

Overlapping and Distinct Roles of STAT4 and T-bet in the Regulation of T Cell Differentiation and Allergic Airway Inflammation¹

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T-bet and STAT4 play critical roles in helper T cell differentiation, especially for Th1 cells. However, it is still unknown about the relative importance and redundancy of T-bet and STAT4 for Th1 differentiation. It is also unknown about their independent role of T-bet and STAT4 in the regulation of allergic airway inflammation. In this study, we addressed these issues by comparing T-bet-deficient (T-bet^{-/-}) mice, STAT4^{-/-} mice, and T-bet- and STAT4-double-deficient (T-bet^{-/-}STAT4^{-/-}) mice on the same genetic background. Th1 differentiation was severely decreased in T-bet^{-/-} mice and STAT4^{-/-} mice as compared with that in wild-type mice, but Th1 differentiation was still observed in T-bet^{-/-} mice and STAT4^{-/-} mice. However, Th1 cells were hardly detected in T-bet^{-/-}STAT4^{-/-} mice. In contrast, the maintenance of Th17 cells was enhanced in T-bet^{-/-} mice but was reduced in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice. In vivo, Ag-induced eosinophil and neutrophil recruitment into the airways was enhanced in T-bet^{-/-} mice but was attenuated in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice. Ag-induced IL-17 production in the airways was also diminished in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice. These results indicate that STAT4 not only plays an indispensable role in T-bet-independent Th1 differentiation but also is involved in the maintenance of Th17 cells and the enhancement of allergic airway inflammation. *The Journal of Immunology*, 2008, 180: 6656–6662.

Activated CD4⁺ T cells differentiate into at least three functionally distinct subsets, Th1 cells, Th2 cells, and IL-17-producing helper T cells (Th17 cells),⁴ as defined by their patterns of cytokine production (1–5). Th1 cells produce IFN- γ and lymphotoxin and play a critical role in protective immunity against intracellular pathogens (1–3). Th2 cells produce IL-4, IL-5, and IL-13 and are essential for the expulsion of parasites (1–3). Recently identified Th17 cells produce IL-17, IL-17F, and IL-22 and play a pivotal role in protective immunity against extracellular pathogens (4, 5).

The accumulating evidence has led to the identification of two major signaling pathways for Th1 differentiation, one involving IL-12/STAT4 and the other involving IFN- γ /STAT1/T-bet (1–3). It has been shown that T-bet, a master regulator of Th1 differen-

tiation (6, 7), induces the expression of IL-12R β 2 and then allows T cells to respond to IL-12 and to differentiate into Th1 cells in STAT4-dependent pathways (8). The importance of IL-12/STAT4 pathways in Th1 differentiation is also supported by the finding that even in the absence of T-bet, developing Th2 cells can differentiate into Th1 cells when the expression of STAT4 and IL-12R β 2 chain is maintained (9). By contrast, it has been demonstrated that retroviral expression of T-bet in STAT4-deficient (STAT4^{-/-}) T cells supports IFN- γ synthesis (8, 10). Therefore, it is suggested that not only T-bet-dependent STAT4-dependent pathways but also T-bet-dependent STAT4-independent pathways and T-bet-independent STAT4-dependent pathways participate in the differentiation of Th1 cells. However, the relative importance and redundancy of STAT4 and T-bet in T cell differentiation are still largely unknown.

In contrast to the considerable overlapping roles of STAT4 and T-bet in Th1 differentiation, it has been demonstrated that Ag-induced airway inflammation, in which Th2 cells play a central role (11, 12), is enhanced in T-bet^{-/-} mice (13) but rather attenuated in STAT4^{-/-} mice (14). These findings raise at least two possible explanations: that T-bet-dependent STAT4-independent pathways participate in the suppression of allergic airway inflammation; or that STAT4-dependent T-bet-independent pathways participate in the enhancement of allergic airway inflammation. However, again, the basis for the distinct roles of STAT4 and T-bet in allergic airway inflammation remains unknown.

In the present study, we addressed these issues by comparing helper T cell differentiation and allergic airway inflammation in STAT4^{-/-} mice, T-bet^{-/-} mice, and T-bet- and STAT4-double-deficient (T-bet^{-/-}STAT4^{-/-}) mice on the same genetic background. Our results indicate that both STAT4 and T-bet independently play a significant role in inducing Th1 differentiation and that either STAT4 or T-bet is required for Th1 differentiation. We

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⁴ Abbreviations used in this paper: Th17 cells, IL-17-producing helper T cells; WT, wild type; PAS, periodic acid-Schiff; BALF, bronchoalveolar lavage fluid.

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also show that STAT4 is significantly involved in the maintenance of Th17 cells and in the enhancement of Th2 cell-mediated eosinophilic inflammation and Th17 cell-mediated neutrophilic inflammation in the airways.

Materials and Methods

Mice

STAT4^{-/-} mice (15) and T-bet^{-/-} mice (6, 16) were backcrossed to BALB/c mice (Charles River Laboratories) for eight generations. T-bet^{+/-} STAT4^{+/-} mice were mated with STAT4^{+/-} T-bet^{+/-} mice to obtain T-bet^{+/-} STAT4^{+/-} mice (wild-type (WT) mice), T-bet^{+/-} STAT4^{-/-} mice, T-bet^{-/-} STAT4^{+/-} mice, and T-bet^{-/-} STAT4^{-/-} mice within the littermate. All mice were housed in microisolator cages under specific pathogen-free conditions and all experiments were performed according to the guidelines of Chiba University (Chiba, Japan).

Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Biosciences: anti-CD3ε FITC (145-2C11); anti-CD4 FITC; PE; allophycocyanin; PerCP (H129.19); anti-CD8 FITC; PE (53-6.7); anti-B220 allophycocyanin (RA3-6B2); anti-IgM FITC (R6-60.2); anti-CD69 FITC (H1.3F3); anti-CD62L FITC; and PE (MEL-14). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Biosciences). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Biosciences).

Cell culture

CD4⁺CD62L^{high} T cells were purified from spleens of WT mice, STAT4^{-/-} mice, T-bet^{-/-} mice, and T-bet^{-/-}STAT4^{-/-} mice by magnetic cell sorting using a CD4⁺CD62L⁺ T cell isolation kit II according to the manufacturer's instructions (Miltenyi Biotec). In some experiments, CD4⁺ T cells were purified from spleen with an EasySep mouse CD4⁺ T cell enrichment kit (StemCell Technologies) using an automated cell separator RoboSep (StemCell Technologies). In both cases, the resultant cells were >98% pure CD4⁺CD62L^{high} T cells or CD4⁺ T cells, respectively, by FACS analysis. Cells were plated at 1×10^6 cells/ml onto plates coated with 1 μg/ml anti-CD3ε mAb (145-2C11; BD Biosciences) in the presence of anti-CD28 mAb (3 μg/ml; clone 37.51; BD Biosciences) in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics at 37°C for 48 h. Where indicated, IL-12 (20 ng/ml; PeproTec), IL-2 (20 ng/ml; PeproTec), and anti-IL-4 mAb (20 μg/ml; clone 11B11; BD Biosciences) were added to polarize toward Th1 cells (Th1-polarizing condition) and IL-4 (20 ng/ml; PeproTec), IL-2, and anti-IFN-γ mAb (20 μg/ml; clone XMG1.2; BD Biosciences) were added to polarize toward Th2 cells (Th2-polarizing condition) (17). Cells were washed with PBS and then cultured for another 3 days in the same condition. To induce Th17 differentiation, CD4⁺ T cells were stimulated with anti-CD3ε mAb plus anti-CD28 mAb for 3 days in the presence of IL-6 (20 ng/ml; PeproTec), TGF-β (2 ng/ml; R&D Systems), anti-IL-4 mAb, and anti-IFN-γ mAb (Th17-polarizing condition). Cells were washed with PBS and then cultured for another 4 days in the presence of anti-IL-4 mAb, anti-IFN-γ mAb, and IL-23 (20 ng/ml; R&D Systems).

Cell proliferation

CD4⁺ T cells from spleen of WT mice, STAT4^{-/-} mice, T-bet^{-/-} mice, and T-bet^{-/-}STAT4^{-/-} were stimulated with anti-CD3ε mAb plus anti-CD28 mAb in Th1-polarizing condition for 72 h, with 1 μCi of [³H]thymidine added for the final 16 h.

Cytokine levels in culture supernatants

CD4⁺ cells cultured in Th0 condition, Th1-polarizing condition, or Th17-polarizing condition were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 6 h. The amounts of IFN-γ and IL-17 in the culture supernatants were determined by enzyme immunoassay according to the manufacturer's instruction (R&D Systems). The detection limits of these assays were 10 pg/ml for IL-17 and 30 pg/ml for IFN-γ.

Intracellular cytokine analysis

Intracellular cytokine staining for IL-4, IFN-γ, and IL-17 was performed as described previously (13). In brief, cultured cells 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 cells were stained with anti-CD4 FITC, cells were fixed,

permeabilized with Perm/Wash buffer (BD Biosciences), and stained with anti-IFN-γ allophycocyanin (XMG1.2; BD Biosciences) and either anti-IL-4 PE (BVD4-1D11; BD Biosciences) or anti-IL-17 PE (TC11-18H10; BD Biosciences) for 30 min at 4°C. The cytokine profile (IL-4 vs IFN-γ or IL-17 vs IFN-γ) on CD4⁺ cells was analyzed on a FACSCalibur using CELLQuest software.

Ag-induced allergic airway inflammation

Allergic airway inflammation was induced by the inhalation of OVA (Sigma-Aldrich) in sensitized mice as described previously (18). Briefly, mice (age 7–8 wk) were immunized i.p. twice with 4 μg of OVA in 4 mg of aluminum hydroxide at a 2-wk interval. Fourteen days after the second immunization, the sensitized mice were given aerosolized OVA (50 mg/ml) dissolved in 0.9% saline with a DeVilbiss 646 nebulizer (DeVilbiss) for 20 min. As a control, 0.9% saline alone was administered by the nebulizer. At 48 h after the OVA inhalation, lungs were excised, fixed in 10% buffered formalin, and embedded in paraffin. The specimens (3 μm thick) of the lung were stained with H&E and periodic acid-Schiff (PAS) according to standard protocols. The number of goblet cells was counted on PAS-stained lung sections as described elsewhere (19). The numbers of eosinophils, lymphocytes, and neutrophils recovered in the bronchoalveolar lavage fluid (BALF) and the levels of cytokines in the BALF were evaluated at 48 h after OVA inhalation as described previously (20).

