IL-4 did not change the morphology of PDCs, whereas CpG ODN changed PDCs to dendritic morphology (data not shown). These results suggest that IL-4 does not change the maturation state of PDCs.

T-bet is not induced by IL-4 in PDCs

T-bet plays an important role in inducing IFN-γ production in $CD4^+$ T cells (29). Recent findings using T-bet-deficient mice have also suggested that T-bet is vital for IFN-γ production from $CD11c^+$ DCs upon IL-12 stimulation (30). To examine the possible involvement of T-bet in IL-4-induced IFN-γ production in PDCs, we examined the expression of T-bet mRNA in PDCs in the presence or in the absence of IL-4 stimulation. The expression of GATA3, an important negative regulator of IFN-γ production (4), was also examined in parallel. As shown in Fig. 4*E*, unstimulated PDCs expressed GATA3 mRNA but not T-bet mRNA. IL-4 did not induce the expression of T-bet mRNA nor alter the expression levels of GATA3 mRNA (Fig. 4*E*). T-bet mRNA was not detected by Taqman PCR analysis even after IL-4 stimulation (data not shown). These results suggest that T-bet may not be involved in IL-4-induced IFN-γ production in PDCs.

IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and Stat4-expressing PDCs produce IFN-γ

It has been demonstrated that Stat4 is required for IFN-γ production in many cell types (31, 32). Stat4 expression has also been demonstrated to be correlated with IFN-γ-producing ability in $CD8^+$ DCs (33). Therefore, we next examined the expression levels of Stat4 in IL-4-stimulated PDCs. As shown in Fig. 5*A*, in the absence of IL-4 stimulation, isolated WT PDCs did not express Stat4 mRNA (*lane 1*). However, Stat4 mRNA was significantly up-regulated in WT PDCs upon IL-4 stimulation (*lane 2*), although the expression level was still lower than that in Th1-polarized cells (*lane 3*) (Fig. 5A, *left panel*). Stat4 mRNA induction by IL-4 stimulation was confirmed by real-time PCR analysis (Fig. 5*A*, *middle panel*). We also examined the expression levels of Stat4 at protein levels using intracellular staining in WT PDCs and $Stat6^{-/-}$ PDCs and found that IL-4 significantly induced Stat4 expression in ~50% of WT PDCs but not in $Stat6^{-/-}$ PDCs (Fig. 5*A*, *right panel*). These results indicate that Stat6 is essential for IL-4-induced Stat4 expression in PDCs. In addition, although IFN-γ has been shown to induce Stat4 expression in some cell types (34), anti-IFN-γ Ab did not affect IL-4-induced Stat4 expression in PDCs (data not shown).

We then examined the correlation between Stat4 expression and IFN-γ production at single-cell levels by double intracellular staining. Interestingly, Stat4-expressing PDCs but not Stat4-nonexpressing PDCs produced IFN-γ upon IL-4 stimulation (Fig. 5*B*). We also examined whether Stat4 was essential for IL-4-induced IFN-γ production in PDCs using $Stat4^{-/-}$ mice. Although PDCs normally developed in $Stat4^{-/-}$ mice (data not shown), IL-4 did not induce IFN-γ production in $Stat4^{-/-}$ PDCs (Fig. 5*C*). Taken together, these results indicate that the induction of Stat4 by IL-4-Stat6 signaling is required for IFN-γ production in PDCs.

IL-4-induced IFN-γ production does not depend on IL-12 or type I IFNs

To determine whether endogenously produced cytokines from PDCs are involved in IL-4-induced IFN-γ production, we examined the effect of blocking Abs against IL-12 (p70), IL-2R β-chain (a shared receptor for IL-2 and IL-15), and IL-18 on IL-4-induced IFN-γ production in PDCs. However, none of the Abs inhibited IL-4-induced IFN-γ production in PDCs even when these Abs were added altogether (Fig. 5*D*). To determine the possible involvement of type I IFNs in IL-4-induced IFN-γ production in PDCs, we also examined the effect of a mixture of neutralizing Abs to IFN-α, IFN-β, and type I IFN receptor on IL-4-induced IFN-γ production in PDCs. However, these Abs did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5*E*). As expected, the addition of anti-IL-4 mAb canceled IL-4-induced IFN-γ production in PDCs (data not shown). These results indicate that none of IL-12, IL-2, IL-15, IL-18, or type I IFNs is required for IL-4-induced IFN-γ production in PDCs.

Discussion

In this study, we show that PDCs are a major IFN-γ-producing cell upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs by a Stat6-dependent mechanism. Moreover, IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and the IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Furthermore, $Stat4^{-/-}$ PDCs do not produce IFN-γ upon IL-4 stimulation. These results suggest that PDCs produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms.

We demonstrate that PDCs are a major IFN-γ-producing cell upon IL-4 stimulation. By searching for $Rag-2^{-/-}$ splenocyte populations that produce IFN-γ upon IL-4 stimulation, we found that the majority of IL-4-induced, IFN-γ-producing cells expressed B220 at high levels and CD11c and Ly6G/C at low levels (Fig. 1). We also found that IL-4 induced IFN-γ production from isolated $B220^+$ PDCs but not from $CD11b^+$ DCs or $CD8^+$ DCs (Fig. 2*A*) and that the depletion of PDCs by anti-Ly6G/C Ab prevented IL-4-induced IFN-γ production in vivo (Fig. 2*B*). Inhibition of IL-4-induced IFN-γ production was similarly observed with the administration of 120G8 Ab, although statistical significance was not achieved due to the limited number of mice examined. On the other hand, IL-4 did not induce IFN-α or IL-12 production in PDCs (Fig. 3). IL-4 also strongly enhanced CpG ODN-induced IFN-γ production but not CpG ODN-induced IFN-α or IL-12 production in PDCs (Fig. 3). Taken together, these results indicate that PDCs are a major IFN-γ producer upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs.

It is well recognized that Stat6 plays a critical role in the production of IL-4 in $CD4^+$ T cells upon IL-4 stimulation through the induction of GATA3, a master regulator of Th2 cell differentiation (4). In contrast, we show here that IL-4 induces IFN-γ production

staining for IFN-γ together with Stat4 staining was performed as described in *Materials and Methods*. Control IgG was used as a negative control of anti-Stat4 Ab. Representative FACS profiles from four independent experiments are shown. *C*, Isolated PDCs from WT splenocytes or $Stat4^{-/-}$ splenocytes were cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. FACS analysis performed as described in Fig. 4*A* indicates normal development of PDCs in $Stat4^{-/-}$ mice (data not shown). Data are means ± SD from three independent experiments. *D*, None of IL-2, IL-12, IL-15, or IL-18 is required for IL-4-induced IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 3 days in the presence of Abs against IL-12, IL-18, or IL-2Rβ. The amount of IFN-γ in the supernatant was measured by ELISA. Data are means ± SD from four independent experiments. *E*, Type I IFNs are not required for IL-4-induced IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 3 days in the presence or in the absence of a mixture of neutralizing Abs to IFN-α, IFN-β, and type I IFN receptor. The amount of IFN-γ in the supernatant was measured by ELISA. Data are means ± SD from four independent experiments.

in PDCs by a Stat6-dependent mechanism (Fig. 4*A*). We also show that IL-4 does not alter the expression levels of GATA3 (Fig. 4*E*) nor induce the expression of T-bet, a key molecule for IFN-γ production in CD4 T cells (29), in PDCs (Fig. 4*E*). Therefore, in contrast to $CD4^+$ T cells, the expression levels of T-bet and GATA3 may not be causatively associated with the production of IFN-γ in PDCs.

Our results show that IL-4, but not other γc-dependent cytokines, induces IFN-γ production from PDCs (Fig. 4*C*). In contrast, it has been demonstrated recently that IL-4 synergistically enhances IL-2-induced IFN-γ production from NK cells, but IL-4 itself does not induce IFN-γ production from NK cells (35). It has also been shown that although IL-4 enhances IL-12-induced IFN-γ production from $CD8^+$ DCs, IL-4 itself does not induce IFN-γ production from $CD8^+$ DCs (36). Moreover, we found that bone marrow-derived PDCs generated with fms-like tyrosine kinase-3 ligand did not produce IFN-γ upon IL-4 stimulation (data not shown). Thus, the IL-4 signaling pathway for IFN-γ production may differ depending not only on cell types but also on the maturation state of the cells.

