was evaluated (Fig. 4). Consistent with the previous studies (12–16), the number of eosinophils recovered in BALF 48 h after the last Ag challenge was significantly diminished in Stat5a$^{-/-}$ mice as well as in Stat6$^{-/-}$ mice compared with that in WT mice (Fig. 4*A*). However, the eosinophil recruitment in BALF was still observed to a considerable extent in both Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice (Fig. 4*A*). In contrast, Ag inhalation induced no significant eosinophil recruitment in BALF in sensitized Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 4*A*). The number of eosinophils in BALF 48 h after the last Ag inhalation was as follows: WT mice, 222.2 ± 75.6; Stat5a$^{-/-}$ mice, 71.2 ± 22.7; Stat6$^{-/-}$ mice, 34.8 ± 13.1; and Stat5a$^{-/-}$Stat6$^{-/-}$ mice, $0.2 \pm 0.2 \times 10^4$/mice ($n = 5$ mice in each group; Fig. 4*A*). Ag-induced eosinophil recruitment in BALF was not observed in Stat5a$^{-/-}$Stat6$^{-/-}$ mice even 96 h after the last Ag inhalation (data not shown). The number of eosinophils infiltrating the submucosal tissue of the trachea 48 h after Ag inhalation was also severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with that in Stat5a$^{-/-}$ or Stat6$^{-/-}$ mice ($n = 5$; $p < 0.01$; Fig. 4*B*).

Ag-induced lymphocyte recruitment in BALF was also significantly decreased in Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice ($n = 5$; $p < 0.05$; Fig. 4*A*). Furthermore, virtually no Ag-induced lymphocyte recruitment in BALF was observed in Stat5a$^{-/-}$Stat6$^{-/-}$ mice ($n = 5$; $p < 0.01$; Fig. 4*A*). Consistent with these data obtained from BALF analysis (Fig. 4*A*), histological analysis showed that inflammatory cell infiltration in the lung after Ag inhalation was significantly decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with Stat5a$^{-/-}$ or Stat6$^{-/-}$ mice ($n = 5$; $p < 0.01$; Fig. 5*A*). In contrast, Ag-induced epithelial goblet cell hyperplasia was severely decreased not only in Stat5a$^{-/-}$Stat6$^{-/-}$ mice; but also in Stat6$^{-/-}$ mice, indicating that Stat6 is absolutely required for Ag-induced epithelial goblet cell hyperplasia ($n = 5$; Fig. 5, *B* and *C*). Taken together, these results suggest that the Stat5a-dependent, Stat6-independent pathway is involved in in vivo Th2 cell differentiation and subsequent allergic airway inflammation, but not in the induction of epithelial goblet cell hyperplasia.

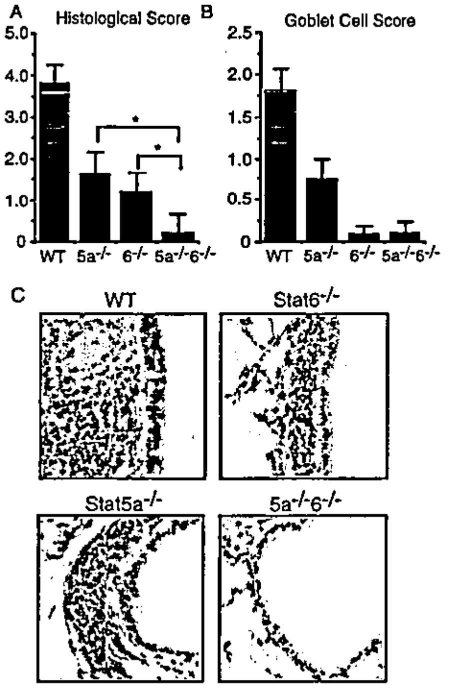

FIGURE 5. The Stat5a-dependent, Stat6-independent pathway induces airway inflammation, but not epithelial goblet cell hyperplasia. OVA-sensitized WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice were challenged with inhaled OVA three times at 24-h intervals. *A*, Forty-eight hours after the last OVA inhalation, lung was removed, and inflammatory cell infiltration into the perivascular and peribronchial spaces was scored as described previously (21). *B*, The degree of goblet cell hyperplasia was scored on PAS-stained sections as described previously (22). Data are the mean ± SD for five mice in each group. *, $p < 0.01$. *C*, Representative photomicrographs of PAS-stained lung sections from these mice are also shown (×100).

