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J Immunol 2007; 179:2170-2179; doi: 10.4049/jimmunol.179.4.2170
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Selective Expansion of Foxp3-Positive Regulatory T Cells and Immunosuppression by Suppressors of Cytokine Signaling 3-Deficient Dendritic Cells

Yumiko Matsumura,* Takashi Kobayashi,* Kenji Ichiyama,* Ryoko Yoshida,* Masayuki Hashimoto,* Tomohito Takimoto,* Kentaro Tanaka,* Takatoshi Chinen,* Takashi Shichita,* Tony Wyss-Coray,‡ Katsuaki Sato,* and Akihiko Yoshimura2* 

Dendritic cells (DCs) induce immunity and immunological tolerance as APCs. It has been shown that DCs secreting IL-10 induce IL-10+ Tr1-type regulatory T (Treg) cells, whereas Foxp3-positive Treg cells are expanded from naïve CD4+ T cells by coculturing with mature DCs. However, the regulatory mechanism of expansion of Foxp3+ Treg cells by DCs has not been clarified. In this study, we demonstrated that suppressors of cytokine signaling (SOCS)-3-deficient DCs have a strong potential as Foxp3+ T cell-inducing tolerogenic DCs. SOCS3−/− DCs expressed lower levels of class II MHC, CD40, CD86, and IL-12 than wild-type (WT)-DCs both in vitro and in vivo, and showed constitutive activation of STAT3. Foxp3+ effector T cells were predominantly expanded by the priming with WT-DCs, whereas Foxp3+ Treg cells were selectively expanded by SOCS3−/− DCs. Adoptive transfer of SOCS3−/− DCs reduced the severity of experimental autoimmune encephalomyelitis. Foxp3+ T cell expansion was blocked by anti-TGF-β Ab, and SOCS3−/− DCs produced higher levels of TGF-β than WT-DCs, suggesting that TGF-β plays an essential role in the expansion of Foxp3+ Treg cells. These results indicate an important role of SOCS3 in determining on immunity or tolerance by DCs. The Journal of Immunology, 2007, 179: 2170–2179.

Received for publication October 16, 2006. Accepted for publication June 12, 2007.

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1 This work was supported by special grants-in-aid from the Ministry of Education, Science, Technology, Sports and Culture of Japan (to A.Y. and K.T.), the Yamanouchi Foundation for Research on Metabolic Disorders (to A.Y.), the Ministry of Education, Culture, Sports, Science, and Technology, Sports and Culture of Japan (to A.Y. and K.T.), the Uehara Memorial Foundation (to K.T.), the Takeda Science Foundation, the Ministry of Education, Culture, Sports, Science, and Technology, Sports and Culture of Japan (to A.Y. and K.T.), the Yamanouchi Foundation for Research on Metabolic Disorders (to A.Y.), and the Uehara Memorial Foundation (to T.C.).

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3 The Journal of Immunology, 2007, 179: 2170–2179.

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receptors for STAT3-activating cytokines, such as IL-6, G-CSF, and leptin, the effect of SOCS3 has been shown to be relatively restricted to STAT3 (12). However, SOCS3 does not inhibit IL-10 signaling, because the Src homology 2 domain of SOCS3 does not bind to the IL-10R (13).

In this study, we investigate the role of SOCS3 in DCs by using SOCS3-deficient bone marrow-derived DCs (BMDCs). We found that constitutive activation of STAT3 and an immature phenotype were maintained in SOCS3+/− DCs after LPS treatment. SOCS3+/− DCs suppressed the development of experimental autoimmune encephalomyelitis (EAE). In addition, Foxp3+ TGF-β1+ Treg cells, but not Foxp3− effector T cells, were expanded by SOCS3+/− DCs. From the effect of anti-TGF-β Ab and the levels of TGF-β in DCs, we propose that SOCS3 negatively regulates Treg expansion by modulating TGF-β secretion from DCs. Our study suggests that SOCS3 in DCs is an important determinant for immunity or tolerance.

Materials and Methods

Mice

Conditional targeting of SOCS3 using the loxP system and crossing with lysosome M (LysM)-Cre mice have been described (13, 14). To delete the SOCS3 gene in hematopoietic stem cells, Tie2-Cre mice were crossed with SOCS3fl/fl mice (14). Age-matched SOCS3fl/fl (SOCS3−/−) and LysM-Cre:SOCS3fl/fl or Tie2-Cre:SOCS3fl/fl (SOCS3−/−) mice were used for analysis. All experiments were approved by the Animal Ethics Committee of Kyushu University.

