

Materials and Methods

Mice

Stat5a-deficient (Stat5a^{-/-}) mice (15) were backcrossed to BALB/c mice (Charles River Breeding Laboratories) for eight generations and littermate wild-type (WT) mice were used as controls. All experiments were performed according to the guidelines of Chiba University (Chiba, Japan).

Cell culture

Splenocytes (2 × 10⁶ cells/ml) from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb (5 μg/ml, clone 145-2C11; BD Pharmingen) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics in a 24-well microtiter plate at 37°C for 48 h. As indicated, IL-12 (15 ng/ml; Pepro-Tech) was added to polarize toward Th1 cells (Th1 condition), and IL-4 (15 ng/ml; PeproTech) and anti-IFN-γ mAb (15 μg/ml, clone XMG1.2; BD Pharmingen) were added to polarize toward Th2 cells (Th2 condition) (11). Cells were washed with PBS and then cultured for another 72 h in Th1 or Th2 condition in the presence of IL-2 (10 ng/ml; PeproTech). In some experiments, either IL-4 (15 ng/ml) or anti-IFN-γ mAb (15 μg/ml) was added to the culture. In separate experiments, the indicated amounts of IL-12 (1–8 ng/ml) were added to the culture.

Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4 FITC, anti-CD4 PE, anti-CD4 PerCP (H129.19), anti-IL-12Rβ1 (clone 114), and anti-IL-12Rβ2 (HAM10B9). Anti-IL-12Rβ1 Ab and anti-IL-12Rβ2 Ab were visualized by anti-mouse IgG2a FITC (BD Pharmingen) and anti-hamster IgG PE (BD Pharmingen), respectively. Before staining, FcRs were blocked with anti-CD16/CD32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, nonspecific Abs (BD Pharmingen).

Intracellular cytokine analysis

Intracellular cytokine staining for IL-4 vs IFN-γ was performed as previously described (11). In brief, cultured splenocytes were restimulated with plate-bound anti-CD3 mAb at 37°C for 6 h, with monensin (2 μM; Sigma-Aldrich) added for the final 4 h. After FcRs were blocked with anti-CD16/CD32 Ab (2.4G2; BD Pharmingen), cells were stained with anti-CD4 PerCP (H129.19; BD Pharmingen), fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IL-4 PE (BVD4-1D11; BD Pharmingen) and anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen) at 4°C for 30 min. Cytokine profile (IL-4 vs IFN-γ) on CD4⁺ cells or CD4⁺GFP⁺ cells (in the case of retrovirus experiments) was analyzed on a FACSCalibur.

Intracellular staining for phosphorylated Stat4

Intracellular staining for tyrosine-phosphorylated Stat4 was performed as described elsewhere (16) with a minor modification. In brief, splenocytes from WT mice and Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb at 37°C for 48 h in Th1 or Th2 condition. Cells were washed and then cultured for another 72 h with fresh medium in Th1 or Th2 condition in the presence of IL-2 (10 ng/ml). Cells were starved from cytokines for 8 h and then stimulated with or without IL-12 (15 ng/ml) at 37°C for 20 min. Cells were stained with anti-CD4 FITC at 4°C, fixed with IC FIX, and permeabilized with 90% methanol and subsequently with IC PERM. Cells were then incubated with rabbit polyclonal anti-phospho-Stat4 Ab (Zymed Laboratories) or normal rabbit serum (as a control) for 30 min and visualized with Alexa Fluor 647 chicken anti-rabbit IgG Ab (Molecular Probes). The levels of anti-phospho-Stat4 staining were evaluated on CD4⁺ population.

Intracellular staining for SOCS3

Splenocytes from WT mice and Stat5a^{-/-} mice were stimulated with anti-CD3 mAb at 37°C for 48 h in Th1 or Th2 condition. Cells were washed and then cultured for another 72 h with fresh medium in Th1 or Th2 condition in the presence of IL-2 (10 ng/ml). Cells were stained with anti-CD4 FITC at 4°C, fixed with IC FIX, and permeabilized with 90% methanol and with IC PERM. Cells were then incubated with biotin-labeled anti-SOCS3 Ab (Medical & Biological Laboratories) or biotin-labeled mouse IgG1 (as a control) for 30 min and visualized with streptavidin-allophycocyanin (BD Pharmingen). The levels of anti-SOCS3 staining were evaluated on CD4⁺ population. To examine the specificity of staining for SOCS3, Plat-E cells that were transfected with SOCS3 expression vector were used as a positive control.

Western blot analysis

Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th2 condition. Cells were cultured for another 72 h in Th2 condition in the presence of IL-2. After CD4⁺ T cells were purified using anti-CD4 FITC and anti-FITC microbeads (BD Pharmingen) (>90% pure by flow cytometry) and rested for 8 h in the fresh medium, cells were stimulated with IL-12 (15 ng/ml) for 20 min and whole cell lysates were subjected to immunoblotting as previously described (17). Anti-phospho-Stat4 Ab and anti-mouse Stat4 Ab were purchased from Zymed Laboratories.