Data analysis

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

Results

Normal T cell and B cell development in T-bet^{-/-}STAT4^{-/-} mice

It has been shown that not only T-bet (6, 7) but also STAT4 (15, 21) play a critical role in Th1 differentiation. To investigate the relative importance of T-bet and STAT4 in the differentiation of helper T cells including Th1 cells in detail, we generated T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-}STAT4^{-/-} mice on the same genetic background and compared the development and differentiation of CD4⁺ T cells in these mice. Consistent with the previous reports (6, 15), the number of splenocytes was normal in T-bet^{-/-} mice and STAT4^{-/-} mice (data not shown). The number of splenocytes was also normal in T-bet^{-/-}STAT4^{-/-} mice (data not shown). FACS analysis revealed that the frequencies of CD3⁺ cells and B220⁺ cells were similar among these mice (Fig. 1). The frequencies of CD4⁺ T cells and CD8⁺ T cells in CD3⁺ cells (Fig. 1) as well as the expression of CD69 and CD62L on CD4⁺ T cells (Fig. 1) was also similar among these mice. These results indicate that T cells and B cells can develop normally even in the absence of T-bet and STAT4.

T-bet-independent Th1 differentiation depends on STAT4

We next examined helper T cell differentiation in WT, T-bet^{-/-} STAT4^{-/-}, and T-bet^{-/-}STAT4^{-/-} mice. Purified CD4⁺CD62L^{high} T cells were stimulated with plate-bound anti-CD3 mAb and anti-CD28 mAb in nonpolarizing (Th0), Th1-polarizing, or Th2-polarizing condition, and the differentiation of CD4⁺ T cells was evaluated by intracellular cytokine staining. In Th0 condition, CD4⁺ T cells that produced IFN-γ but not IL-4 (Th1 cells) were significantly decreased in T-bet^{-/-} mice and STAT4^{-/-} mice as compared with those in WT mice, and Th1 cells were undetectable in T-bet^{-/-}STAT4^{-/-} mice (Fig. 2A). In Th1-polarizing condition, the induction of Th1 cells was significantly less effective in T-bet^{-/-} mice and STAT4^{-/-} mice than in WT mice (Fig. 2A), but Th1 cells still developed in T-bet^{-/-} mice and STAT4^{-/-} mice (Fig. 2A). However, Th1 cells were hardly detected in T-bet^{-/-}STAT4^{-/-} mice even in Th1-polarizing condition (Fig. 2A). The levels of IFN-γ in the culture supernatants were

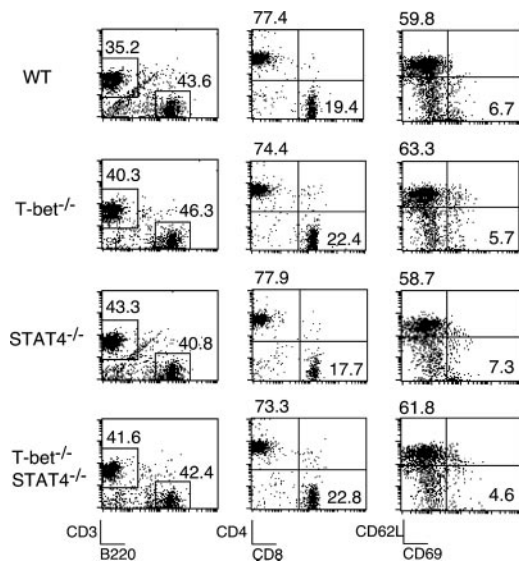


FIGURE 1. Normal T cell and B cell development in T-bet^{-/-} STAT4^{-/-} mice. Splenocytes from WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-} STAT4^{-/-} mice were stained either with anti-CD3 mAb and anti-B220 mAb, with anti-CD4 mAb, anti-CD8 mAb, and anti-CD3 mAb or with anti-CD4 mAb, anti-CD69 mAb, and anti-CD62L mAb. Shown are representative FACS profiles (CD3 vs B220 staining (left), CD4 vs CD8 staining gating on CD3⁺ cells (middle), and CD69 vs CD62L staining gating on CD4⁺ cells (right) from five mice in each group.

also severely decreased in T-bet^{-/-} mice and STAT4^{-/-} mice as compared with those in WT mice in Th0 condition as well as in Th1-polarizing condition ($n = 5$, each; $p < 0.01$; Fig. 2B). These results indicate that both STAT4 and T-bet independently play a significant role in inducing Th1 differentiation and that either STAT4 or T-bet is required for Th1 differentiation. In contrast, no significant difference was observed in the proliferative responses of T cells among these mice even in Th1-polarizing condition (data not shown), suggesting that the impaired Th1 differentiation in T-bet^{-/-} STAT4^{-/-} mice does not result from a defect in cell proliferation. As expected, CD4⁺ T cells that produced IL-4 but not IFN- γ (Th2 cells) developed similarly among these mice in Th2-polarizing condition (Fig. 2A).