DC preparation

BMDCs were prepared from bone marrow suspensions obtained from the femurs and tibias, as described (15). Bone marrow cells were cultured in 20 ng/ml murine GM-CSF (PeproTech) or culture supernatant from J558L cells transfected with the murine GM-CSF gene. On day 8, LPS (Sigma-Aldrich) was added at 100 ng/ml for 16 h. IL-10-treated DCs (IL-10-DCs) were generated from bone marrow cells cultured with murine GM-CSF (20 ng/ml) for 8 days and murine IL-10 (20 ng/ml; PeproTech) for 2 days, and then stimulated with LPS. Flowed SOCS3 gene deletion was evaluated by PCR using genomic DNA obtained from BMDCs, as described (13, 14).

Preparation of CD4+ T cells and allogeneic MLR

Spleen and lymph node (LN) cells of BALB/c or OT-II transgenic mice were incubated with anti-CD4-coated MACS magnetic beads (Miltenyi Biotec) and were positively selected. The purity of CD4+ T cells was >95%, as determined by flow cytometry, CD4+ T cells (2 × 10⁶) from BALB/c mice (H-2b) were cocultured with BMDCs (1 × 10⁷) from SOCS3+/− or SOCS3−/− mice (H-2b) in RPMI 1640 medium with 10% FCS and antibiotics for 3–4 days. Expanded T cells (4 × 10⁶) were re-stimulated with indicated concentrations of plate-bound anti-CD3 Ab (0.1 mg/ml), and then cell proliferation was assessed after 72 h of culture by [3H]thymidine incorporation assay. Cytokine concentration in the supernatant was measured by ELISA. For Ab-blocking experiments, anti-CD4 Abs, fixed, and permeabilized, according to the manufacturer's instructions, were added to 105 μg/ml of mouse IgG1 as control for each experiment.

Suppression assay

To isolate naturally occurring Treg cells (CD4+ CD25+ T cells), CD4+ T cells were positively selected with anti-CD25-coated MACS beads (purity >98%). CD4+ T cells or CD4+ CD25− T cells were expanded by BMDCs for 4 days, as described above, and then CD25+ cells were collected by MACS beads (Miltenyi Biotec) and used as suppressor cells. To isolate responder CD4+ CD25− T cells, splenocytes were labeled with biotin conjugated anti-CD3 (53-6-7), anti-CD11b (M1/70), anti-B220 (RA3-6B2), anti-DOI-5, anti-Ter-119, and anti-CD25 (PC61) mAbs (eBioscience); incubated with streptavidin magnetic beads; and loaded onto MACS separation columns. Number of responder CD4+ CD25− T cells and suppressor CD25+ T cells expanded by DCs was cultured with irradiated whole spleen cells (1 × 10⁶) with 1.0 μg/ml anti-CD3 Ab. The number of responder cells was fixed (1 × 10⁶) and that of suppressors was varied. [3H]Thymidine was added for the last 16 h of a 72-h assay.

Flow cytometric analysis

Cells were stained with FITC-, PE-, Biotin-, and allophycocyanin-conjugated anti-CD86 (GL1), and I-A (AF6-120.1) from BD Pharmingen, and CD40 (1C10), CD80 (16-10A1), CD4 (RM-45), and CD25 (PC61) from eBioscience. Biotinylated Ab staining was followed by streptavidin-PE-Cy5.5 (BD Pharmingen). For anti-mouse allophycocyanin-Foxp3 (FKH-16s) (eBioscience) intracellular staining, cells were labeled with anti-CD4 and anti-CD25 Abs, fixed, and permeabilized, according to the manufacturer’s protocol.

Western blotting, ELISA, and NO measurement

Western blotting for detection of phosphorylated STATs was performed, as described (16). IL-4, IL-6, IL-10, and IFN-γ were detected in culture supernatants with OptEIA ELISA sets (BD Biosciences), and IL-17 was detected with DuoSet ELISA Development Systems (R&D Systems), as per the manufacturer’s instructions. NO was measured as the accumulation of nitrite in the incubation medium, as described (16).