TaqMan PCR analysis

Total cellular RNA was prepared as previously described (18). The expression levels of SOCS3 mRNA were determined by real-time PCR using a standard protocol on ABI PRISM 7000 instrument (Applied Biosystems). PCR primers and a fluorogenic probe for SOCS3 were previously described (13). The levels of SOCS3 mRNA were normalized to the levels of GAPDH mRNA (Applied Biosystems).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were conducted using the ChIP Assay kit (Upstate Biotechnology) according to the manufacturer's instruction with some modifications. Briefly, splenocytes from WT mice and Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb at 37°C for 48 h. CD4⁺ T cells were purified (>90% pure by flow cytometry) using a CD4⁺ T cell enrichment column (R&D Systems), starved from cytokines in fresh RPMI 1640 medium for 3 h, and then stimulated with IL-2 (20 ng/ml) at 37°C for 30 min. Cells were fixed with 1% formaldehyde to cross-link chromatin at room temperature for 15 min and then at 4°C for 45 min. Cells were lysed with SDS lysis buffer and then sonicated on ice to shear DNA to lengths between 500 and 700 bp. Sonicated lysates were centrifuged at 13,000 rpm at 4°C for 10 min, and the sonicated cell pellet was suspended in ChIP dilution buffer. The sonicated chromatin was then immunoprecipitated with anti-Stat5a antisera (R&D Systems) or control rabbit serum at 4°C for 12 h. The anti-Stat5a immunoprecipitates were purified with protein A-agarose. After deproteination and reversal of cross-links, the amounts of selected DNA sequences in the immunoprecipitates were assessed by PCR. The sequences of the primers for SOCS3 promoter are 5'-TTTGCTCCTCTCGGTGA-3' and 5'-GTGTAGAGTACAGAGTTAGAG-3'. The sequences of the primers for β-globin promoter (as a control) were described elsewhere (19).

Luciferase assay

Stat6-dependent reporter plasmid, TPU474 (20), was a kind gift from Dr. U. Schindler (Tularik, San Francisco, CA). Stat5-dependent reporter plasmid, mammary gland factor luciferase (MGF-Luc) (21), was a kind gift from Dr. H. Wakao (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan). Murine SOCS3 promoter (-273 to +160) (22) was amplified by PCR using murine genomic construct as a template and inserted into *KpnI/BglII* site of pGL3-basic vector (Promega) to generate SOCS3 WT Luc. Putative Stat-binding sequences of SOCS3 WT Luc were mutated (mt) in SOCS3 mutagenesis (mt1) Luc, SOCS3 mt2 Luc, and SOCS3 mt3 Luc (see Fig. 5a) using a PCR-based site-directed mutagenesis kit (Stratagene). Mutation was confirmed by DNA sequencing. COS7 cells were transiently transfected with either TPU474, MGF Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3 mt2 Luc, or SOCS3 mt3 Luc with pRL-TK in the presence or absence of the expression vectors of a constitutively active form of Stat5a (1*6 Stat5a) (23) (pcDNA 1*6 Stat5a) or a constitutively active form of Stat6 (Stat6VT) (24) (pcDNA Stat6VT) using FuGENE6 transfection reagents (Roche Diagnostics). Twenty-four hours after transfection, the luciferase activity was measured by the dual luciferase assay system (Promega). Firefly luciferase activity of TPU474, MGF Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3 mt2 Luc, or SOCS3 mt3 Luc was normalized by Renilla luciferase activity of pRL-TK. All values were obtained from experiments conducted in triplicate and repeated at least four times.

Retrovirus-mediated gene expression

Bicistronic retrovirus vector pMX-IRES-GFP (23) was a kind gift from Dr. T. Kitamura (Tokyo University, Tokyo, Japan). pMX-SOCS3-IRES-GFP was previously described (14). Retroviruses were produced with a transient retrovirus packaging cell line, Plat-E (25), and stored at -80°C until use. For infection to T cells, after splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 40 h in the presence or absence of IL-4 (15 ng/ml), cells were incubated with 500 μl of the retrovirus in the presence of IL-2 (20 ng/ml) in a 24-well microtiter plate

that was coated with RetroNectin (27 $\mu\text{g/ml}$; Takara Shuzo). After 4 h of infection, 500 μl of fresh medium was added to the culture and cells were allowed to grow for another 72 h in the presence or absence of IL-4 before being subjected to intracellular cytokine analysis. Under these conditions, the efficiency of infection to CD4⁺ T cells was 15–30% as assessed by GFP⁺ cells by FACS.

Data analysis

Data are summarized as mean \pm SD. The statistical analysis of the results was performed by the unpaired *t* test. Values for *p* < 0.05 were considered significant.

Results

IFN- γ plays a dominant inhibitory role in the down-regulation of Th2 cell differentiation in Stat5a^{-/-} CD4⁺ T cells

We have previously shown that Th2 cell differentiation is impaired but Th1 cell differentiation is increased in Stat5a^{-/-} CD4⁺ T cells (11). Because Th1 cells suppress Th2 cell differentiation by producing cytokines such as IFN- γ (1–3), it is possible that the enhanced Th1 cell differentiation is a principal reason for the decreased Th2 cell differentiation of Stat5a^{-/-} CD4⁺ T cells. To determine whether Th1 cytokines participate in the decreased Th2 cell differentiation in Stat5a^{-/-} CD4⁺ T cells, we first examined the effect of neutralizing anti-IFN- γ Ab on Th2 cell differentiation in Stat5a^{-/-} CD4⁺ T cells. After a 5-day culture of anti-CD3-stimulated splenocytes, Th2 cell differentiation was significantly decreased but Th1 cell differentiation was increased in Stat5a^{-/-} CD4⁺ T cells as compared with those in WT CD4⁺ T cells (Fig. 1, *a* and *b*). IL-4 alone could not significantly induce Th2 cell differentiation of Stat5a^{-/-} CD4⁺ T cells (Fig. 1, *b* vs *f*). In contrast, in the presence of anti-IFN- γ Ab, IL-4 strongly induced Th2 cell differentiation in Stat5a^{-/-} CD4⁺ T cells (Fig. 1*h*). Also, IL-4 itself significantly induced Th2 cell differentiation of WT CD4⁺ T cells (Fig. 1, *a* vs *e*) and the Th2 cell differentiation was further increased in the presence of anti-IFN- γ Ab (Fig. 1*g*). These results indicate that enhanced Th1 cell differentiation and the resultant IFN- γ production play a dominant inhibitory role in the down-regulation of IL-4-induced Th2 cell differentiation of Stat5a^{-/-} CD4⁺ T cells. In addition, even in the presence of anti-IFN- γ Ab, the frequency of Th2 cells was still lower in Stat5a^{-/-} CD4⁺ T

cells than that in WT CD4⁺ T cells (Fig. 1, *g* vs *h*), suggesting that other mechanisms are also involved in Stat5a-induced Th2 cell differentiation.

