Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4 FITC, PE (H129.19), anti-CD8 FITC, PE (53-6.7), anti-B220 FITC, PE, allophycocyanin, PerCP, biotin (RA3-6B2), anti-CD3 PE (145-2C11), anti-CD19 PE (ID3), anti-CD11b (Mac-1) PE (M1/70), anti-CD11c FITC (HL3), anti-Ly6G/C PE (RB6-8C5), anti-erythroid PE (TER-119), anti-pan NK PE (DX5), anti-CD80 PE (16-10A1), anti-CD86 PE (GL-1), and anti-I-A^d PE (AMS-32.1). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Isolation of DC subtypes

Splenic DCs were prepared using OptiPrep (Axis Shield) according to the manufacturer's instructions. In brief, spleens were cut into small fragments and then digested with collagenase A (0.5 mg/ml; Roche) for 10 min at 37°C with continuous agitation. Digested fragments were filtered through a stainless steel sieve, and cells were resuspended in 3 ml of HBSS and then mixed with 1 ml of OptiPrep to make 15% iodixanol solution (density 1.085 g/ml). Cell suspension was overlaid with 5 ml of 12% iodixanol solution (density 1.069 g/ml) and subsequently with 3 ml of HBSS. Low-density cells were collected by centrifugation at 600 × *g* for 15 min at room temperature. Low-density cells were stained with anti-CD11c FITC and FITC-stained cells were positively collected using anti-FITC microbeads (Miltenyi Biotec), according to the manufacturer's instructions. The resultant cells were routinely >95% pure CD11c$^+$ cells by FACS analysis.

To isolate PDCs, low-density cells were prepared from wild-type (WT), Stat6$^{-/-}$ splenocytes, or Stat4$^{-/-}$ splenocytes and then stained with a mixture of PE-labeled Abs to CD3, CD19, CD11b, DX5, and TER-119. After PE-stained cells were depleted using anti-PE microbeads (Miltenyi Biotec), cells in flow-through were stained with anti-B220 biotin and subsequently B220$^+$ cells were positively collected using streptavidin microbeads (Miltenyi Biotec). Alternatively, PDCs were purified using a PDC isolation kit according to the manufacturer's instructions (Miltenyi Biotec). In both cases, the resultant cells were >95% pure B220$^+$CD19$^-$ cells by FACS analysis.

For CD11b$^+$ DCs purification, low-density cells were prepared from WT splenocytes and stained with a mixture of PE-labeled Abs to CD3, B220, DX5, TER-119, and CD8. After PE-stained cells were depleted using anti-PE microbeads, cells in flow-through were stained with anti-CD11c FITC and CD11c$^+$ cells were positively collected using anti-FITC microbeads. The resultant cells were routinely >95% pure CD11b$^+$CD8$^-$CD11c$^+$ cells by FACS analysis.

For CD8$^+$ DCs purification, low-density cells were prepared from WT splenocytes and stained with a mixture of PE-labeled Abs against CD3, B220, DX5, TER-119, and CD11b and PE-stained cells were depleted using anti-PE microbeads. Cells in flow-through were stained with anti-CD11c FITC and CD11c$^+$ cells were positively collected using anti-FITC microbeads. The resultant cells were routinely >95% pure CD11b$^-$CD8$^+$CD11c$^+$ cells by FACS analysis.

Cell culture

Isolated PDCs, CD11b$^+$ DCs, or CD8$^+$ DCs were cultured (5 × 10^5/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics (complete RPMI 1640 medium) at 37°C for 72 h in the presence or in the absence of IL-4 (20 ng/ml). In some experiments, PDCs were stimulated with CpG ODN (10 μg/ml) for 48 or 72 h. PDCs were also stimulated with IL-2, IL-7, IL-9, IL-13, or IL-15 (20 ng/ml each) for 72 h to determine whether these cytokines induce IFN-γ production from PDCs. In other experiments, anti-IL-12 (p40/p70) Ab (10 μg/ml, clone C17.8; BD Pharmingen), anti-IL-2R β-chain Ab (10 μg/ml, clone TM-β1; BD Pharmingen), or anti-IL-18 Ab (5 μg/ml, clone 93-10C; MBL) was added to neutralize IL-12, IL-2 and IL-15, or IL-18, respectively. A mixture of anti-murine IFN-α Ab (20 μg/ml, clone 4EA1) (17), anti-murine IFN-β Ab (20 μg/ml, clone 7DF3) (17), and anti-type I IFN receptor antisera (10 μg/ml, R&D Systems) was used to neutralize type I IFNs.

ELISAs

The amounts of IFN-γ and IL-12 in the culture supernatant were measured by the enzyme immunoassay using murine IFN-γ and IL-12 (p70) ELISA kits from BD Pharmingen. The amounts of IFN-α in the culture supernatant were measured by an IFN-α ELISA kit from PBL. The assays were performed in duplicate according to the manufacturers' instruction. The minimum significant values of these assays were 31.3 pg/ml IFN-γ, 62.5 pg/ml IL-12, and 12.5 pg/ml IFN-α.

Intracellular staining for IFN-γ

Cells were stimulated with IL-4 (20 ng/ml) in the complete RPMI 1640 medium for the indicated periods (48 or 72 h). Monensin (2 μM; Sigma-Aldrich) was added for final 4 h to prevent cytokine release. After surface staining, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen) as described previously (18).

Intracellular Stat4 staining

Intracellular staining for Stat4 was performed as described elsewhere (19) with minor modifications. In brief, isolated PDCs from WT splenocytes or Stat6$^{-/-}$ splenocytes were cultured for 48 h in the presence or in the absence of IL-4 (20 ng/ml). Cells were harvested, washed with PBS, fixed with IC FIX, and permeabilized with 90% methanol and subsequently with IC PERM. Cells were then incubated with anti-Stat4 Ab (Zymed) or control rabbit IgG (Serotec) for 30 min at room temperature. After washing, cells were incubated with Alexa Fluor 647-conjugated anti-rabbit IgG Ab (Molecular Probes) and analyzed on a FACSCalibur. In the case of double

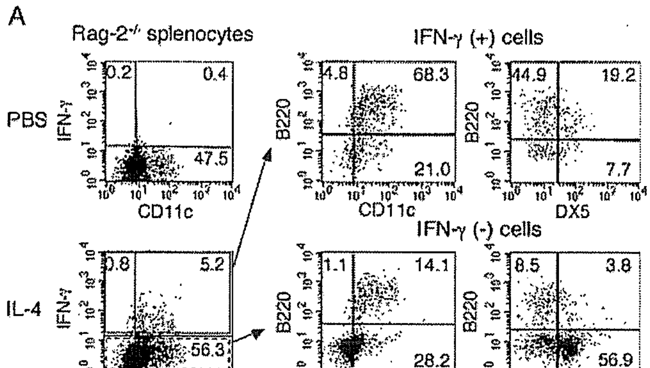

FIGURE 1. B220$^+$ PDCs produce IFN-γ upon IL-4 stimulation. *A*, Splenocytes from Rag-2$^{-/-}$ mice were cultured with or without IL-4 (20 ng/ml) for 3 days with monensin added for the final 4 h. After cells were stained with anti-B220 and either anti-CD11c or anti-DX5, intracellular staining for IFN-γ was performed. Representative FACS profiles of anti-CD11c vs anti-IFN-γ staining (*left panels*) and anti-CD11c vs anti-B220 or anti-DX5 vs anti-B220 staining gating on either IFN-γ$^+$ cells or IFN-γ$^-$ cells (*right panels*) are shown. *B*, CD11c$^+$ low-density splenocytes were isolated from WT mice as described in the *Materials and Methods*. Cells were then cultured with or without IL-4 for 3 days and analyzed for the expression of CD11c, B220, CD19, CD8, CD11b, Ly6G/C, and I-A^d together with the intracellular IFN-γ. Shown are representative FACS profiles of anti-CD11c vs anti-IFN-γ staining from four independent experiments (*left panels*). Representative FACS profiles of anti-CD19 vs anti-B220, anti-CD3 vs anti-B220, and anti-CD8 vs anti-B220 staining on IFN-γ$^+$ cells, as well as histograms for anti-Ly6G/C, anti-CD11b, and anti-I-A^d staining on IFN-γ$^+$ cells are shown in the *right panels*. Dashed lines indicate the staining with isotype-matched control Abs.

