Cytokines play essential roles in the control of immune systems; they not only act as growth factors but also regulate the differentiation, maintenance, and activation of naive, effector, and memory state of immune cells. Their cytoplasmic signal transduction pathways are well defined. Upon binding of cytokines to their receptors and subsequent receptor dimerization, receptor-associated JAKs become activated and phosphorylate tyrosine residues in the cytoplasmic domains of receptors, which serve as the binding sites for Src homology 2 (SH2) domain of STAT molecules. After phosphorylation of STATs by JAKs, STATs dimerize and translocate into the nucleus to induce transcription of cytokine-responsive genes (1, 2).

The cytokine milieu and their intracellular signaling molecules are also involved in naive CD4+ T cell differentiation. It is well established that IL-12/STAT4 and IL-4/STAT6 are necessary for Th1 and Th2 differentiation, respectively. In addition, IFN-γ–STAT1 pathway is also necessary for Th1 differentiation (3, 4). The molecular mechanism for generating Th3 regulatory cells, which is a unique Th cell subset that primarily secretes TGF-β1, is poorly understood. TGF-β1 secreted from Th3 cells provides help for IgA induction and has suppressive properties for both Th1 and Th2 cells (5, 6). Because TGF-β1 KO mice exhibited severe multiorgan inflammations (7, 8), TGF-β1 has been thought to be an important immune regulatory cytokine. TGF-β1 has been shown to be involved in the regulatory function of CD4+ CD25+ regulatory T cells (9, 10), though the molecular mechanism of TGF-β1 induction is not thoroughly elucidated. Because production of TGF-β1

Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor–β1

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Suppressor of cytokine signaling (SOCS)3 is a major negative feedback regulator of signal transducer and activator of transcription (STAT)3–activating cytokines. Transgenic mouse studies indicate that high levels of SOCS3 in T cells result in type 2 T helper cell (Th2) skewing and lead to hypersensitivity to allergic diseases. To define the physiological roles of SOCS3 in T cells, we generated T cell–specific SOCS3 conditional knockout mice. We found that the mice lacking SOCS3 in T cells showed reduced immune responses not only to ovalbumin–induced airway hyper responsiveness but also to Leishmania major infection. In vitro, SOCS3–deficient CD4+ T cells produced more transforming growth factor (TGF)–β1 and interleukin (IL)–10, but less IL–4 than control T cells, suggesting preferential Th3–like differentiation. We found that STAT3 positively regulates TGF–β1 promoter activity depending on the potential STAT3 binding sites. Furthermore, chromatin immunoprecipitation assay revealed that more STAT3 was recruited to the TGF–β1 promoter in SOCS3–deficient T cells than in control T cells. The activated STAT3 enhanced TGF–β1 and IL–10 expression in T cells, whereas the dominant–negative form of STAT3 suppressed these. From these findings, we propose that SOCS3 regulates the production of the immunoregulatory cytokines TGF–β1 and IL–10 through modulating STAT3 activation.

The online version of this article contains supplemental material.
is greatly enhanced by IL-4 and IL-10 in Th cells, while suppressed by IFN-γ (11), cytokine signals may play critical roles in the induction and regulation of TGF-β1 production.