IL-12-induced Th1 cell differentiation is enhanced in Stat5a^{-/-} CD4⁺ T cells

IL-12 plays a critical role in the induction of Th1 cell differentiation (26). To determine whether IL-12/Stat4 signaling plays a causative role in the enhanced Th1 cell differentiation in Stat5a^{-/-} CD4⁺ T cells, we first examined the sensitivity of Stat5a^{-/-} CD4⁺ T cells to IL-12-induced Th1 cell differentiation. As shown in Fig. 2, even in the absence of exogenous IL-12, the frequency of Th1 cells after a 5-day culture of anti-CD3-stimulated splenocytes was significantly higher in Stat5a^{-/-} CD4⁺ T cells than that in WT CD4⁺ T cells (*n* = 4, *p* < 0.05). Importantly, a low concentration (1 ng/ml) of IL-12 significantly increased the number of Th1 cells in Stat5a^{-/-} CD4⁺ T cells but not in WT CD4⁺ T cells (*n* = 4, *p* < 0.01), whereas a high concentration of IL-12 (4 or 8 ng/ml) increased the number of Th1 cells not only in Stat5a^{-/-} CD4⁺ T cells but also in WT CD4⁺ T cells (Fig. 2). In contrast, the levels of IL-12 production in CpG ODN-stimulated CD11c⁺ dendritic cells were similar in Stat5a^{-/-} mice and WT mice (data not shown). These results indicate that enhanced IL-12 responsiveness of CD4⁺ T cells but not the capacity of IL-12 production from APCs is responsible for the increased Th1 cell differentiation in Stat5a^{-/-} CD4⁺ T cells.

IL-12-induced Stat4 phosphorylation is enhanced in Stat5a^{-/-} CD4⁺ T cells

It is well recognized that Stat4 activation is essential for IL-12-induced Th1 cell differentiation (26). Therefore, we next examined IL-12-induced Stat4 phosphorylation in Stat5a^{-/-} CD4⁺ T cells. Interestingly, Stat5a^{-/-} CD4⁺ T cells that were cultured in Th2-polarizing condition exhibited the enhanced IL-12-induced Stat4 phosphorylation as compared with WT CD4⁺ T cells (*n* = 4, *p* < 0.01) (Fig. 3*a*). In contrast, IL-12-induced Stat4 phosphorylation was not significantly enhanced in Stat5a^{-/-} CD4⁺ T cells that were cultured in Th1-polarizing condition (Fig. 3*a*). Enhanced IL-12-induced Stat4 phosphorylation of Stat5a^{-/-} CD4⁺ T cells in

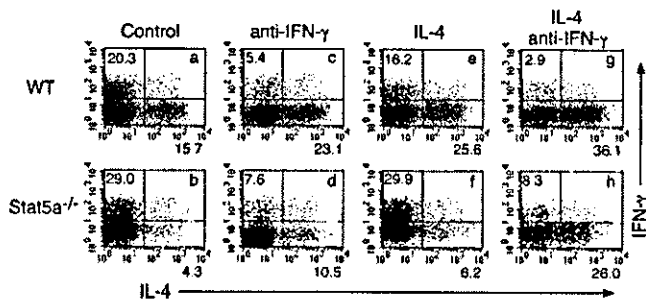


FIGURE 1. IFN- γ plays a dominant inhibitory role in the down-regulation of Th2 cell differentiation in Stat5a^{-/-} CD4⁺ T cells. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of IL-4 (15 ng/ml) and/or neutralizing anti-IFN- γ Ab (15 $\mu\text{g/ml}$). After washing, cells were cultured in the presence of IL-4 and/or neutralizing anti-IFN- γ Ab for another 72 h. IL-2 (10 ng/ml) was added in the second culture to prevent apoptosis. The number of apoptotic annexin V⁺CD4⁺ cells was not significantly different between WT mice and Stat5a^{-/-} mice in this condition (data not shown), consistent with our previous finding that Stat5a^{-/-} CD4⁺ T cells proliferate normally in the presence of a high concentration of IL-2 (36). Cells were then restimulated with plate-bound anti-CD3 mAb for 6 h and intracellular cytokine profiles for IL-4 vs IFN- γ were determined on CD4⁺ T cells. Shown are representative FACS profiles from five mice in each group.

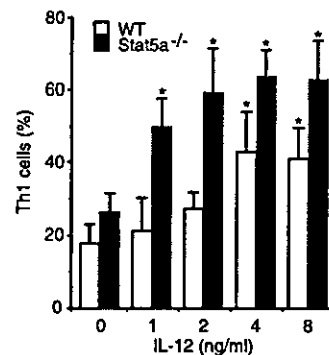


FIGURE 2. IL-12-induced Th1 cell differentiation is enhanced in Stat5a^{-/-} CD4⁺ T cells. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of the indicated amounts of IL-12 (1–8 ng/ml). Cells were cultured for another 72 h in the presence of the same amounts of IL-12 and IL-2 (10 ng/ml). Cells were then restimulated with plate-bound anti-CD3 mAb for 6 h and intracellular cytokine profiles for IL-4 vs IFN- γ were determined on CD4⁺ T cells. Data are mean \pm SD of percentage of Th1 cells (IL-4⁻IFN- γ ⁺ cells) from four independent experiments. *, *p* < 0.01. Significantly different from the mean value of the corresponding control response (no IL-12).

