IL-7/STAT5 Cytokine Signaling Pathway Is Essential but Insufficient for Maintenance of Naive CD4 T Cell Survival in Peripheral Lymphoid Organs

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Constitutive expression of suppressors of cytokine signaling (SOCS)1 in T lineage in vivo attenuated cytokine signaling and resulted in a dramatic reduction in the number of naive CD44highCD62Lhigh CD4 T cells in the spleen. After adoptive transfer of thymocytes from SOCS1 transgenic mice into normal recipients, naive CD4 T cells rapidly disappeared from the spleen within 1 wk. Likewise, T cell-specific deletion of STAT5a/b in vivo resulted in a similar phenotype characterized by loss of naive CD4 T cells. Thus, STAT5-mediated signaling is crucial for promoting naive T cell survival. However, forced expression of constitutively active STAT5 failed to rescue CD4 T cells in SOCS1 transgenic mice, implying that STAT5 activation is necessary but not sufficient for naive CD4 T cell survival. Although blockade of the IL-7R, a SOCS1 target, resulted in clear inhibition of naive T cell survival, the effect occurred 3 wk after anti-IL-7R Ab treatment, but not at earlier time points. These results suggest that IL-7-mediated STAT5 activation is essential for long-term survival of naive CD4 cells after export from thymus, and that another SOCS1-sensitive cytokine is critical for short-term naive T cell survival.

To maintain a constant level of lymphocytes, the total number of T cells in peripheral tissues is tightly controlled by homeostatic mechanisms (1). High avidity autoreactive thymocytes are eliminated in the thymus, whereas positively selected T cells emigrate and accumulate as naive T cells in the periphery. Studies from many laboratories have addressed signals and factors that maintain naive T cell numbers in peripheral tissues without cell proliferation (2, 3). MHC molecules appear to be implicated in the survival of naive CD4 T cells because the lifespan of newly formed CD4 T cells is considerably shorter in the absence of MHC class II. In contrast, memory T cells undergo expansion even in lymphopenic MHC class II-deficient recipients, implying that the interaction of TCR with MHC class II-peptide ligands is not essential for either survival or expansion of CD4 memory T cells (4, 5).

The signals delivered through MHC molecules appear to be insufficient for the survival of naive T cells, which undergo apoptosis in vitro in the absence of cytokines (6). The cells can be rescued by IL-2, IL-4, IL-6, and IL-7 (7) and, similarly, treatment with IL-4 or IL-7 has been shown to enhance resistance to gamma irradiation-induced apoptosis in human peripheral blood T cells. IL-7 is the most well-characterized cytokine known to regulate the development and maintenance of T cells, and blocking IL-7 function severely reduces the number of T cells in the thymus and periphery (8, 9). More recently, it has been demonstrated that IL-7 is also required for homeostatic survival of CD8 T cells (10, 11). This survival effect may be ascribed to an antiapoptotic function because IL-7 has been shown to regulate three members of the Bcl2 family at different levels: synthesis of Bcl2, phosphorylation of Bad, and cytosolic retention of Bax. Therefore, cytokines play a role in regulating cell survival by striking a balance between proapoptotic and antiapoptotic members of the Bcl2 family (12). Receptors that share the common γ chain (γc), including those for IL-2, IL-7, and IL-15, activate STAT5 through Jak3, and activated STAT5 is strongly implicated in cell survival via the control of the Bcl2 family members (13, 14). Recent characterization of the lymphoid compartment in STAT5a/bnull/null mice showed marked reduction of B cell and CD8 T cell numbers, indicating that STAT5 regulates many aspects of lymphocyte development (15, 16).

The suppressors of cytokine signaling (SOCS) are a family of proteins that act to negatively regulate cytokine signaling (17). SOCS1 is a powerful negative regulator capable of inhibiting a wide range of cytokines through binding to all family members of the Jak family of tyrosine kinases via its Src homology 2 domain (18, 19). SOCS1 is highly expressed in double positive population of thymocytes and to a lesser extent in the spleen. Thus, it has been postulated that T cell-specific deletion of the SOCS1 gene should have a major effect on thymic T cell development (20, 21). However, T cell-specific SOCS1-deficient mice have only a slight increase of CD8 single-positive (SP) cells in thymus. In the periphery, the proportion of memory CD8 T cells increases, whereas CD4 T cells remain unchanged (20, 22). Interestingly, overexpression of SOCS1...
in T cells resulted in most peripheral CD4 and CD8 T cells having a CD44<sup>high</sup>CD62L<sup>low</sup> memory phenotype (MP) (23). Thus, it is possible that the inhibition of cytokine signals due to constitutive SOCS1 expression might induce the transition from naive to MP in the periphery.