Discussion

In this study we show that Stat5a plays an indispensable role in Stat6-independent Th2 cell differentiation and subsequent allergic airway inflammation. We found that Th2 cell differentiation was severely decreased in Stat6$^{-/-}$ CD4$^+$ T cells, but that Stat6-independent Th2 cell differentiation was still observed in Stat6$^{-/-}$ CD4$^+$ T cells (Figs. 2 and 3). However, even in the Th2-polarizing condition, Th2 cells did not significantly develop in Stat5a$^{-/-}$ Stat6$^{-/-}$ CD4$^+$ T cells (Figs. 2 and 3), suggesting that the residual Th2 cell differentiation in Stat6$^{-/-}$ CD4$^+$ T cells depends on Stat5a. We also found that Ag-induced eosinophil and lymphocyte recruitment in the airways was severely decreased in Stat5a$^{-/-}$ Stat6$^{-/-}$ mice compared with that in Stat6$^{-/-}$ mice (Fig. 4). Taken together, our results suggest that the Stat5a-dependent, Stat6-independent pathway participates not only in in vitro Th2 cell differentiation, but also in in vivo Th2 cell-mediated allergic airway inflammation.

We show that Stat6 is not necessarily required for Stat5a-mediated Th2 cell differentiation. We found that the impairment of Th2 cell differentiation was more severe in Stat5a$^{-/-}$Stat6$^{-/-}$ CD4$^+$ T cells than that in Stat6$^{-/-}$ CD4$^+$ T cells (Fig. 3), indicating that Stat5a can induce Th2 cell differentiation even in the absence of Stat6 activation. This observation is consistent with a recent finding by Zhu et al. (18) demonstrating that the enforced expression of a constitutively active form of Stat5a induces IL-4 production even in Stat6$^{-/-}$ CD4$^+$ T cells. Because the induction of IL-4R α-chain expression requires IL-4/Stat6-mediated signaling (8–10, 25), it is possible that the Stat5a-dependent pathway plays a role in the initiation of Th2 cell differentiation before developing Th2 cells begin to up-regulate IL-4R α-chain to increase the sensitivity to IL-4/Stat6-mediated signaling. It is also possible that the Stat5a-dependent pathway may function as an amplifier of IL-4/Stat6-mediated Th2 cell differentiation.

Regarding the molecular mechanisms of Stat5a-mediated Th2 cell differentiation, it has recently been shown that activated Stat5a directly interacts with HSII and HSIII sites of the IL-4 gene and then up-regulates the accessibility of the IL-4 gene (18). These results suggest that Stat5a functions as a direct inducer of IL-4 production. In contrast, we found that the enhanced Th1 cell differentiation was responsible in part for the impaired Th2 cell differentiation in Stat5a$^{-/-}$ CD4$^+$ T cells.[4] We also found that the expression pattern of SOCS family proteins was different between WT CD4$^+$ T cells and Stat5a$^{-/-}$ CD4$^+$ T cells (see Footnote 4). Because accumulating evidence suggests that some of SOCS family proteins are involved in cross-regulation of the cytokine network and then regulate Th1 and Th2 cell differentiation (26, 27), the different expression of SOCS family proteins in Stat5a$^{-/-}$ CD4$^+$ T cells may also be involved in the regulation of Th1/Th2 balance.

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[4] H. Takatori, H. Nakajima, S. Kagami, K. Hirose, A. Suto, K. Suzuki, M. Kubo, A. Yoshimura, Y. Saito, and I. Iwamoto. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of SOCS3 expression. *Submitted for publication.*

We also demonstrate that Stat5a, independently of Stat6, contributes to the induction of Th2 cell-mediated allergic airway inflammation. It has been shown that Ag-induced eosinophil and lymphocyte recruitment in the airways is mediated by Th2 cells secreting IL-5 (20, 28) and IL-4 (29, 30), respectively. Although it is apparent that Stat6 plays an important role in causing allergic airway inflammation (31), it has been demonstrated that in vivo Th2 cell differentiation and allergic airway inflammation are still substantial in Stat6$^{-/-}$ mice (12–15), suggesting that a Stat6-independent mechanism is involved in the development of allergic airway inflammation. In the present study we found that the residual Th2 cell-mediated allergic airway inflammation in Stat6$^{-/-}$ mice was abrogated by the additional deletion of the Stat5a gene (Fig. 4). Therefore, in addition to the Stat6-dependent pathway, the Stat5a-dependent, Stat6-independent pathway participates in in vivo Th2 cell-mediated immune responses such as allergic airway inflammation.