We also show that IL-4 induces Stat4 expression in PDCs by a Stat6-dependent mechanism (Fig. 5*A*), that only the Stat4-expressing PDCs produce IFN-γ at single-cell levels (Fig. 5*B*), and that $Stat4^{-/-}$ PDCs do not produce IFN-γ upon IL-4 stimulation (Fig. 5*C*). Therefore, it is indicated that Stat4 is required for IL-4-induced IFN-γ production in PDCs. Interestingly, we also found that when PDCs were stimulated with CpG ODN for 48 h, Stat4 induction was detected by intracellular FACS analysis (data not shown). This finding may account for the synergistic effect of CpG ODN on IL-4-induced IFN-γ production in PDCs (Fig. 3).

The mechanisms leading to Stat4 activation could not be yet identified. Indeed, the phosphorylation status of Stat4 could not be clearly defined in IL-4-stimulated PDCs presumably for technical reasons (data not shown). However, as tyrosine phosphorylation is required for the activity of STAT proteins (37), a Stat4-activating cytokine seems to be involved in IL-4-induced IFN-γ production in PDCs. Because IL-12 is a representative cytokine that activates Stat4 (38) and because it has been reported that IL-4 enhances IL-12 production from $CD11c^+$ DCs (39) or $CD8\alpha^+$ DCs (40) in some situations, it was suggested that IL-12 might be responsible for activating Stat4 in PDCs. However, we found that IL-4 by itself does not induce IL-12 production from PDCs (Fig. 3) and that a neutralizing Ab against IL-12 did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5*D*), suggesting that IL-12 is not responsible for the activation of Stat4 in PDCs.

Recently, it has also been demonstrated that in addition to IL-12, type I IFNs activate Stat4 and induce IFN-γ production in some cell types such as $CD8^+$ T cells (41). However, again, we found that IL-4 by itself did not induce IFN-α production from PDCs (Fig. 3) and that neutralizing Abs against type I IFNs did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5*E*). These findings suggest that type I IFNs are not responsible for the activation of Stat4 in PDCs. Recent studies have also demonstrated that IL-23 (42) and IL-21 (43) use Stat4 as a signaling molecule in some cell types. Therefore, IL-23, IL-21, or an undefined Stat4-activating cytokine may function as a Stat4-activating cytokine and then may contribute to IL-4-induced IFN-γ production in PDCs. Further studies that identify the cytokine responsible for Stat4 activation are required for the understanding of the mechanism leading to IL-4 induced IFN-γ production in PDCs.

The effect of IL-4 on the expression of Stat4 in DCs seems different depending on the subtypes of DCs, as well as the maturation state of each DC subtype. Recently, Fukao et al. (33) have shown that when IL-4 is present during the maturation of $CD11c^+$ DCs, IL-4 suppresses Stat4 induction and subsequent IL-12-induced IFN-γ production in $CD11c^+$ DCs. On the other hand, the same group has shown that IL-4 does not alter the expression levels of Stat4 in mature $CD8^+$ DCs (36). However, we showed that IL-4-Stat6 signaling induced Stat4 expression in PDCs (Fig. 5). Moreover, we found that the maturation state of PDCs, assessed by the expression levels of CD80 and $I\text{-}A^d$, was similar between Stat4-expressing PDCs and Stat4-nonexpressing PDCs (data not shown). Therefore, our results indicate that IL-4 specifically induces Stat4 expression and IFN-γ-producing ability in PDCs without affecting their maturation state.

In the present study, we showed that a typical Th2 cytokine IL-4 induced the production of a typical Th1 cytokine IFN-γ in PDCs in BALB/c mice. IL-4-induced IFN-γ production in PDCs was also observed in C57BL/6 mice (data not shown), suggesting that this phenomenon is a general one observed beyond strain differences. Because IL-4 is produced in an early phase in immune responses by NK T cells (44) and/or basophils (45, 46), the IL-4-induced IFN-γ production by PDCs may function in the negative-feedback regulation against a Th2-type deviation in an early phase of immune responses. In this regard, de Heer et al. (14) have demonstrated recently that PDCs inhibit typical Th2 responses such as IgE production and allergic airway inflammation. Although the authors indicated the induction of regulatory T cells as the mechanism underlying the PDC-mediated Th2 cell suppression (14), IL-4-induced IFN-γ production in PDCs may also contribute to the PDC-mediated inhibition of allergic airway inflammation because IFN-γ inhibits Ag-induced Th2 cell differentiation (1–3) and allergic airway inflammation (47).

In conclusion, we have shown that PDCs preferentially produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms. Although further studies are required to address the physiological importance of IL-4-induced IFN-γ production in PDCs, our results would give a new insight into PDC-mediated immune regulation of cytokine network.

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Disclosures

The authors have no financial conflict of interest.

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Stat5a Is Essential for the Proliferation and Survival of Murine Mast Cells

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Key Words
Stat5a · Mast cell · Bcl-x(L) · Apoptosis

Abstract
The regulatory role of signal transducer and activator of transcription (Stat) 5a in the proliferation and survival of mast cells was determined using Stat5a-deficient ($Stat5a^{-/-}$) mice. First, although the mast cells in $Stat5a^{-/-}$ mice were morphologically indistinguishable from those in wild-type (WT) mice, the number of peritoneal mast cells was significantly decreased in $Stat5a^{-/-}$ mice as compared with that in WT mice. Furthermore, the interleukin-3 (IL-3)-dependent development of bone marrow-derived mast cells (BMMCs) was markedly decreased in $Stat5a^{-/-}$ mice. Second, IL-3-induced but not stem cell factor (SCF)-induced proliferation of BMMCs was significantly diminished in $Stat5a^{-/-}$ mice as compared with that in WT mice. Moreover, survival rates of both peritoneal mast cells and BMMCs were significantly decreased with increased apoptotic cells in $Stat5a^{-/-}$ mice as compared with those in WT mice. Finally, mRNA of Bcl-x(L) was induced after IL-3 stimulation in WT BMMCs but not in $Stat5a^{-/-}$ BMMCs, which may account for the accelerated apoptosis in $Stat5a^{-/-}$ mast cells. These results indicate that Stat5a plays an important role in mast cell development, proliferation, and survival.

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Introduction

Mast cells are recognized as the major effector cells of the type I hypersensitivity reactions by virtue of their possessing high-affinity receptors for immunoglobulin E (IgE). They are known to play a pivotal role in allergic diseases, such as asthma, atopic rhinitis, and atopic dermatitis, and are also known to play an essential role in parasite infection in mice [1, 2]. Mature mast cells are distributed throughout all vascularized tissues, and the development and proliferation of mast cells require proper signaling from several cytokines, among which the c-kit/stem cell factor (SCF) system and interleukin-3 (IL-3) are the best studied [1–4].

Although IL-3 is not essential for the generation of murine mast cells under physiological conditions, it does contribute to increased numbers of tissue mast cells and enhanced immunity in mice infected with the nematode *Strongyloides venezuelensis* [5]. IL-3 is also known to play a central role in the development of bone marrow-derived mast cells (BMMCs) in mice [1].

Signal transducer and activator of transcription (Stat) 5a and Stat5b are cytosolic latent transcription factors that are activated by a very wide range of cytokines, including IL-3 [6, 7]. Under IL-3/Jak2 activation, Stat5a and Stat5b directly regulate the gene expression of a number of important genes. Among Stat5-inducible genes, pim-1 is essential for proliferation, Bcl-x(L) is essential

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for survival, and CIS and SOCS-1 play an important role in termination of cytokine signaling in proB cell lines [8]. Although Stat5a and Stat5b are highly homologous, the different phenotypes of Stat5a-deficient (Stat5a$^{-/-}$) mice and Stat5b$^{-/-}$ mice underscore the distinctive roles of Stat5a and Stat5b [9]. Recently, the study of Stat5a/b-deficent mice showed that Stat5 expression is critical for mast cell development [10]. However, the distinctive role of either Stat5a or Stat5b in mast cell development and survival remains to be determined.

In this study, in order to determine the importance of Stat5a in mast cell development, survival, and proliferation, we analyzed the differentiation and expansion of mast cells in Stat5a$^{-/-}$ mice. We present the data that demonstrate an important role of Stat5a in the proliferation and survival of murine mast cells. Our data also suggest the role of Bcl-x(L), which is induced by Stat5a, in the survival of murine mast cells against apoptosis.