The maintenance but not the differentiation of Th17 cells is impaired in the absence of STAT4

It has been shown that IL-23 is involved in the maintenance of Th17 cells (4, 5) and that STAT4 is involved in IL-23 signaling (22). Recently, it has also been reported that STAT4 is involved in the maintenance of Th17 cells (23). Therefore, we next evaluated the differentiation and the maintenance of IL-17-producing CD4⁺ T cells (Th17 cells) in WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-} STAT4^{-/-} mice. As shown in Fig. 3A, when CD4⁺ T cells were cultured in Th17-polarizing condition (IL-6, TGF- β , anti-IL-4 mAb, and anti-IFN- γ mAb) for 3 days, CD4⁺ T cells differentiated into Th17 cells similarly in WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-} STAT4^{-/-} mice, suggesting that neither T-bet nor STAT4 is essential for the differentiation of Th17 cells. However, when Th17 cells were maintained in the presence of IL-23 for additional 4 days, Th17 cells were significantly decreased not only in STAT4^{-/-} mice but also in T-bet^{-/-} STAT4^{-/-} mice as compared with those in WT mice (Fig. 3A). The levels of IL-17 in the culture supernatants were also decreased in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice as compared

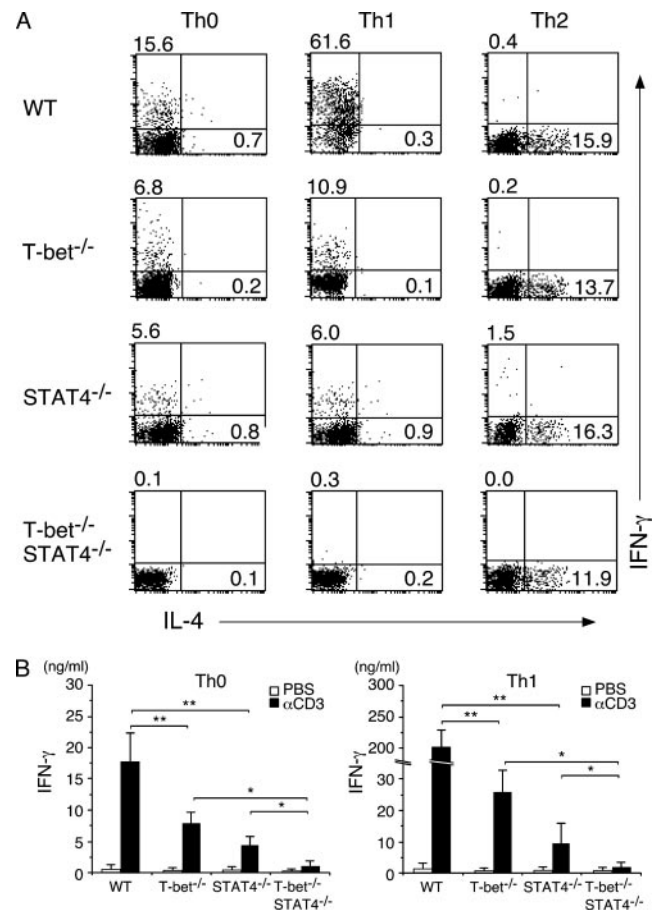


FIGURE 2. Th1 differentiation is severely decreased in T-bet^{-/-} STAT4^{-/-} mice. *A*, Splenic CD4⁺CD62L^{high} T cells were purified from WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-} STAT4^{-/-} mice and stimulated with plate-bound anti-CD3 mAb plus anti-CD28 mAb in nonpolarizing Th0 condition, Th1-polarizing condition, or Th2-polarizing condition for 5 days as described in *Materials and Methods*. Cells were restimulated with anti-CD3 mAb for 6 h in the absence of exogenous cytokines, and intracellular cytokine profiles for IL-4 vs IFN- γ were determined. Shown are representative FACS profiles from five mice in each group. *B*, Splenic CD4⁺ T cells from WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-} STAT4^{-/-} mice were stimulated with plate-bound anti (α)-CD3 mAb plus anti-CD28 mAb in Th0 condition or Th1-polarizing condition for 5 days as described previously. After a washing, cells (1×10^6 /ml) were restimulated with plate-bound anti-CD3 mAb for 6 h in the absence of exogenous cytokines. The amounts of IFN- γ in the culture supernatants were determined by ELISA. Data are means $\pm$ SD for five mice in each group. *, $p < 0.05$, **, $p < 0.01$.

with those in WT mice ($n = 4$; $p < 0.01$) (Fig. 3B). In contrast, Th17 cells (Fig. 3A) and IL-17 levels in the culture supernatants (Fig. 3B) were significantly increased in T-bet^{-/-} mice as compared with those in WT mice ($n = 4$; $p < 0.05$), consistent with the previous findings that T-bet exerts an inhibitory effect on Th17 differentiation (13, 24, 25). These results suggest that STAT4 independently of T-bet is required for the maintenance of Th17 cells. Interestingly, in Th17-polarizing condition, the number of IFN- γ -producing CD4⁺ T cells (Fig. 3A) and IFN- γ levels in the culture supernatants (Fig. 3B) were decreased in T-bet^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice but not in STAT4^{-/-} mice, suggesting that T-bet independently of STAT4 is required for the differentiation of IFN- γ -producing CD4⁺ T cells in Th17-polarizing condition.