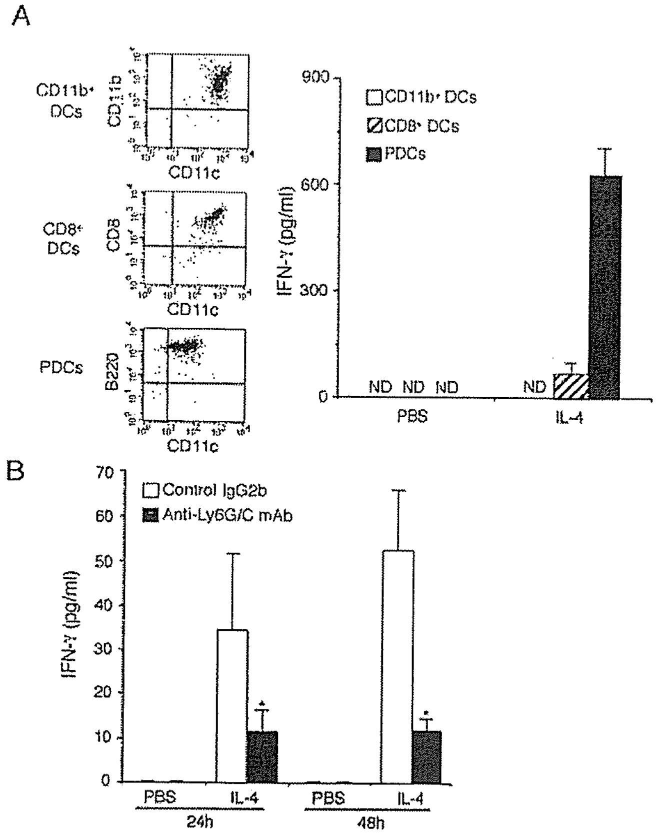

FIGURE 2. PDCs but not CD11b$^+$ or CD8$^+$ DCs produce IFN-γ upon IL-4 stimulation. *A*, PDCs, CD11b$^+$ DCs, and CD8$^+$ DCs were prepared from WT splenocytes as described in *Materials and Methods*. Each DC subtype was cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Representative FACS profiles of isolated each DC subtype are shown in the *left panels*. Data are means ± SD from four independent experiments. ND = not detectable. *B*, PDCs produce IFN-γ upon IL-4 stimulation in vivo. Rag-2$^{-/-}$ mice were injected i.p. with anti-Ly6G/C Ab (500 μg/mouse) or rat IgG2b (as a control). Twenty-four hours later, rIL-4 (10 μg/mouse) or saline (as a control) was injected i.v. in the retro-orbital vein of mice. The levels of IFN-γ in the serum were determined by ELISA at 24 and 48 h after IL-4 injection. Data are means ± SD for four mice in each group. ND, not detectable. *, Significantly different from the mean value of the corresponding control response (control IgG2b); $p < 0.01$.

intracellular staining for Stat4 and IFN-γ, FITC-conjugated anti-rabbit IgG Ab (Zymed) was used as a second Ab.

RT-PCR analysis

Total cellular RNA was prepared and RT-PCR analysis was performed as described previously (20). The primer pairs for Stat4 were 5′-CTTGGGT GGACCAATCTGAA-3′ and 5′-TGGTCTTGAGACTTCGCACG-3′. The primer pairs for GATA3 and T-bet were described elsewhere (21). RT-PCR for β-actin was performed as a control. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

Taqman PCR analysis

The expression levels of Stat4 mRNA were determined by real-time PCR using a standard protocol on ABI PRISM 7000 instrument (Applied Biosystems). PCR primers and fluorogenic probes for Stat4, T-bet, and GATA3 were described previously (22). The levels of Stat4 mRNA were normalized to the levels of GAPDH mRNA (Applied Biosystems).

Effect of in vivo depletion of PDCs on IFN-γ production induced by IL-4 administration

To deplete PDCs in vivo, anti-Ly6G/C Ab (500 μg/mouse; BD Pharmingen) was injected i.p. to Rag-2$^{-/-}$ mice as described previously (12). As a control, purified rat IgG2b (BD Pharmingen) was injected to Rag-2$^{-/-}$ mice. In some experiments, 120G8 Ab (500 μg/mouse; a gift from Drs. G. Trinchieri and D. La Face, Schering-Plough Research Institute, Dardilly, France) (23) was injected to Rag-2$^{-/-}$ mice to deplete PDCs. Twenty-four hours later, rIL-4 (10 μg/mouse) or saline (as a control) was injected i.v. in the retro-orbital vein of the mice. The levels of IFN-γ in sera were determined by ELISA using a highly sensitive mouse IFN-γ ELISA kit (AN-18; BD Pharmingen) at 24 and 48 h after IL-4 injection. The minimum significant value of this assay was 3 pg/ml IFN-γ.

Th1 and Th2 cell differentiation

Splenic CD4$^+$ T cells from DO11.10$^+$ mice were purified (>90% pure by flow cytometry) using T cell enrichment columns (R&D Systems) and stimulated with plate-bound anti-CD3ϵ mAb (5 μg/ml, clone 145-2C11; BD Pharmingen) plus anti-CD28 mAb (5 μg/ml, clone 37.51; BD Pharmingen) at 37°C for 48 h in the presence of IL-12 (7.5 ng/ml; R&D Systems) (Th1 condition) or IL-4 (15 ng/ml; R&D Systems) and anti-IFN-γ mAb (15 μg/ml, clone XMG1.2; BD Pharmingen) (Th2 condition) as described previously (18).