Materials and Methods

Mice and Genetic Analysis

Stat5a-deficient (Stat5a$^{-/-}$) mice [11] were backcrossed to BALB/c mice (Charles River Laboratories, Kanagawa, Japan) for at least 8 generations and littermate wild-type (WT) mice were used as controls. Mice were housed in microisolator cages under pathogen-free conditions. All animal experiments were performed under the guidelines approved by the Chiba University.

Culture of BMMCs

Primary culture of IL-3-dependent BMMCs was prepared from 8- to 10-week-old WT or Stat5a$^{-/-}$ mice and maintained as described previously [12]. Briefly, the mice were sacrificed and the bone marrow was aseptically flushed from femurs and tibias into RPMI 1640 medium containing 10% heat-inactivated FCS, 50 μ*M* β-mercaptoethanol, 2 m*M* *L*-glutamine, 1 m*M* sodium pyruvate, 0.1 m*M* nonessential amino acids, antibiotics, and 10% (vol/vol) of murine IL-3 transfectant X63 cell-conditioned medium [13] (X63-IL-3, a kind gift from Dr. H. Karasuyama, Tokyo Medical and Dental University) as a source of IL-3. The nonadherent bone marrow cells were then maintained at 37°C at a density of 2–5 × 10^5 cells/ml in the same medium, with biweekly replacement of old media with fresh ones. BMMCs were used for experiments at 4–5 weeks of culture unless otherwise stated.

Peritoneal Lavage Cells

Peritoneal lavage was performed by injecting 10 ml of ice-cold PBS into the peritoneal cavity of the mouse. After cells were centrifuged (×400 *g*), resuspended in 1 ml of PBS, and counted using a hemocytometer, differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution. A fraction of the cells was subjected to flow-cytometric analysis as described below.

Flow-Cytometric Analysis

Cells from the peritoneal cavity and BMMCs were stained and analyzed on a FACScalibur (Becton Dickinson, San Jose, Calif., USA) using CellQuest software. FACS analysis was performed with anti-CD117 (c-kit) FITC (2B8, BD PharMingen, San Diego, Calif., USA) and anti-CD16/32 (FcγR II/III) PE (2.4G2, BD PharMingen). Before anti-CD117 staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2, BD PharMingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific antibodies (BD PharMingen).

IgE Receptors on Mast Cells

To quantify the levels of IgE receptors expressed on the cell surface, cells were first incubated with mouse anti-TNP IgE (IgE3, BD PharMingen) at 4°C for 60 min to saturate IgE receptors, and were then labeled with anti-IgE FITC (R35-72, BD PharMingen).

Cell Survival Assay

BMMCs were washed 3 times with PBS and cultured at 1 × 10^6 cells/ml in triplicate at 37°C for 48 h in RPMI 1640 medium without IL-3. Peritoneal lavage cells were cultured at 1 × 10^6 cells/ml in triplicate at 37°C for 24 h in RPMI 1640 medium without IL-3. The viability of those cells was determined by FACS with anti-CD117 FITC and 5 μg/ml of propidium iodide (PI; Boehringer Mannheim, Indianapolis, Ind., USA) [14].

Annexin V Staining of BMMCs

BMMCs were washed twice with PBS containing 1% BSA, stained with Annexin V FITC (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions, and analyzed on a FACScalibur with 5 μg/ml of PI.

Proliferation Assay

BMMCs (2 × 10^5/well) were cultured in triplicate at 37°C for 36 h in 96-well plates in RPMI 1640 medium containing the indicated amounts of murine IL-3 (10^{-5} to 1 μg/ml; R&D Systems) or SCF (10^{-5} to 1 ng/ml; R&D Systems) with 0.5 μCi of [^{3}H]-thymidine added for the final 12 h.

Cell Division Assay

BMMCs were incubated with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, 10 μ*M*, Molecular Probes, Eugene, Oreg., USA) in PBS at 37°C for 10 min, and then washed with RPMI 1640 medium. CFSE-labeled BMMCs were cultured at 37°C for 72 h with IL-3. Cells were harvested and analyzed by FACS.

RT-PCR Assay

BMMCs were washed twice with PBS and total RNA was extracted using Isogen reagent (Nippon Gene Co., Tokyo, Japan). The first-strand complementary DNA (cDNA) was then synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT) primers (Pharmacia Biotech, Buckinghamshire, UK). cDNAs encoding Bcl-x(L) [8] and β-actin (as a control) were amplified by PCR.

Data Analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t test. p values <0.05 were considered significant.

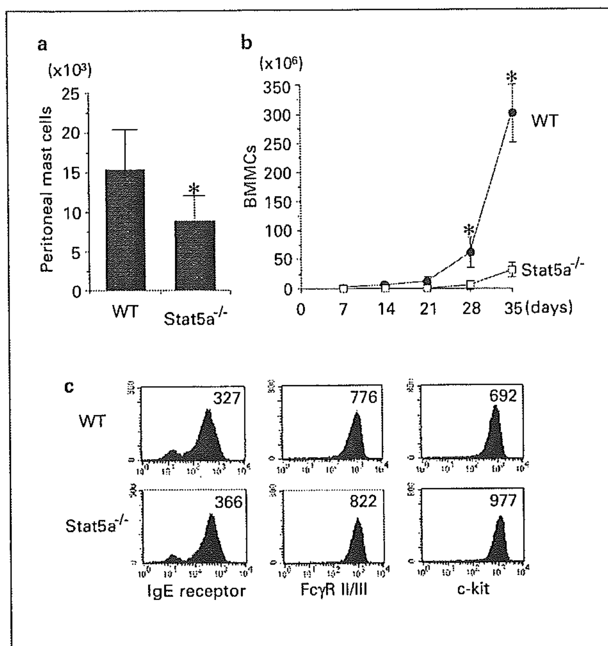

Fig. 1. Stat5a is required for the development of mast cells. **a** The number of peritoneal mast cells is decreased in Stat5a$^{-/-}$ mice. Peritoneal cells were recovered by the lavage in 8- to 10-week-old WT and Stat5a$^{-/-}$ mice and the number of mast cells in the lavage was evaluated. Peritoneal mast cells were identified morphologically on cytospin cell preparations stained with Wright-Giemsa solution. Data are means ± SD for 6–8 mice in each group. The mean value of Stat5a$^{-/-}$ mice is significantly different from that of WT mice. * $p<0.01$. **b** Development of IL-3-dependent BMMCs is decreased in Stat5a$^{-/-}$ mice. Bone marrow cells from WT mice and Stat5a$^{-/-}$ mice were cultured in the presence of IL-3 at 37°C and the number of mast cells was determined at day 7, day 14, day 21, day 28, and day 35 using a hemocytometer and cytospin cell preparations stained with Wright-Giemsa solution. Data are means ± SD for 5 mice in each group. * $p < 0.001$. **c** BMMCs normally develop in Stat5a$^{-/-}$ mice. Expression of IgE receptors, FcγR II/III, and c-kit on WT and Stat5a$^{-/-}$ BMMCs was determined by FACS using anti-IgE FITC, anti-FcγR II/III PE, and anti-c-kit FITC, respectively, as described in the Materials and Methods. Representative FACS profiles and the mean fluorescence intensities from four independent experiments are shown.

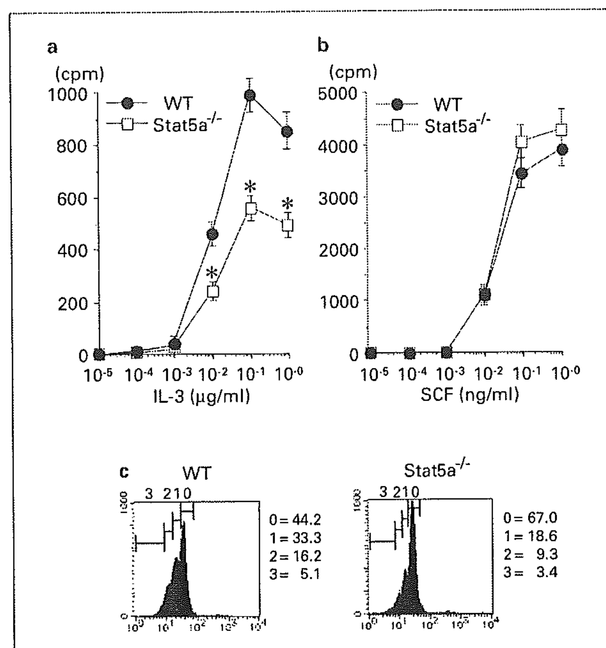

Fig. 2. Stat5a is crucial for IL-3-induced but not SCF-induced proliferation of BMMCs. **a, b** IL-3-induced but not SCF-induced proliferation of BMMCs is decreased in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were cultured in the presence of either IL-3 (10^{-5} to 1 μg/ml) or SCF (10^{-5} to 1 ng/ml) at 37°C for 48 h and the proliferative responses were evaluated by the addition of [^{3}H]-thymidine for the final 12 h. Data are means ± SD for 5 mice in each group. * $p < 0.001$. **c** IL-3-induced cell division of BMMCs is decreased in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were labeled with CFSE. These cells were cultured with IL-3 at 37°C for 48 h and then analyzed by FACS. Representative FACS profiles for the intensity of CFSE (n = 4) are shown. The figures indicate percent of cell numbers after 0–3 cell divisions.