EAE induction and treatment by DCs

Myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MEVGW YRRSPSRVVHLRNGK) (BEX) was used to induce EAE in C57BL/6 mice. Briefly, mice were injected i.c. with 200 μg of MOG peptide in 100 μl of PBS emulsified in 100 μl of CFA that was further enriched with 5 mg/ml Mycobacterium tuberculosis (H37Ra; Difco/BD Pharmingen). In addition, 500 ng of pertussis toxin (Calbiochem) was injected i.p. on days 0 and 2. Paralysis was evaluated according to the following scores: 0 = no signs, 1 = full tail, 2 = hind limbs, 3 = complete back, 4 = fore limbs, and 5 = dead. Three hours after stimulation and peptide pulse, DCs were injected (1 × 10⁶ cells) i.v. three times on days 7, 5, and 3 before EAE induction (day 0) (17), DC-treated and control mice were immunized with MOG peptide, according to the protocol for EAE induction. After indicated days, splenic CD4+ T cells were isolated and cultured (2 × 10⁵ cells/well) with plate-bound anti-CD3 Ab in a 96-well plate.

RT-PCR

The cells were lysed in TRIzol reagent (Invitrogen Life Technologies) for RNA preparation. RT-PCR was performed with a standard procedure. The expression level of G3PDH was first evaluated as an internal control. The primer sequences and PCR cycles were as follows: IFN-γ, 5'-gca tgg ttg gtt gtc ttc tgt ggt acct gc-3' and 5'-cct ctt cgg ctc gct tta gtt tta gtt gc-3' (30 cycles); IL-10, 5'-tac tgc gta gaa gtt gtc cc-3' and 5'-cat cag cat gta ttc tgc gc-3' (30 cycles); and TGF-β1, 5'-aaa tgt gtc aac aca aca cag c-3' and 5'-gac gga ata cag ggc ttc g-3' (30 cycles). IL-17, 5'-cag cag cag cag cag cag cag cag-3' (30 cycles); Foxp3, 5'-cag ctc cct aca gta gtt ccc caa g-3' and 5'-cat cag tgc cca gtg gtt gc-3' (32 cycles); CTLA-4, 5'-tgg act ccc cag gta cca gaa ag-3' and 5'-cag ttc cag gat gtt ggt ggc gc-3' (30 cycles); G3PDH, 5'-acc aca gtc ccc gtt gct ac-3' and 5'-tcc acc acct ctc gtc tgt ca-3' (28 cycles).

Transfer of Ag-pulsed DCs into mice

DCs from SOCS3+/− and SOCS3−/− mice were incubated in culture medium with 50 μg/ml keyhole limpet hemocyanin (KLH) for 16 h, resuspended in PBS (5 × 10⁶ cells of DCs in 40 μl), and then administered into the hind footpads. The draining LNs were removed and teased into a cell suspension on day 5. The lymphocytes were cultured with or without Ag (20 μg/ml KLH) at 5 × 10⁶ cells in 96-well plates for 96 h, and cytokine levels in the culture supernatant were determined by ELISA (15).

For adoptive transfer into OT-II mice, DCs from SOCS3+/− and SOCS3−/− mice were cultured in the presence of 1 μg/ml OVA peptide 323–339 (IQSVAHAAHINAEAGR) for 16 h, then 4 × 10⁶ DCs were injected i.v. into OT-II transgenic mice. After 7 days, splenic CD4+ T cells were analyzed by Foxp3 intracellular staining.

TGF-β bioassay

MBF-F11 cells (18) were seeded at 1–4 × 10⁶ cells/well in 96-well plates. After overnight incubation, cells were washed twice with PBS and incubated in 50 μl of serum-free DMEM supplemented with penicillin/streptomycin for 2 h before test samples were added in 50 μl vol. Ten-μl aliquots of the culture supernatants were collected after 24-h incubation with MBF-F11 cells. Secreted alkaline phosphatase (SEAP) activity was measured using Reporter Assay Kit SEAP (Toyobo), according to the manufacturer’s instructions, and measured with a Lumat LB 9507 tube luminometer (EG & G Berthold).
Tie2-Cre:SOCS3 fl/fl mice. Deletion efficiency is almost 100%. A band of 250 bp indicates Cre-mediated deletion of the SOCS3 gene in LysM-Cre and Tie2-Cre:SOCS3 fl/fl-derived BMDCs. The position of PCR primers A and B are also shown. B, PCR analysis of genomic DNA from BMDCs. The PCR product of the SOCS3 allele is 2000 bp. A band of 250 bp indicates Cre-mediated deletion of the SOCS3 gene in LysM-Cre and Tie2-Cre:SOCS3 fl/fl-derived BMDCs. Deletion efficiency is almost 100%. C, Effects of LPS on STAT3 activation in SOCS3−/− DCs. DCs from SOCS3−/+ mice and SOCS3−/− mice were cultured with LPS for 24 h. Whole cell extracts were immunoblotted with the indicated Abs. Data represent one of three similar experiments.