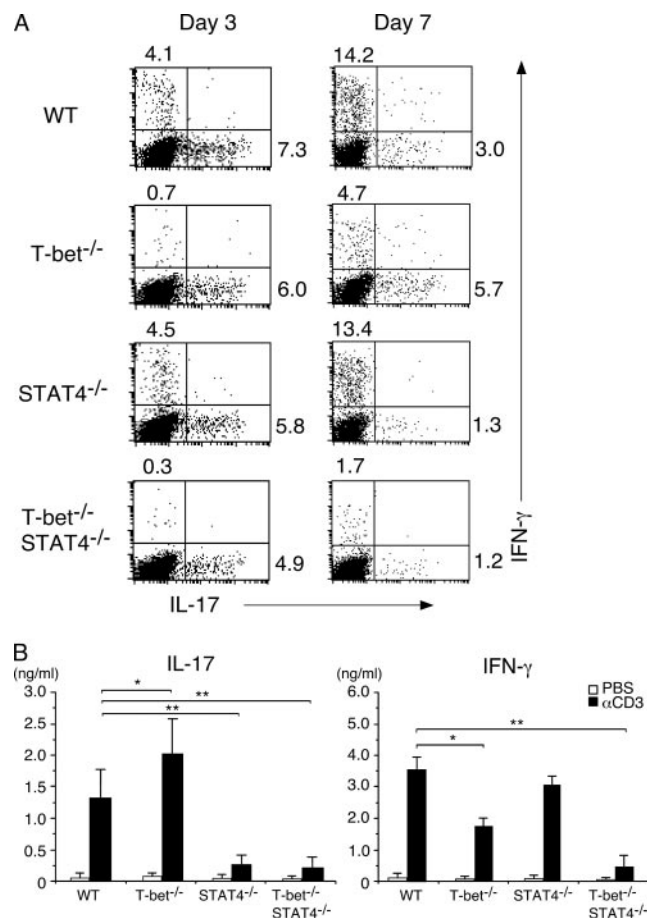


FIGURE 3. The maintenance of Th17 cells is impaired in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice. *A*, Splenic CD4⁺ T cells from WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-}STAT4^{-/-} mice were stimulated with plate-bound anti-CD3 mAb plus anti (α)-CD28 mAb in Th17-polarizing condition for 3 days (*left*). Subsequently, a fraction of cells was cultured in the presence of IL-23 for additional 4 days (*right*). Cells were restimulated with plate-bound anti-CD3 mAb for 6 h, and intracellular cytokine profiles for IL-17 vs IFN-γ were determined. Shown are representative FACS profiles from four mice in each group. *B*, Splenic CD4⁺ T cells were purified from WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-}STAT4^{-/-} mice and stimulated with plate-bound anti-CD3 mAb plus anti-CD28 mAb in Th17-polarizing condition for 7 days with IL-23 added for the final 4 days. After a washing, cells (1 × 10⁶/ml) were restimulated with plate-bound anti-CD3 mAb for 6 h in the absence of exogenous cytokines. The amounts of IL-17 and IFN-γ in the culture supernatants were measured by ELISA. Data are means ± SD for four mice in each group. *, *p* < 0.05; **, *p* < 0.01.

Ag-induced eosinophil and neutrophil recruitment into the airways is diminished in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice

To determine the consequence of T-bet- and STAT4-dependent signaling in the regulation of allergic airway inflammation, we next examined Ag-induced eosinophil and neutrophil recruitment into the airways in T-bet^{-/-} mice, STAT4^{-/-} mice, T-bet^{-/-}STAT4^{-/-} mice, and littermate WT mice. These mice were immunized i.p. twice with OVA; 2 wk after the second immunization, airway inflammation was induced by OVA inhalation (Fig. 4). Consistent with our previous report (13), the number of eosinophils recovered from BALF at 48 h after the Ag challenge was significantly increased in T-bet^{-/-} mice as compared with that in WT mice (*n* = 5; *p* < 0.05; Fig. 4A). In contrast, Ag-induced eosinophil recruitment into the airways was significantly attenuated in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (*n* = 5; *p* < 0.05; Fig. 4A).