Data analysis

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

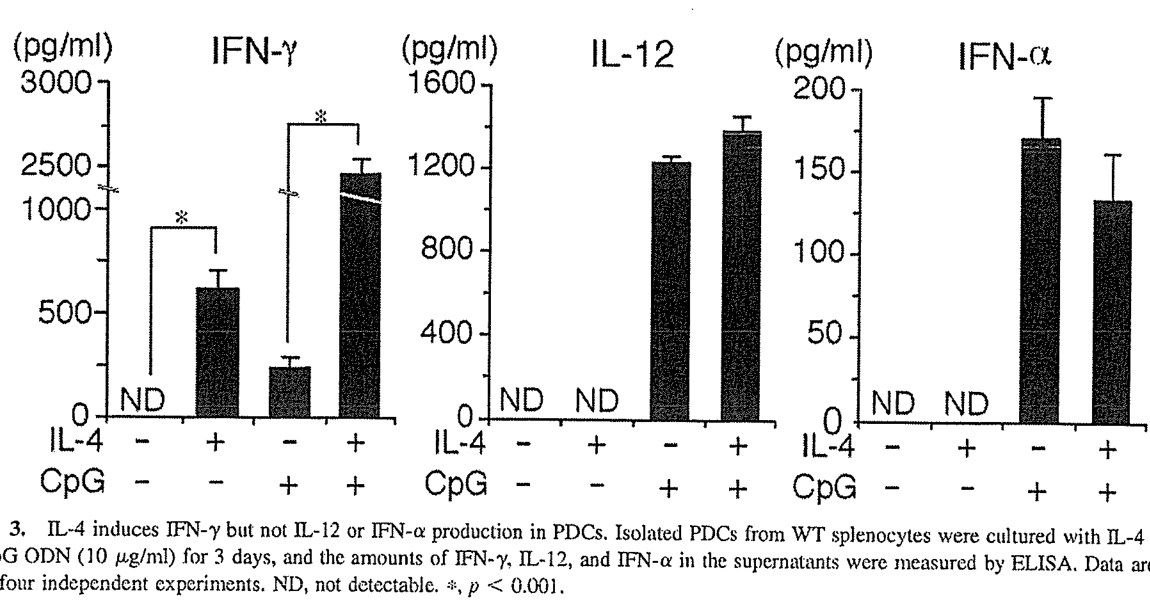

FIGURE 3. IL-4 induces IFN-γ but not IL-12 or IFN-α production in PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 (20 ng/ml) and/or CpG ODN (10 μg/ml) for 3 days, and the amounts of IFN-γ, IL-12, and IFN-α in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. ND, not detectable. *, $p < 0.001$.

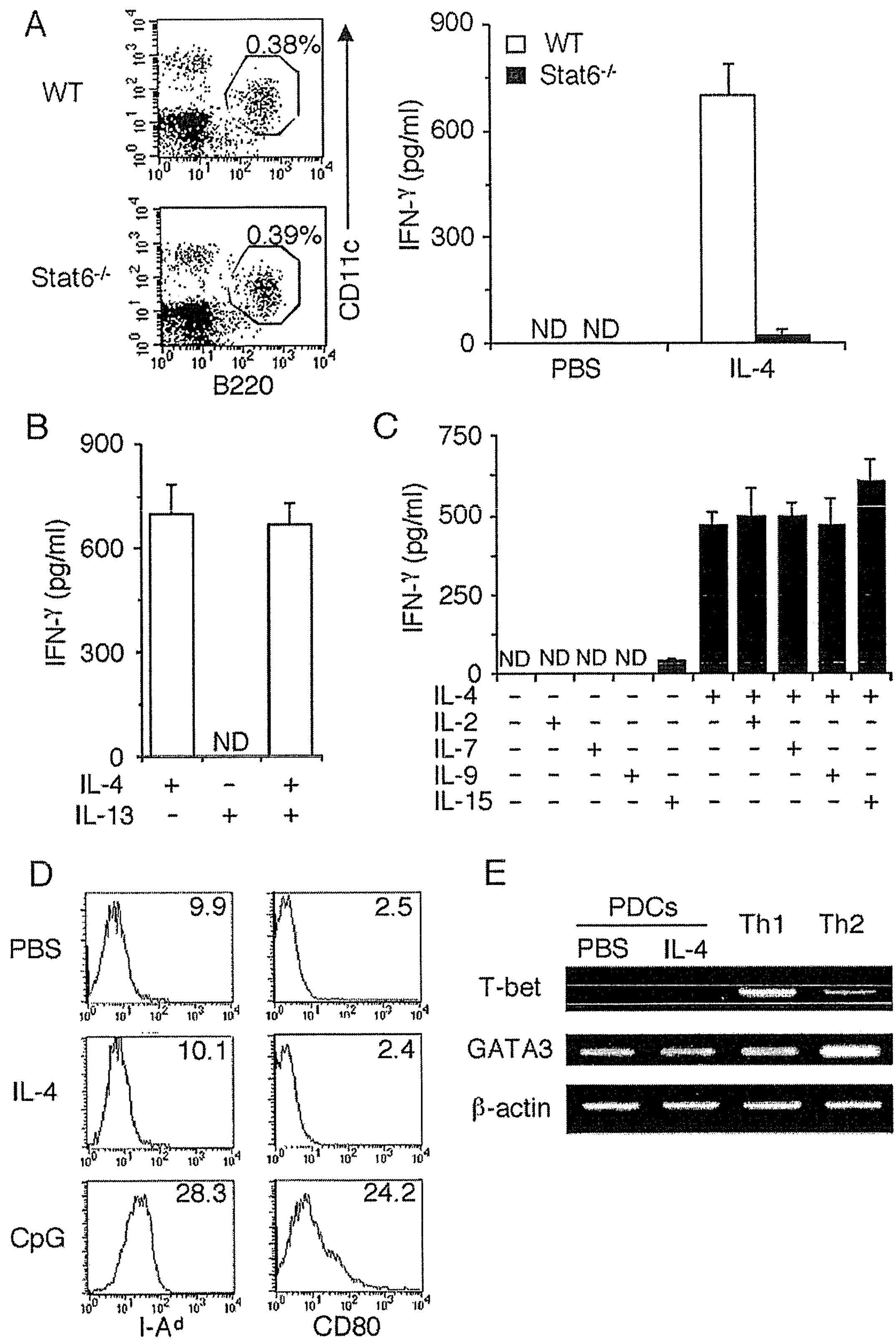

FIGURE 4. IL-4 induces IFN-γ production in PDCs by a Stat6-dependent mechanism. *A*, Stat6 is essential for IL-4-induced IFN-γ production in PDCs. Representative anti-B220 vs anti-CD11c staining on $CD3^-CD11b^-CD19^-DX5^-TER\text{-}119^-$ splenocytes from WT mice and $Stat6^{-/-}$ mice are shown in the *left panels* (n = 6 mice for each genotype), indicating normal development of PDCs in $Stat6^{-/-}$ mice. Isolated PDCs from WT splenocytes or $Stat6^{-/-}$ splenocytes were cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. *B*, IL-13 does not induce IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 and/or IL-13 (20 ng/ml) for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. ND, not detectable. *C*, Other γc-dependent cytokines do not enhance IL-4-induced IFN-γ production from PDCs. Isolated PDCs from WT splenocytes were cultured with IL-2 (20 ng/ml), IL-7 (20 ng/ml), IL-9 (20 ng/ml), or IL-15 (20 ng/ml) in the presence or in the absence of IL-4 (20 ng/ml) for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent (*Figure legend continues*)

Results

B220⁺ PDCs produce IFN-γ upon IL-4 stimulation

To examine the negative-feedback regulation of cytokine networks, we searched for cell populations that produce IFN-γ upon IL-4 stimulation. We found that ~5% of IL-4-stimulated Rag-$2^{-/-}$ splenocytes became positive for intracellular IFN-γ staining (Fig. 1*A*, *left panels*). Multicolor FACS analyses revealed that the majority of IL-4-induced, IFN-γ-producing cells expressed CD11c at low levels, expressed B220 at high levels, but lacked the expression of DX5 (Fig. 1*A*, *right panels*). In contrast, the majority of IFN-γ-nonproducing cells in IL-4-stimulated Rag-$2^{-/-}$ splenocytes were positive for DX5 but negative for B220 (Fig. 1*A*), suggesting that these IFN-γ-nonproducing cells are NK cells.

To further characterize cell populations that produce IFN-γ upon IL-4 stimulation, $CD11c^{+}$ low-density splenocytes were isolated from WT mice and then stimulated with IL-4. Again, the majority of IL-4-induced, IFN-γ-producing cells expressed B220 at high levels and expressed CD11c at low levels (Fig. 1*B*). IFN-γ-producing cells were also positive for anti-Ly6G/C staining (Fig. 1*B*). Moreover, IFN-γ-producing cells expressed class II MHC molecules (I-A^{d}) at very low levels but lacked the expression of CD19, CD3, CD8, and CD11b (Fig. 1*B*). These results suggest that the IL-4-induced, IFN-γ-producing cells are very similar to type I IFN-producing PDCs (10–12).