Results

Stat5a Is Required for the Development of Mast Cells

To determine whether Stat5a is essential for mast cell development in vivo, we first analyzed the number of peritoneal mast cells in Stat5a$^{-/-}$ mice. The number of mast cells recovered from the peritoneal cavity was significantly decreased in Stat5a$^{-/-}$ mice as compared with that in WT mice (n = 6–8 mice, $p < 0.01$) (fig. 1a). We also found reduced numbers of mast cells in tissue sections of ear and stomach in Stat5a$^{-/-}$ mice (data not shown). Furthermore, IL-3-dependent development of BMMCs was markedly decreased in Stat5a$^{-/-}$ mice (n = 5, $p < 0.001$) (fig. 1b), suggesting that Stat5a plays an important role in the development of mast cells both in vivo and in vitro. However, Stat5a$^{-/-}$ BMMCs normally expressed IgE receptors, FcγR II/III, and c-kit (fig. 1c), and Stat5a$^{-/-}$ BMMCs were morphologically indistinguishable from WT BMMCs (data not shown). These results suggest that mast cells mature normally, but the expansion of mast cells is impaired in Stat5a$^{-/-}$ mice.

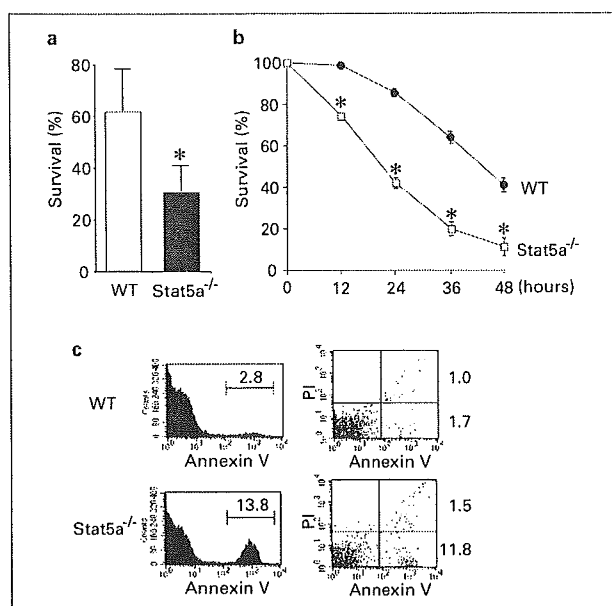

Fig. 3. The survival of mast cells is diminished in Stat5a$^{-/-}$ mice. **a** The survival of peritoneal mast cells is diminished in Stat5a$^{-/-}$ mice. Freshly isolated peritoneal cells from WT mice or Stat5a$^{-/-}$ mice were cultured for 24 h in RPMI 1640 medium without IL-3. The viability of c-kit+ cells was determined by FACS using anti-c-kit FITC and 5 μg/ml of PI. Data are means ± SD of the percent survival (n = 4 mice in each group). The mean value of Stat5a$^{-/-}$ mice is significantly different from the mean value of WT mice. * $p < 0.01$. **b** The survival of BMMCs is diminished in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were cultured at 1×10^6 cells/ml at 37°C for 48 h in RPMI 1640 medium without IL-3. Cells were harvested and the viability was determined by FACS using 5 μg/ml of PI. n = 4 experiments, * $p < 0.001$. **c** Apoptosis of BMMCs is increased in Stat5a$^{-/-}$ mice. BMMCs from WT mice or Stat5a$^{-/-}$ mice were cultured in the presence of IL-3 and stained with Annexin V and 5 μg/ml of PI. Representative FACS profiles from five independent experiments are shown.

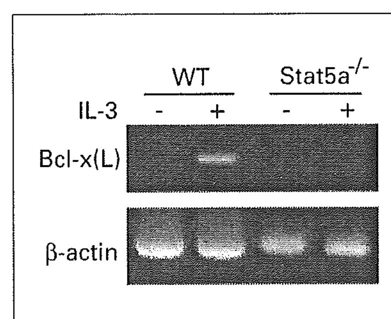

Fig. 4. Bcl-x(L) expression is decreased in Stat5a$^{-/-}$ BMMCs. BMMCs from WT mice or Stat5a$^{-/-}$ mice were washed with PBS and total RNA was extracted using Isogen reagent. The first strand complementary DNA (cDNA) was then synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers. cDNAs encoding Bcl-x(L) and β-actin (as a control) were amplified by PCR.

Stat5a Is Crucial for IL-3-Induced but Not SCF-Induced Proliferation of Mast Cells

To clarify the mechanism of the reduced number of mast cells in Stat5a$^{-/-}$ mice, we examined the proliferation of BMMCs. IL-3-induced but not SCF-induced proliferation of BMMCs was significantly diminished in Stat5a$^{-/-}$ mice as compared with that in WT mice (n = 5, at 0.01–1 μg/ml of IL-3, $p < 0.001$) (fig. 2a, b). In addition, IL-3-induced cell division of Stat5a$^{-/-}$ BMMCs was less frequent than that of WT BMMCs (fig. 2c). These results indicate that IL-3-induced proliferation of mast cells is decreased in Stat5a$^{-/-}$ mice.

Apoptosis Is Increased in Stat5a$^{-/-}$ Mast Cells

We next examined the survival and apoptosis of mast cells in Stat5a$^{-/-}$ mice. Survival rates of both peritoneal mast cells and BMMCs were significantly decreased in Stat5a$^{-/-}$ mice as compared with those in WT mice (n = 4 experiments each, $p < 0.01$ and $p < 0.001$, respectively) (fig. 3a, b). Interestingly, apoptotic cells were significantly increased in Stat5a$^{-/-}$ BMMCs even when they were cultured with IL-3 (n = 5, $p < 0.001$) (fig. 3c). Furthermore, mRNA of Bcl-x(L), an antiapoptotic molecule [15–17], was expressed in WT BMMCs but not in Stat5a$^{-/-}$ BMMCs (fig. 4), suggesting that Stat5a may suppress apoptosis of mast cells by inducing the expression of the antiapoptotic gene, Bcl-x(L).

Discussion

In this study, we show that Stat5a plays an important role in the development, proliferation, and survival of murine mast cells. We found that the lack of Stat5a resulted in reduced numbers of peritoneal mast cells in vivo and impaired development of BMMCs (fig. 1). We also found that IL-3-induced but not SCF-induced proliferation was decreased in Stat5a$^{-/-}$ BMMCs (fig. 2). Finally, we found that apoptosis was increased and the expression of an antiapoptotic molecule Bcl-x(L) was diminished in Stat5a$^{-/-}$ BMMCs (fig. 3, 4). These results indicate that Stat5a is crucial for IL-3-induced proliferation and survival of murine mast cells.

We show that Stat5a mediates IL-3-induced proliferation of murine mast cells. IL-3 has been shown to be an important growth factor for murine mast cells [3, 4]. IL-3 promotes the growth of multipotential mast cell progenitors, whereas SCF induces unipotential mast cell progenitors and also supports the growth of them [3, 4]. IL-3 has also been shown to be required for mast cell expansion in the tissues during immune responses to parasitic infection in mice [5]. Because it has been shown that IL-3, but not SCF, leads to activation of Jak2 and Stat5 and induces pim-1 expression in mast cells [18], Stat5a-mediated pim-1 expression is possibly involved in their proliferation [8].