It is still uncertain which cytokine is upstream of Stat5a-mediated Th cell differentiation. A number of immunologically important cytokines, including IL-2, IL-7, and IL-15, have been shown to activate Stat5a in many cell types (32). IL-4 has also been reported to activate Stat5 in some circumstances (33, 34), but we have previously shown that IL-4 does not phosphorylate Stat5a in CD4$^+$ T cells (17). Therefore, it is unlikely that IL-4 is an upstream cytokine for Stat5a-mediated Th2 cell differentiation. In contrast, it has recently been shown that developing Th2 cells express higher levels of IL-2R α-chain and exhibit stronger Stat5 activation than developing Th1 cells (35). This is consistent with a previous finding that Stat5a functions as an enhancer of IL-2 signaling by inducing the expression of IL-2R α-chain (23). Moreover, it has been demonstrated that Th2 cell differentiation is decreased by the neutralization of IL-2 or the blocking of IL-2R (18, 35, 36). Furthermore, it has been demonstrated that IL-2, but not IL-4, IL-9, IL-15, or IL-21, induces Stat5 phosphorylation and IL-4 production in activated CD4$^+$ T cells (37). Therefore, IL-2 is likely to be a cytokine responsible for Stat5a activation during Th2 cell differentiation.

Given that Stat5b is highly homologous to Stat5a (32) and that Stat5a/Stat5b double-deficient mice exhibit a severe defect in T cell responses compared with Stat5a$^{-/-}$ or Stat5b$^{-/-}$ mice (38), it is apparent that Stat5a and Stat5b have overlapping functions. However, the different phenotypes of Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice underscore the distinctive roles of Stat5a and Stat5b (17, 23, 39). For example, it has been demonstrated that although Stat5a$^{-/-}$ T cells exhibit no detectable defect in anti-CD3-induced proliferation, Stat5b$^{-/-}$ T cells are defective in anti-CD3-induced proliferation (17, 23, 39). These observations suggest 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 previously shown that both Th1 and Th2 cells are decreased in Stat5b$^{-/-}$ mice, whereas Th2, but not Th1, cells are decreased in Stat5a$^{-/-}$ mice (16). Nevertheless, because the number of CD4$^+$ T cells recovered from the culture was significantly lower in Stat5b$^{-/-}$ mice than in Stat5a$^{-/-}$ or WT mice (17), these data on Th cell differentiation in Stat5b$^{-/-}$ mice might be inconclusive. However, our finding that Th2 cells cannot develop in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3) suggests that Stat5b cannot compensate for the role of Stat5a in Stat6-independent Th2 cell differentiation, because Stat5b can be normally expressed and activated in response to IL-2 even in the absence of Stat5a (23, 39).

In conclusion, we have shown that Stat5a activation is required for proper Th2 cell differentiation, and that Stat5a plays an indispensable role in Th2 cell differentiation in the absence of Stat6 activation. Although additional studies are required for complete understanding of the molecular mechanisms of Stat5a-mediated Th2 cell differentiation, our findings provide new insight into the mechanism of Stat6-independent Th2 cell differentiation and allergic airway inflammation.

Acknowledgments

We thank Dr. L. Hennighausen for Stat5a$^{-/-}$ mice, and Drs. S. Akira and K. Takeda for Stat6$^{-/-}$ mice.

Disclosures

The authors have no financial conflict of interest.

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The Journal of Immunology

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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Received for publication September 28, 2004. Accepted for publication January 23, 2005.

[1] This work was supported in part by grants from Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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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.

0022-1767/05/$02.00

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

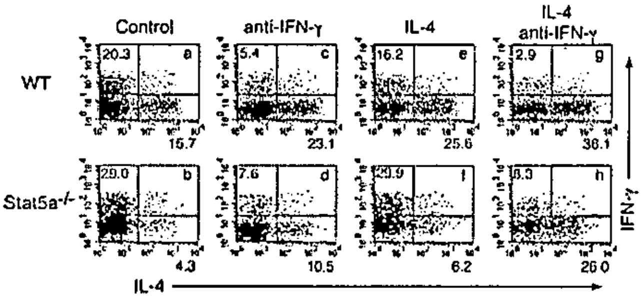

FIGURE 1. IFN-γ plays a dominant inhibitory role in the down-regulation of Th2 cell differentiation in Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of IL-4 (15 ng/ml) and/or neutralizing anti-IFN-γ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