LPS injection and in vivo DC and T cell analysis
LPS (10 µg/head) derived from Escherichia coli O55:B5 (Sigma-Aldrich) was i.p. injected into SOCS3−/− (LysM-Cre:SOCS3 fl/fl or Tie2-Cre: SOCS3 fl/fl) and SOCS3−/+ mice. Sixteen hours later, spleens and mesenteric LNs were dissected. Single-cell suspensions were prepared and stained for flow cytometric analysis with anti-CD86, I-A<sup>δ</sup>, CD40, and CD11c Abs. Permeabilized T cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs.

Results

**Hyperactivation of STAT3 in SOCS3-deficient DCs**
SOCS3-deficient mice die during embryonic development as a result of placental deficiency. Thus, to delete the SOCS3 gene in BMDCs, SOCS3-flox/flox mice were crossed with either Tie2-Cre mice or LysM-Cre mice (Fig. 1A) (13, 14). Deletion of the SOCS3 gene was confirmed in BMDCs from both Tie2-Cre:SOCS3<sup>fl/fl</sup> mice and LysM-Cre:SOCS3<sup>fl/fl</sup> mice, in which the SOCS3 gene has been deleted in all hemopoietic lineages and monocytes/monocytes, respectively. The SOCS3 gene was efficiently deleted in BMDCs from these mice (Fig. 1B). Because similar data were obtained in BMDCs from Tie2-Cre:SOCS3<sup>fl/fl</sup> mice and LysM-Cre:SOCS3<sup>fl/fl</sup> mice, and we used only BMDCs as DCs for all experiments, data of BMDCs from Tie2-Cre:SOCS3<sup>fl/fl</sup> mice are shown and designated as SOCS3−/− DCs throughout the text, except for notification. Loss of the SOCS3 protein was confirmed by Western blotting in DCs stimulated with LPS. SOCS3 has been shown to be an important negative regulator for STAT3 (12). Therefore, we examined the effect of SOCS3 gene disruption on STAT3 activation. STAT3 was constitutively activated in SOCS3−/− DCs and further hyperactivated in response to LPS (Fig. 1C). These data were consistent with the previous observations that SOCS3 negatively regulates STAT3.

**Effect of SOCS3 deletion on DC maturation**
We then examined LPS-induced maturation of DCs. LPS up-regulates class II MHC (I-A<sup>δ</sup>), CD40, and costimulators (CD80 and CD86), and induces inflammatory cytokines, such as IFN-γ, IL-12, and IL-6 in wild-type (WT) (SOCS3<sup>+/+</sup>) DCs (Fig. 2A and B). However, up-regulation of these surface molecules and inflammatory cytokines was severely repressed in SOCS3−/− DCs (Fig. 2A and B). In contrast, secretion of IL-10 and production of NO were enhanced in SOCS3−/− DCs compared with SOCS3−/+ DCs. All these immature phenotypes were observed to be similar to those in tolerogenic IL-10-treated DCs (IL-10-DCs) (10, 19) (data not shown).

To confirm that SOCS3-deficient DCs are resistant to LPS-induced maturation in vivo, we i.p. injected LPS into SOCS3−/− mice, and I-A<sup>δ</sup> CD11c<sup>+</sup> DC fractions were analyzed. As shown in Fig. 2C, the levels of CD80 and CD40 were not significantly different between WT and SOCS3−/− splenic and LN DCs without LPS administration. As shown previously (20), injection of LPS strongly up-regulated CD86 and CD40 expression on splenic DCs in WT mice, whereas their up-regulation was partly repressed in SOCS3−/− splenic DCs. In LN DCs, CD86 up-regulation was also impaired in SOCS3−/− LN DCs (Fig. 2C). CD40 was not significantly up-regulated by LPS administration in both WT and SOCS3−/− LN DCs (data not shown). Thus, these data suggest that SOCS3-deficient DCs are resistant to LPS-induced maturation not only in vitro, but also in vivo.