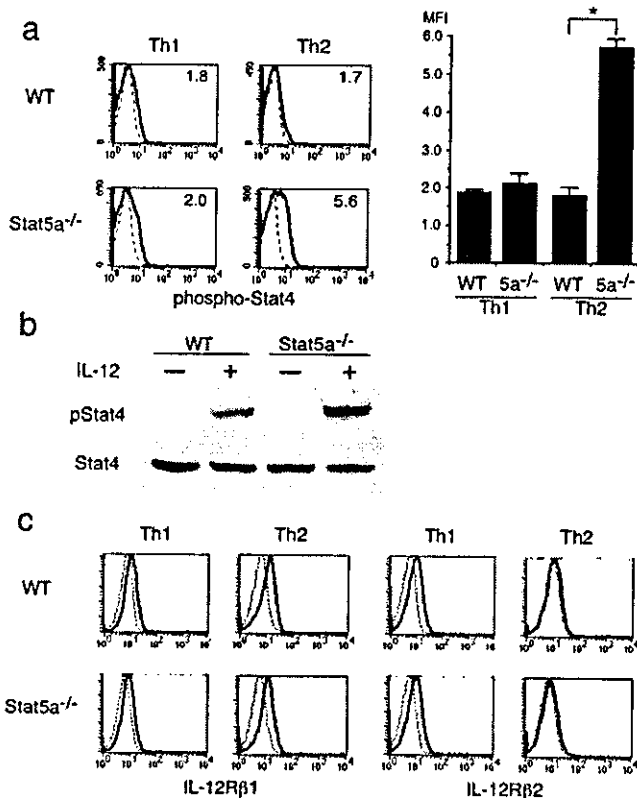


FIGURE 3. IL-12-induced Stat4 phosphorylation is enhanced in Stat5a^{-/-} CD4⁺ T cells. *a*, IL-12-induced Stat4 phosphorylation in Stat5a^{-/-} CD4⁺ T cells. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th1-polarizing condition (15 ng/ml, in the presence of IL-12) or Th2-polarizing condition (15 ng/ml, in the presence of IL-4 and 15 μg/ml, anti-IFN-γ Ab). Cells were cultured for another 72 h in Th1 or Th2 condition in the presence of IL-2. After cells were washed and rested for 8 h in the fresh medium, cells were then stimulated with IL-12 (15 ng/ml) for 20 min, and intracellular staining for the phosphorylated form of Stat4 was performed. Representative anti-phospho-Stat4 staining gated on CD4⁺ T cells (*left*) and the mean fluorescence intensity (MFI) of anti-phospho-Stat4 staining (*right*) are shown. Dashed lines are FACS profiles of anti-phospho-Stat4 staining without IL-12 stimulation. Data are mean ± SD from four experiments. *, *p* < 0.01. *b*, Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb in Th2 condition. After CD4⁺ T cells were purified using anti-CD4 FITC and anti-FITC microbeads (>90% pure by flow cytometry) and rested for 8 h in the fresh medium, cells were stimulated with or without IL-12 for 20 min and whole cell lysates were subjected to immunoblotting with anti-phospho-Stat4 Ab (*top*) and anti-Stat4 Ab (*bottom*). Shown is a representative immunoblot from four independent experiments. *c*, Expression of IL-12R on Stat5a^{-/-} CD4⁺ T cells. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th1 or Th2 condition and for another 72 h in Th1 or Th2 condition in the presence of IL-2. The expression of IL-12Rβ1 and IL-12Rβ2 on CD4⁺ T cells was evaluated by FACS. Shown are representative FACS profiles of anti-IL-12Rβ1 or anti-IL-12Rβ2 staining from four independent experiments. Dashed lines are FACS profiles for the isotype-matched controls.

Th2-polarizing condition was confirmed by immunoblotting (Fig. 3*b*). Yet, IFN-γ-induced Stat1 phosphorylation was similarly observed in WT CD4⁺ T cells and Stat5a^{-/-} CD4⁺ T cells in both Th1- and Th2-polarizing condition (data not shown). These results suggest that the inhibitory machinery that prevents IL-12/Stat4 signaling in developing Th2 cells is impaired in Stat5a^{-/-} CD4⁺ T cells.

Because IL-12 responsiveness is regulated in part by the expression levels of its receptor (27), we next examined the expression of

IL-12Rβ1 and IL-12Rβ2 on Stat5a^{-/-} CD4⁺ T cells. However, FACS analysis revealed that both IL-12Rβ1 and IL-12Rβ2 were normally expressed in Stat5a^{-/-} CD4⁺ T cells even in Th2-polarizing condition (Fig. 3*c*), suggesting that the expression levels of IL-12Rs are not likely to be responsible for the enhanced IL-12-induced Stat4 phosphorylation in developing Th2 cells by the absence of Stat5a.

SOCS3 expression is decreased in Stat5a^{-/-} CD4⁺ T cells

Increasing evidence indicates that SOCS family proteins negatively regulate JAK/STAT signaling pathways (28–30). Recently, it has been demonstrated that one of SOCS family proteins, SOCS3, is preferentially expressed in Th2 cells and inhibits IL-12-induced Stat4 phosphorylation (13, 14). To determine whether SOCS3 is involved in the enhanced IL-12 responsiveness in Stat5a^{-/-} CD4⁺ T cells (Figs. 2 and 3*a*), we first examined the expression levels of SOCS3 mRNA in Stat5a^{-/-} CD4⁺ T cells. Consistent with previous reports (13, 14), SOCS3 mRNA was detectable in freshly isolated WT CD4⁺ T cells by real-time PCR analysis (Fig. 4*a*) and the expression levels were enhanced when