To understand how the abrogation of cytokine signaling by SOCS1 affects the development and maintenance of naive and memory CD4 T cells, we investigated the generation of these cells in SOCS1 transgenic (Tg) mice. Cell surface phenotype analysis confirmed that most peripheral CD4 T cells in SOCS1 Tg mice had a memory CD4 T cell phenotype. We also showed that most peripheral CD4 and CD8 T cells having a CD44<sup>high</sup>CD62L<sup>low</sup> memory phenotype (MP) (23). Thus, it is possible that the inhibition of cytokine signals due to constitutive SOCS1 expression might induce the transition from naive to MP in the periphery. The data presented indicate that cytokine signaling plays an important role in the survival of post-thymic naive T cells.

**Materials and Methods**

**Mice**

Transgenic mice were generated using a vector containing a myc-tagged wild-type SOCS1 cDNA that was expressed under the control of the lck proximal promoter and the intronic enhancer (E<sub>A</sub>) of Ig H chain locus. These promoter Tg mice (SOCS1 Tg) mice were backcrossed with C57BL/6 mice for over 10 generations (24). The SOCS1 Tg mice were also backcrossed with OVA-specific TCR Tg mice (DO11.10 Tg) on a B10.D2 background for over six generations to generate DO11.10 Tg mice. The lck receptor-deficient, stat6-deficient, ifn-g deficiency, and constitutively active STAT5 (CA-STAT5) Tg mice were previously described (25–27). CD4-cre Tg mice were obtained from Dr. C. Wilson (University of Washington, Seattle, WA) and STAT5<sup>F/F</sup> mice were provided by Dr. L. Hennighausen and colleagues (15).

**Isolation and adoptive transfer of naive and memory CD4 T cells and in vivo treatment with IL-7R mAb**

CD4 T cells were isolated from spleenocytes and lymph node cells using CD4 magnetic beads (MACS; Miltenyi Biotec). Purity of CD4 T cells was 94–96%. NKT and CD25<sup>+</sup> regulatory T cells were depleted using depletion with anti-CD1d-<i>cd28</i>-deficient, and constitutive SOCS1 mice were provided by Dr. L. Hennighausen and colleagues (15).

For adoptive transfer, T cells were prepared from the thymus and spleen of C57BL/6 and SOCS1 Tg mice, and cells were labeled with CFSE (Molecular Probes) as previously described (28). Labeled cells (3 x 10<sup>5</sup>) were injected i.v. into sublethally irradiated (6 Gy) or nonirradiated C57BL/6 mice. After 7–10 days, labeled CD4 T cells were purified from host lymph node spleen cells and analyzed for levels of CFSE expression. For treatment with anti-IL-7R mAb, C57BL/6 mice were injected i.p. every other day for 4 wk with purified anti-IL-7Rα Ab (A7R34, 500 μg/mouse) that was provided by Dr. S.-I. Nishikawa (RIKEN, Center for Developmental Biology, Kobe, Japan).

**Measurement of cytokines by ELISA**

The reagents for ELISA, including anti-IFN-γ (R4-6A2 and XMG1.2 biotin), anti-IL-4 (BVD4-1D11 and BVD6-24G2 biotin), and anti-IL-2 (JES6-1A12 and JES6-5H4 biotin), were purchased from BD Biosciences. T cells were stimulated with plate-bound anti-TCR plus anti-CD28 Abs. After 48 h, the culture supernatants were harvested, and the concentrations of IL-2, IL-4, and IFN-γ were analyzed. Results are representative of three independent experiments.