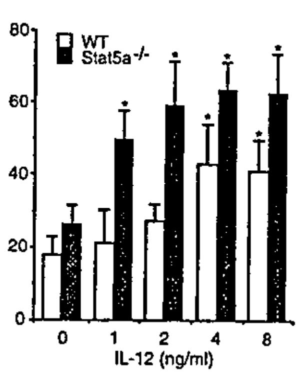

FIGURE 2. IL-12-induced Th1 cell differentiation is enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of the indicated amounts of IL-12 (1–8 ng/ml). Cells were cultured for another 72 h in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

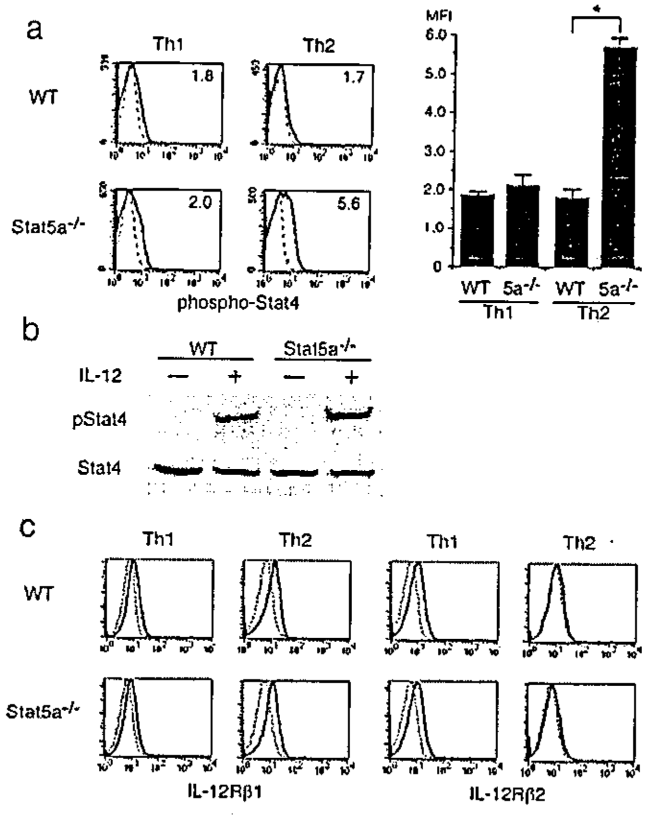

FIGURE 3. IL-12-induced Stat4 phosphorylation is enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells. *a*, IL-12-induced Stat4 phosphorylation in Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th1-polarizing condition (15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

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

Because IL-12 responsiveness is regulated in part by the expression levels of its receptor (27), we next examined the expression of IL-12Rβ1 and IL-12Rβ2 on Stat5a$^{-/-}$ CD4$^+$ T cells. However, FACS analysis revealed that both IL-12Rβ1 and IL-12Rβ2 were normally expressed in Stat5a$^{-/-}$ CD4$^+$ T cells even in Th2-polarizing condition (Fig. 3*c*), suggesting that the expression levels of IL-12Rs are not likely to be responsible for the enhanced IL-12-induced Stat4 phosphorylation in developing Th2 cells by the absence of Stat5a.

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

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

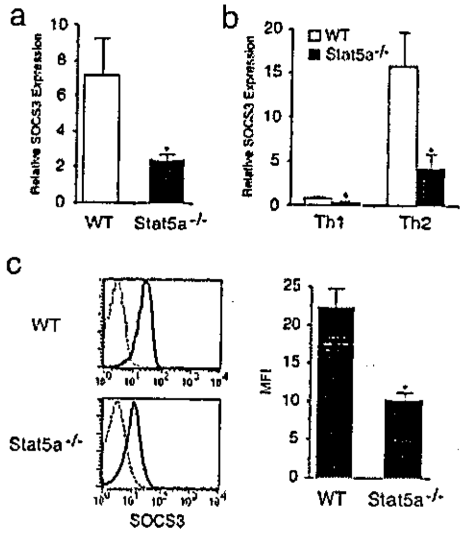

FIGURE 4. SOCS3 is diminished in Stat5a$^{-/-}$ CD4$^+$ T cells. *a*, Expression levels of SOCS3 mRNA in freshly isolated CD4$^+$ T cells. Total RNA was prepared from freshly isolated splenic WT CD4$^+$ T cells or Stat5a$^{-/-}$ CD4$^+$ T cells. TaqMan PCR analysis for SOCS3 and GAPDH (as a control)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

cells were cultured in Th2-polarizing condition (Fig. 4*b*). As shown in Fig. 4*a*, the expression levels of SOCS3 mRNA were significantly decreased in freshly isolated Stat5a$^{-/-}$ CD4$^+$ T cells ($n = 4$, $p < 0.01$). The expression of SOCS3 mRNA was upregulated 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.