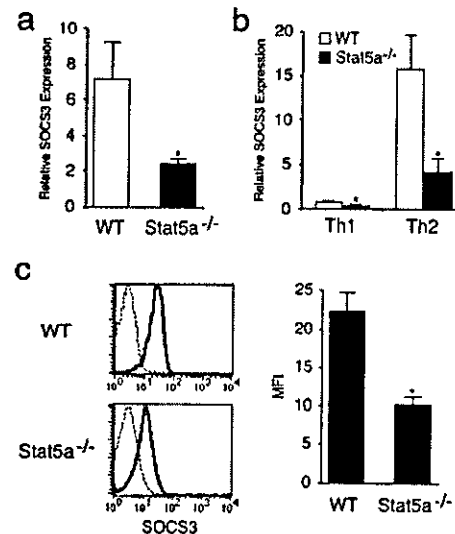


FIGURE 4. SOCS3 is diminished in Stat5a^{-/-} CD4⁺ T cells. *a*, Expression levels of SOCS3 mRNA in freshly isolated CD4⁺ T cells. Total RNA was prepared from freshly isolated splenic WT CD4⁺ T cells or Stat5a^{-/-} CD4⁺ T cells. TaqMan PCR analysis for SOCS3 and GAPDH (as a control) mRNA was performed and the levels of SOCS3 mRNA were normalized to the levels of GAPDH mRNA. Data are means ± SD from four experiments. *, *p* < 0.01, significantly different from the mean value of WT CD4⁺ T cells. *b*, Expression levels of SOCS3 mRNA in Th1- or Th2-polarized CD4⁺ T cells. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb in Th1 or Th2 condition for 48 h and then for another 72 h in Th1 or Th2 condition in the presence of IL-2. After CD4⁺ T cells were purified using a CD4⁺ T cell enrichment column, total RNA was prepared from these cells and TaqMan PCR for SOCS3 and GAPDH mRNA was performed. Data are mean ± SD from four experiments. *, *p* < 0.01, significantly different from the mean value of the corresponding WT CD4⁺ T cells. *c*, Expression of SOCS3 at protein levels. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb in Th2 condition for 48 h and then for another 72 h in Th2 condition in the presence of IL-2. Intracellular staining for SOCS3 was performed as described in *Materials and Methods*. Representative anti-SOCS3 staining gated on CD4⁺ T cells (*left*) and the mean fluorescence intensity (MFI) of anti-SOCS3 staining (*right*) are shown. Dashed lines are FACS profiles for the isotype-matched controls. Data are mean ± SD from four experiments. *, *p* < 0.01, significantly different from the mean value of WT CD4⁺ T cells.

cells were cultured in Th2-polarizing condition (Fig. 4*b*). As shown in Fig. 4*a*, the expression levels of SOCS3 mRNA were significantly decreased in freshly isolated Stat5a^{-/-} CD4⁺ T cells (*n* = 4, *p* < 0.01). The expression of SOCS3 mRNA was up-regulated in Th2-polarizing condition even in Stat5a^{-/-} CD4⁺ T cells, but the expression levels were still lower than those in WT CD4⁺ T cells (*n* = 4, *p* < 0.01) (Fig. 4*b*). Decreased expression of SOCS3 of Stat5a^{-/-} CD4⁺ T cells in Th2-polarizing condition was confirmed at protein levels by intracellular FACS analysis (*n* = 4, *p* < 0.01) (Fig. 4*c*). These results suggest that Stat5a regulates the expression levels of SOCS3 in CD4⁺ T cells.

Stat5a activates SOCS3 promoter

It has been shown that the SOCS3 promoter contains putative tandem STAT-binding sequences (Fig. 5*a*) (22). We therefore investigated whether Stat5a activated SOCS3 promoter. Either a constitutively active form of Stat5a (1*6 Stat5a) or of Stat6 (Stat6VT) was expressed in COS7 cells, and SOCS3 WT Luc was determined by a reporter assay (Fig. 5*b*). The activity of SOCS3 WT Luc was significantly enhanced by the expression of 1*6 Stat5a but not of Stat6VT (Fig. 5*b*). When one of the putative STAT-binding sequences located in the SOCS3 promoter was mutated (SOCS3 mt1 Luc or SOCS3 mt2 Luc) (Fig. 5*a*), 1*6 Stat5a-induced activation was largely abolished (Fig. 5*b*). 1*6 Stat5a-induced activation of the SOCS3 promoter was more severely decreased when both STAT-binding sequences were simultaneously mutated (SOCS3

mt3 Luc) (Fig. 5*b*). As positive controls, we confirmed that the expression of 1*6 Stat5a preferentially activated a Stat5-dependent reporter construct (MGF-Luc) (21), whereas Stat6VT preferentially activated a Stat6-dependent reporter construct TPU474 (Fig. 5*b*) (20). These results indicate that Stat5a but not Stat6 activates the SOCS3 promoter.

Stat5a binds to the SOCS3 promoter in CD4⁺ T cells

To determine whether Stat5a binds to the SOCS3 promoter in CD4⁺ T cells, we next examined Stat5a binding to the SOCS3 promoter by a ChIP. Activated CD4⁺ T cells from WT mice or Stat5a^{-/-} mice were stimulated with IL-2 for 30 min, fixed with formaldehyde, and sonicated to reduce the DNA length between 500 and 700 bp. After the sonicated chromatin from these cells were immunoprecipitated with anti-Stat5a Ab, the amount of DNA sequences derived from the SOCS3 promoter (from -214 to +13) or the β-globin promoter (as a control) in the immunoprecipitates was assessed by PCR. As shown in Fig. 6, anti-Stat5a Ab precipitated DNA derived from the SOCS3 promoter but not from the β-globin promoter in IL-2-stimulated WT CD4⁺ T cells. As anticipated, anti-Stat5a Ab did not precipitate DNA derived from the SOCS3 promoter in Stat5a^{-/-} CD4⁺ T cells even when stimulated with IL-2 (Fig. 6). These results indicate that Stat5a binds to the SOCS3 promoter in CD4⁺ T cells.