In the physiologic condition as well as in pathological conditions, functions of cytokines are strictly controlled. Cytokine signaling pathways are negatively regulated by the family of proteins called suppressors of cytokine signaling (SOCSs), which are characterized by the presence of an SH2 domain and a COOH terminal conserved domain termed the SOCS-box. Several reports have indicated that SOCS proteins are necessary for regulation of normal immune responses (12). Among them, SOCS3, which associates with the tyrosine kinase Lck, calcineurin, and CD28, has been shown to inhibit IL-2 production during T cell activation (13–16). During Th differentiation, SOCS3 is selectively expressed in Th2 cells, whereas SOCS1 expression is higher in Th1 than in Th2 cells (17, 18). In the analysis of Lck promoter-driven SOCS3-transgenic mice, the high expression of SOCS3 in Th cells led to skewing to Th2-type differentiation. This is probably because SOCS3 binds to IL-12Rβ2 and inhibits IL-12–mediated STAT4 activation, thereby blocking Th1 development (18, 19). Importantly, SOCS3 levels were high in T cells from allergic disease patients (18). These observations implied that SOCS3 might be crucial for Th cell differentiation and activation. However, as most of these conclusions have been drawn by overexpression studies or in pathological conditions such as asthma and atopy, analysis of SOCS3-deficient mice has been necessary to clarify the physiological function of SOCS3 in T cells more precisely. Because mice lacking SOCS3 die during embryogenesis as the result of a placental defect by an enhanced activation of the leukemia inhibitory factor (LIF) signaling pathway (20, 21), we generated T cell–specific SOCS3-deficient (conditional KO [cKO]) mice by a conditional gene targeting approach using Cre-loxP system (22). We showed that not only Th2-type responses in OVA-induced asthma model but also immune responses against Leishmania major infection were reduced in cKO mice. In vitro analysis of T cells demonstrated that SOCS3-deficient CD4+ T cells produced more TGF-β1 and IL-10, but less IL-4 than WT T cells, suggesting a preferential Th3-like differentiation. We found that STAT3 bound to the TGF-β1 promoter and elevated the promoter activity, and SOCS3 deletion enhanced STAT3 recruitment to the promoter. It has been shown that STAT3 also binds to the IL-10 promoter and elevates IL-10 gene expression (23). In conclusion, we propose that STAT3 and SOCS3 reciprocally regulate Th cell function and differentiation by controlling the induction of the immunosuppressive cytokine, TGF-β1, and IL-10.

RESULTS

Generation of T cell–specific SOCS3-deficient mice

To delete the SOCS3 gene in a T cell–specific manner, proximal p56Lck promoter-cre transgenic mice were crossed with SOCS3-flox/flox mice (22) (Fig. 1 A). Resulting SOCS3-flox/flox: Lck-cre Tg mice are designated as cKO mice. SOCS3 WT alleles, floxed alleles, and the Lck-cre transgene were determined by PCR using genomic DNA from tails and CD4+ T cells. A 380-bp product corresponding to the nondeleted floxed allele was amplified by primer set of a and b from tail DNA of SOCS3-cKO and WT-flox/flox mice (C57BL/6 showed a 280-bp fragment because of the lack of floxed alleles). An approximately 1.2-kbp fragment was amplified from DNA isolated from CD4+ T cells of WT-flox/flox mice by the primer set of a and c, whereas a 250-bp fragment corresponding to the deleted allele was amplified from SOCS3-cKO mice CD4+ T cells (Fig. 1 B). Deletion of the SOCS3-flox gene was specific to T cells, and no deletion was observed in B cells and DCs in cKO mice (Fig. 1 C). Next, to confirm the deletion of mRNA, we performed RT-PCR analysis in IL-6–stimulated splenic CD4+ T cells from SOCS3-cKO mice. Although SOCS3 was
induced after IL-6 stimulation in WT CD4$^+$ T cells and the
expression of IL-6 receptor was at an almost equal level,
SOCS3 mRNA was undetectable in CD4$^+$ T cells from
SOCS3-cKO mice (Fig. 1 D). Western blotting analysis us-
ing antibody specific for SOCS3 also confi rmed the absence
of SOCS3 protein in CD4$^+$ T cells from SOCS3-cKO mice
(Fig. 1 E). Thus, we concluded that cre-mediated deletion of
SOCS3 occurred effi ciently and specifi cally in T cells in
SOCS3-cKO mice.