PDCs but not CD11b⁺ DC or CD8⁺ DCs produce IFN-γ upon IL-4 stimulation

To determine whether PDCs specifically produce IFN-γ upon IL-4 stimulation, isolated PDCs, $CD11b^{+}$ DCs, and $CD8^{+}$ DCs were examined for their ability of IFN-γ production upon IL-4 stimulation. Consistent with the data obtained by intracellular IFN-γ staining (Fig. 1), isolated PDCs produced a considerable amount of IFN-γ upon IL-4 stimulation (625.5 ± 79.9 pg/ml, means ± SD, $n = 4$) (Fig. 2*A*). On the other hand, IL-4-stimulated $CD8^{+}$ DCs produced little IFN-γ (74.3 ± 32.2 pg/ml, $n = 4$) and IL-4-stimulated $CD11b^{+}$ DCs did not produce IFN-γ (Fig. 2*A*). Together with the data shown in Fig. 1, these results indicate that among DC subtypes, PDCs specifically produce IFN-γ upon IL-4 stimulation.

We also examined whether PDCs produced IFN-γ upon IL-4 stimulation in vivo. As shown in Fig. 2*B*, when rIL-4 was administered i.v. to Rag-$2^{-/-}$ mice, a considerable amount of IFN-γ was detected in the serum after 24 and 48 h. Importantly, the depletion of PDCs with preinjection of anti-Ly6G/C Ab significantly decreased the IL-4-induced IFN-γ production ($n = 4$ mice, $p < 0.01$) (Fig. 2*B*). A similar trend was observed with 120G8 Ab, which depletes PDCs more specifically (23), although statistical significance was not achieved due to the limited number of mice examined (data not shown). These results suggest that PDCs produce IFN-γ upon IL-4 stimulation in vivo.

IL-4 preferentially induces IFN-γ production in PDCs

PDCs have been identified as a potent producer of IFN-α and IL-12 upon viral or bacterial infection (8–12). Therefore, we examined whether IL-4-induced IFN-α and IL-12 production in isolated PDCs. However, IL-4 did not induce the production of IFN-α or IL-12 (p70) in PDCs ($n = 4$) (Fig. 3). In contrast, PDCs produced considerable amounts of IFN-γ, IFN-α, and IL-12 upon CpG ODN stimulation, a potent stimulator of PDCs through TLR9 (24, 25) (Fig. 3). Furthermore, IL-4 strikingly enhanced CpG ODN-induced IFN-γ production ~10-fold but not CpG ODN-induced IFN-α or IL-12 production in PDCs ($n = 4, p < 0.001$) (Fig. 3). These results indicate that IL-4 preferentially induces IFN-γ production in PDCs.

IL-4 induces IFN-γ production in PDCs by a Stat6-dependent mechanism

It is well established that IL-4 uses Stat6 as a signaling molecule (26). Therefore, we next studied whether Stat6 was required for IL-4-induced IFN-γ production in PDCs using Stat6-deficient (Stat$6^{-/-}$) mice. The number of PDCs ($CD19^{-}B220^{+}CD11c^{low}$ cells) in spleen was similar between Stat$6^{-/-}$ mice and WT mice (Fig. 4*A*), suggesting that Stat6 is not essential for the development of PDCs. However, when isolated PDCs were stimulated with IL-4, WT PDCs but not Stat$6^{-/-}$ PDCs produced IFN-γ (Fig. 4*A*). On the other hand, CpG ODN-induced IFN-γ production was similarly observed between WT PDCs and Stat$6^{-/-}$ PDCs (data not shown). These results indicate that among signaling molecules under IL-4, Stat6 is essential for IFN-γ production in PDCs. We also examined the effect of IL-13, which shares type II IL-4R with IL-4 and activates Stat6 (26), on IFN-γ production in PDCs. However, IL-13 did not induce IFN-γ production in PDCs (Fig. 4*B*) nor enhance IL-4-induced IFN-γ production in PDCs (Fig. 4*B*), suggesting that type I IL-4R but not type II IL-4R is involved in IL-4-induced IFN-γ production in PDCs. Moreover, another representative Th2 cytokine, IL-5, did not induce IFN-γ production nor enhance IL-4-induced IFN-γ production in PDCs (data not shown).

Other γc-dependent cytokines do not induce IFN-γ production nor enhance IL-4-induced IFN-γ production in PDCs

To determine whether other γc-dependent cytokines induce IFN-γ production in PDCs, isolated PDCs were stimulated with IL-2, IL-7, IL-9, and IL-15 in the presence or in the absence of IL-4 for 3 days. As shown in Fig. 4*C*, none of γc-dependent cytokines, except for IL-4 induced IFN-γ production in PDCs (Fig. 4*C*). In addition, none of them significantly enhanced IFN-γ production in IL-4-stimulated PDCs (Fig. 4*C*).

IL-4 does not alter the maturation state of PDCs

It has been shown that the ability of DCs for cytokine production depends on their maturation state (27, 28). We then examined whether IL-4 changed the maturation state of PDCs and thus induced IFN-γ-producing ability. Consistent with previous reports (8–12), isolated PDCs expressed I-A^{d} at very low levels, and lacked the expression of CD80 (Fig. 4*D*) and CD86 (data not shown). IL-4 did not alter the expression levels of I-A^{d}, CD80, and CD86 of PDCs (Fig. 4*D* and data not shown). In contrast, when PDCs were stimulated with CpG ODN, the expression levels of I-A^{d} and CD80 were significantly increased (Fig. 4*D*). In addition,

experiments. ND, not detectable. *D*, IL-4 does not alter the maturation state of PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 (20 ng/ml) or CpG ODN (10 μg/ml) for 48 h, and the levels of I-A^{d} and CD80 on PDCs were analyzed by FACS. Shown are representative histograms, and the mean fluorescent intensities for anti-I-A^{d} and anti-CD80 staining on live cells (propidium iodide $(PI)^{-}$ cells) from four independent experiments. Forty-three percent of PDCs could survive in the presence of IL-4, whereas 69% of PDCs could survive in the presence of CpG ODN (data not shown). *E*, T-bet is not induced by IL-4 in PDCs. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 16 h. As controls, Th1-polarized cells or Th2-polarized cells were prepared from DO11.10 TCR transgenic mice as described in the *Materials and Methods*. Shown are representative data of RT-PCR analysis for T-bet, GATA3, and β-actin mRNA from four independent experiments.