Enforced expression of SOCS3 restores Th cell differentiation in Stat5a^{-/-} CD4⁺ T cells

We finally examined the effect of SOCS3 expression on the impaired Th cell differentiation of Stat5a^{-/-} CD4⁺ T cells. We used bicistronic retrovirus-mediated gene expression system, in which infected cells were identified by coexpressed GFP. Splenocytes from Stat5a^{-/-} mice were stimulated with anti-CD3 mAb and infected with pMX-SOCS3-IRES GFP retrovirus or pMX-IRES-GFP retrovirus (as a control) in the presence or absence of IL-4. As a control, splenocytes from WT mice were stimulated with anti-CD3 mAb and infected with pMX-IRES-GFP retrovirus. Three days after infection, intracellular cytokines (IL-4 vs IFN-γ) were analyzed on GFP-expressing CD4⁺ T cells. As shown in Fig. 7, the enforced expression of SOCS3 decreased Th1 cell differentiation but increased Th2 cell differentiation in Stat5a^{-/-} CD4⁺ T cells even in the absence of exogenous cytokines. Interestingly, IL-4, even in the absence of anti-IFN-γ mAb, significantly induced Th2 cell differentiation of Stat5a^{-/-} CD4⁺ T cells that expressed SOCS3 (Fig. 7). Taken together, these results suggest that the diminished SOCS3 expression is involved in the impaired Th1/Th2 balance in Stat5a^{-/-} CD4⁺ T cells.

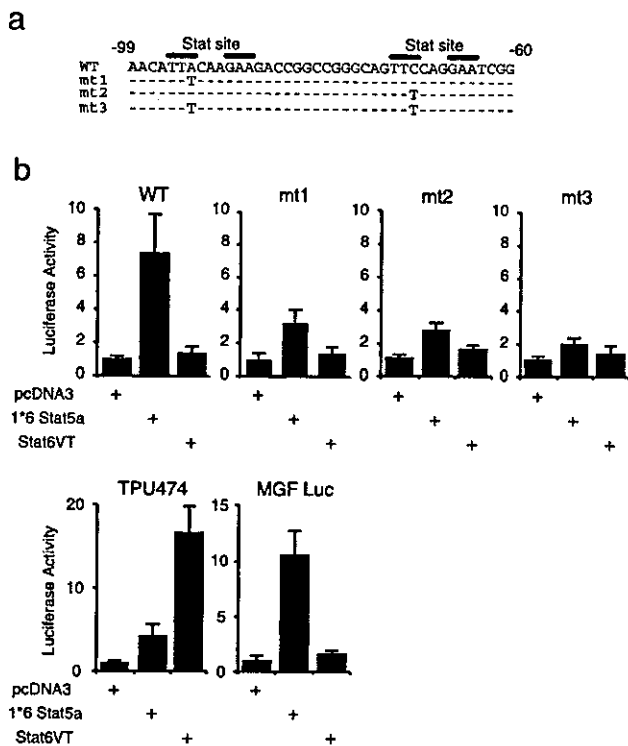


FIGURE 5. Stat5a directly activates the SOCS3 promoter. *a*, The murine SOCS3 promoter (WT) and the mutants (mt1, mt2, and mt3) of putative STAT-binding sequences. *b*, Stat5a preferentially activates the SOCS3 promoter. COS7 cells were transfected with TPU474, MGF-Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3 mt2 Luc, or SOCS3 mt3 Luc in the presence or absence of the expression vectors for the constitutively active form of Stat6 (pcDNA3 Stat6VT) or the constitutively active form of Stat5a (pcDNA3 1*6 Stat5a). Twenty-four hours after transfection, the luciferase activity of TPU474, MGF-Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3 mt2 Luc, or SOCS3 mt3 Luc was evaluated by the dual luciferase reporter system. Data are mean ± SD from four experiments.

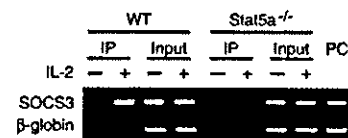


FIGURE 6. Stat5a binds to SOCS3 promoter in CD4⁺ T cells. Splenocytes from WT mice or Stat5a^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h. CD4⁺ T cells were purified (>90% pure by flow cytometry) using a CD4⁺ T cell enrichment column, starved from cytokines in fresh medium for 3 h, and then stimulated with IL-2 (20 ng/ml) for 30 min. Cells were fixed with formaldehyde, lysed, and sonicated to reduce the DNA length between 500 and 700 bp. The sonicated chromatin was immunoprecipitated with anti-Stat5a antisera. After deproteination and reversal of cross-links, the amounts of DNA sequence for the SOCS3 promoter and the β-globin promoter (as a control) in the immunoprecipitates were assessed by PCR. The input DNA and genomic DNA (as a positive control (PC)) were also subjected to PCR analysis. Shown are representative data from four independent experiments.