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**FIGURE 1.** Effect of SOCS1 on cytokine induced STAT-activation in CD4 T cells. A. CD4 T cells from C57BL/6 (WT) and SOCS1 Tg mice were stimulated with IL-2, IL-4, IL-6, IL-7, IL-12, IL-15, and IFN-γ at different time points indicated in minutes. Phosphorylation of STAT1, STAT3, STAT4, STAT5, and STAT6 was analyzed by anti-phospho-STAT (pYSTAT) Abs. WT SOCS expression was detected with a myc mAb. B, CD4 SP thymocytes isolated from C57BL/6 and SOCS1 Tg mice were stimulated with soluble anti-CD3 mAb for 0 to 15 min. Phosphorylation of ERK were detected by anti-phospho-ERK (pERK) Ab. C. Sorted CD4 SP cells from C57BL/6 and SOCS1 Tg thymus were stimulated with various concentrations of a TCR mAb for 36 h, and proliferative responses were analyzed. Results are representative of three independent experiments.
Flow cytometry analysis

The following reagents purchased from BD Biosciences were used for flow cytometry analysis: FITC-labeled anti-CD4 (GK1.5) and anti-OVA-specific TCR (KJ1-26) and PE-labeled anti-CD8 (53-6.7), anti-CD25 (3C7), anti-CD44 (IM7), anti-CD62L (10E9.6), and anti-CD69 (H1.2F3). Cells were surface-stained in Abs, and FACS data were analyzed using CellQuest software (BD Biosciences).

Western blot analysis

Cells were extracted in radioimmunoprecipitation assay solution (0.15 mM NaCl, 0.05 mM Tris-HCl (pH 7.2), 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 0.1% SDS). Tyrosine phosphorylation was detected by phospho-specific Abs to STAT1, STAT3, STAT4, STAT5, STAT6, and p42/44 MAPK (Cell Signaling Technology) and by HRP-conjugated RC20 Ab (HRP-RC20; BD Transduction Laboratories). The myc-tagged SOCS1 was detected with the 9E10 myc mAb.

Proliferation

Cells were stimulated with either plate-bound TCR or TCR/CD28 mAbs for 36 h. During the last 6 h, cells were pulsed with 1 μCi of [H3]thymidine, and [H3]thymidine incorporation was measured by liquid scintillation counting.

Results

Constitutive SOCS1 expression leads to the accumulation of MP CD4 T cells

SOCS1 is known to act as a strong negative regulator of a wide range of cytokine signaling pathways. The SOCS1 Tg mice exhibit an MP in SOCS1 Tg mice. A, Cell numbers of thymocytes were compared between C57BL/6 (WT) mice (n = 8) and SOCS1 Tg mice (n = 11). **, p < 0.01 by Student’s t test analysis. Thymocytes from C57BL/6 (WT) and SOCS1 Tg mice were stained with CD4, CD8, CD44, CD69, and αβ TCR mAbs. Gated CD4 SP cells were analyzed for expression of the markers indicated (right panels). B, Splenocytes from C57BL/6 and SOCS1 Tg mice were stained with Abs against CD4, CD8, CD44, CD62L, CD25, and CD69. The gated CD4 T cells were analyzed for expression of the indicated markers. The number of splenic CD44<sup>lo</sup> and CD44<sup>hi</sup> CD4 T cells was compared between C57BL/6 and SOCS1 Tg mice. The results are representative of three independent experiments, and the mean and SD of five mice are illustrated. **, p < 0.01 by Student’s t test analysis.

Generation of MP CD4 T cells in SOCS1 Tg mice is independent of T cell activation.

A, Expression levels of CD44 were analyzed on CD4<sup>+</sup> cells on KJ1-26<sup>+</sup> thymocytes sorted from DO11.10 Tg and DO11.10 Tg × SOCS1 Tg mice on a B10.D2 background. B, Splenocytes from DO11.10 Tg and DO11.10 Tg × SOCS1 Tg mice were stained with anti-CD4 and anti-CD8 Abs. CD44 expression was analyzed in the sorted KJ1-26<sup>+</sup> and KJ1-26<sup>-</sup> gated CD4 T cells. C, The number of CD44<sup>lo</sup> and CD44<sup>hi</sup> splenic CD4 T cells was compared between DO11.10 and DO11.10 Tg × SOCS1 Tg mice. The results shown are the mean ± SD of three independent experiments. *, p < 0.05 by Student’s t test analysis. D, CD44 expression profile on splenic CD4 T cells isolated from CD28<sup>+/+</sup>, CD28<sup>-/-</sup>, and SOCS1 Tg × CD28<sup>-/-</sup> mice on a C57BL/6 background. Results are representative of three independent experiments.
allowed us to study how the inhibition of cytokine signaling affects the development of naive and memory CD4 T cells. To confirm the function of the SOCS1 Tg in T cells, CD4 T cells from SOCS1 Tg mice were stimulated with IFN-γ, IL-2, IL-4, IL-6, IL-7, IL-12, and IL-15 and assessed for phosphorylation of STAT1 through STAT6. SOCS1 overexpression inhibited IL-2-, IL-7-, and IL-15-mediated STAT3, IL-6-mediated STAT5, and IFN-γ-mediated STAT1 activation, but not IL-12-mediated STAT4 activation (Fig. 1A). Although previous reports demonstrated that SOCS1 impacts TCR-mediated signaling (30–32), constitutive SOCS1 expression did not alter phosphorylation of ERK (Fig. 1B) and cell proliferation (Fig. 1C) following anti-TCR-induced activation of CD4 SP thymocytes, indicating that SOCS1 expression does not inhibit TCR-mediated signaling in vitro.

