

Indispensable Role of Stat5a in Stat6-Independent Th2 Cell Differentiation and Allergic Airway Inflammation¹

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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.

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 V β 8.1,2-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 (5 μ g/ml; clone 145-2C11; BD Pharmingen) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μ M 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 μ g/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 μ M) (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 μ g 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 μ m 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 $\pm$ 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. 1A). The number of splenocytes in Stat5a $^{-/-}$ Stat6 $^{-/-}$ mice was also decreased compared with that in Stat6 $^{-/-}$ mice (Fig. 1A). 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. 1B). 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. 1B). 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

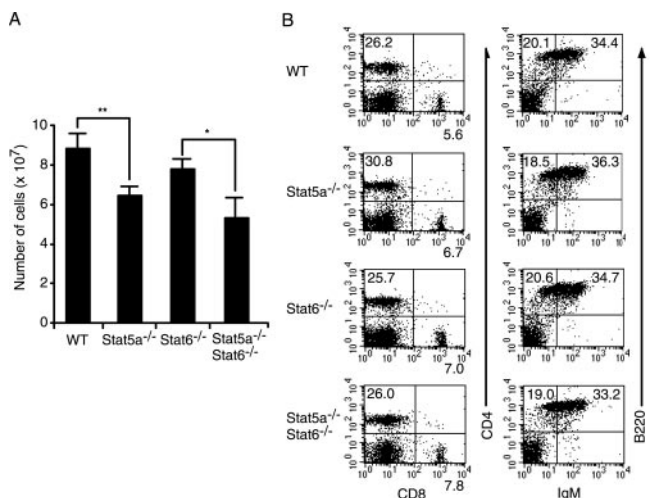


FIGURE 1. Normal T cell and B cell development in Stat5a $^{-/-}$ Stat6 $^{-/-}$ mice. **A**, Number of splenocytes in WT, Stat5a $^{-/-}$, Stat6 $^{-/-}$, and Stat5a $^{-/-}$ Stat6 $^{-/-}$ mice. Data are the mean $\pm$ SD from eight mice for each genotype. *, *p* < 0.05; **, *p* < 0.01. **B**, Flow 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