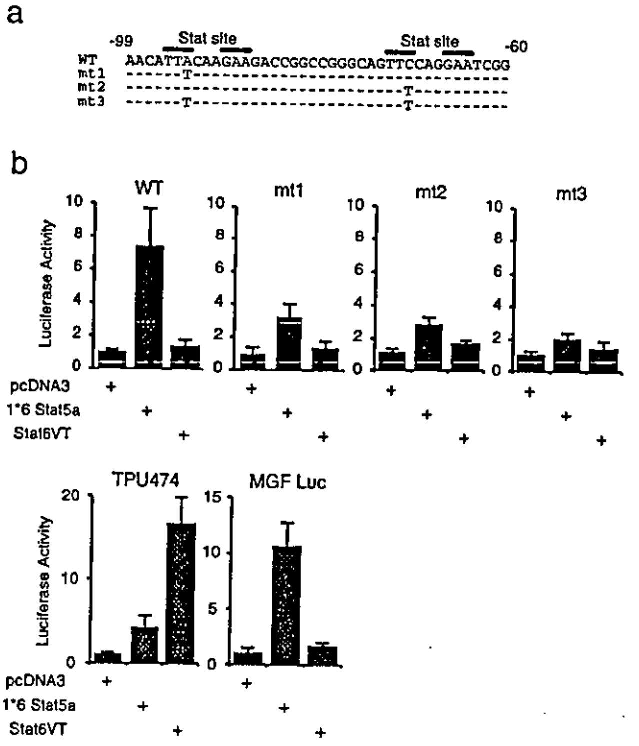

FIGURE 5. Stat5a directly activates the SOCS3 promoter. *a*, The murine SOCS3 promoter (WT) and the mutants (mt1, mt2, and mt3) of putative STAT-binding sequences. *b*, Stat5a preferentially activates the SOCS3 promoter. COS7 cells were transfected with TPU474, MGF-Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3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

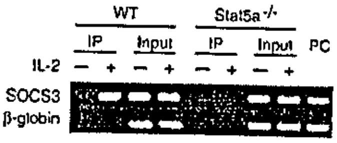

FIGURE 6. Stat5a binds to SOCS3 promoter in CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h. CD4$^+$ T cells were purified (>90% pure by flow cytometry) using a CD4$^+$ T cell enrichment column, starved from cytokines in fresh medium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

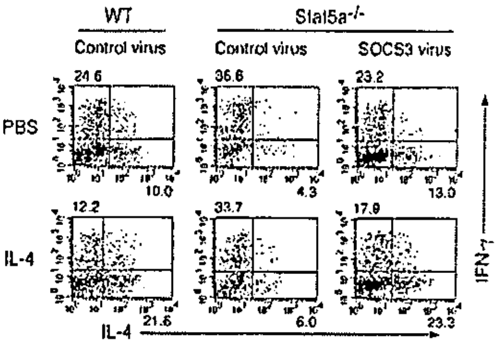

FIGURE 7. Retrovirus-mediated gene transduction of SOCS3 restores Th cell differentiation of Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 40 h in the presence or absence of IL-4 (15 ng/ml) and then infected with retroviruses 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.

Acknowledgments

We thank Dr. L. Hennighausen for Stat5a$^{-/-}$ mice, Dr. T. Kitamura for pMX-1*6 Stat5a-IRES-GFP and pMX-IRES-GFP, Dr. H. Wakao for MGF-Luc, Dr. U. Schindler for TPU474, and Dr. K. Ikuta for valuable discussion.

Disclosures

The authors have no financial conflict of interest.

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In press in Int. Arch. Allergy Immunol.

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

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Running head: Role of Stat5a in mast cell development
Key words: Stat5a, Mast cell, Bcl-x(L), Apoptosis

ABSTRACT

The regulatory roles of signal transducer and activator of transcription (Stat) 5a in the proliferation and survival of mast cells were 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, interleukin (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 apoptic 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.