SCF is also a critical growth factor for the proliferation and suppression of apoptosis in mast cells. Activation of the receptor tyrosine kinase c-kit by SCF induces receptor autophosphorylation and association with various signaling molecules including phosphatidylinositol 3-kinase (PI 3-kinase) and Src kinases. Timokhina et al. [19] have shown that the activation of PI 3-kinase and Src kinases contribute to c-kit-mediated proliferation and suppression of apoptosis induced by factor deprivation in BMMCs. Furthermore, the Rac1/JNK pathway has been shown to be critical for SCF-induced proliferation of mast cells [19]. Although it has been reported that SCF-induced proliferation of Stat5a/b-deficient BMMCs is impaired [10], our findings indicate that SCF normally induces the proliferation of Stat5a$^{-/-}$ BMMCs (fig. 2b), suggesting that Stat5a is not involved in SCF-induced proliferation of mast cells.

We also show that Stat5a is essential for the induction of Bcl-x(L) transcript after IL-3 stimulation in murine BMMCs (fig. 4). Bcl-x is a gene of the Bcl family and its longer isoform, Bcl-x(L), is known to have an antiapoptotic function [15–17]. Thus, diminished expression of Bcl-x(L) can account for the increased apoptosis in Stat5a$^{-/-}$ BMMCs in our study. On the other hand, we found normal expression levels of Bcl-2 gene in Stat5a$^{-/-}$ BMMCs as compared with that in WT BMMCs (data not shown). Our findings are in agreement with the observation that in the bone marrow-derived Ba/F3 cell line, IL-3-induced Stat5 activation induces Bcl-x(L) expression and IL-3-dependent suppression of apoptosis [20].

We found that the IL-3-induced proliferation of BMMCs in Stat5a$^{-/-}$ mice was reduced to about half of that in WT mice (fig. 2a). We also found that the number of apoptotic BMMCs was increased 4-fold in Stat5a$^{-/-}$ mice as compared with that in WT mice even in the presence of IL-3 (fig. 3c). As the results of the decreased proliferation and the increased apoptosis, we found that IL-3-induced mast cell development from bone marrow was strikingly impaired in Stat5a$^{-/-}$ mice (fig. 1b). On the other hand, we found that the decrease of mast cells in the peritoneal cavity and in ear and stomach was not so severe in Stat5a$^{-/-}$ mice (fig. 1a and data not shown), suggesting that Stat5a-independent pathways also participate in the in vivo development of mast cells in mice.

Acknowledgments

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Stat5a Inhibits IL-12-Induced Th1 Cell Differentiation through the Induction of Suppressor of Cytokine Signaling 3 Expression[1]

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In previous studies, we have shown that Th2 cell differentiation is diminished but Th1 cell differentiation is increased in Stat5a-deficient (Stat5a$^{-/-}$) CD4$^+$ T cells. In the present study, we clarified the molecular mechanisms of Stat5a-mediated Th cell differentiation. We found that enhanced Th1 cell differentiation and the resultant IFN-γ production played a dominant inhibitory role in the down-regulation of IL-4-induced Th2 cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells. We also found that IL-12-induced Stat4 phosphorylation and Th1 cell differentiation were augmented in Stat5a$^{-/-}$ CD4$^+$ T cells. Importantly, the expression of suppressor of cytokine signaling (SOCS)3, a potent inhibitor of IL-12-induced Stat4 activation, was decreased in Stat5a$^{-/-}$ CD4$^+$ T cells. Moreover, a reporter assay showed that a constitutively active form of Stat5a but not Stat6 activated the SOCS3 promoter. Furthermore, chromatin immunoprecipitation assays revealed that Stat5a binds to the SOCS3 promoter in CD4$^+$ T cells. Finally, the retrovirus-mediated expression of SOCS3 restored the impaired Th cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells. These results suggest that Stat5a forces the Th1/Th2 balance toward a Th2-type by preventing IL-12-induced Th1 cell differentiation through the induction of SOCS3. *The Journal of Immunology,* **2005, 174: 4105–4112.**

Over the last several years, significant progress has been made in the regulatory mechanisms of the transition of naive CD4$^+$ T cells into mature Th2 cells (1–3). Whereas early studies have demonstrated that Th2 cell differentiation is essentially a Stat6-dependent process (4–6), recent studies have revealed that Stat6-independent pathways also participate not only in in vitro Th2 cell differentiation (7) but also in in vivo Th2 cell-mediated allergic airway inflammation (8, 9). Because the presence of IL-4-producing cells during T cell activation induces subsequent Stat6-dependent Th2 cell differentiation (1–3), it is inferred that Stat6-independent IL-4 production enhances the Stat6-dependent process of Th2 cell differentiation.

Regarding the Stat6-independent pathway, recent studies including ours indicate that Stat5a is involved in Th2 cell differentiation. We have previously shown that Ag-induced Th2 cytokine production and subsequent allergic airway inflammation are decreased in Stat5a-deficient (Stat5a$^{-/-}$) mice (10). We have also shown that Th cell differentiation is biased toward a Th1-type at single cell levels in Stat5a$^{-/-}$ CD4$^+$ T cells (11) and that the retrovirus-mediated expression of Stat5a restores the impaired Th2 cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells (11). Moreover, it has recently been demonstrated that the enforced expression of a constitutively active form of Stat5a induces IL-4 production in CD4$^+$ T cells by regulating the accessibility of the IL-4 gene (12). These results suggest that the intrinsic expression of Stat5a within CD4$^+$ T cells plays a critical role in Th2 cell differentiation and in the induction of allergic airway inflammation and that Stat5a may function as a direct inducer of IL-4 production. In addition, we have found that, by comparing Stat6$^{-/-}$ mice to Stat5a- and Stat6-double deficient mice, Stat5a is indispensable in Stat6-independent Th2 cell differentiation of Stat6$^{-/-}$ CD4$^+$ T cells (44). However, the molecular mechanisms underlying Stat5a-mediated Th cell differentiation are still largely unknown.

In the present study, we determined the molecular mechanisms underlying Stat5a-mediated Th cell differentiation. First, we found that IL-12-induced Stat4 phosphorylation and Th1 cell differentiation were enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells. Second, we found that the expression of suppressor of cytokine signaling (SOCS)[4] 3, a potent inhibitor of IL-12/Stat4 signaling (13, 14), was decreased in Stat5a$^{-/-}$ CD4$^+$ T cells. Third, we found that Stat5a bound to SOCS3 promoter in CD4$^+$ T cells and directly induced SOCS3 expression. Finally, we found that the retrovirus-mediated expression of SOCS3 restored the Th1/Th2 balance of Stat5a$^{-/-}$ CD4$^+$ T cells. Taken together, our results indicate that Stat5a induces SOCS3 expression in CD4$^+$ T cells and thus inhibits IL-12-induced Th1 cell differentiation, forcing the Th1/Th2 balance toward a Th2-type.

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[4] Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; WT, wild type; ChIP, chromatin immunoprecipitation; LUC, luciferase; MGF, mammary gland factor.

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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 × 10^6 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; PeproTech) 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′-TTTGTCTC CCTCTCGGTGA-3′ and 5′-GTGTAGAGTCAGAGTTAGAG-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. 5*a*) 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 μg/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 ± 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

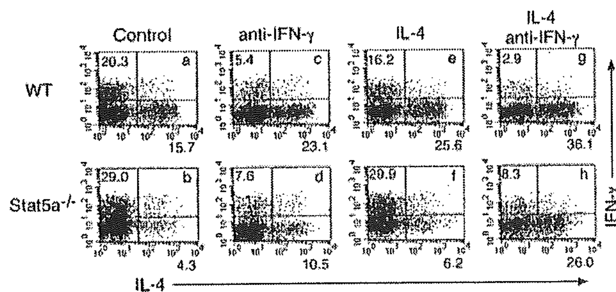

FIGURE 1. IFN-γ plays a dominant inhibitory role in the down-regulation of Th2 cell differentiation in $Stat5a^{-/-}$ $CD4^+$ T cells. Splenocytes from WT mice or $Stat5a^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of IL-4 (15 ng/ml) and/or neutralizing anti-IFN-γ Ab (15 μg/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.

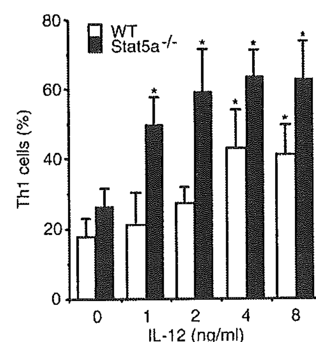

FIGURE 2. IL-12-induced Th1 cell differentiation is enhanced in $Stat5a^{-/-}$ $CD4^+$ T cells. Splenocytes from WT mice or $Stat5a^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of the indicated amounts of IL-12 (1–8 ng/ml). Cells were cultured for another 72 h in 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 ± SD of percentage of Th1 cells ($IL-4^-$ $IFN-\gamma^+$ cells) from four independent experiments. *, $p < 0.01$. Significantly different from the mean value of the corresponding control response (no IL-12).