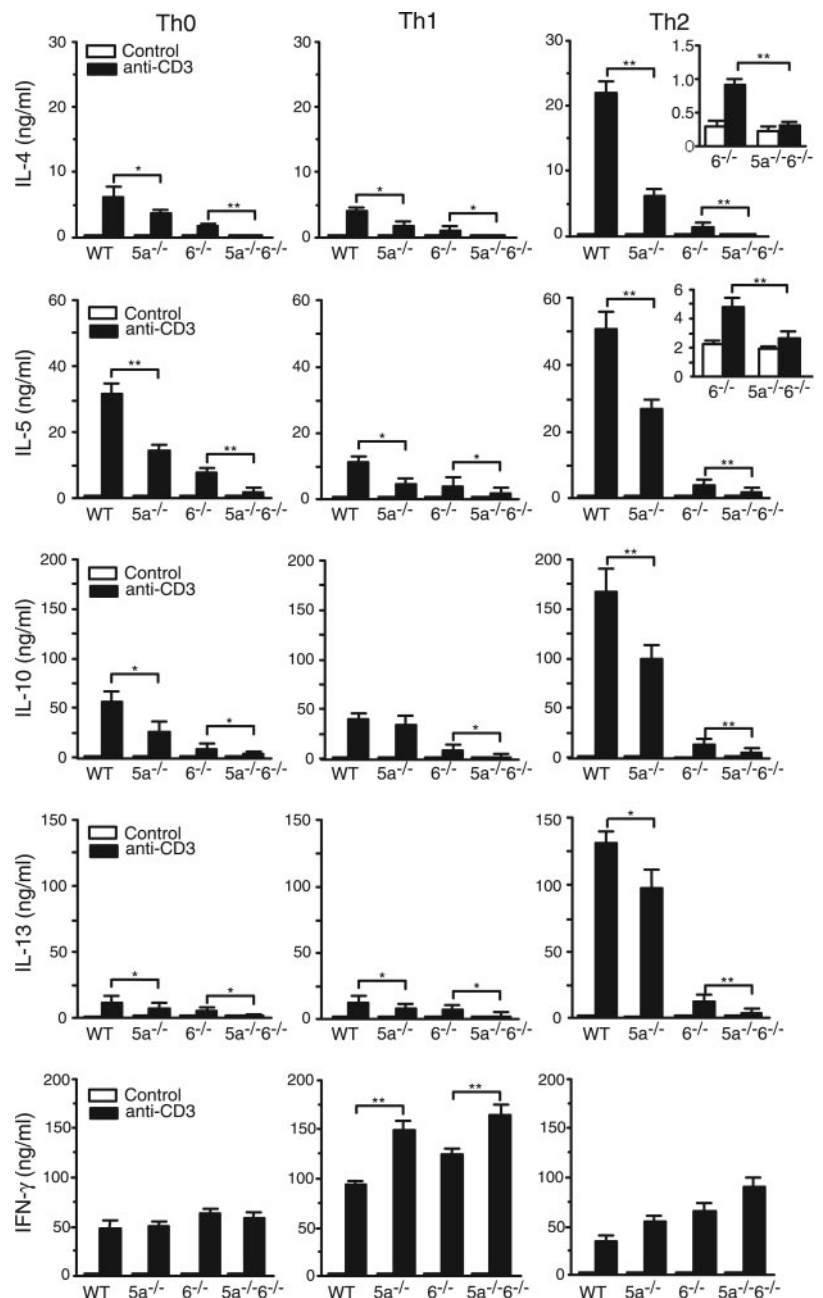


FIGURE 2. Th2 cytokine production is severely decreased in Stat5a^{-/-}Stat6^{-/-} mice. Splenocytes from WT, Stat5a^{-/-} (5a^{-/-}), Stat6^{-/-} (6^{-/-}), or Stat5a^{-/-}Stat6^{-/-} (5a^{-/-}6^{-/-}) mice were stimulated with plate-bound anti-CD3 mAb in the nonpolarizing Th0 condition (no exogenous 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 $\pm$ SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$.

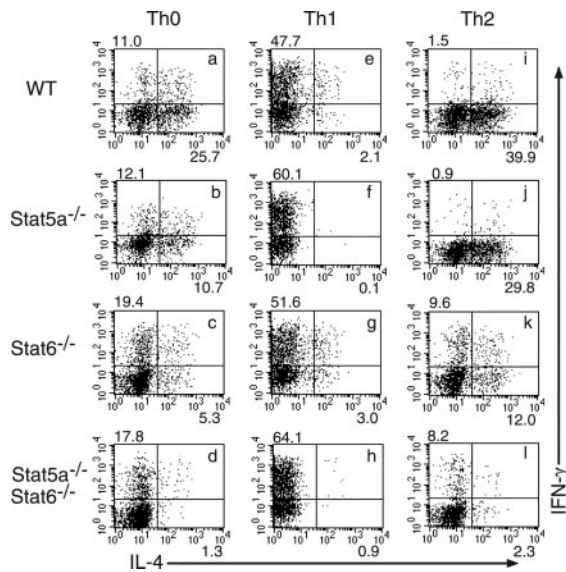


FIGURE 3. Th2 cell differentiation is severely decreased in Stat5a^{-/-}Stat6^{-/-} mice. Splenocytes from WT, Stat5a^{-/-}, Stat6^{-/-}, or Stat5a^{-/-}Stat6^{-/-} mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th0, Th1, or Th2 conditions and cultured for another 72 h in Th0, Th1, or 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. 3c). 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. 3d). 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. 3l). These results indicate that Stat5a is essential for Stat6-independent Th2 cell differentiation and vice versa.