**Low proliferation capacity of T cells expanded by SOCS3−/− DCs**
It has been shown that IL-10-DCs induce an Ag-specific anergy and suppressor activity in CD4<sup>+</sup> T cells (4, 5, 10). Thus, we examined the nature of T cells expanded by SOCS3−/− mice, and I-A<sup>δ</sup> CD11c<sup>+</sup> DCs were examined for all experiments. Data of BMDCs from Tie2-Cre:SOCS3 fl/fl mice. Sixteen hours later, spleens and mesenteric LNs were dissected. Single-cell suspensions were prepared and stained for flow cytometric analysis with anti-CD86, I-A<sup>δ</sup>, CD40, and CD11c Abs. Permeabilized T cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs.

**Preferential expansion of Foxp3-positive T cells by SOCS3−/− DCs**
Then we investigated the expression of Foxp3, which is essential for Treg development. Approximately 5–8% of naive CD4<sup>+</sup> T cells were CD25<sup>+</sup> Foxp3<sup>+</sup> (data not shown). It has been demonstrated that Foxp3<sup>+</sup> Treg cells are expanded by mature DCs in vitro (6). As shown in Fig. 3D, ~50–70% of CD25<sup>+</sup> T cells became Foxp3<sup>+</sup> positive after priming with SOCS3−/− DCs, whereas Foxp3-positive CD25<sup>+</sup> T cells were ~20–30% after priming with WT-DCs. Very high percentage of Foxp3<sup>+</sup> T cells after coculture with SOCS3−/− DCs is mostly due to lower expansion of CD25<sup>+</sup> T cells.
Foxp3− effector T cells by SOCS3−/− DCs (Fig. 3D). IL-10-DCs also hardly induced expansion of CD25+ Foxp3− effector T cells; however, CD25+ Foxp3+ T cells was not also expanded by IL-10-DCs. Thus, SOCS3−/− DC was apparently different from IL-10-DC, and preferentially promoted expansion of Foxp3+ T cells.
To examine whether SOCS3

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DCs can promote stronger ex-

pansion of Foxp3

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T cells with Ag-specific manner than WT-DCs in vitro and in vivo, we used T cells from OTII-TCR transgenic mice. Naive T cells from TCR transgenic mice have been shown to express low levels of Foxp3 (7). Syngeneic BMDCs were pulsed with OVA and cocultured with CD4

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T cells from OTII mice. Similar to allogeneic responses, proliferation of T cells restimu-

lated with anti-CD3 Ab was severely repressed by coculture with SOCS3

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DCs (Fig. 4A). In addition, expression of TGF-

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and Foxp3 was high in T cells after coculture with Ag-loaded SOCS3

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DCs (Fig. 4B).

In vivo administration of WT and SOCS3

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DCs resulted in an enhancement of Foxp3

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T cell fractions in splenic CD4

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T cells (Fig. 4C). SOCS3

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DCs induced expansion of Foxp3

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T cells more strongly than WT DCs in vivo (Fig. 4C). These data suggest that SOCS3

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DC has a higher potential for expansion of Foxp3

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Treg cells compared with Foxp3

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effector T cells both in vitro and in vivo.

To confirm higher Foxp3

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T cell expansion in vivo, we compared splenic and LN T cells from WT and SOCS3

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mice (Fig. 4D). Thymic CD4

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CD25

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T cell population was not different between WT and SOCS3

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mice (5.64% in Tie2-Cre:SOCS3fl/fl mice and 4.98% LysM-Cre:SOCS3fl/fl mice, whereas 5.33% in WT mice). However, the fractions of CD25

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Foxp3

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T cells in the spleen and LN of SOCS3

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mice were higher than those of WT mice. Especially in LN, CD25

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Foxp3

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T cells in SOCS3

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mice were 1.5 times more than those in WT mice (15.0 vs 10.9%). Similar results were obtained for both Tie2-Cre:SOCS3fl/fl and LysM-Cre:SOCS3fl/fl mice. Thus, our data suggest that Treg cells were expanded more efficiently at the periphery in SOCS3

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mice than in WT mice.