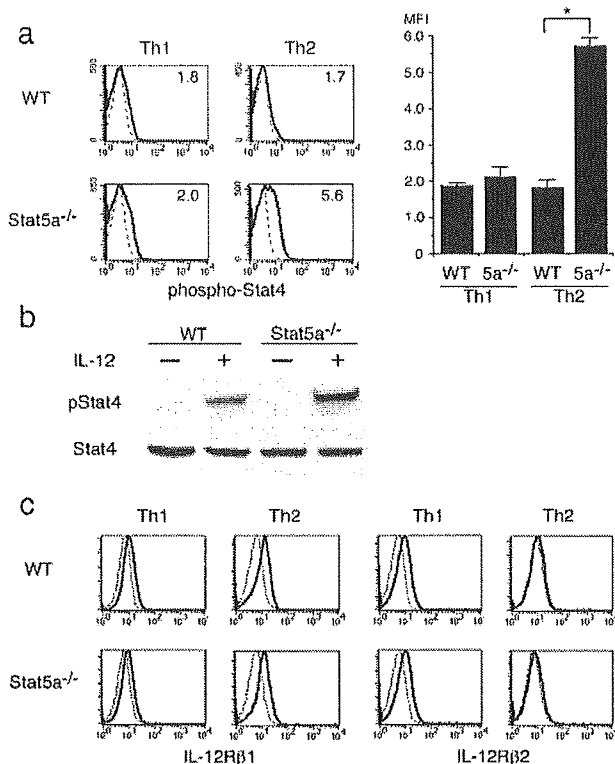

FIGURE 3. IL-12-induced Stat4 phosphorylation is enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells. *a*, IL-12-induced Stat4 phosphorylation in Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th1-polarizing condition (15 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

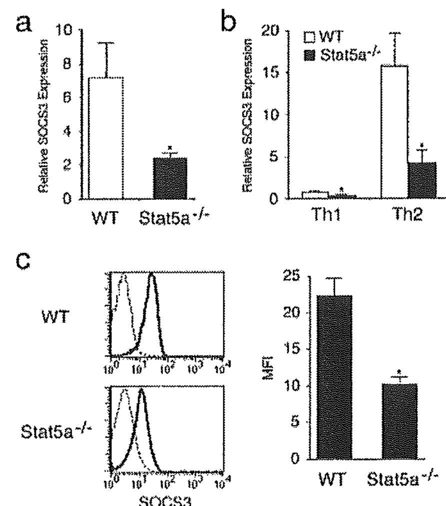

FIGURE 4. SOCS3 is diminished in Stat5a$^{-/-}$ CD4$^+$ T cells. *a*, Expression levels of SOCS3 mRNA in freshly isolated CD4$^+$ T cells. Total RNA was prepared from freshly isolated splenic WT CD4$^+$ T cells or Stat5a$^{-/-}$ CD4$^+$ T cells. TaqMan PCR analysis for SOCS3 and GAPDH (as a control) 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.

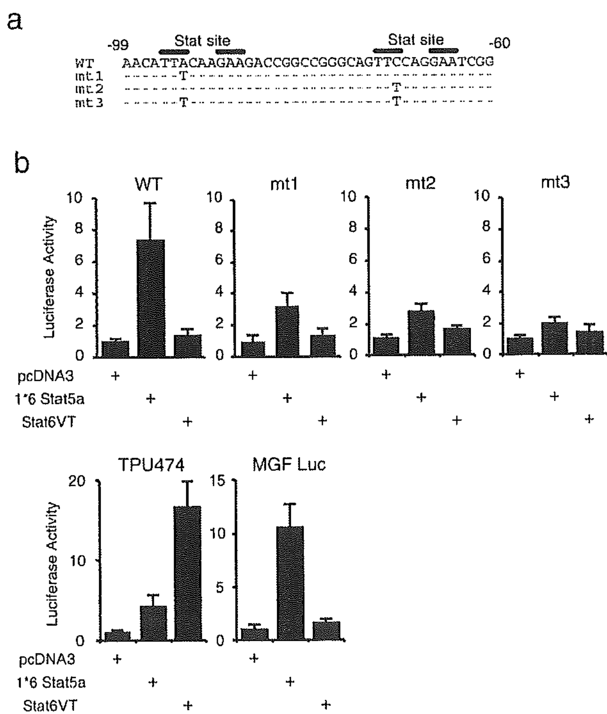

FIGURE 5. Stat5a directly activates the SOCS3 promoter. *a*, The murine SOCS3 promoter (WT) and the mutants (mt1, mt2, and mt3) of putative STAT-binding sequences. *b*, Stat5a preferentially activates the SOCS3 promoter. COS7 cells were transfected with TPU474, MGF-Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3 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.

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.

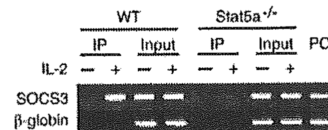

FIGURE 6. Stat5a binds to SOCS3 promoter in CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h. CD4$^+$ T cells were purified (>90% pure by flow cytometry) using a CD4$^+$ T cell enrichment column, starved from cytokines in fresh medium 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.

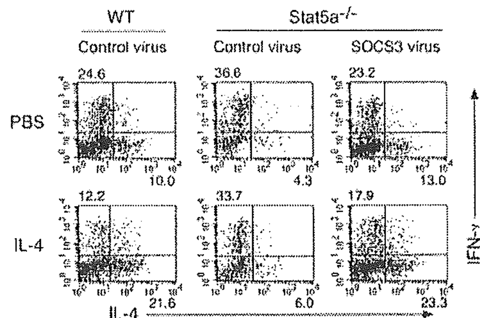

FIGURE 7. Retrovirus-mediated gene transduction of SOCS3 restores Th cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 40 h in the presence or absence of IL-4 (15 ng/ml) and then infected with retroviruses 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 disregulated 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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Indispensable Role of Stat5a in Stat6-Independent Th2 Cell Differentiation and Allergic Airway Inflammation[1]

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It is well-recognized that Stat6 plays a critical role in Th2 cell differentiation and the induction of allergic inflammation. We have previously shown that Stat5a is also required for Th2 cell differentiation and allergic airway inflammation. However, it is the relative importance and redundancy of Stat6 and Stat5a in Th2 cell differentiation and allergic airway inflammation are unknown. In this study we addressed these issues by comparing Stat5a-deficient (Stat5a$^{-/-}$) mice, Stat6$^{-/-}$ mice, and Stat5a- and Stat6 double-deficient (Stat5a$^{-/-}$ Stat6$^{-/-}$) mice on the same genetic background. Th2 cell differentiation was severely decreased in Stat6$^{-/-}$CD4^{+} T cells, but Stat6-independent Th2 cell differentiation was still significantly observed in Stat6$^{-/-}$CD4^{+} T cells. However, even in the Th2-polarizing condition (IL-4 plus anti-IFN-γ mAb), no Th2 cells developed in Stat5a$^{-/-}$Stat6$^{-/-}$ CD4^{+} T cells. Moreover, Ag-induced eosinophil and lymphocyte recruitment in the airways was severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with that in Stat6$^{-/-}$ mice. These results indicate that Stat5a plays an indispensable role in Stat6-independent Th2 cell differentiation and subsequent Th2 cell-mediated allergic airway inflammation. *The Journal of Immunology,* **2005, 174: 3734–3740.**

Newly activated CD4^{+} T cells differentiate into at least two functionally distinct subsets, Th1 and Th2 cells, as defined by their patterns of cytokine production (1, 2). Th1 cells produce IFN-γ and lymphotoxin and are responsible for delayed-type hypersensitivity reactions, promoting control of intracellular pathogens (1, 2). Th2 cells produce IL-4, IL-5, and IL-13 and provide an excellent helper function for Ab production, particularly of IgE (1, 2). Th2 cells are essential for promoting host defense against helminths, but uncontrolled Th2 cell activation to noninvasive Ags (allergen) causes atopic disorders, including asthma (3, 4).