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 (Ig) E. Mast cells 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 c-kit/stem cell factor (SCF) system and interleukin (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 *Stronglyoides 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 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 back-crossed 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 Chiba University.

Culture of BMMCs

Primary culture of IL-3-dependent bone marrow-derived cells (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 μM β-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 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 non-adherent bone marrow cells were then maintained at 37°C at a density of 2-5 x 10^5 cells/mL in the same medium, with biweekly replacement of old media with fresh ones. BMMCs were used for experiments at 4 to 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 (x 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, CA) using CELLQuest software. FACS analysis was performed with anti-CD117 (c-kit) FITC (2B8, BD PharMingen, San Diego, CA) 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 cell surface, cells were first incubated with mouse anti-TNP IgE (IgE3, BD PharMingen) at 4°C for 60 minutes to saturate IgE receptors, and were then labeled with anti-IgE FITC (R35-72, BD PharMingen).

Cell survival assay

BMMCs were washed three times with PBS and cultured at 1 x 10^6 cells/mL in triplicate at 37°C for 48 hours in RPMI 1640 medium without IL-3. Peritoneal lavage cells were cultured at 1 x 10^6 cells/mL in triplicate at 37°C for 24 hours 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, IN) [14].

Annexin V staining of BMMCs

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

Proliferation assay

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

Cell division assay

BMMCs were incubated with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, 10 μM, Molecular Probes Inc., Eugene, OR) in PBS at 37°C for 10 minutes, and then washed with RPMI 1640 medium. CFSE-labeled BMMCs were cultured at 37°C for 72 hours 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, Buckinghamshice, 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.

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) (Figure 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) (Figure 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 (Figure 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.

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, $p<0.001$) (Figures 2A and 2B). In addition, IL-3-induced cell division of Stat5a$^{-/-}$ BMMCs was less frequent than that of WT BMMCs (Figure 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) (Figures 3A and 3B). Interestingly, apoptotic cells were significantly increased in Stat5a$^{-/-}$ BMMCs even when they were cultured with IL-3 (n = 5, $p<0.001$) (Figure 3C). Furthermore, mRNA of Bcl-x(L), an anti-apoptotic molecule [15-17], was expressed in WT BMMCs but not in Stat5a$^{-/-}$ BMMCs (Figure 4), suggesting that Stat5a may suppress apoptosis of mast cells by inducing the expression of anti-apoptotic 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 (Figure 1). We also found that IL-3-induced but not SCF-induced proliferation was decreased in Stat5a$^{-/-}$ BMMCs (Figure 2). Finally, we found that apoptosis was increased and the expression of an anti-apoptotic molecule Bcl-x(L) was diminished in Stat5a$^{-/-}$ BMMCs (Figures 3 and 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 (Figure 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 (Figure 4). Bcl-x is a gene of the Bcl family and its longer isoform, Bcl-x(L), is known to have an anti-apoptotic 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 a half of that in WT mice (Figure 2A). We also found that the number of apoptotic BMMCs was increased four-fold in Stat5a$^{-/-}$ mice as compared with that in WT mice even in the presence of IL-3 (Figure 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 (Figure 1B). On the other hand, we found that the decrease of mast cells in peritoneal cavity and in ear and stomach was not so severe in Stat5a$^{-/-}$ mice (Figure 1A and data not shown), suggesting that Stat5a-independent pathways also participate in the in vivo development of mast cells in mice.

ACKNOWLEDGMENTS

We thank Dr. L. Hennighausen for Stat5a-/- mice and Dr. H. Karasuyama for X63-IL-3 cells.

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FIGURE LEGENDS

Figure 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 wild-type (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 bone marrow-derived mast cells (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. Shown are representative FACS profiles and the mean fluorescence intensities from four independent experiments.

Figure 2. Stat5a is crucial for IL-3-induced but not SCF-induced proliferation of BMMCs.

(A and 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}-1 μg/mL) or SCF (10^{-5}-1 ng/mL) at 37°C for 48 hours and the proliferative responses were evaluated by the addition of [^{3}H] thymidine for the final 12 hours. 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 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE). These cells were cultured with IL-3 at 37°C for 48 hours and then analyzed by FACS. Shown are representative FACS profiles for the intensity of CFSE (n = 4). The numbers indicate % of cell numbers after 0 to 3 cell divisions, respectively.

Figure 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 hours 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 propidium iodide (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 x 10^6 cells/ml at 37°C for 48 hours 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. Shown are representative FACS profiles from five independent experiments.

Figure 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.