We examined IL-6–mediated STAT3 activation in
SOCS3-defi cient T cells. As shown in Fig. 1 E, IL-6–induced
STAT3 activation was enhanced and prolonged in SOCS3-
deficient CD4$^+$ T cells. This confi rmed a negative regulatory
function of SOCS3 for the gp130–STAT3 pathway. As in
macrophages (22), SOCS3 defi ciency in T cells did not much
affect IL-10–induced STAT3 activation (unpublished data).

Next, we examined development of T cells in SOCS3
cKO mice. Total mononuclear cell numbers of lymphoid or-
gans such as thymus, spleen, and lymph nodes in SOCS3–cKO
mice were almost the same as those in WT mice. Flow cyto-
metric analysis revealed that the ratio of CD4$^+$ or CD8$^+$ SP
cells was not altered in SOCS3-cKO mice, although the num-
bers of CD4$^+$ CD8$^-$ DN cells were slightly higher in SOCS3-
cKO mice (unpublished data). T cell numbers, the CD4/CD8

Figure 2. Reduced Th2 responses of SOCS3-defi cient T cells in
OVA/alum immunized mice. (A) Analyses of serum OVA-specific IgG1,
IgG2a, and IgE titers in cKO and WT mice. Plasma samples were taken from
mice ($n = 5$) at indicated days after immunization with OVA/alum on days
0 and 14. Ab titers were measured by ELISA and endpoint analysis. Data
indicate mean ± SD. (B) Mice ($n = 9$ for each group) immunized with
OVA/alum were aerosol challenged with OVA. Airway responsiveness was
determined by the acetylcholine-dependent change in airway pressure in
saline-treated control and OVA-sensitized/challenged WT and SOCS3-cKO
mice. Provocative concentration 200 (PC$_{200}$), the concentration
at which airway pressure is 200% of its baseline value. Data indicate
mean ± SD. (C) Cell counts in bronchoalveolar lavage fi luid. *, $P < 0.05$ by
analysis of variance with Bonferroni correction. Data indicate mean ± SD.
(D) Cytokine profi les of Th1 type (IFN-$\gamma$), Th2 type (IL-4 and IL-5), and
TGF-$\beta$1 and IL-10. Splenic CD4$^+$ T cells isolated from OVA-immunized mice
were restimulated with or without OVA ex vivo for 48 h. Cytokine levels
were determined by ELISA. Data indicate mean ± SD in one representative
experiment with fi ve mice per group out of three independent experiments.
ratio, and other T cell markers (TCRβ, CD25, CD69, CD62L) were not altered in the spleen and lymph node of cKO mice (unpublished data). Therefore, we concluded that SOCS3 does not play an essential role in T cell development.

**Reduced Th2-type response in SOCS3-cKO mice**

Previously, we reported that constitutive expression of SOCS3 in T cells causes preferential Th2 differentiation of CD4+ T cells, resulting in hyper IgE production and enhanced OVA-induced airway hypersensitiveness (18). Thus, we investigated the effect of SOCS3 deletion in T cells on OVA immunization. After mice were immunized with OVA and alum as an adjuvant on days 1 and 14, we examined the Ig levels and cytokine production. Total IgG1 and IgG2a levels before immunization were almost the same between WT and cKO mice (unpublished data). As shown previously (24), OVA/alum immunization significantly enhanced Th2-mediated Ig (IgG1 and IgE) production (Fig. 2 A). Interestingly, SOCS3-cKO mice produced lower levels of IgG1 and IgE than WT mice did, although Th1-mediated IgG2a production was similarly low between WT and cKO mice (Fig. 2 A). Reflecting reduced IgE levels, SOCS3-cKO mice exhibited lower sensitivity to airway responsiveness and reduced eosinophil infiltration in BAL fluids in cKO mice after OVA challenge compared with WT mice (Fig. 2, B and C). These data confirmed that SOCS3 levels in Th cells alter type 2 responses in vivo.