Over the last several years, significant progress has been made in the molecular mechanisms for Th2 cell differentiation (5–7). Although early studies have indicated that Stat6 (8–10), a cytosolic latent transcription factor that is rapidly activated after cellular exposure to IL-4 and IL-13, is essential for Th2 cell differentiation through the induction of GATA3 (5–7), recent studies have revealed that Stat6-deficient (Stat6$^{-/-}$) CD4^{+} T cells make a considerable amount of IL-4 upon stimulation with TCR (11). In addition, it has been demonstrated that Th2 cell-mediated allergic airway inflammation is still observed in Stat6$^{-/-}$ mice (12–15). Therefore, in addition to the Stat6-dependent pathway, the Stat6-independent pathway participates in in vitro Th2 cell differentiation as well as in vivo Th2 cell-mediated immune responses.

In contrast, we have shown that Ag-induced IL-5 production and eosinophil recruitment in the airways are decreased in Stat5a$^{-/-}$ mice (16). In addition, we have shown that Th cell differentiation in Stat5a$^{-/-}$ mice is biased toward the Th1 type at single cell levels and that retrovirus-mediated expression of Stat5a restores the impaired Th2 cell differentiation of Stat5a$^{-/-}$CD4^{+} T cells (17). Consistent with these findings, it has recently been shown that the enforced expression of a constitutively active form of Stat5a induces IL-4 production in CD4^{+} T cells by enhancing the accessibility of the IL-4 gene (18). These findings suggest that the intrinsic expression of Stat5a in CD4^{+} T cells plays an important role in Th2 cell differentiation and the induction of allergic airway inflammation. However, the relative importance and redundancy of Stat5a-mediated Th2 cell differentiation and Stat6-mediated Th2 cell differentiation are still unclear.

In the present study we addressed these issues by comparing Th2 cell differentiation in Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a and Stat6 double-deficient (Stat5a$^{-/-}$Stat6$^{-/-}$) mice in the same genetic background. We also examined allergic airway inflammation in these mice as a model of in vivo Th2 cell-mediated immune responses. We found that Th2 cell differentiation and allergic airway inflammation were severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in Stat5a$^{-/-}$ mice or Stat6$^{-/-}$ mice. Our results suggest that Stat5a is essential for Th2 cell differentiation in the absence of Stat6 activation and vice versa.

Materials and Methods

Mice

Stat5a-deficient (Stat5a$^{-/-}$) mice (19) and Stat6-deficient (Stat6$^{-/-}$) mice (8) were backcrossed to BALB/c mice (Charles River Laboratories) for eight generations. Stat5a$^{+/-}$Stat6$^{+/-}$ male mice were mated with Stat5a$^{+/-}$Stat6$^{+/-}$ female mice to obtain Stat5a$^{+/+}$Stat6$^{+/+}$ mice (wild-type (WT)[3] mice), Stat5a$^{-/-}$Stat6$^{+/+}$ mice (Stat5a$^{-/-}$ mice), Stat5a$^{+/+}$Stat6$^{-/-}$ mice (Stat6$^{-/-}$ mice), and Stat5a$^{-/-}$Stat6$^{-/-}$ mice within the litter. All experiments were performed according to the guidelines of Chiba University.

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[3] Abbreviations used in this paper: WT, wild type; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff.

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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, -PE, -allophycocyanin, and -PerCP (H129.19); anti-CD8-FITC and -PE (53-6.7); anti-B220-allophycocyanin (RA3-6B2); anti-IgM-FITC (R6-60.2); anti-CD69-FITC (H1.3F3); anti-CD62L-FITC (MEL-14); anti-TCR Vβ8.1,2-FITC (MR5-2); and anti-pan-NK-PE (DX5). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Cell culture

Splenocytes (2×10^6 cells/ml) from WT mice, Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (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. Where indicated, IL-12 (15 ng/ml; PeproTech EC) was added to polarize toward Th1 cells (Th1 condition), and IL-4 (15 ng/ml; PeproTech EC) and anti-IFN-γ mAb (15 μg/ml; clone XMG1.2; BD Pharmingen) were added to polarize toward Th2 cells (Th2 condition) (17). Cells were washed with PBS, then cultured for another 3 days in Th0 (no exogenous cytokines), Th1, or Th2 conditions in the presence of IL-2 (20 U/ml; PeproTech).

Intracellular cytokine analysis

Intracellular cytokine staining for IL-4 vs IFN-γ was performed as described previously (17). In brief, cultured splenocytes were washed with PBS and 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 being stained with anti-CD4-PerCP, cells were 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) for 30 min at 4°C. The cytokine profile (IL-4 vs IFN-γ) of CD4$^+$ cells was analyzed on a FACSCalibur using CellQuest software.

Ag-induced allergic inflammation in the airways

Allergic airway inflammation was induced by the inhalation of OVA (Sigma-Aldrich) in sensitized mice as described previously (20). Briefly, mice (aged 7–8 wk) were immunized i.p. twice with 4 μg of OVA in 4 mg of aluminum hydroxide at a 2-wk interval. Twelve to 14 days after the second immunization, the sensitized mice were given aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer three times, for 20 min each time, at 24-h intervals. As a control, 0.9% saline alone was administered by the nebulizer. Forty-eight hours after the last inhalation, trachea and lung were excised, fixed in 10% buffered-formalin, and embedded in paraffin. The specimens (3 μm thick) of the trachea were stained with Luna and H&E solutions. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per length of the basement membrane of trachea, which was measured with a digital curvimeter.

Lung sections were stained with H&E and periodic acid-Schiff (PAS) according to standard protocols. The magnitude of inflammatory cell infiltration in the perivascular and peribronchial spaces on H&E-stained lung sections was evaluated by a semiquantitative scoring system as described previously (21): +5 signified a large (more than three cells deep) widespread infiltrate around the majority of vessels and bronchioles, and +1 signified a small number of inflammatory foci. The H&E-stained sections were coded and then examined by two observers in a blind manner, the sum of the scores from each lung was divided by the number of airways examined for the score, and the average of the two determinations for each section was used for subsequent calculations. PAS-stained lung sections were also categorized according to the abundance of PAS$^+$ goblet cells and assigned numerical scores as described previously (22): 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%.

The numbers of eosinophils, lymphocytes, and macrophages recovered in the bronchoalveolar lavage fluid (BALF) were evaluated as described previously (16). In short, after bronchoalveolar lavage was performed with 2 ml of PBS, BALF was centrifuged at $400 \times g$ for 5 min at 4°C, and differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution.

ELISA

Cultured splenocytes were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 12 h. The amounts of IL-4, IL-5, IL-10, and IFN-γ in the culture supernatant were measured by enzyme immunoassay using murine IL-4, IL-5, IL-10, and IFN-γ ELISA kits (BD Pharmingen). The amount of IL-13 in the culture supernatant was measured using an ELISA kit from R&D Systems. The assays were performed in duplicate according to the manufacturer's instructions. The minimum significant values of these assays were 15 pg/ml IL-4 and IL-5 and 30 pg/ml IFN-γ, IL-10, and IL-13.

Data analysis

Data are summarized as the mean ± SD. The statistical analysis of the results was performed by unpaired t test. A value of $p < 0.05$ was considered significant.

Results

Normal CD4$^+$ T cell development in Stat5a$^{-/-}$Stat6$^{-/-}$ mice

It has been shown that not only Stat6 (8–10), but also Stat5a (16–18), play critical roles in Th2 cell differentiation. To investigate the relative importance of Stat5a- and Stat6-mediated signaling in Th2 cell differentiation in detail, we generated Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice on the same genetic background and compared the development and differentiation of CD4$^+$ T cells among these mice. Consistent with the previous reports (16, 23), the number of splenocytes in Stat5a$^{-/-}$ mice was modestly, but significantly, decreased compared with that in WT mice (Fig. 1*A*). The number of splenocytes in Stat5a$^{-/-}$Stat6$^{-/-}$ mice was also decreased compared with that in Stat6$^{-/-}$ mice (Fig. 1*A*). However, FACS analysis revealed that the frequencies of CD4$^+$ T cells and CD8$^+$ T cells were similar among WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 1*B*). The expression of CD69 and CD62L on CD4$^+$ T cells was also similar among these mice (data not shown). Based on B220 vs IgM staining, B cells in the spleen exhibited normal maturation in these mice (Fig. 1*B*). These results indicate that T and B cells can develop even in the absence of Stat5a and Stat6.