SOCS1 Tg mice showed drastic reduction in thymocyte numbers, ranging from one-third to one-fourth that of normal mice (Fig. 2A). The reduction in cell number might be due to the impaired IL-7 signaling pathway, which regulates the expansion of double negative cells. Overexpression of SOCS1 resulted in slight reduction of CD4 SP, substantial reduction of CD8 SP, and slight gain of double positive cell number. However, the CD4 SP cells had normal levels of TCR, CD69, and CD44 expression (Fig. 2A).

The number of splenic CD4 and CD8 T cells was drastically reduced in the SOCS1 Tg mice from one-fourth to one-fifth that found in normal mice (data not shown), and most of the peripheral CD4 T cells highly expressed CD44 but not CD62L, CD25, or CD69, which is indicative of MP cells (Fig. 2B). Despite the reduction in total T cells, the absolute number of splenic MP CD4 cells was nearly identical in SOCS1 Tg and wild-type mice (Fig. 2B). These results suggest that constitutive SOCS1 expression leads to a dramatic alteration in the proportion of naive vs MP CD4 T cells, with a selective decrease of naive CD4 T cells in spleen.

SOCS1 expression promotes the predominance of MP CD4 T cells in TCR Tg and cd28-deficient mice

The expression of CD44 is used as a marker for MP CD4 cells because increased CD44 expression is indicative of cell activation. Therefore we examined the SOCS1-mediated predominance of MP cells in two environments for which TCR-mediated activation is limited, TCR Tg mice and cd28-deficient mice. The SOCS1 Tg mice were crossed with DO11.10 TCR Tg or cd28 gene targeted mice and a similar phenotypic alteration was observed (Fig. 3). The major population of KJ1-26 CD4 T cells from DO11.10 Tg mice on a B10.D2 background exhibits a naive phenotype both in thymus and spleen. However, DO11.10 Tg × SOCS1 Tg again showed accumulation of MP CD4 spleen cells even though positive selection occurred normally and CD4 SP in the thymus retained a naive phenotype (Fig. 3, A and B). Moreover, the absolute number of peripheral MP CD4 T cells from DO11.10 Tg × SOCS1 Tg and DO11.10 TCR Tg mice was similar, although the number of CD44low/CD4 T cells was greatly reduced by SOCS1 expression (Fig. 3C).

CD28 costimulation is essential for primary T cell activation, thus T cells from cd28-deficient mice cannot be activated by Ag (33, 34). As a result, the majority of splenic CD4 T cells in cd28-deficient mice exhibit a naive phenotype. In marked contrast, most
CD4 T cells from $cd28^{-/-}$ SOCS1 Tg acquired a MP (Fig. 3D). These results indicate that SOCS1 expression preferentially induced the CD44 activation marker on CD4 T cells even in an environment in which Ag-induced activation is minimal.

**MP CD4 cells in SOCS1 Tg mice are not memory T cells derived from homeostatic proliferation**

The transition from naive to MP seems to occur during the passage from the thymus to the spleen. Particular subsets of memory T cells can be generated by homeostasis driven proliferation when there is available space for T cell expansion in the periphery (28). Consequently, we asked whether the generation of MP CD4 cells is the result of cell division by naive T cells. For these studies, we labeled thymocytes with CFSE and transferred them into sublethally irradiated syngeneic mice, in which space is available for homeostatic proliferation. The generation of memory T cells was assessed from day 7 to 10 after adoptive transfer by examining the expression of CD44. Both thymus- and spleen-derived naive CD4 T cells from wild-type mice underwent CD44 up-regulation along with successive cell divisions (Fig. 4A, a and c). In contrast, splenic CD4 T cells derived from SOCS1 Tg mice already showed a MP before the first cell division (Fig. 4Ab).