To further determine the role of SOCS3 in Th cell differentiation, we compared the immune responses against L. major infection with WT mice (Fig. 2, B and C). These data confirmed that loss of SOCS3 expression in T cells resulted in lower Th2-type immune responses, which was accompanied with reduced IL-4 levels; however, production of TGF-β1 and IL-10, but not IFN-γ, was enhanced. Moreover, serum antibody levels after L. major infection were lower in cKO mice than in WT mice (Fig. 2 B). Therefore, we concluded that SOCS3-deficient T cells possess higher potential to produce IL-10 and TGF-β1 than WT T cells.

**Cytokine production from in vitro–differentiated SOCS3-deficient T cells**

To elucidate the reason why IL-10 and TGF-β1 were elevated in CD4+ T cells from SOCS3-cKO mice, we analyzed cytokine production in CD4+ T cells from SOCS3-cKO mice, suggesting that loss of SOCS3 in Th cells did not enforce Th1 skewing. In contrast with Th2-type cytokines, TGF-β1 and IL-10 levels were higher in SOCS3-cKO mice than in WT mice (Fig. 2 D). We also confirmed high mRNA expression levels of TGF-β1 and IL-10 by RT-PCR (unpublished data). During in vitro restimulation, no significant difference in the proliferation was observed between WT and SOCS3-deficient T cells (unpublished data). These results indicate that loss of SOCS3 expression in T cells resulted in lower Th2-type immune responses, which was accompanied with reduced IL-4 levels; however, production of TGF-β1 and IL-10, but not IFN-γ, was enhanced.
We next examined Th3 differentiation, which has been induced in vitro by culturing CD4+ T cells in the presence of IL-4, IL-10, and TGF-β1 (11, 27). As previously described, TGF-β1 levels were especially enhanced in the Th3 condition compared with the Th2 condition (Fig. 4 B). Under this Th3 condition, SOCS3-deficient CD4+ T cells produced higher levels of IL-10 and TGF-β1 than WT CD4+ T cells (Fig. 4 B). Collectively, SOCS3 deficiency caused enhanced production of TGF-β1 and IL-10, but reduced production of IL-4 in CD4+ T cells not only in vivo but also in vitro.

### STAT3 elevates TGF-β1 promoter activity

The inhibitory effect of SOCS3 is relatively specific to STAT3 among six STATs. Therefore, we next investigated whether STAT3 could directly regulate the TGF-β1 promoter activity. The 4.1-kb fragment of the 5′-flanking region of the murine TGF-β1 gene was fused to the luciferase expression vector, and promoter activity was examined in HEK293 cells by transient transfection. Luciferase gene expression was induced not only by high glucose and TGF-β1 itself as described previously (28) but also by LIF, suggesting that this 4.1-kb 5′-fragment of the TGF-β1 gene contained STAT3 responsive elements (Fig. 5 A). Co-expression of exogenous...
WT STAT3 enhanced LIF-mediated TGF-β1 promoter activity in a dose-dependent manner (Fig. 5 A). Furthermore, constitutive active form of STAT3 (STAT3c) (29) also enhanced TGF-β1 promoter activity similar to LIF stimulation (Fig. 5, A and B), suggesting that STAT3 positively regulates TGF-β1 promoter. We also confirmed that STAT3 elevated TGF-β1 promoter activity in the lymphoid cell line by using Jurkat cell (unpublished data).

As shown in Fig. 5 B, a reporter assay using a series of 5′-deletion mutants revealed that the LIF-responsive elements were present upstream of −1755. By searching for potential STAT3-binding sites with the consensus sequences, TTC/A(N)3G/TAA (30), two candidates of STAT3-binding sites were identified in the 4.1-kb TGF-β1 5′-flanking region. The two sites were at positions −3155 and −2515 upstream of the transcription initiation site in the TGF-β1 promoters designated STAT3-binding element (SBE-1) and SBE-2, respectively. To determine the significance of these elements, mutations were introduced into the SBE-1 and/or SBE-2 sites. A mutant promoter lacking both SBE-1 and SBE-2 did not respond to LIF stimulation anymore, whereas constructs containing a single SBE site still responded to STAT3 (Fig. 5, B and C). These results indicate that the two SBE sites of the TGF-β1 promoter are important for STAT3-dependent activation.