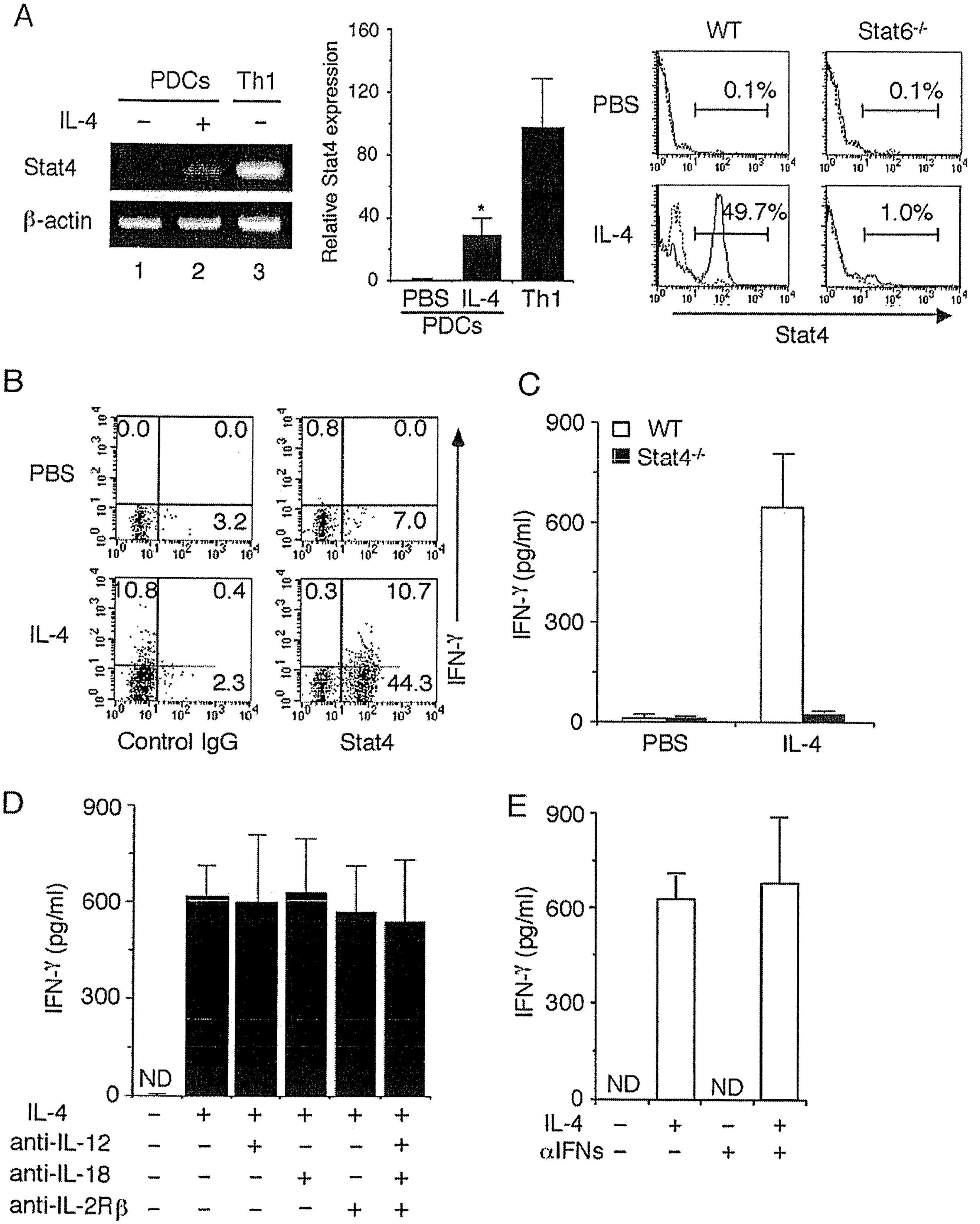

FIGURE 5. IL-4 induces Stat4 expression in PDCs by a Stat6-dependent mechanism. *A*, Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 16 h. As s control for Stat4-expressing cells, Th1-polarized cells were prepared from DO10$^+$ mice as described previously (15). Shown are representative data of RT-PCR analysis for Stat4 and β-actin mRNA from four independent experiments (*left panels*). Taqman PCR analysis for Stat4 and GAPDH (as a control) mRNA was performed, and the levels of Stat4 mRNA were normalized to the levels of GAPDH mRNA (*middle panel*). Data are means ± SD from four independent experiments. *, Significantly different from the mean value of control response (PBS); $p < 0.01$. Isolated PDCs from WT splenocytes or Stat6$^{-/-}$ splenocytes were stimulated with or without IL-4 (20 ng/ml) for 48 h, and the expression levels of Stat4 were evaluated by intracellular staining. Shown are representative FACS profiles from four independent experiments (*right panels*). *B*, IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 48 h. Intracellular (*Figure legend continues*)

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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Disclosures

The authors have no financial conflict of interest.

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Indispensable Role of Stat5a in Stat6-Independent Th2 Cell Differentiation and Allergic Airway Inflammation[1]

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

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

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

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

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

Materials and Methods

Mice

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

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

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Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4-FITC, -PE, -allophycocyanin, and -PerCP (H129.19); anti-CD8-FITC and -PE (53-6.7); anti-B220-allophycocyanin (RA3-6B2); anti-IgM-FITC (R6-60.2); anti-CD69-FITC (H1.3F3); anti-CD62L-FITC (MEL-14); anti-TCR Vβ8.1,2-FITC (MR5-2); and anti-pan-NK-PE (DX5). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Cell culture

Splenocytes (2×10^6 cells/ml) from WT mice, Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (mAb) (5 μg/ml; clone 145-2C11; BD Pharmingen) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics in a 24-well microtiter plate at 37°C for 48 h. Where indicated, IL-12 (15 ng/ml; PeproTech EC) was added to polarize toward Th1 cells (Th1 condition), and IL-4 (15 ng/ml; PeproTech EC) and anti-IFN-γ mAb (15 μg/ml; clone XMG1.2; BD Pharmingen) were added to polarize toward Th2 cells (Th2 condition) (17). Cells were washed with PBS, then cultured for another 3 days in Th0 (no exogenous cytokines), Th1, or Th2 conditions in the presence of IL-2 (20 U/ml; PeproTech).

Intracellular cytokine analysis

Intracellular cytokine staining for IL-4 vs IFN-γ was performed as described previously (17). In brief, cultured splenocytes were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 6 h, with monensin (2 μM) (Sigma-Aldrich) added for the final 4 h. After being stained with anti-CD4-PerCP, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IL-4-PE (BVD4-1D11; BD Pharmingen) and anti-IFN-γ-allophycocyanin (XMG1.2; BD Pharmingen) for 30 min at 4°C. The cytokine profile (IL-4 vs IFN-γ) of CD4$^+$ cells was analyzed on a FACSCalibur using CellQuest software.

Ag-induced allergic inflammation in the airways

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

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

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

ELISA

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

Data analysis

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

Results

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

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

Stat6-independent Th2 cell differentiation depends on Stat5a

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

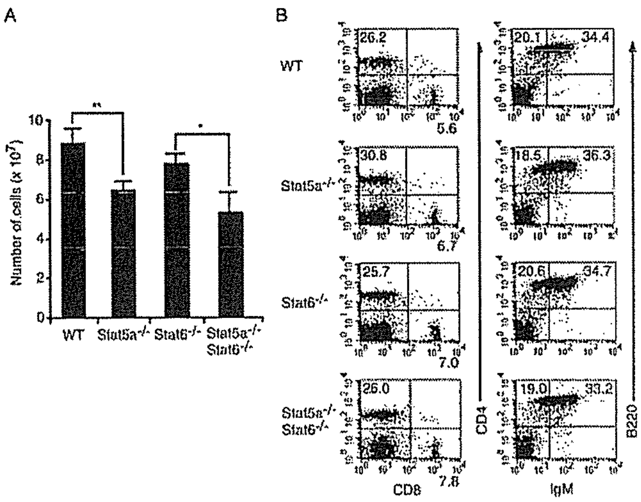

FIGURE 1. Normal T cell and B cell development in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, Number of splenocytes in WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/}$-Stat6$^{-/-}$ mice. Data are the mean ± SD from eight mice for each genotype. *, $p < 0.05$; **, $p < 0.01$. *B*, Flow cytometric analysis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