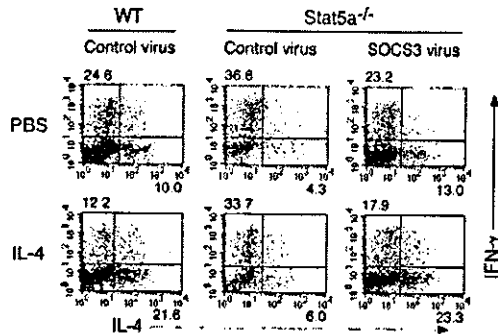


FIGURE 7. Retrovirus-mediated gene transduction of SOCS3 restores Th cell differentiation of $Stat5a^{-/-}$ $CD4^{+}$ T cells. Splenocytes from WT mice or $Stat5a^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 40 h in the presence or absence of IL-4 (15 ng/ml) and then infected with retroviruses of pMX-SOCS3-IRES-GFP or pMX-IRES-GFP (as a control) as described in *Materials and Methods*. Cells were cultured with IL-2 in the presence or absence of IL-4 for another 72 h. Cells were restimulated with plate-bound anti-CD3 mAb for 6 h and intracellular cytokine profiles for IL-4 vs IFN- γ were evaluated on infected $CD4^{+}$ T cells (GFP $^{+}$ $CD4^{+}$ cells). In these conditions, we found that the levels of SOCS3 evaluated by intracellular FACS analysis were ~ 1.5 -fold higher in SOCS3 retrovirus-infected $Stat5a^{-/-}$ $CD4^{+}$ T cells as compared with those in WT $CD4^{+}$ T cells cultured in Th2 condition. Shown is a representative intracellular cytokine staining from four independent experiments.

Discussion

In this study, we show that Stat5a regulates IL-12-induced Th1 cell differentiation through SOCS3 induction. We found that enhanced Th1 cell differentiation and the IFN- γ -mediated suppression were a principal reason for the decreased Th2 cell differentiation of $Stat5a^{-/-}$ $CD4^{+}$ T cells (Fig. 1). We then found that IL-12-induced Th1 cell differentiation (Fig. 2) and Stat4 phosphorylation (Fig. 3, *a* and *b*) were enhanced in $Stat5a^{-/-}$ $CD4^{+}$ T cells. Moreover, SOCS3, a potent inhibitor of IL-12/Stat4 signaling (14), was decreased in $Stat5a^{-/-}$ $CD4^{+}$ T cells (Fig. 4). Furthermore, the reporter assay showed that Stat5a but not Stat6 directly activated the SOCS3 promoter (Fig. 5) and ChIP assay revealed that Stat5a bound to the SOCS3 promoter in $CD4^{+}$ T cells (Fig. 6). Finally, the retrovirus-mediated expression of SOCS3 restored the altered Th cell differentiation of $Stat5a^{-/-}$ $CD4^{+}$ T cells (Fig. 7). These results suggest that Stat5a induces SOCS3 expression in $CD4^{+}$ T cells and thus inhibits IL-12-induced Th1 cell differentiation, resulting in the increase in Th2 cell differentiation.

We show that IL-12/Stat4 signaling and subsequent IL-12-induced Th1 cell differentiation are up-regulated in $Stat5a^{-/-}$ $CD4^{+}$ T cells. We found that $Stat5a^{-/-}$ $CD4^{+}$ T cells but not WT $CD4^{+}$ T cells differentiated into Th1 cells in response to a low concentration of IL-12 (Fig. 2). We also found that IL-12-induced Stat4 phosphorylation was enhanced in $Stat5a^{-/-}$ $CD4^{+}$ T cells (Fig. 3, *a* and *b*). In contrast, IL-12 production from APCs was not significantly altered in $Stat5a^{-/-}$ mice (data not shown). These results suggest that the increased sensitivity to IL-12/Stat4 signaling is responsible in part for the enhanced Th1 cell differentiation and subsequent Th2 cell suppression in $Stat5a^{-/-}$ $CD4^{+}$ T cells. However, even in the presence of anti-IFN- γ Ab, Th2 cell differentiation was still decreased in $Stat5a^{-/-}$ $CD4^{+}$ T cells as compared with that in WT $CD4^{+}$ T cells (Fig. 1), suggesting that the increased IFN- γ production cannot account for all of the impairment in Th2 cell differentiation of $Stat5a^{-/-}$ $CD4^{+}$ T cells. Direct induction of the IL-4 gene by Stat5a (12) may account for the difference between WT $CD4^{+}$ T cells and $Stat5a^{-/-}$ $CD4^{+}$ T cells in Th2 cell differentiation in the presence of an anti-IFN- γ Ab.

We also show that Stat5a regulates the expression of SOCS3 in $CD4^{+}$ T cells. Increasing evidence has revealed that SOCS family proteins are involved in a negative feedback loop of JAK/STAT signaling (28–30). Among SOCS family proteins, SOCS3 has been shown to be preferentially expressed in Th2-polarized cells and to prevent IL-12-induced Th1 cell differentiation (13, 14). In this study, we found that the expression of SOCS3 was decreased not only in freshly isolated $CD4^{+}$ T cells but also in Th2-polarized $CD4^{+}$ T cells in $Stat5a^{-/-}$ mice (Fig. 4). We also found that Stat5a bound to the SOCS3 promoter in $CD4^{+}$ T cells upon IL-2 stimulation (Fig. 6). In addition, we found that a constitutively active form of Stat5a but not a constitutively active form of Stat6 could activate the SOCS3 promoter in a STAT-binding sequence-dependent fashion (Fig. 5*b*), which is in agreement with a previous finding that Stat5a preferentially recognizes TTC-N3-GAA STAT-binding sequence, whereas Stat6 preferentially recognizes TTC-N4-GAA STAT-binding sequence (31). Taken together, these results suggest that Stat5a but not Stat6 induces SOCS3 expression in the developing Th2 cells. Moreover, because the retrovirus-mediated expression of SOCS3 restored the altered Th cell differentiation of $Stat5a^{-/-}$ $CD4^{+}$ T cells (Fig. 7), the reduced expression of SOCS3 is likely to be involved in the dysregulated Th1/Th2 balance in $Stat5a^{-/-}$ $CD4^{+}$ T cells.