Because MP cells appeared independent of cell division, we transferred CFSE-labeled cells into nonirradiated syngeneic mice for which no “space” is available for homeostatic proliferation. After transfer of splenic CD4 T cells from both C57BL/6 and SOCS1 Tg mice, the naive vs MP ratio remained the same as that of the cells that were originally transferred (Fig. 4B, a and b). Likewise, the majority of transferred CD4 T cells from both C57BL/6 and SOCS1 Tg mice were stimulated with 1000 U/ml IL-2 and 50 ng/ml IL-7 for 30 min. Results are representative of three experiments.

**SOCS1 expression selectively impairs survival of naive CD4 T cells in the periphery**

We next asked whether there is a phenotypic transition from naive to MP CD4 T cells or a selective loss of naive T cells. To investigate this issue, we studied the survival kinetics of both naive and MP CD4 populations in parallel with absolute cell numbers. The CFSE-labeled thymocytes from C57BL/6 or SOCS1 Tg mice were adoptively transferred into nonirradiated syngeneic mice for which no “space” is available for homeostatic proliferation. The expression of CD44high and CD44low CD4 T cells from spleen were analyzed at days 1, 3, and 5.

FIGURE 6. Impairment of naive CD4 T cell survival in CD4Cre $\times$ STAT5F/F mice. A, Thymocytes from CD4Cre $\times$ STAT5$^{+/+}$ and CD4Cre $\times$ STAT5$^{+/+}$ mice were stained with CD4, CD8, and CD44 mAbs. The gated CD4 SP cells were analyzed for CD44 expression. B, Splenocytes from CD4Cre $\times$ STAT5$^{+/+}$ and CD4Cre $\times$ STAT5$^{+/+}$ mice were stained with CD4, CD8, CD44, and CD62L mAbs. The gated CD4 T cells were analyzed for the indicated markers. C, The numbers of CD44high and CD44low CD4 T cells from spleen were compared between CD4Cre $\times$ STAT5$^{+/+}$ mice ($n = 8$) and CD4Cre $\times$ STAT5F/F mice ($n = 9$). **, $p < 0.01$ by Student’s t test analysis. D, CD44low and CD44high CD4 T cells from CD4Cre $\times$ STAT5$^{+/+}$ and CD4Cre $\times$ STAT5F/F mice were stimulated with 1000 U/ml IL-2 and 50 ng/ml IL-7 for 30 min. Results are representative of three experiments.
cells from wild-type (WT) and SOCS1 Tg mice. The number of CD4 T cells from spleen was compared among wild-type (n = 3), SOCS1 Tg (n = 3), and SOCS1 × STAT5-CA (n = 3) mice. **p < 0.01 by Student’s t test analysis. C, CD4, CD8, and CD44 expression profiles of thymocytes and spleen cells from wild-type (WT) and SOCS1 × CA-STAT5 mice. CD44 expression was examined in the gated CD4 SP thymocytes (left), and the CD4/CD44 profile was analyzed using whole spleen (right). Results are representative of three experiments.

FIGURE 7. Overexpression of CA-STAT5 partially recovers naive CD4 T cell survival in SOCS1 Tg mice. A, Thymocytes and spleen cells were obtained from wild-type (WT) and SOCS1 × CA-STAT5 mice, and constitutive phosphorylation of STAT5 was analyzed by anti-phospho-STAT5 (pYSTAT5) Abs. Tg SOCS expression (SOCS1) was detected with myc mAb. The number of peripheral CD4 T cells was examined among C57BL/6, SOCS1 Tg, and SOCS1 Tg × CA-STAT5 Tg mice. The number of CD4 T cells from spleen was compared among wild-type (n = 3), SOCS1 Tg (n = 3), and SOCS1 × STAT5-CA (n = 3) mice. **p < 0.01 by Student’s t test analysis. C, CD4, CD8, and CD44 expression profiles of thymocytes and spleen cells from wild-type (WT) and SOCS1 × CA-STAT5 mice. CD44 expression was examined in the gated CD4 SP thymocytes (left), and the CD4/CD44 profile was analyzed using whole spleen (right). Results are representative of three experiments.

total cell numbers, to those of wild-type mice. However, the number of naive cells from SOCS1 Tg but not C57BL/6 mice was rapidly reduced by day 3 after transfer, and by day 5 posttransfer, the number of naive CD4 T cells was 10 times lower than that of control mice (Fig. 5, A and B). However, MP CD4 cell numbers remained at the same levels in both groups at all three time points. As a result, the MP vs naive ratio was drastically increased in the group receiving SOCS1 Tg thymocytes. These results indicate that thymus-derived T cells from SOCS1 Tg mice fail to survive in the periphery, thereby resulting in a proportional increase of MP CD4 cells. Therefore, we postulate that a survival signal controlled by SOCS1-sensitive cytokines is required for maintaining the size of the naive CD4 T cell pool in peripheral organs.