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

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

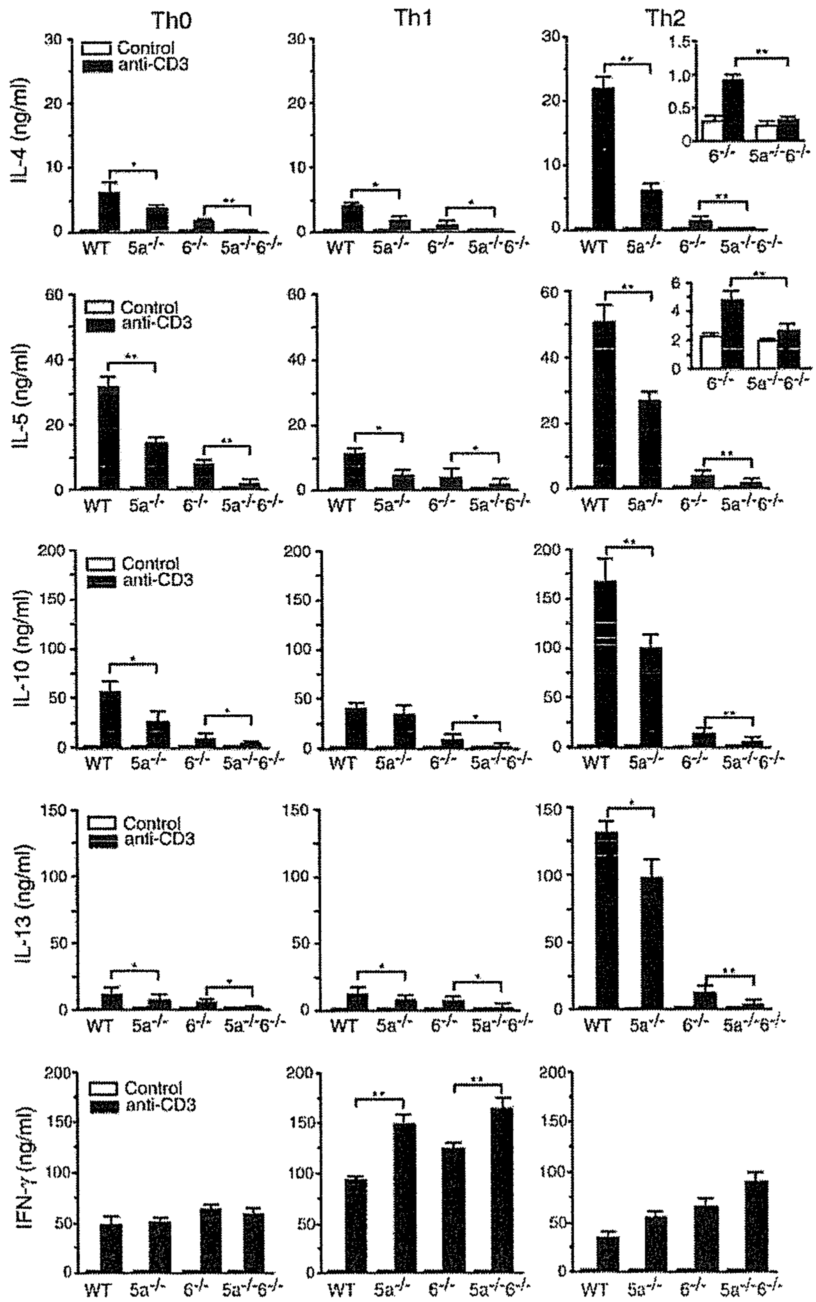

FIGURE 2. Th2 cytokine production is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$ (5a$^{-/-}$), Stat6$^{-/-}$ (6$^{-/-}$), or Stat5a$^{-/-}$Stat6$^{-/-}$ (5a$^{-/-}$ 6$^{-/-}$) mice were stimulated with plate-bound anti-CD3 mAb in the nonpolarizing Th0 condition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

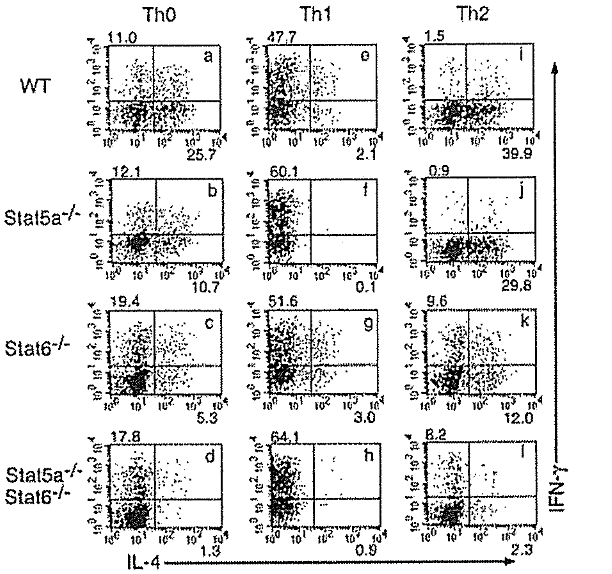

FIGURE 3. Th2 cell differentiation is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, or Stat5a$^{-/-}$Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th0, Th1, or Th2 conditions and cultured for another 72 h in Th0, Th1, or Th2 conditions in the presence of IL-2. Cells were washed and restimulated with plate-bound anti-CD3 mAb for 6 h. 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.

Stat6$^{-/-}$ mice (Fig. 3*c*). Consistent with a previous report (11), IL-4-producing CD4$^+$ cells in Stat6$^{-/-}$ mice lacked the expression of DX5, and the frequency of TCR Vβ8$^+$ cells was not significantly increased in these cells (data not shown), suggesting that the majority of IL-4-producing CD4$^+$ cells in Stat6$^{-/-}$ mice were conventional Th2 cells, but not NK T cells. Importantly, Th2 cells were hardly detected in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3*d*). The frequency of Th2 cells in the Th0 condition was as follows: WT mice, 24.7 ± 3.4%; Stat5a$^{-/-}$ mice, 10.2 ± 2.6%; Stat6$^{-/-}$ mice, 5.5 ± 1.1%; and Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 1.2 ± 0.3% (mean ± SD; n = 5 experiments in each group).

When splenocytes were cultured in Th2-polarizing conditions, the frequency of Th2 cells increased in Stat5a$^{-/-}$ mice and Stat6$^{-/-}$ mice, although the frequency of Th2 cells was still significantly lower in Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice than that in WT mice (Fig. 3). However, even in the Th2 condition, the frequency of Th2 cells did not significantly increase in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3*l*). These results indicate that Stat5a is essential for Stat6-independent Th2 cell differentiation and vice versa.

In contrast, in the Th1 condition, CD4$^+$ T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in Stat5a$^{-/-}$ and Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in WT and Stat6$^{-/-}$ mice, respectively (WT mice, 44.9 ± 8.2%; Stat5a$^{-/-}$ mice, 62.3 ± 11.9% ($p < 0.05$); Stat6$^{-/-}$ mice, 50.8 ± 12.9%; Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 66.4 ± 12.3% ($p < 0.05$); $n = 5$; Fig. 3). In contrast, in the Th0 or Th2 condition, Th1 cells were significantly increased in Stat6$^{-/-}$ and Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in WT mice and Stat5a$^{-/-}$ mice, respectively (Fig. 3). These results suggest that Stat5a and Stat6 are differently involved in the suppression of Th1 cell differentiation, depending on the cytokine environment.

Interestingly, CD4$^+$ T cells that produced both IFN-γ and IL-4 tended to be increased in Stat6$^{-/-}$ mice, but not in Stat5a$^{-/-}$ mice (Fig. 3). These results suggest that Stat6 may also play a role in the suppression of IFN-γ production in developing Th2 cells; this idea is consistent with the previous finding that Stat6 induces the expression of GATA3 (24), a master regulator of Th2 cells that induces Th2 cytokine production and inhibits IFN-γ production in T cells (5–7).