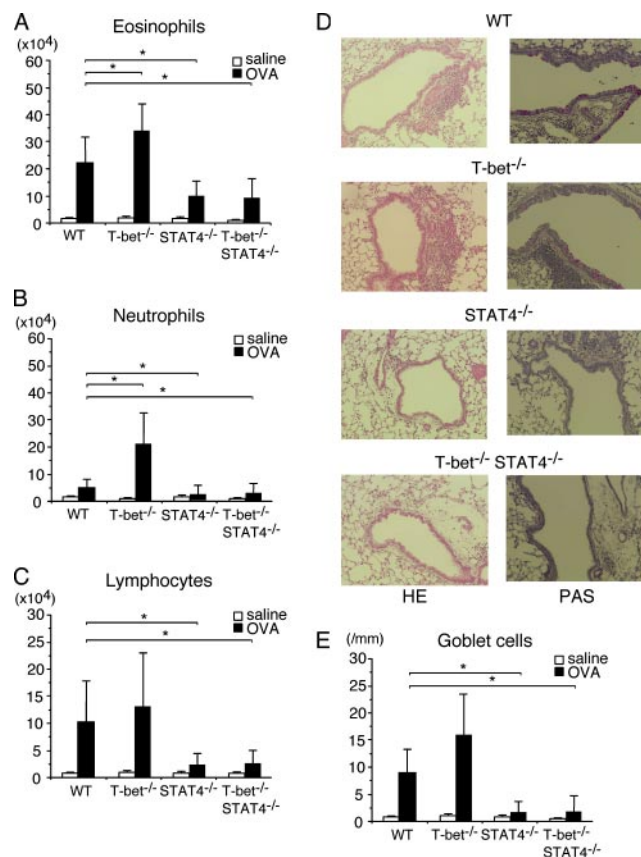
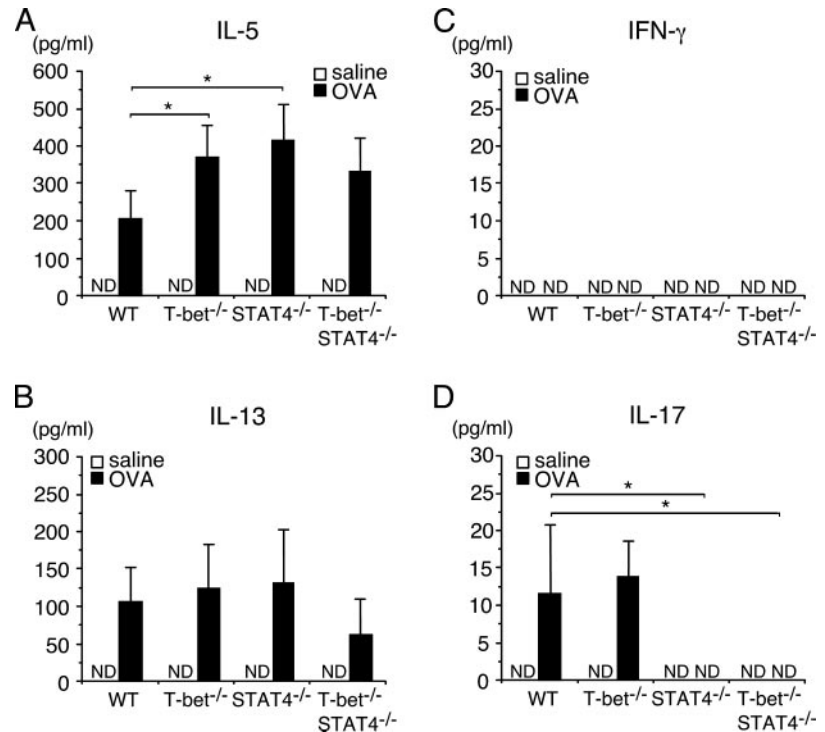


FIGURE 4. Ag-induced eosinophil and neutrophil recruitment into the airways are decreased in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice. *A–C*, OVA-sensitized WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-}STAT4^{-/-} mice were challenged with the inhalation of OVA or saline (as a control). The numbers of eosinophils (*A*), neutrophils (*B*), and lymphocytes (*C*) in the BALF were evaluated at 48 h after the OVA inhalation. Data are means ± SD for five mice in each group. *, *p* < 0.05. *D–E*, Similar to results in *A*, OVA-sensitized mice were challenged with the inhaled OVA. Forty-eight hours after the OVA inhalation, a sagittal block of left lung was excised and stained with H&E or PAS. Representative photomicrographs of lung sections stained with H&E or PAS. ×100 (*D*), and the number of goblet cells evaluated on PAS-stained lung sections (*E*) are shown. Data are means ± SD for five mice in each group. *, *p* < 0.05.

ated in STAT4^{-/-} mice as compared with WT mice (*n* = 5; *p* < 0.05; Fig. 4A). Ag-induced eosinophil recruitment into the airways was also attenuated in T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (*n* = 5; *p* < 0.05; Fig. 4A). In addition, even at 96 h after the OVA inhalation, Ag-induced eosinophil recruitment into the airways was attenuated in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (data not shown). Ag-induced neutrophil recruitment into the airways was significantly enhanced in T-bet^{-/-} mice but was attenuated in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (*n* = 5; *p* < 0.05; Fig. 4B). Ag-induced lymphocyte recruitment into the airways was also significantly attenuated in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (*n* = 5; *p* < 0.05; Fig. 4C). Histological analysis showed that Ag-induced inflammatory cell infiltration in the lung was significantly enhanced in T-bet^{-/-} mice but was attenuated in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (*n* = 5; Fig. 4D). Furthermore, Ag-induced epithelial goblet cell hyperplasia was diminished in STAT4^{-/-} mice and T-bet^{-/-}STAT4^{-/-} mice as compared with WT mice (*n* = 5, *p* < 0.05; Fig. 4E).