Signal pathway of CD44high population may be distinct from naive CD4 T cells. Thus, we examined the function of the STAT5-dependent pathway and the SOCS1 Tg in CD44high T cells. CD44highCD4 T cells from wild-type and SOCS1 Tg mice were stimulated with IL-7 and assessed for phosphorylation of STAT5. Wild-type CD44highCD4 T cells showed clear phosphorylation of STAT5 in the comparable level of naive CD4 T cells as seen in Fig. 1A, whereas SOCS1 Tg CD44high population again showed marked reduction in the phosphorylation (Fig. 5C). CD44high population expressed detectable levels of transgene, indicating that expressed SOCS1 was able to inhibit the cytokine signal in CD44highCD4 T cells.

T cell-specific deletion of STAT5 results in the loss of naive CD4 T cells in the periphery

The previous results raise the question of whether cytokines may be required for the maintenance of the naive T cell pool in the periphery. STAT5 is a common signal transducer for IL-2, IL-7, and IL-15, which share the γc and Jak3. IL-2 and IL-15 are anti-apoptotic regulators that control the expression of Bcl2 family members through the activation of STAT5, and SOCS1 acts as a negative regulator for both IL-2R and IL-15R signaling (Fig. 1). To determine whether STAT5 regulates survival of naive and MP CD4 T cells, we made use of mice in which the STAT5a and STAT5b genes have been flanked by loxP sites (STAT5<sup>F/F</sup>) (35). These mice were crossed with CD4-cre Tg mice, which results in CD4 T cell-specific deletion of STAT5. CD4Cre × STAT5<sup>F/F</sup> mice showed indistinguishable CD4 SP development and the CD44 expression profile from thymus of normal mice (Fig. 6A). Proportional reduction of CD8 cells was found in the spleen of CD4Cre × STAT5<sup>F/F</sup> mice because the cre gene was expressed in double positive stage (Fig. 6B). This finding might be due to a higher requirement of CD8 cells for STAT5. Importantly, the number of CD4 cells from CD4Cre × STAT5<sup>F/F</sup> spleen showed a significant reduction of naive CD4<sup>+</sup>CD44high T cells (Fig. 6C). In CD4Cre × STAT5<sup>F/F</sup> mice, STAT5 activation was diminished in both naive and CD44highCD4 T cells from spleen (Fig. 6D). These results indicated that STAT5-dependent signals are essential for the survival of naive CD4 T cells in peripheral organs after export from thymus. These proportional alterations in naive and MP CD4 cells are quite similar to those observed in SOCS1 Tg mice. Therefore, it is likely that the impairment in survival of naive T cells in SOCS1 Tg is due to the inhibition of a STAT5-dependent pathway.

CA-STAT5 partially rescues the reduction of naive CD4 T cells in SOCS1 Tg mice

If STAT5 is a target of SOCS1, reconstitution of STAT5 activation might prevent the reduction of naive CD4 T cells observed in SOCS1 Tg mice. To reconstitute STAT5 activity, we crossed SOCS1 Tg mice with CA-STAT5 Tg mice. Spleen cells from the CA-STAT5 Tg mice exhibited constitutive phosphorylation of
STAT5 even in the presence of SOCS1 expression (Fig. 7A). Previous studies reported that CA-STAT5 Tg mice exhibited expansion of memory CD8 T cells (27), and similar results were observed in the SOCS1 Tg × CA-STAT5 Tg mice (data not shown). Moreover, in SOCS1 Tg × CA-STAT5 Tg mice, the reduction of total CD4 T cell number in the thymus and spleen (Fig. 7B) was reversed, indicating that the expression of CA-STAT5 can reconstitute T cell numbers in SOCS1 Tg mice, although the substantial reduction of CD8 SP was retained in SOCS1 Tg × CA-STAT5 Tg mice. However, even in the presence of CA-STAT5, the reconstitution of CD44low naive T cell numbers in the spleen was only partial (Fig. 7C). These results identify distinct effects on naive T cell survival in loss and gain of function STAT5 mutant mice. Thus, we speculate that STAT5 may not be the only target of SOCS1 involved in the regulation of naive CD4 T cell survival, and that other cytokine-dependent signaling pathways are also involved.