To confirm STAT3 binding to the TGF-β1 promoter in T cells, chromatin immunoprecipitation (ChIP) assay was performed (Fig. 5 D). The chromatin–DNA complex was immunoprecipitated with anti-STAT3 antibody; then, STAT3 binding to the TGF-β1 promoter was analyzed using pairs of specific primers spanning the STAT3 binding sites. The SBE site of the c-fos promoter was used as a positive control of STAT3 recruitment (31). As shown in Fig. 5 D, STAT3 was actually bound to the TGF-β1 promoter region containing SBE-1 site in T cells in an IL-6-dependent manner. These data indicate that TGF-β1 is a direct downstream target of STAT3.

**DISCUSSION**

Previously, we reported that forced expression of SOCS3 in T cells resulted in Th2 skewing. SOCS3 expression levels are high in T cells from patients with asthma and atopy. Therefore, we concluded that high SOCS3 levels are related to pathological conditions, especially Th2-type diseases (18). However, the role of SOCS3 in physiological conditions has not been clarified. Here, we generated T cell–specific SOCS3-cKO mice and found that the Th2 immune responses in SOCS3-cKO mice were actually reduced. However, this is not the result of higher Th1 responses. Our SOCS3-deficient CD4+ T cells showed higher TGF-β1 and IL-10 production compared with control WT CD4+ T cells. Thus, we suspect that reduced Th2 responses in SOCS3-cKO mice may be the result of immunosuppression by these two immunoregulatory cytokines.

We proposed that SOCS3 inhibits Th1 differentiation by suppressing IL-12–mediated signaling (18). We found that IL-12–induced STAT4 phosphorylation was actually enhanced in SOCS3-deficient T cells compared with WT T cells (unpublished data). However, similar or only slightly reduced IFN-γ production occurred in CD4+ T cells from SOCS3 cKO mice compared with WT mice (Figs. 3 D and 4 A). Furthermore, delay of parasite clearance and reduced production of antibodies were observed in cKO mice during L. major infection (Fig. 3, B and C). This may be the result of immunosuppressive effect of TGF-β1 and IL-10 produced from T cells during infection. Regulatory roles of SOCS3-deficient T cells in other immune reactions should be defined in future studies.

Recently, regulatory functions of Th cells have been extensively studied. CD4+ CD25+ regulatory T (T reg) cells are recognized as naturally occurring T reg cells and exhibit immunosuppressive abilities by a mechanism that is dependent on cell-to-cell contact through the interaction of CTLA-4 with CD86 (34). Though TGF-β1 is shown to be one of the mechanisms of the immunosuppressive effects of T reg cells (10) and Foxp3 has been shown to be an essential transcription factor in the generation and function of T reg cells (35), we did not find any change in the number of CD4+ CD25+ T reg population or Foxp3 expression between SOCS3-deficient and WT T cells (unpublished data).
However, regulatory function of SOCS3-deficient T reg cells remains to be investigated.

Previous studies have identified another subset of T reg cells; Tr1 cells (T reg cell 1), which are induced in vitro by repeated antigen stimulation of T cells in the presence of IL-10 (36, 37). Tr1 cells produce high levels of IL-10 rather than TGF-β1 (38). The additional subset of T reg cells is Th3, which is induced by orally administered antigens. Th3 cells
negative STAT3 suppressed TGF-β1 production (Fig. 6 C). Therefore, STAT3 could be a positive regulator of Th3-like differentiation. STAT3 being required for Th3 is unlike STAT4 and STAT6 being required for Th1 and Th2, respectively, because basal transcription of TGF-β1 and IL-10 is not completely dependent on STAT3. However, STAT3 is an important regulatory factor for Th3 differentiation because STAT3 is essential for the immunosuppressive function of IL-10 in macrophages (40) and IL-10 is usually necessary for induction of Th3 in vitro. Collectively, STAT3 seems to positively regulate induction and/or differentiation of Th3.