Stat5a-dependent, Stat6-independent Th2 cell differentiation participates in Ag-induced eosinophil and lymphocyte recruitment into the airways

To clarify the in vivo role of Stat5a-dependent, Stat6-independent Th2 cell differentiation, we examined Ag-induced airway inflammation as a model of Th2 cell-mediated in vivo immune responses. Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and control WT mice were immunized twice with OVA; 2 wk later, these mice were challenged with aerosolized OVA three times at 24-h intervals. Forty-eight hours after the last Ag challenge, airway inflammation

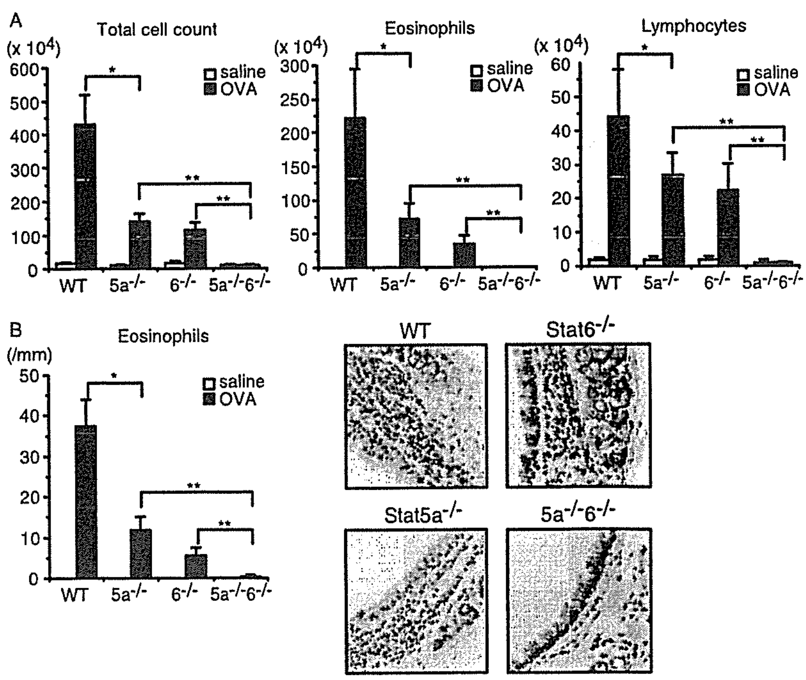

FIGURE 4. Ag-induced eosinophil and lymphocyte recruitment into the airways is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, OVA-sensitized Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and littermate WT mice were challenged with the inhalation of OVA or saline (as a control) three times at 24-h intervals. The numbers of total cells, eosinophils, and lymphocytes in BALF were evaluated 48 h after the last inhalation. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$. *B*, Similar to *A*, OVA-sensitized mice were challenged with inhaled OVA or saline, and the number of eosinophils infiltrating the submucosal tissue of trachea was evaluated 48 h after the last inhalation. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$. Representative photomicrographs of trachea sections stained with Luma solution are also shown (×100).

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 ± 0.2 × 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,

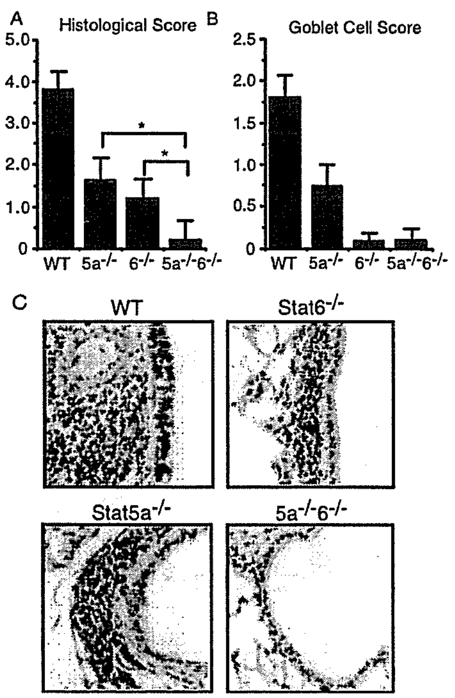

FIGURE 5. The Stat5a-dependent, Stat6-independent pathway induces airway inflammation, but not epithelial goblet cell hyperplasia. OVA-sensitized WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice were challenged with inhaled OVA three times at 24-h intervals. *A*, Forty-eight hours 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).

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.

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.

[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.

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Disclosures

The authors have no financial conflict of interest.

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

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

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

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

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

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

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Materials and Methods

Mice

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

Cell culture

Splenocytes (2 × 10^6 cells/ml) from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (5 μg/ml, clone 145-2C11; BD Pharmingen) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics in a 24-well microtiter plate at 37°C for 48 h. As indicated, IL-12 (15 ng/ml; PeproTech) was added to polarize toward Th1 cells (Th1 condition), and IL-4 (15 ng/ml; PeproTech) and anti-IFN-γ mAb (15 μg/ml, clone XMG1.2; BD Pharmingen) were added to polarize toward Th2 cells (Th2 condition) (11). Cells were washed with PBS and then cultured for another 72 h in Th1 or Th2 condition in the presence of IL-2 (10 ng/ml; PeproTech). In some experiments, either IL-4 (15 ng/ml) or anti-IFN-γ mAb (15 μg/ml) was added to the culture. In separate experiments, the indicated amounts of IL-12 (1–8 ng/ml) were added to the culture.

Flow cytometric analysis

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

Intracellular cytokine analysis

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

Intracellular staining for phosphorylated Stat4

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

Intracellular staining for SOCS3

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

Western blot analysis

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

TaqMan PCR analysis

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

Chromatin immunoprecipitation (ChIP) assay

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

Luciferase assay

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

Retrovirus-mediated gene expression

Bicistronic retrovirus vector pMX-IRES-GFP (23) was a kind gift from Dr. T. Kitamura (Tokyo University, Tokyo, Japan). pMX-SOCS3-IRES-GFP was previously described (14). Retroviruses were produced with a transient retrovirus packaging cell line, Plat-E (25), and stored at −80°C until use. For infection to T cells, after splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 40 h in the presence or absence of IL-4 (15 ng/ml), cells were incubated with 500 μl of the retrovirus in the presence of IL-2 (20 ng/ml) in a 24-well microtiter plate that was coated with RetroNectin (27 μg/ml; Takara Shuzo). After 4 h of infection, 500 μl of fresh medium was added to the culture and cells were allowed to grow for another 72 h in the presence or absence of IL-4 before being subjected to intracellular cytokine analysis. Under these conditions, the efficiency of infection to $CD4^+$ T cells was 15–30% as assessed by GFP^+ cells by FACS.

Data analysis

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

Results

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

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

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

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

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

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

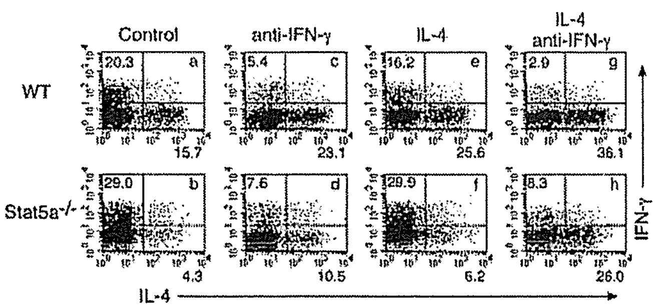

FIGURE 1. IFN-γ plays a dominant inhibitory role in the down-regulation of Th2 cell differentiation in Stat5a$^{-/-}$ $CD4^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of IL-4 (15 ng/ml) and/or neutralizing anti-IFN-γ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