Accumulating evidence suggests that the Stat5a-induced SOCS3 expression is also involved in the regulation of Th2 cell-mediated allergic inflammation *in vivo*. First, we have previously shown that Th2 cell-mediated allergic airway inflammation is decreased in $Stat5a^{-/-}$ mice (10), indicating that Stat5a is involved in the induction of *in vivo* Th2 cell-mediated immune responses. Second, a recent study has shown that SOCS3 expression is increased in peripheral T cells in asthma patients and that the constitutive expression of SOCS3 within T cells results in the enhanced airway hyperreactivity in a mouse model of asthma (14), suggesting that SOCS3 also plays an important role in the induction of Th2 cell-mediated allergic airway inflammation. Third, in the present study, we show that Stat5a is essential for the appropriate expression of SOCS3 in $CD4^{+}$ T cells, especially in developing Th2 cells (Figs. 4–6). Therefore, although further studies are required, it is suggested that the Stat5a-mediated SOCS3 induction participates in the induction of Th2 cell-mediated allergic airway inflammation.

Because Stat5 has been shown to up-regulate a number of SOCS family proteins (28) and because it is suggested that, in addition to SOCS3, some of SOCS family proteins may regulate Th1 cell and Th2 cell differentiation (30), it is possible that other SOCS family proteins are also involved in Stat5a-mediated Th cell differentiation. For example, SOCS1, an important negative regulator of IFN- γ signaling (32, 33), has been shown to be induced by Stat5 activation (23). However, we found that IFN- γ -induced Stat1 phosphorylation was not enhanced in $Stat5a^{-/-}$ $CD4^{+}$ T cells (data not shown), suggesting that SOCS1 may not be involved in Stat5a-mediated suppression of Th1 cell differentiation. The possible involvement of other SOCS family proteins in Stat5a-induced Th cell differentiation needs to be determined in future.

Recently, progress has been made on an upstream cytokine for Stat5a activation during Th cell differentiation. Among a number of cytokines that activate Stat5a, it has been demonstrated that blocking of IL-2, either by the neutralization of IL-2 itself or the blocking of IL-2R, decreases Th2 cell differentiation (12, 34, 35). It has also been shown that the developing Th2 cells express higher levels of the IL-2R α -chain and exhibit stronger Stat5 activation than the developing Th1 cells (34), consistent with a previous finding that Stat5a functions as an amplifier of IL-2 signaling by inducing the expression of the IL-2R α -chain (36). Moreover, it has recently been demonstrated that IL-2 but not IL-9, IL-15, or IL-21

induces Stat5 phosphorylation and IL-4 production in activated CD4⁺ T cells (37). Therefore, it is suggested that IL-2 is most likely to be a cytokine responsible for Stat5a activation during Th cell differentiation.

It is well recognized that Stat5a regulates the expression of CD25 by directly binding to the 5' regulatory region of the CD25 gene (38, 39). Consistent with this observation, we have previously shown that the number of CD4⁺ T cells that express CD25 (CD25⁺CD4⁺ T cells) is decreased in Stat5a^{-/-} mice and we have suggested that the decreased number of CD25⁺CD4⁺ T cells may account for the altered Th cell differentiation of Stat5a^{-/-} CD4⁺ T cells to some extent (11). In addition, it has been demonstrated that Stat5a directly induces IL-4 production by regulating the accessibility of the IL-4 gene (12). Moreover, we show in this study that the induction of SOCS3 expression by Stat5a in conventional CD4⁺ T cells is important for Stat5a-mediated Th cell differentiation. Therefore, it is suggested that Stat5a regulates Th cell differentiation in multiple pathways. Further studies are required for the understanding of the relative importance of these pathways in Stat5a-mediated Th cell differentiation.

Because Stat5b is highly homologous to Stat5a (40) and because the mice lacking both Stat5a and Stat5b exhibit a severe defect in T cell responses as compared with Stat5a^{-/-} mice or Stat5b^{-/-} mice (41), it is suggested that the function of Stat5a and Stat5b is somehow overlapped. However, the different phenotypes of Stat5a^{-/-} mice and Stat5b^{-/-} mice underscore the distinctive roles of Stat5a and Stat5b. For example, it has been demonstrated that, although Stat5a^{-/-} T cells exhibit no detectable defects in anti-CD3-induced proliferation, Stat5b^{-/-} T cells are defective in anti-CD3-induced proliferation (10, 36, 42), suggesting that Stat5b is likely to play a role in the proliferation and/or survival of activated T cells and that this function of Stat5b may not be shared with Stat5a.

Regarding Th cell differentiation, we have recently found that Stat5a is essential for the residual Th2 cell differentiation in Stat6^{-/-} CD4⁺ T cells by comparing Stat6^{-/-} mice to Stat5a- and Stat6-double deficient mice in the same genetic background (44). Because Stat5b is normally expressed and activated in response to IL-2 even in the absence of Stat5a (36, 42), the results suggest that Stat5b cannot compensate the role of Stat5a in Stat6-independent Th2 cell differentiation. In contrast, we have previously shown that in addition to Th2 cell differentiation, Th1 cell differentiation is also decreased in Stat5b^{-/-} mice (10). Because it has recently been shown that Stat5 activates the distal region of the human IFN- γ promoter (43), Stat5b may be involved in the induction of IFN- γ production during Th1 cell differentiation.

In conclusion, we show that Stat5a forces the Th1/Th2 balance toward a Th2-type by preventing IL-12-induced Th1 cell differentiation through the induction of SOCS3. Because it has been demonstrated that SOCS3 regulates the onset and maintenance of Th2 cell-mediated allergic diseases such as asthma and atopic dermatitis (14), it is suggested that Stat5a-mediated SOCS3 induction could be a target for the treatment of Th2 cell-mediated allergic diseases.

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Disclosures

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