A question that remains unsolved is what kind of cytokines are regulated by SOCS3 during Th3-like phenotype induction. Previously, IL-4 has been shown to induce SOCS3 expression in Th2 cells (17). However, IL-4–induced STAT6 phosphorylation levels were not affected in SOCS3-deficient T cells (unpublished data). Therefore, it is unlikely that SOCS3 directly regulates IL-4 signaling. Because STAT3 is strongly activated by IL-10, we compared IL-10–induced STAT3 activation between WT and SOCS3-deficient T cells. In SOCS3-deficient T cells, however, IL-10–mediated STAT3 activation was not much affected (unpublished data). This is probably because SOCS3 does not bind to the IL-10 receptor (22). In contrast, we observed stronger and prolonged STAT3 activation in response to IL-6 and IL-27 in SOCS3-deficient T cells (Fig. 1 E and not depicted). Furthermore, STAT3 recruitment to the TGF-β1 promoter under Th3 differentiation condition was enhanced in SOCS3-deficient CD4+ T cells. Although we could not conclude that IL-6 is responsible for the Th3-like phenotype of SOCS3-deficient CD4+ T cells, these results suggest that STAT3 is hyperactivated in SOCS3-deficient T cells during T cell differentiation, and this is the result of the hypersensitivity to autocrine or paracrine cytokines that activate STAT3. Identification of these cytokines other than IL-10, which modulate TGF-β1 and IL-10 production, will be important for understanding of the regulation of Th3 differentiation.

Another possibility for answering the unsolved question is that SOCS3 affects TCR signaling. SOCS3 has been shown to be able to interact with tyrosine kinase Lck, calcineurin, and CD28 (13–16). The level of SOCS3 expression is significantly high in resting CD4+ T cells and rapidly decreased after TCR stimulation (unpublished data). Some reports have shown that the strength of TCR stimulation is an important factor for Th differentiation. Although we could not detect apparent differences in proliferation, tyrosine phosphorylation of cellular proteins, and ERK activation between SOCS3-deficient and WT T cells in response to TCR stimulation (Fig. S1 and not depicted), the absence of SOCS3 in naive CD4+ T cells may permit some stronger TCR signalings, which might lead to higher IL-10 and TGF-β1 secretion at an early stage of T cell activation, thereby leading to large differences at later stages of T cell differentiation.

Although the more detailed molecular basis of the hyperproduction of TGF-β1 and IL-10 in SOCS3-deficient T cells has remained elusive, our biochemical analyses suggest that STAT3 directly binds to the promoter region of TGF-β1 and elevates TGF-β1 production in T cells. It has already been shown that IL-10 is up-regulated by STAT3 (23). We showed that constitutive active form of STAT3 enhanced TGF-β1 and IL-10 production in T cells. Furthermore, we showed that a dominant

Figure 6. Retroviral transduction of STAT3 mutants modulates TGF-β1 and IL-10 production. (A) Schematic structure of the retroviral pMX vectors containing mutant STAT3, either myc-STAT3c (constitutive active form) or myc-dNSTAT3 (dominant negative form). (B) GFP-positive cells were sorted from infected T cells and the expression levels of exogenous myc-STAT3 were examined by Western blotting. (C) IL-10 and TGF-β1 production from infected CD4+ T cells after Th3 differentiation. GFP-positive cells were cultured in the presence of IL-4, IL-10, and TGF-β1 for 7 d and restimulated with anti-CD3 mAb and anti-CD28 mAb and cytokines in the culture supernatants were measured by ELISA. Data shown are mean ± SD of triplicate samples from four independent experiments. (D) ChIP assay to compare STAT3 recruitment to TGF-β1 promoter (SBE-1 site) between Th0 and Th3 differentiated T cells from WT and cKO mice. Anti-STAT3 Abs immunoprecipitates were used as templates for PCR products. A non-SBE region near the transcription initiation sites was amplified as a negative control. Ratios of the bands intensity of SBE-1 PCR products and those of control (G3PDH) in two independent experiments are plotted (right).
that SOCS3 regulates TGF-β1 and IL-10 production by suppressing STAT3 activity. Thus, we propose that STAT3 and SOCS3 reciprocally regulate Th2/Th3 differentiation. Therefore, suppression of SOCS3 expression in T cells may possibly be one of the ways to introduce tolerance for autoimmune diseases or to ameliorate allergic diseases.

