytokine in the absence of SOCS-1 or defective cytokine secretion by the T cells themselves.

A second phenotype observed is that T cells in the periphery of SOCS-1-deficient mice express high levels of CD44, proliferate at a faster rate, and are larger in size, all features typical of activated cells. However, these cells were not found to display significant functional activation, because T cells from SOCS-1−/−IFN-γ−/− mice did not show direct CTL activity ex vivo, nor did they have substantial increases of other activation markers such as CD69, CD25, or IL-12R, nor did they down-regulate CD62L.

An increase in the expression of activation markers was also seen to a lesser extent in the thymus of SOCS-1−/−IFN-γ−/− mice. However, no difference was observed in CD44 expression in FTOC generated from SOCS-1−/−IFN-γ−/− embryos, suggesting that the apparently activated thymocytes may represent mature T cells that have become activated in the periphery and recirculated to the thymus, or activation of thymocytes by perturbed levels of immunoregulatory factors.

What could be inducing this up-regulation of CD44? A CD44high phenotype is characteristic of memory and activated cells (33). SOCS-1 is expressed in virtually all cells in the thymus, suggesting that it may play a role in thymic differentiation (14, 17). It is conceivable that in the absence of SOCS-1, negative selection in the thymus may be impaired, allowing autoreactive T cells to exit the thymus and migrate to the periphery where they encounter Ag and become activated. Although this may contribute to the observed phenotype, it is unlikely to be a full explanation. Aside from the finding that the CD44high cells in SOCS-1-deficient mice do not have full effector function, expression of CD44 by SOCS-1−/− T cells does not require stimulation with foreign Ag, because this phenotype was also observed in OT-I SOCS-1−/− mice in which CD8+ T cells have not encountered their specific Ag.

Recent reports have shown that naive T cells can transiently acquire features of memory cells including up-regulation of CD44 during homeostatic proliferation (34–36). T cells undergo homeostatic proliferation to expand the T cell pool under lymphopenic conditions. Both naive and memory T cells undergo homeostatic proliferation although the mechanisms appear to be different (44, 45). In naive T cells, homeostatic proliferation is thought to be stimulated by low-affinity interactions with self-MHC molecules in combination with self-peptides, and these signals are also thought to be essential for maintaining cell survival (46–48). Cytokines that signal through the common γ-chain are thought to be important both for maintaining survival of naive cells and for stimulating homeostatic proliferation (44, 49). Several reports have described a role for SOCS-1 in the regulation of this family of cytokines (13, 21, 40). In the absence of SOCS-1, homeostatic signals may not be regulated appropriately. Intriguingly, T cell homeostasis is also perturbed in SOCS-1 transgenic mice, and T cells from these mice show features of activation, including CD44 expression (21).

T cells appear to be crucial for mediating disease in SOCS-1−/− mice (17). To explore further the role of T cells in this disease, we generated TCR transgenic SOCS-1−/− mice. The improved lifespan of both OT-I and OT-II SOCS-1−/− mice indicates that recognition of high-affinity Ag by the TCR contributes to the inflammatory disease in SOCS-1−/− mice. T cells still appear activated in TCR transgenic SOCS-1−/− mice, further supporting the idea that the up-regulation of CD44 is occurring in response to homeostatic signals rather than high-affinity Ag interaction. These mice succumb to inflammatory disease as young adults which is similar to that of SOCS-1−/−IFN-γ−/− mice, suggesting that the delayed phenotype may result from limiting IFN-γ levels (28).

In addition to establishing that Ag-dependent mechanisms contribute to the SOCS-1−/− disease, this study clearly indicates that Ag-independent mechanisms are also important. In a germ-free environment, the survival of OT-I SOCS-1−/−RAG-1−/− mice is strikingly different to that of SOCS-1−/−RAG-1−/− mice. The difference between these two mice strains is limited to the presence of T and NK cells in the OT-I SOCS-1−/−RAG-1−/− mice. However, these cells are restricted in their normal function by the absence of reactivity to specific Ag, but somehow still are able to induce disease. Although autoimmune processes (Ag-specific) may contribute to disease, we have provided evidence that autoimmunity alone is insufficient to account for the SOCS-1−/− phenotype, and that T and/or NK cells can induce disease by means that are distinct from classical autoimmunity.

In summary, we have shown that SOCS-1 has physiological roles in addition to its regulation of IFN-γ, and which involve the maintenance of T cell homeostasis. Studies are underway to identify which additional cytokine responses are dysregulated in the absence of SOCS-1, and which may contribute to the immune abnormalities seen in SOCS-1−/−IFN-γ−/− mice. Cytokines that signal through the common γ-chain are prime candidates for this role.

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