Recently, increased number of Treg cells has been observed in septic spleens (21). Therefore, we next examined in vivo effect of LPS administration on Treg expansion in WT and SOCS3

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mice. Consistent with previous report, the faction of CD25

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Foxp3

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T cells was increased from 8.14 to 11.1% in the spleen and
10.9 to 17.7% in the LN of WT mice. We noticed more CD25\(^+\)/Foxp3\(^+\) T cells were present in SOCS3\(^{-/-}\) mice (12.8% in the spleen and 19.6% in the LN) compared with WT mice. These data further support our hypothesis that SOCS3-deficient DCs have a stronger potential to expand Foxp3\(^+\) T cells not only in vitro, but also in vivo.

**SOCS3\(^{-/-}\) BMDC suppresses T cell activation in vivo**

Next, to assess the initiation of T cell responses in vivo, SOCS3\(^{-/-}\) and SOCS3\(^{+/-}\) DCs were pulsed with KLH and then injected into each footpad of the same mouse. Swelling of the popliteal LNs was observed on the side of the SOCS3\(^{+/-}\) DC-injected footpad, but little LN swelling was observed on the side of the SOCS3\(^{-/-}\) DC-injected footpad 5 days after injection (Fig. 5A). KLH-induced IFN-\(\gamma\) production from LN cells was lower in SOCS3\(^{-/-}\) DC-injected mice than WT-DC-injected mice (Fig. 5B), suggesting that SOCS3\(^{-/-}\) DC is less immunogenic than WT-DC.

Then we investigated the in vivo immunosuppressive effect of SOCS3\(^{-/-}\) DCs on experimental autoimmune encephalomyelitis (EAE). MOG-peptide-pulsed DCs were i.v. injected before immunization of the mice. Control mice receiving SOCS3\(^{-/-}\) DCs (WT-DCs) as well as untreated mice exhibited characteristic signs of EAE starting on day 8. In contrast, mice receiving SOCS3\(^{-/-}\) DCs developed significantly less severe EAE, indicating that SOCS3\(^{-/-}\) DC is immunosuppressive in vivo (Fig. 5C).

Then the nature of CD4\(^+\) T cells from mice with EAE was examined. The CD4\(^+\) T cells were isolated from the spleen of mice on day 15 and stimulated with anti-CD3 Ab. Proliferation and IFN-\(\gamma\) production of restimulated T cells from SOCS3\(^{-/-}\) DC-treated mice were severely reduced (Fig. 5D). However, levels of IL-17, which is important for EAE development (22), were not...
significantly different in restimulated T cells. Therefore, suppression of EAE by SOCS3−/− DCs was mainly due to reduced T cell activation. These data suggest a tolerogenic nature of SOCS3−/− DCs in vivo.

**T cells expanded by SOCS3−/− DCs have suppressor activity**

Then to examine a tolerogenic nature of SOCS3−/− DCs, suppression assay was conducted using T cells expanded by DCs. Expanded T cells primed with WT-DCs or SOCS3−/− DCs were cocultured with freshly isolated CD4+CD25− BALB/c responder T cells and γ-irradiated spleen cells as APCs. Proliferation of T cells (including not only responder T cells, but also DC-expanded T cells) in response to anti-CD3 Ab was measured (Fig. 6A). [3H]Thymidine incorporation was strongly enhanced when responder T cells were cocultured with WT-DCs (Fig. 6A, center). In contrast, T cells expanded by SOCS3−/− DC marginally proliferated and suppressed proliferation of responder T cells (Fig. 6A, right). These data confirmed that SOCS3−/− DCs predominantly expand Foxp3+ T cells and poorly expand Foxp3− effector T cells (see Fig. 3D).

Then, to compare the suppression activity, Treg cells were expanded from naive CD4+CD25− T cells by coculturing with WT or SOCS3−/− DCs. WT-DCs and SOCS3−/− DCs showed similar expansion of CD4+CD25− T cells (data not shown), and expanded Treg cells possessed similar suppression activity (Fig. 6B). Thus, Foxp3+ T cells expanded by SOCS3−/− DCs can be taken as Treg cells, although we could not compare suppressor activity of CD4+CD25+ T cells expanded by SOCS3−/− DCs and naturally occurring Treg cells because former fraction contained 30–40% Foxp3− effector T cells. Because SOCS3−/− DCs predominantly expanded Foxp3+ Treg cells from CD4+ T cells, whereas WT-DCs expanded Foxp3− effector T cells, only CD25+ T cells expanded by SOCS3−/− DCs could show suppression activity in vitro (Fig. 6A). This implies that SOCS3 is an important factor for determining immunity or tolerance in DC.