MATERIALS AND METHODS

Generation of T cell–specific SOCS3–disrupted mice. SOCS3−/− mice were generated by breeding back SOCS3+/− mice with a 129 background to generate SOCS3−/− mice in which SOCS3 was deleted in a T cell–specific manner. Genotyping was performed by PCR as described previously (22). Offspring carrying both Lck-cre and floxed SOCS3 genes (Lck-Cre;SOCS3−/−/floxed; cKO) and the floxed SOCS3 gene (SOCS3−/−/floxed; WT) were used for intercrossing and further analyses. Littermate controls were used for all experiments. CD4+ T cells, splenic B cells, and DCs were isolated by MACS sorting as described previously (42). Mice were kept in specific-pathogen-free facilities in the Collaborative Station Animal Facility of Kyushu University. All experiments using these mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Kyushu University, Fukuoka, Japan.

OVA/alum immunization and assay for airway hyperresponsiveness. Alhydrogel (alum; Al(OH)3) gel (LSL) was mixed with a predetermined quantity of OVA grade V (Sigma-Aldrich) and incubated at room temperature for 20 min. After centrifugation of the mixture at 14,000 × g for 10 min, supernatants were used for immunization as described previously (24). Mice (8–12 wk old) were immunized with 0.1 ml of OVA (10 μg) in PBS and absorbed to alum. Boosting inoculations were performed in the same fashion 2 wk later. For airway hyperresponsiveness (AHR) and eosinophil infiltration assay, mice received aerosol challenge containing either saline or 1% OVA for 10 min. Following challenge, mice received aerosol challenge containing either saline or 1% OVA for 10 min. Following challenge, mice were ventilated and AHR to acetylcholine aerosol was measured. For airway hyperresponsiveness (AHR) and eosinophil infiltration assay, mice received aerosol challenge containing either saline or 1% OVA for 10 min. Following challenge, mice were ventilated and AHR to acetylcholine aerosol was measured. Serum levels of total and OVA–specific Ig was analyzed by ELISA with rat anti–mouse Ig (Serotec Ltd.). Ab titers were determined by endpoint analysis. Additional cytokines. Supernatants were collected 24 h after secondary stimulation for measurement of TGF-β1 measurement using mTGF-β1 ELISA kit (Promega).