**IL-7 regulates long-term, but not short-term survival of naive CD4 cells**

IL-7 has been reported to be a critical factor for regulating survival of naive T cells in vitro and in vivo (8, 9). Thus, the IL-7-STAT5 signaling pathway is a major potential candidate for regulating the maintenance of the naive T cell population size. Thus, we examined the in vivo kinetics of the IL-7 requirement for the survival of naive T cells. Syngeneic thymocytes were transferred into recipient C57BL/6 mice that were treated with neutralizing anti-IL-7R mAb. The treatment with anti-IL-7R mAb inhibited survival of CD44low naive CD4 T cells; however, significant reduction was only observed at 3–4 wk after continuous anti-IL-7R Ab treatment without affecting the survival cell number of CD4 cells (Fig. 8A, left, and B). By contrast, treated mice exhibited a dramatic reduction of pro- and pre-B cell development in the bone marrow even after only 1 wk of treatment (Fig. 8A, right). These results suggest that IL-7-STAT5 signaling mainly affects long-term survival of the naive T cells. However, the survival of naive CD4 T cells was rapidly terminated in the presence of SOCS1. Therefore, it seems likely that alternative SOCS1-sensitive, but STAT5 unrelated, cytokines regulates the short-term survival of naive T cells.

In an attempt to identify these SOCS1-sensitive cytokines, we further studied the role of IL-4 and IL-6 in peripheral T cell survival. Mice deficient in IL-4R, STAT6, or IL-6 showed a normal naive to MP ratio (data not shown). Moreover, transfer of stats6-deficient thymocytes into syngeneic recipients (Fig. 8C), or normal control thymocytes into ifn-6-deficient mice (Fig. 8D), did not result in any increase in the number of memory T cells when compared with numbers in their normal counterparts. Additional blockade of IL-7 signaling using anti-il7 Abs again showed no inhibition of naive T cell survival (Fig. 8, C and D). These results indicate that cytokine signaling through IL-4-STAT6, IL-6-STAT3, and IL-7-STAT5 pathways are not targets of SOCS1 that are crucial for the maintenance of the naive CD4 T cell pool in peripheral lymphoid organs.

**FIGURE 8.** Effect of blocking IL-7-IL-7R signaling on the survival of CD44low naive CD4 T cells. A, Thymocytes from C57BL/6 donors were labeled with CFSE and injected i.v. into syngeneic recipient mice (3 × 10^6 cells/mouse). Recipient C57BL/6 mice were treated with multiple injections as (described in Materials and Methods) of anti-IL-7R mAb or PBS. CD44 expression was examined in CFSE-labeled lymph node- and spleen-derived CD4 T cells 1–4 wk after cell transfer. Data are representative of two independent experiments. To confirm the efficacy of the anti-IL-7R mAb treatment, bone marrow cells from IL-7R mAb treated and control mice were examined for B220 and IgM expression 1 and 3 wk after the transfer. Pro- and pre-B cell populations are enclosed in oval. B, The number of CD4 cells in the anti-IL-7R treated C57BL/6 recipient mice. The number of CD4 cells in spleen from the anti-IL-7R treated C57BL/6 recipient mice were counted 1 and 3 wk after the transfer. The mean of three individual mice is indicated by vertical bar. C, The role of the IL-4-STAT6 pathway in the maintenance of naive T cells. Thymocytes from BALB/c and anti-IL-7R treated C57BL/6 recipient mice were counted 1 and 3 wk after the transfer. Data are representative of two independent experiments. To confirm the efficacy of the anti-IL-7R mAb treatment, bone marrow cells from IL-7R mAb treated and control mice were examined for B220 and IgM expression 1 and 3 wk after the transfer. Pro- and pre-B cell populations are enclosed in oval. D, The role of the IL-6 signaling pathway in the maintenance of naive T cells. Thymocytes from C57BL/6 mice were labeled with CFSE and injected i.v. into normal C57BL/6 or IL-6-deficient mice on a C57BL/6 background. In B and C, recipient mice were treated with repeated injections of an IL-7R mAb, while controls received PBS. Seven days after cell transfer, spleen and lymph node (data not shown) derived CD4 T cells of recipient mice were stained with a CD44 mAb.
Discussion
The present study highlights the requirement of both IL-7-STAT5 signaling and a SOCS1-sensitive cytokine signaling pathway for the maintenance of naive CD4 T cells in the periphery. In SOCS1 Tg mice, in which there is a broad impairment of cytokine signaling, we demonstrated that naïve CD4 T cells are unable to survive. Specifically, following adoptive transfer of thymocytes derived from SOCS1 Tg mice, the number of MP CD4 T cells was readily sustained in the spleen, whereas naïve CD4 T cells disappeared within 1 wk. A similar reduction of naïve CD4 T cells was found in STAT5-deficient mice, suggesting that STAT5 is necessary for naïve CD4 T cell survival. However, crossing a CA-STAT5b Tg with SOCS1 Tg mice did not restore the defect observed in naïve CD4 T cell numbers indicating that STAT5 activation is not sufficient for survival of these cells. This discrepancy leads to the possibility that multiple cytokine signaling pathways affected by SOCS1 may regulate survival of naïve CD4 cells after their export from the thymus. Finally, IL-7 blockade also inhibited naïve T cell survival. However, IL-7 appears to be responsible for late phase survival because anti-IL-7 treatment reduced naïve CD4 T cell numbers only after 3 wk of treatment. Therefore, we speculate that an uncharacterized SOCS1-sensitive cytokine is required in the early phase of naïve CD4 T cell survival.