Stat6-independent Th2 cell differentiation depends on Stat5a

We then examined cytokine production from WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ T cells. Splenocytes were stimulated with plate-bound anti-CD3 mAb in Th0 (no exogenous cytokines), Th1 (in the presence of IL-12), or Th2 (in the presence of

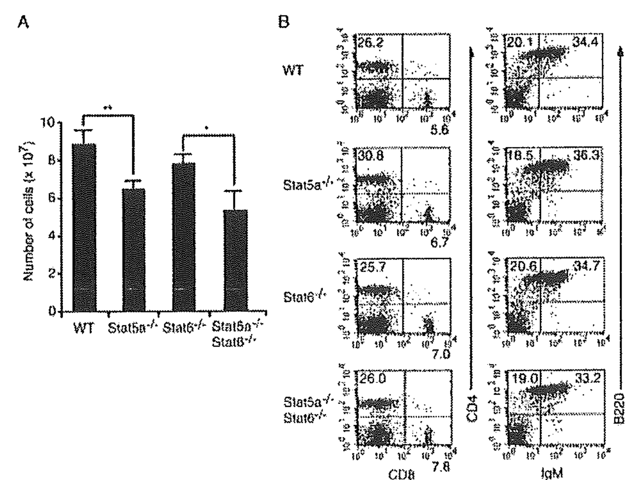

FIGURE 1. Normal T cell and B cell development in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, Number of splenocytes in WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$-Stat6$^{-/-}$ mice. Data are the mean ± SD from eight mice for each genotype. *, $p < 0.05$; **, $p < 0.01$. *B*, Flow cytometric analysis of splenocytes from 6-wk-old mice. Cells were stained with anti-CD4-PE vs anti-CD8-FITC or anti-B220-allophycocyanin vs anti-IgM-FITC. Shown are representative FACS profiles from five mice in each group.

IL-4 and anti-IFN-γ mAb) conditions for 2 days, then cultured for another 3 days in Th0, Th1, or Th2 conditions in the presence of IL-2. After washing, cells were restimulated with plate-bound anti-CD3 mAb for 12 h, and the amounts of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatant were determined. In the Th0 condition, IL-4 and IL-5 production was significantly decreased in $Stat5a^{-/-}$ splenocytes compared with that in WT splenocytes (Fig. 2), consistent with our previous report (17). IL-4 and IL-5 production was more severely decreased in $Stat6^{-/-}$ splenocytes (Fig. 2). However, significant IL-4 and IL-5 production was still detected in $Stat6^{-/-}$ splenocytes (Fig. 2). In contrast, almost no IL-4 or IL-5 was detected in $Stat5a^{-/-}Stat6^{-/-}$ splenocytes in the Th0 condition (Fig. 2). Furthermore, even when $Stat5a^{-/-}Stat6^{-/-}$ splenocytes were stimulated with anti-CD3 Ab in Th2 condition, they did not significantly produce IL-4 and IL-5 ($n = 5$; $p < 0.01$; Fig. 2). Similarly, IL-10 and IL-13 production was significantly decreased in $Stat5a^{-/-}Stat6^{-/-}$ splenocytes compared with that in $Stat5a^{-/-}$ or $Stat6^{-/-}$ splenocytes in the Th2 condition (Fig. 2). By contrast, IFN-γ production did not change in $Stat5a^{-/-}Stat6^{-/-}$ splenocytes in the Th0 condition and, instead, was increased in the Th1 condition compared with that in WT splenocytes or $Stat6^{-/-}$ splenocytes ($n = 5$; $p < 0.01$; Fig. 2). In contrast, no significant differences were observed in the proliferative responses of T cells among these mice in Th0, Th1, and Th2 conditions (data not shown), suggesting that the impaired Th2 cytokine production in $Stat5a^{-/-}Stat6^{-/-}$ splenocytes does not result from possible defects in cell proliferation.

Next, we examined Th1/Th2 cell differentiation at single-cell levels (Fig. 3). Splenocytes were stimulated with plate-bound anti-CD3 mAb in Th0, Th1, or Th2 conditions, and the cytokine profile (IL-4 vs IFN-γ) of $CD4^+$ T cells was evaluated by intracellular cytokine analysis. In the Th0 condition, $CD4^+$ T cells that produced IL-4, but not IFN-γ, were significantly decreased in $Stat5a^{-/-}$ mice compared with those in WT mice (Fig. 3, *a* vs *b*). IL-4-producing $CD4^+$ cells were more severely decreased in $Stat6^{-/-}$ mice but IL-4-producing $CD4^+$ cells still developed in

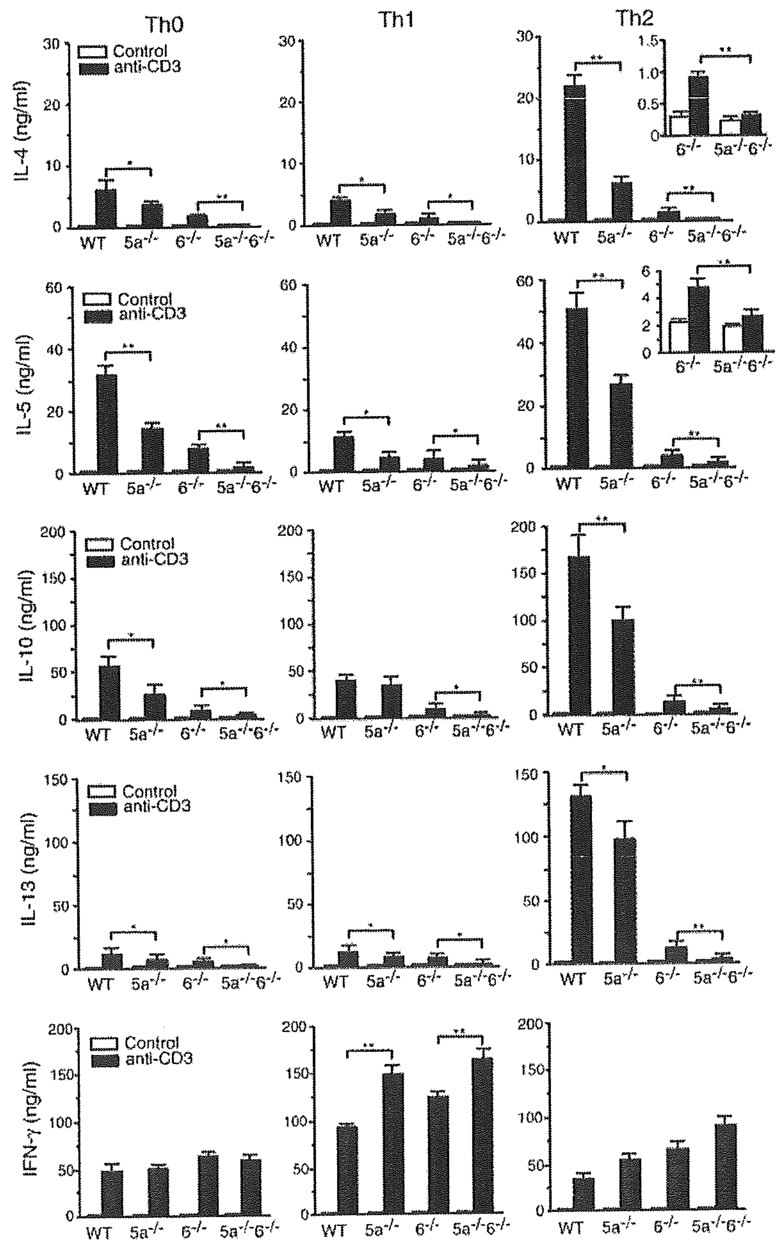

FIGURE 2. Th2 cytokine production is severely decreased in $Stat5a^{-/-}Stat6^{-/-}$ mice. Splenocytes from WT, $Stat5a^{-/-}$ ($5a^{-/-}$), $Stat6^{-/-}$ ($6^{-/-}$), or $Stat5a^{-/-}Stat6^{-/-}$ ($5a^{-/-}6^{-/-}$) mice were stimulated with plate-bound anti-CD3 mAb in the nonpolarizing Th0 condition (no exogenous cytokines), the Th1 condition (in the presence of IL-12), or the Th2 condition (in the presence of IL-4 and anti-IFN-γ mAb) for 48 h, then cultured for another 72 h in Th0, Th1, or Th2 conditions in the presence of IL-2. After washing, cells (1×10^6/ml) were restimulated with plate-bound anti-CD3 mAb for 12 h in the absence of exogenous cytokines. The amounts of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatant were determined by ELISA. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$.