Construction of reporter plasmids. PCR was done to generate the TGF-β1 promoter plasmid by using mouse genomic DNA as a template. The nucleotide sequence of the mTGF-β1 promoter has been submitted to GenBank (TIGR/EBI) under accession no. L42456.1. A 4.1-kb XhoI–EcoRI fragment corresponding to nucleotides from −3245 to +845 relative to the determined transcriptional start site of TGF-β1 gene was subcloned into a pGv-basc2 vector (TOYOINK), pTG4.1-luc. Reporter plasmids, including a series of deletion mutants of the TGF-β1 promoter, were generated by excision at restriction enzyme recognition sites as follows: −2977 (NcoI), −1755 (HindIII), −1072 (SmaI), −585 (NcoI). To construct SBE-1mt, SBE-2mt, and SBE-1mt/2mt, point mutations were introduced to the following sites of the TGF-β1 promoter DNA from −1029 to −965 for SBE-1mt, −1029 to −965 for SBE-2mt, and −1029 to −965 for SBE-1mt/2mt. The subsequent SBE-1mt, SBE-2mt, and SBE-1mt/2mt fragments were cloned into pGL2 basic vector. The resulting plasmids were purified and used for transfection. Transfection and luciferase assay. HEK293 (106 cells) were seeded on six-well plates, cultured for 24 h, and transfected with various amounts of an expression vector of WT–STAT3–pCDNA3 or STAT3–pCDNAMV along with 0.2 μg of TGF-β1–pGvbas2 and 0.1 μg of β-galactosidase (β-gal) plasmid by the calcium phosphate coprecipitation method. Some of them were stimulated with LIF (10 ng/ml) or TGF-β1 (10 ng/ml) for 8 h. Cells were harvested in 40 μl lysis buffer. Luciferase assay was performed using a luciferase substrate kit (Promega) and luciferase activity was read in Packard luminoimeter. Luciferase activity was normalized by the internal control β-gal activity, and shown as the means ± SD of three to five experiments.

ChIP assay. ChIP assay was performed in 106 mouse T lymphocytes. Cells were fixed with 1% formaldehyde at 37°C for 10 min after IL-6 stimulation as described previously (31). Cells were washed, suspended in SDS lysis buffer, and sonicated for 30 s pulses four times using a sonicator (Bi-ruptor; Cosmo Bio Co.). Samples were incubated with 5 μg anti-STAT3 antibody (C-20; Santa Cruz Biotechnology, Inc.) overnight at 4°C. After adding salmon sperm DNA and protein A–Agarose Slurry (UBI), the immunoprecipitates were sequentially washed with low-salt buffer, high-salt buffer, LiCl buffer and twice with TE buffer. The DNA–protein complex was eluted into elution buffer at 65°C for 6 h. Proteins were digested by proteinase K and RNA was removed by addition of 10 μg of RNase A. DNA was recovered by extraction with phenol and chloroform and ethanol precipitation and subjected to PCR analysis. To estimate the DNA content in the soluble chromatin samples, DNA was similarly extracted from sonicated samples and used as a template for G3PDH gene amplification. Promoter–specific primers were as follows; mTGF-β1, SBE-1 forward: 5′-TGGCTCAGGCTTGGACCCGACACC-3′; SBE-1 reverse: 5′-TGGGAAACCGGTCAGTAAC-3′, which amplify 311-bp fragments flanking the STAT3 binding element. For negative control, 5′-franking region close to the transcription initiation site was amplified by forward:
Retroviral constructs and transduction to primary T cells. The STAT3c-IRES-GFP-pMX, dSTAT3T3-IRES-GFP-pMX, and empty GFP-pMX plasmids (a gift from T. Kitamura, Tokyo University, Tokyo, Japan) were transfected into a packaging cell line, Plat-E (33), using FuGENE6 (Roche Diagnostic), and after incubation for 48 h, the culture supernatant was harvested. CD4+ enriched T cells were stimulated with 1 μg/ml anti-CD3e mAb and 1 μg/ml anti-CD28 mAb for 24 h and infected with the viruses by adding the viral containing supernatants in the presence of 0.6 μg/ml polybrene (Sigma-Aldrich). The infected CD4+ T cells were expanded in the medium supplemented with 100 U/ml rIL-2 for 4 d. GFP-positive cells were collected by a cell sorter (EPICS ALTRA; Beckman Coulter) and restimulated with 1 μg/ml anti-CD3e mAb and 1 μg/ml anti-CD28 mAb or Th3-inducing condition for 72 h. Culture supernatants were harvested after 48 h to analyze TGF-β1 and IL-10 production by ELISA.

Online supplemental material. Fig. S1 shows tyrosine phosphorylation of cellular proteins (anti-pY blot) and ERK activation in CD4+ T cells from WT and cKO mice after TCR stimulation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052333/DC1.

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