TCR-MHC interactions appear to be essential for some aspects of the survival of naïve CD4 T cells as the lifespan of newly developing CD4 T cells is considerably shorter in the absence of MHC molecules (36). However, in SOCS1 Tg mice, the number of naïve T cells decreased rapidly, despite the fact that SOCS1 overexpression did not affect TCR signaling or TCR-dependent thymocyte development. Therefore, we propose that the drastic decrease of naïve T cell numbers observed in SOCS1 Tg mice is not due to a defect in TCR-mediated signaling but rather is the result of an inhibitory affect on cytokine signaling. This hypothesis is supported by the observation that the profile shift into MP CD4 T cell dominance is also observed in peripheral T cells from DO11.10 Tg × SOCS1 Tg mice and cd28-deficient SOCS1 Tg mice.

SOCS1 inhibits multiple cytokine signaling pathways through binding to Jak in a phosphotyrosine-dependent manner (19). The impairment observed in SOCS1 Tg mice is quite similar to that of mice lacking γc and Jak3, and cytokines necessary for the survival of T lymphocytes use γc and Jak3 and activate STAT5a and STAT5b (37, 38). In mice with a T-cell-specific deletion of STAT5a and STAT5b, CD4 T cells are clearly reduced. Moreover, most naïve CD4 T cells could survive for at least 2 wk without IL-7 help. IL-7 is the best-characterized cytokine known to regulate the homeostasis and maintenance of naïve T cells. Our results are consistent with previous observations that treatment of normal or thymectomized mice with IL-7 mAb affected the survival of CD4 naïve T cells at 3 or 4 wk after the treatment (39). Therefore, the IL-7γc-Jak3-STAT5 pathway is essential for long-term survival of naïve CD4 T cells.

However, most naïve CD4 T cells from SOCS1 Tg mice disappear within 7 days in the periphery, and blockade of IL-7 pathway does not initiate such a rapid inhibition. These results suggest that the early phase of survival is regulated by cytokines distinct from the IL-7/STAT5-mediated signaling. An alternative interpretation might be that STAT5 signaling is necessary but not sufficient for short-term survival of naïve CD4 T cells, and that other SOCS-sensitive signaling pathways are also necessary.

IL-4 and IL-6 have been reported as alternative candidate cytokines for regulating the survival of naïve T cells in vitro (39, 40). However, our results indicate that IL-4 and IL-6 are unlikely to be responsible for maintaining the naïve T cell pool in peripheral lymphoid organs because IL-4R, STAT6-deficient, and IL-6-deficient mice show no abnormality in the number of naïve T cells. This apparent discrepancy in the results might be result of in vivo vs in vitro experiments. The cytokine signal responsible for the early phase of naïve CD4 T cell survival still remains unresolved, and further investigations are clearly needed.

In conclusion, this study suggests that long- and short-term survival of naïve CD4 T cells in peripheral tissues is regulated by an IL-7γc-Jak3-STAT5 pathway and a SOCS1-sensitive cytokine, respectively. A more in depth understanding of the role of SOCS1-sensitive cytokine-mediated signaling may lead to new insights into the survival mechanisms of distinct T cell subsets.

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Disclosures
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