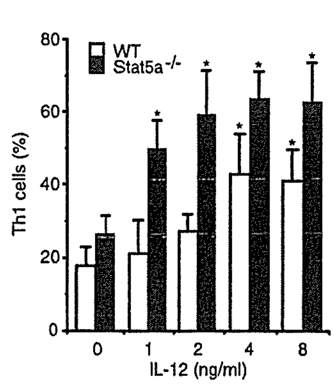

FIGURE 2. IL-12-induced Th1 cell differentiation is enhanced in Stat5a$^{-/-}$ $CD4^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in the presence of the indicated amounts of IL-12 (1–8 ng/ml). Cells were cultured for another 72 h in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

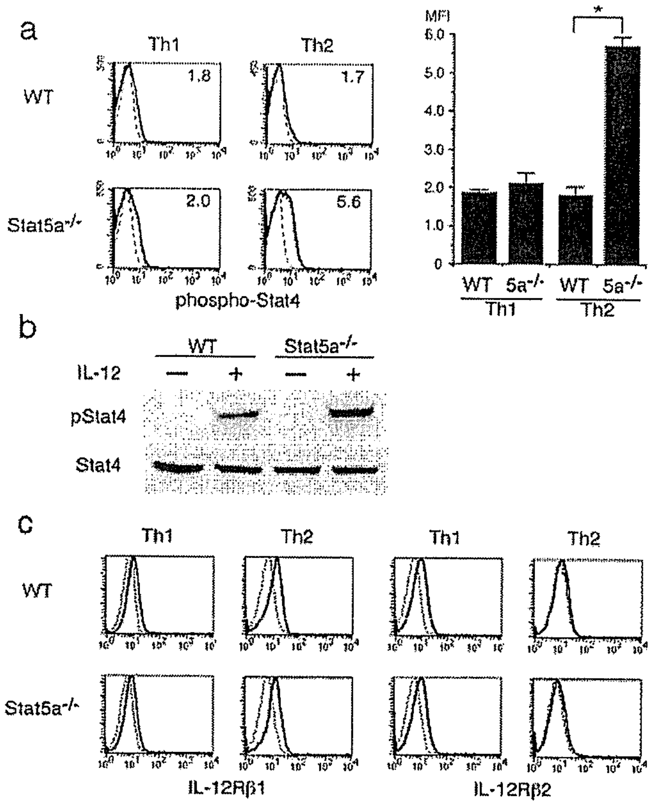

FIGURE 3. IL-12-induced Stat4 phosphorylation is enhanced in Stat5a$^{-/-}$ CD4$^+$ T cells. *a*, IL-12-induced Stat4 phosphorylation in Stat5a$^{-/-}$ CD4$^+$ T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th1-polarizing condition (15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

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

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

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

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

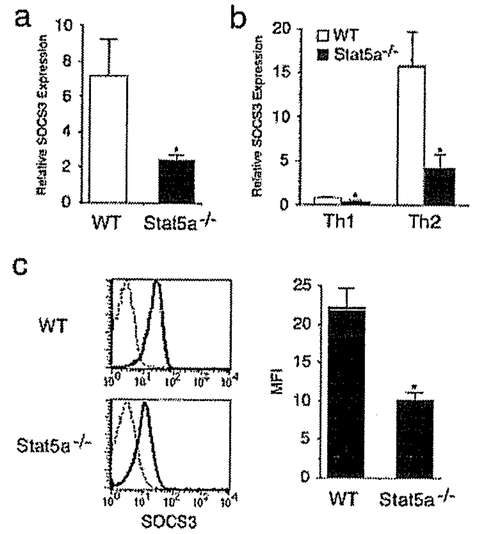

FIGURE 4. SOCS3 is diminished in Stat5a$^{-/-}$ CD4$^+$ T cells. *a*, Expression levels of SOCS3 mRNA in freshly isolated CD4$^+$ T cells. Total RNA was prepared from freshly isolated splenic WT CD4$^+$ T cells or Stat5a$^{-/-}$ CD4$^+$ T cells. TaqMan PCR analysis for SOCS3 and GAPDH (as a control)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

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

Stat5a activates SOCS3 promoter

It has been shown that the SOCS3 promoter contains putative tandem STAT-binding sequences (Fig. 5*a*) (22). We therefore investigated whether Stat5a activated SOCS3 promoter. Either a constitutively active form of Stat5a (1*6 Stat5a) or of Stat6 (Stat6VT) was expressed in COS7 cells, and SOCS3 WT Luc was determined by a reporter assay (Fig. 5*b*). The activity of SOCS3 WT Luc was significantly enhanced by the expression of 1*6 Stat5a but not of Stat6VT (Fig. 5*b*). When one of the putative STAT-binding sequences located in the SOCS3 promoter was mutated (SOCS3 mt1 Luc or SOCS3 mt2 Luc) (Fig. 5*a*), 1*6 Stat5a-induced activation was largely abolished (Fig. 5*b*). 1*6 Stat5a-induced activation of the SOCS3 promoter was more severely decreased when both STAT-binding sequences were simultaneously mutated (SOCS3 mt3 Luc) (Fig. 5*b*). As positive controls, we confirmed that the expression of 1*6 Stat5a preferentially activated a Stat5-dependent reporter construct (MGF-Luc) (21), whereas Stat6VT preferentially activated a Stat6-dependent reporter construct TPU474 (Fig. 5*b*) (20). These results indicate that Stat5a but not Stat6 activates the SOCS3 promoter.

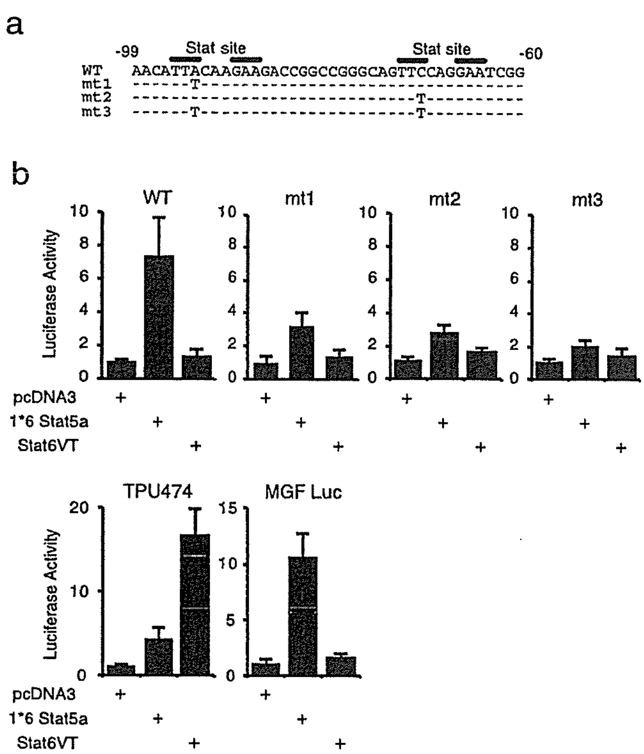

FIGURE 5. Stat5a directly activates the SOCS3 promoter. *a*, The murine SOCS3 promoter (WT) and the mutants (mt1, mt2, and mt3) of putative STAT-binding sequences. *b*, Stat5a preferentially activates the SOCS3 promoter. COS7 cells were transfected with TPU474, MGF-Luc, SOCS3 WT Luc, SOCS3 mt1 Luc, SOCS3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

Stat5a binds to the SOCS3 promoter in CD4^{+} T cells

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

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

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

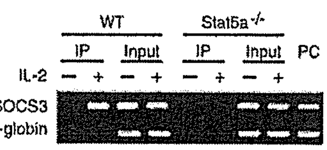

FIGURE 6. Stat5a binds to SOCS3 promoter in CD4^{+} T cells. Splenocytes from WT mice or Stat5a$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h. CD4^{+} T cells were purified (>90% pure by flow cytometry) using a CD4^{+} T cell enrichment column, starved from cytokines in fresh medium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