FIGURE 5. Ag-induced IL-17 production in the airways is diminished in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice. Similar to results in Fig. 4, OVA-sensitized WT mice, T-bet^{-/-} mice, STAT4^{-/-} mice, and T-bet^{-/-} STAT4^{-/-} mice were challenged with the inhalation of OVA or saline (as a control). The levels of IL-5 (A), IL-13 (B), IFN- γ (C), and IL-17 (D) in the BALF were evaluated at 48 h after the OVA inhalation. Data are means $\pm$ SD for five mice in each group. *, $p < 0.05$. ND, Not detectable.



0.05; Fig. 4E). Taken together, these results indicate that STAT4-dependent pathways are involved in the enhancement of Ag-induced allergic airway inflammation.

Ag-induced IL-17 production in the airways is decreased in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice

We finally examined the levels of cytokines in the BALF of OVA-sensitized T-bet^{-/-} mice, STAT4^{-/-} mice, T-bet^{-/-} STAT4^{-/-} mice, and littermate WT mice with the inhaled OVA or saline challenge. Consistent with our previous report (13), Ag-induced IL-5 production in the airways was significantly enhanced in T-bet^{-/-} mice as compared with that in WT mice ($n = 5$; $p < 0.05$; Fig. 5A). Ag-induced IL-5 production in the airways was also enhanced in STAT4^{-/-} mice as compared with that in WT mice ($n = 5$; $p < 0.05$; Fig. 5A). Ag-induced IL-13 production also tended to be enhanced in T-bet^{-/-} mice and STAT4^{-/-} mice, although the difference did not reach statistical significance (Fig. 5B). In contrast, in this experimental setting, IFN- γ was undetectable in the airways even in OVA-inhaled WT mice (Fig. 5C). Importantly, Ag-induced IL-17 production in the airways was significantly decreased in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice as compared with that in WT mice ($n = 5$; $p < 0.05$; Fig. 5D). These results suggest that STAT4 is required for Ag-induced IL-17 production in the airways.

Discussion

In this study, we addressed the overlapping and distinct roles of STAT4 and T-bet in the differentiation of helper T cells including Th1 cells and Th17 cells and in the regulation of allergic airway inflammation. We found that Th1 differentiation was severely decreased in T-bet^{-/-} mice and STAT4^{-/-} mice and was hardly detected in T-bet^{-/-} STAT4^{-/-} mice even in Th1-polarizing condition (Fig. 2), suggesting that STAT4 is essential for T-bet-independent Th1 differentiation and vice versa. In contrast, IL-23-induced maintenance of Th17 cells was enhanced in T-bet^{-/-} mice but was diminished in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice (Fig. 3). In vivo, Ag-induced eosinophil and neutrophil re-

cruitment into the airways was enhanced in T-bet^{-/-} mice but was attenuated in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice (Fig. 4). Ag-induced IL-17 production in the airways was also diminished in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice (Fig. 5). These results indicate that STAT4 not only plays an indispensable role in T-bet-independent Th1 differentiation but also is involved in the maintenance of Th17 cells and the enhancement of allergic airway inflammation.

We show that STAT4 and T-bet induce Th1 differentiation by complementary mechanisms. We found that Th1 differentiation was severely impaired in both T-bet^{-/-} CD4⁺ T cells and STAT4^{-/-} CD4⁺ T cells and was almost completely abrogated in T-bet^{-/-} STAT4^{-/-} CD4⁺ T cells even in Th1-polarizing condition (Fig. 2). We also found that the residual IFN- γ -producing CD4⁺ T cells in T-bet^{-/-} mice and STAT4^{-/-} mice lacked the expression of DX5 and that the frequency of TCR V β 8⁺ cells was not significantly increased in these cells (data not shown), suggesting that the majority of IFN- γ -producing CD4⁺ cells in T-bet^{-/-} mice and STAT4^{-/-} mice were conventional Th1 cells but not NK T cells. Taken together, it is indicated that both STAT4 and T-bet independently play a significant role in inducing Th1 differentiation and that the expression of either STAT4 or T-bet is required for Th1 differentiation. In contrast, we found that CD8⁺ T cells in T-bet^{-/-} STAT4^{-/-} mice still produced a considerable amount of IFN- γ upon stimulation with anti-CD3 mAb (S.-i. Kagami et al., manuscript in preparation), suggesting that the requirement for T-bet and STAT4 in the production of IFN- γ differs depending on cell lineages.

We also show that STAT4 exerts a dominant enhancing effect over T-bet-mediated inhibition on the maintenance of Th17 cells, as evidenced by the decrease in Th17 cells similarly observed in STAT4^{-/-} mice and T-bet^{-/-} STAT4^{-/-} mice but the increase in these cells in T-bet^{-/-} mice (Fig. 3). IL-23 has been shown to use STAT4 as a signaling molecule (22) and play an important role in the maintenance of Th17 cells (4, 5, 23). Previous studies showed that STAT4 significantly contributed to the induction of Th17 cells

in neutrophilic inflammation in the peritoneal cavity and collagen-induced arthritis (26, 27). It has recently been shown that STAT4 is required for the maintenance of Th17 cells (23) but not for Th17 differentiation (23, 24). Our findings are in agreement with a recent study reporting that STAT4 is required for IL-23-induced maintenance of Th17 cells but not IL-6- and TGF- β -induced Th17 differentiation (23). In contrast, T-bet has been shown to prevent IL-23-induced Th17 differentiation (24, 25), which is consistent with the findings of our present study (Fig. 3). Our findings that the maintenance of Th17 cells is decreased in T-bet $^{-/-}$ STAT4 $^{-/-}$ mice but is rather enhanced in T-bet $^{-/-}$ mice (Fig. 3) suggest that STAT4 deficiency cancels the effect of T-bet deficiency in enhancing Th17 differentiation.