**Higher production of TGF-β1 is important for higher Foxp3+ Treg expansion by SOCS3−/− DCs**

TGF-β1 and IL-2 have been implicated in the induction of Foxp3+ Treg cells by DCs in vitro (23–25). IL-2 has been shown to be important for Foxp3+ T cells expansion by DCs (23–25). Higher production of TGF-β1 and IL-2 from DCs (26) to define the molecular basis for Foxp3+ Treg cell expansion by SOCS3−/− DCs, we examined the effect of Abs against cytokines. Anti-IFN-γ Ab showed little effect on Foxp3+ Treg expansion by DCs (Fig. 7A). Anti-IL-2 Ab showed profound effect on Foxp3+ Treg cell expansion by WT-DCs or SOCS3−/− DCs, suggesting an important role of IL-2 in effector T cell and Treg expansion (Fig. 7A). However, we could not find any differences in the IL-2 production between WT and SOCS3−/− DCs (Fig. 7B). Thus, IL-2 from DCs may not be able to account for the differences between WT and SOCS3−/− DCs.
TGF-β1 levels were higher in SOCS3-deficient T cells and hepatocytes than in normal cells (27). Therefore, we measured production of biologically active TGF-β1 by using a reporter cell line, MFB-F11 (18). MFB-F11 cells were stably transfected with a reporter plasmid consisting of TGF-β-responsive Smad-binding elements coupled to a SEAP reporter gene. This cell line can detect a biologically active form of TGF-β1 (all three forms of TGF-β) with extremely high sensitivity (detectable as little as 1 pg/ml.)

**FIGURE 6.** Suppressor activity of T cells primed with SOCS3-deficient DCs. A, CD25⁺ T cells (suppressors) were isolated from CD4⁺ T cells expanded by SOCS3⁺/⁺ or SOCS3⁻/⁻ DCs. These cells were further cocultured with CD4⁺ CD25⁺ cells (3 × 10⁶) from BALB/c mice (responders) and APCs in the presence of soluble anti-CD3 Ab (1 μg/ml) for 72 h. Proliferation was assessed by [³H]thymidine uptake. In the left three columns, proliferation of suppressors and responders alone (3 × 10⁶ cells) is shown. The numbers of responder and APCs were fixed, and the ratio of suppressor/responder is shown on the right of the panel. Similar results were obtained in two independent experiments. B, Suppressor activity by DC-expanded CD4⁺CD25⁺ T cells. CD4⁺CD25⁺ T cells were isolated from BALB/c mice and expanded by coculturing with SOCS3⁻/⁻ or SOCS3⁺/⁺ DCs. These cells were further cocultured with CD4⁺CD25⁺ cells (1 × 10⁶) from BALB/c mice (responders) and allogeneic APCs from C57BL/6 for 72 h. In the left three columns, proliferation of suppressors and responders alone (1 × 10⁵ cells) is shown as in the left columns (*, p < 0.01; Student’s t test, vs responder).

**FIGURE 7.** Essential role of TGF-β1 in selective Foxp3⁺ T cell expansion by SOCS3⁻/⁻ DCs. A, Effect of anti-cytokine Abs. Allogeneic CD4⁺ T cells were stimulated with DCs in the presence of 10 μg/ml indicated Abs for 4 days and analyzed by FACS. B, Production of IL-2 and TGF-β1 from LPS-stimulated DCs for 24 h. IL-2 and TGF-β1 levels were determined by ELISA and a bioassay on MFB-F11 cells, respectively. One representative experiment of three is shown (*, p < 0.05; Student’s t test). C, Total RNA (1 μg) of DCs treated with LPS for 24 h was extracted, and the mRNA levels of indicated genes were determined by RT-PCR. IL-10 treatment was done 48 h before LPS stimulation (IL-10-DC). One representative experiment of three is shown.
active TGF-β1). As shown in Fig. 7B, WT-DCs secreted TGF-β at below detectable levels by MFB-F11. In contrast, SOCS3−/− DCs secreted biologically active TGF-β at ~10 pg/ml, and the secretion was slightly enhanced by LPS treatment. Up-regulation of TGF-β1 in SOCS3−/− DCs was confirmed by RT-PCR (Fig. 7C). TGF-β1 levels in IL-10-DC were as low as those in WT-DCs (Fig. 7C). These data suggest that higher expression of TGF-β in SOCS3−/− DCs is one of an important mechanism for enhanced Foxp3+ T cell expansion by SOCS3−/− DCs.