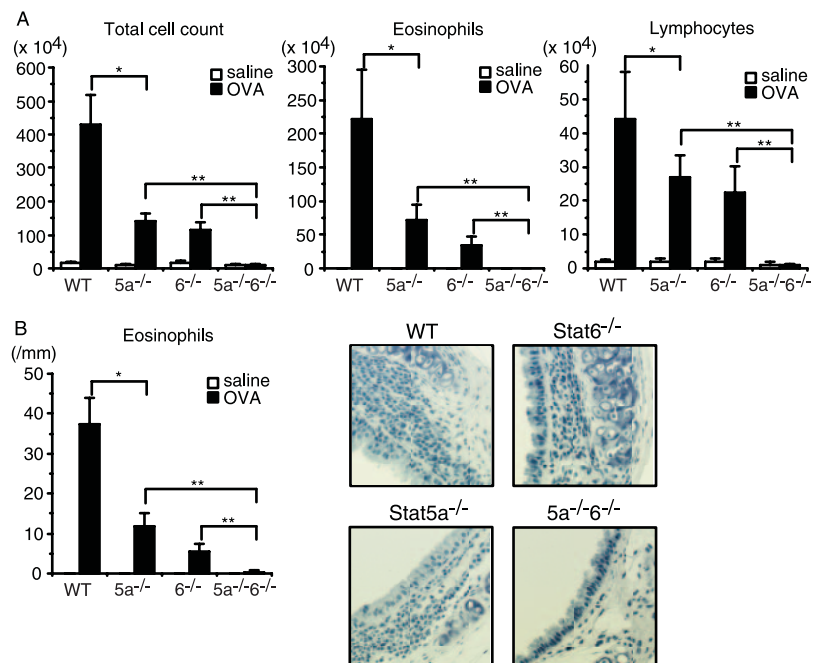
In contrast, in the Th1 condition, CD4⁺ T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in Stat5a^{-/-} and Stat5a^{-/-}Stat6^{-/-} mice compared with those in WT and Stat6^{-/-} mice, respectively (WT mice, 44.9 ± 8.2%; Stat5a^{-/-} mice, 62.3 ± 11.9% (*p* < 0.05); 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. 4A). However, the eosinophil recruitment in BALF was still observed to a considerable extent in both Stat5a^{-/-} and Stat6^{-/-} mice (Fig. 4A). In contrast, Ag inhalation induced no significant eosinophil recruitment in BALF in sensitized Stat5a^{-/-}Stat6^{-/-} mice (Fig. 4A). 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 × 10⁴/mice (*n* = 5 mice in each group; Fig. 4A). 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. 4B).

Ag-induced lymphocyte recruitment in BALF was also significantly decreased in Stat5a^{-/-} and Stat6^{-/-} mice (*n* = 5; *p* < 0.05; Fig. 4A). Furthermore, virtually no Ag-induced lymphocyte recruitment in BALF was observed in Stat5a^{-/-}Stat6^{-/-} mice (*n* = 5; *p* < 0.01; Fig. 4A). Consistent with these data obtained from BALF analysis (Fig. 4A), 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. 5A). In contrast,

Ag-induced epithelial goblet cell hyperplasia was severely decreased not only in Stat5a^{-/-}Stat6^{-/-} mice; but also in Stat6^{-/-} mice, indicating that Stat6 is absolutely required for Ag-induced epithelial goblet cell hyperplasia (*n* = 5; Fig. 5, B and C). Taken together, these results suggest that the Stat5a-dependent, Stat6-independent pathway is involved in *in vivo* Th2 cell differentiation and subsequent allergic airway inflammation, but not in the induction of epithelial goblet cell hyperplasia.

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.

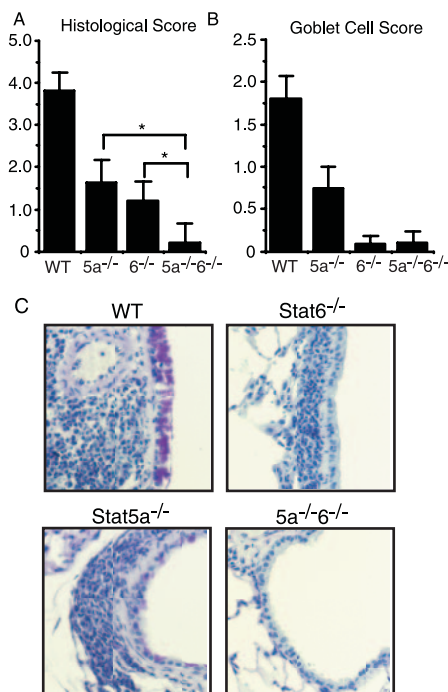


FIGURE 5. The Stat5a-dependent, Stat6-independent pathway induces airway inflammation, but not epithelial goblet cell hyperplasia. OVA-sensitized WT, Stat5a^{-/-}, Stat6^{-/-}, and Stat5a^{-/-}Stat6^{-/-} mice were challenged with inhaled OVA three times at 24-h intervals. A, Forty-eight hours after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

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 indis-

pensable 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

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