Interestingly, we found that the differentiation of IFN- γ -producing CD4 $^{+}$ T cells was reduced in T-bet $^{-/-}$ mice but not in STAT4 $^{-/-}$ mice in Th17-polarizing condition (Fig. 3). At present, the reason for the differential requirement of T-bet and STAT4 for the development of IFN- γ -producing CD4 $^{+}$ T cells in Th17-polarizing condition is unknown. In this regard, it has recently been shown that CD4 $^{+}$ T cells produce a large amount of IL-21 in Th17-polarizing condition (28–30). In addition, we have shown that IL-21 inhibits IFN- γ production in developing Th1 cells even in the absence of T-bet (31). Therefore, it is possible that IL-21-mediated suppression of the development of IFN- γ -producing CD4 $^{+}$ T cells may be enhanced in T-bet $^{-/-}$ CD4 $^{+}$ T cells in Th17-polarizing condition because the T-bet-independent pathways for IFN- γ production seem to be significant in T-bet $^{-/-}$ CD4 $^{+}$ T cells.

We also demonstrate that STAT4 has a dominant enhancing effect over T-bet-mediated inhibition on Th2 cell-mediated eosinophilic airway inflammation, as evidenced by the findings that Ag-induced eosinophil recruitment into the airways is attenuated in STAT4 $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice but is enhanced in T-bet $^{-/-}$ mice (Fig. 4). It is well established that Ag-induced eosinophil recruitment into the airways is mediated by Th2 cells secreting IL-4, IL-5, and IL-13 (11, 12). In contrast, we and others have shown that a blocking of IFN- γ enhances Ag-induced eosinophil recruitment into the airways in a mouse model of asthma (32, 33). In accordance with these findings, we found that T-bet $^{-/-}$ mice, in which IFN- γ production from CD4 $^{+}$ T cells is reduced (Fig. 2), exhibited the enhanced Ag-induced eosinophil recruitment into the airways (Fig. 4 and Ref. 13). However, we found that T-bet $^{-/-}$ STAT4 $^{-/-}$ mice, in which IFN- γ production from CD4 $^{+}$ T cells is more severely reduced than in T-bet $^{-/-}$ mice (Fig. 2), exhibited the decreased Ag-induced eosinophil recruitment into the airways (Fig. 4). Because Th2 responses were similarly mounted in T-bet $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice (Figs. 2 and 5), it is unlikely that the balance between Th1 cells and Th2 cells can account for the difference in Ag-induced eosinophil recruitment into the airways between T-bet $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice and is rather suggested that STAT4-dependent T-bet-independent pathways are involved in the enhancement of allergic airway inflammation.

Regarding the mechanisms underlying STAT4-dependent enhancement of airway inflammation, our findings that the maintenance of Th17 cells and Ag-induced IL-17 production in the airways are significantly decreased in STAT4 $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice but not in T-bet $^{-/-}$ mice (Figs. 3 and 5) suggest that IL-17 may be involved in the STAT4-dependent enhancement of airway inflammation. In this regard, our findings of decreased Ag-induced airway inflammation together with decreased IL-17 levels in the airways in STAT4 $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice (Figs. 4 and 5) are consistent with the decreased Ag-induced airway inflammation in IL-17-deficient mice (34). In addition, it

has been shown that Th17 cells produce TNF- α besides IL-17 (35) and that TNF- α induces eosinophil recruitment into the airways in part through the induction of VCAM-1 expression (36). Thus, it is suggested that the decreased maintenance of Th17 cells by the absence of STAT4 may be responsible for the decreased Ag-induced eosinophil and neutrophil recruitment into the airways. In contrast, it has been shown that decreased peribronchial eosinophilia in STAT4 $^{-/-}$ mice in response to a cockroach Ag is associated with the decreased levels of chemokines including CCL11 (eotaxin-1; Ref. 14). However, we found that the levels of CCL11 and CCL24 (eotaxin-2) were decreased in STAT4 $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice as well as in T-bet $^{-/-}$ mice (data not shown), suggesting that the decreased levels of CCL11 and CCL24 in the airways of STAT4 $^{-/-}$ mice and T-bet $^{-/-}$ STAT4 $^{-/-}$ mice may not be responsible for the diminished Ag-induced eosinophil recruitment into the airways in these mice.

In conclusion, we have shown that STAT4 is capable of inducing Th1 differentiation independently of T-bet and is essential for Th1 differentiation in the absence of T-bet and vice versa. We have also shown a significant involvement of STAT4 in the maintenance of Th17 cells and in the enhancement of Th2 cell-mediated eosinophilic inflammation and Th17 cell-mediated neutrophilic inflammation in the airways. Our findings illustrate unique and complementary functions of STAT4 and T-bet in the regulation of helper T cell differentiation and Ag-induced allergic airway inflammation.

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Disclosures

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