Discussion

DCs have an important role in the control of the adaptive immune response. They simultaneously induce not only Ag-specific effector T cells, but also Treg cells (24). However, little is known how DCs balance induction of these functionally opposite T cells. In this study, we found that SOCS3 in BMDCs plays a critical role in the balance of Foxp3+ Treg cells and effector T cells. Although the mechanism is still not clear at present, predominant Foxp3+ Treg expansion by SOCS3−/− DCs is striking. Enhanced Foxp3+ Treg expansion was observed not only in vitro, but also in vivo (Fig. 4). We described in this study that SOCS3-deficient DCs were tolerogenic probably because of selective expansion of Treg cells. TGF-β1 has been shown to strongly enhance Foxp3+ Treg expansion from naive CD4+ T cell (8, 23). Our Ab-blocking experiments (Fig. 7A) confirmed that Foxp3+ Treg expansion by SOCS3−/− DC is dependent on TGF-β1. We showed that TGF-β1 production was suppressed by expression of dominant-negative STAT3 and enhanced by STAT3c (27, 28). Furthermore, the TGF-β1 promoter contains two potential STAT3 binding sites, and STAT3 enhanced TGF-β1 promoter activity (28). These data suggest that STAT3 positively and SOCS3 negatively regulates the production of TGF-β1 from DCs. However, the levels of TGF-β1 in the culture supernatant of SOCS3−/− DCs were as low as 10 pg/ml. This level was too low to induce Foxp3+ T cells from CD4+CD25− naive T cells by anti-TCR stimulation in vitro (data not shown). Thus, we suspect that local TGF-β1 activation at cell-cell contact sites or cell surface-bound active TGF-β1 is important for the induction of Foxp3+ T cells. In addition to higher TGF-β1, other phenotypes of SOCS3−/− DCs may be also necessary to selectively expand Foxp3+ T cells.

We suspect that lower levels of class II MHC and costimulators in SOCS3−/− DCs are also important for selective Foxp3+ Treg expansion, because effector T cells require higher amounts of anti-TCR stimulation for proliferation than Treg cells (Y. Matsumura, unpublished data). However, because IL-10-DCs also have an immature phenotype, but did not strongly induce Foxp3+ Treg cells (Fig. 3D), the immature nature of DCs is not sufficient to explain the ability of SOCS3−/− DCs. IL-10-DC did not express high levels of TGF-β1 (Fig. 7C). Probably, both the immature phenotype of DCs and the high levels of TGF-β1 are necessary for the predominant expansion of Foxp3+ Treg cells by SOCS3−/− DCs. Effector T cell expansion was reduced because of lower levels of MHC and higher levels of TGF-β1 in SOCS3−/− DCs, whereas the expansion of Treg cells may not be so strongly affected by these. This idea is consistent with a recent paper showing a predominant induction of Foxp3+ cells by immature splenic DCs in the presence of small amount of TGF-β1 (23).

Recently, Li et al. (29) reported that DCs overexpressing SOCS3 exhibit a tolerogenic phenotype that directs Th2 differentiation and suppresses EAE. This situation resembles the phenotypes of T cells overexpressing or lacking SOCS3. We have shown that forced expression of SOCS3 in T cells promotes Th2 differentiation, whereas deletion of the SOCS3 gene in T cells induces TGF-β1-secreting Th3 cells (28). Li et al. (29) demonstrated that enhanced SOCS3 expression in DCs blocked the IL-12 and IL-23 responses, and that SOCS3-transduced DCs expressed a low level of MHC class II and CD86 on their surface, producing a high level of IL-10, but low levels of IL-12. Thus, SOCS3-overexpressing DCs resemble IL-10-DCs (30). It is still not very clear how SOCS3 in DCs regulates the induction of Foxp3+ Treg and Th2. Our data and these works indicate that the regulation of intracellular signaling pathways is extremely important for the decision of Th cell fates.

Acknowledgments

We thank Mr. Ohtsu, Y. Honda, and T. Yoshioka for technical assistance; Y. Nishi for manuscript preparation; and Dr. Y. Fukui and Dr. T. Hanada for discussion and comments.

Disclosures

The authors have no financial conflict of interest.

References


