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T-bet and Eomesodermin Play Critical Roles in Directing T Cell Differentiation to Th1 versus Th17

Yu Yang,*§ Jiangnan Xu,* Yanyan Niu,* Jonathan S. Bromberg,*†§§ and Yaozhong Ding2*†§§

Th1 and Th17 cells are crucial in immune regulation and autoimmune disease development. By adding Stat6 deficiency to T-bet deficiency, and thus negating effects from elevated levels of IL-4/Stat6/GATA3 Th2 signals in T-bet-deficient cells, we investigated the signals important for Th1 and Th17 cell differentiation and their role in colitis development. The data reveal that Eomesodermin compensates T-bet deficiency for IFN-γ and Th17 development. However, without T-bet, IFN-γ production and Th1 differentiation are susceptible to inhibition by IL-6 and TGFβ. As a result, Th17 development is strongly favored, the threshold for TGFβ requirement is lowered, and IL-6 drives Th17 differentiation, elucidating a critical role for T-bet in directing T cell differentiation to Th1 vs Th17. In contrast to IL-6 plus TGFβ-driven Th17, IL-6-driven Th17 cells do not express IL-10 and they induce a more intense colitis. Naive CD4 T cells deficient in Stat6 and T-bet also induce a Th17-dominant colitis development in vivo. Our data provide new insights into the choice between Th1 and Th17 development and their roles in autoimmunity. The Journal of Immunology, 2008, 181: 8700 – 8710.

Regulation of IFN-γ production and Th1 development of CD4 T cells is mediated by IFN-γ/Stat1/T-bet and IL-12/Stat4 signal pathways (1–10). Recent studies have argued that T-bet may not have a direct role in positively regulating IFN-γ and Th1 differentiation, and its principal function in developing Th1 cells may be to prevent the Th2 transcription factor GATA-3 from inhibiting the IL-12/Stat4 signal pathway (10). In addition to T-bet, Eomesodermin (Eomes), a molecule that belongs to the same subfamily of T-box factors, has been shown to regulate IFN-γ expression in CD8 T cells (11–13). Eomes is expressed at very low levels in naive CD4 T cells, and it thus was suggested to be restricted in its activities to CD8 and NK cells (11, 12). While studies on Listeria monocytogenes infection suggested that Eomes might not play a role in generating IFN-γ producing Ag-specific CD4 T cells (14), another report showed that Eomes expression is up-regulated after TCR stimulation and was involved in IL-21-mediated IFN-γ regulation (15). The role of Eomes in CD4 effector development remains unclear.

Th1 cells are implicated in inflammatory responses and autoimmune disease, as mice deficient in Th1 transcription factors T-bet or Stat4 are severely impaired in their ability to produce IFN-γ and Th1 cells, and they are resistant to the development of experimental autoimmune encephalomyelitis or colitis (16–19). Since IFN-γ deficiency, IFN-γR deficiency, and Stat1 deficiency failed to confer resistance to autoimmunity, the role of Th1 responses in autoimmunity was questioned (18, 20, 21). Recent studies on IL-12 and IL-23, which share the common p40 subunit but differ in p35 and p19, respectively, demonstrated that autoimmune models previously attributed to IL-12 and Th1 cells are actually due to the effects of IL-23 and IL-17 producing helper T (Th17) cells (22).

Differentialization of Th17 cells was initially suggested to be driven by IL-23 (23, 24). Later studies demonstrated that the generation of Th17 cells requires the combination of TCR stimulation, IL-6 and TGFβ (25–29), and transcriptional factor RORγt activation (30), while IL-23 plays a role in promoting Th17 survival, expansion, and phenotype maintenance. Very recently, studies have shown that in addition to IL-17, IL-6 combined with TGFβ also drives IL-10 production in these cells to modulate their activities (31, 32). It is not understood how pathogenic Th17 cells develop and how IL-10 is regulated during TGFβ- and IL-6-driven Th17 lineage commitment.

T-bet has been shown to negatively regulate Th17 differentiation, and mice deficient in T-bet or Stat4 have decreased Th1 and increased numbers of Th17 cells (25, 26, 33). However, while mice lacking T-bet and Th1 development favor Th17 development, they are often resistant to autoimmune diseases (16–19), indicating that Th1 cells may still be involved in the pathogenesis of autoimmunity. It is not known if Th1 and Th17 play different roles, act at different stages, or coordinate with each other during autoimmune disease pathogenesis. Additionally, in many of these gene-deficient strains, unopposed actions of Th1 or Th2 cells and cytokines obscure the interpretation of the results.

Here, we introduced Stat6 deficiency into T-bet-deficient mice to limit interference from elevated levels of IL-4/Stat6/GATA3 Th2 polarizing signals, and to examine the signals important for Th1 and Th17 cell differentiation and their role in colitis development. Our data show that both T-bet and Eomes play direct roles in IFN-γ production and Th1 development, and they reveal a critical role for T-bet in the choice between Th1 and Th17 development. In the absence of T-bet, IFN-γ expression and Th1 responses are highly susceptible to suppression by IL-6 and TGFβ, and Th17 responses are strongly favored.
Materials and Methods

**Mice**

BALB/c, Stat6 knockout (BALB/c background), and T-bet knockout (BALB/c background) mice were purchased from The Jackson Laboratory. Stat6/T-bet double knockout (DKO) mice were bred and selected by PCR. CB17-SCID (BALB/c background) mice were purchased from Taconic.

**Cytokines, Abs, and other reagents**

Recombinant cytokines IL-2, IL-4, IL-6, IL-12, IFN-γ, PE-IL-17A, and PE-CD25 mAbs were purchased from BD PharMingen. Recombinant cytokines IL-21, TGFB, and anti-TGFβ Ab were purchased from R&D Systems. Recombinant mouse IL-23, anti-CD3, anti-CD28, FITC-anti-CD4, PE-anti-CD4, allophycocyanin-CD4, allophycocyanin-anti-CD8, FITC-anti-CD8 mAbs, PE-IFN-γ, FITC-IFN-γ, FITC-IL-10, anti-IL-6, and anti-IL-17A mAbs and monesin and intracellular staining kits were all purchased from eBioscience. CD4, CD8, and CD25 microbeads were purchased from Miltenyi Biotec. PMA and ionomycin were purchased from Sigma-Aldrich.

**T cell purification, activation, and retroviral infection**

Splenocytes were harvested from various strains of mice, and CD4+CD25− or CD8 T cells were positively selected by using CD4+CD25+ or CD8+ microbeads by following the manufacturer’s protocol. Cells were seeded into 24-well flat-bottom plates. For APC-dependent assay, 2 × 10^5 cells were cultured with irradiated T-depleted splenocytes (1 × 10^6), soluble anti-CD3 (1 μg/ml), mouse IL-2 (1 ng/ml), in combination with various cytokines. For the APC-independent assay, 5 × 10^5 cells were cultured with plate-bound anti-CD3 mAb (5 μg/ml) plus soluble anti-CD28 (1 μg/ml), along with various cytokines.

Bicistronic MigR1-T-bet or MigR1-Eomes retrovirus was produced by transfecting EcoPack 2-293 cells (BD Biosciences). After stimulating with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) mAbs in combination with IL-2 (1 ng/ml) for 24–30 h, Stat6/T-bet DKO CD4+CD25− T cells were infected with retroviral supernatant by spin infection. A second spin infection was performed on the following day. GFP+ or GFP− cells were purified by cell sorting 48 h after the first infection. Purified GFP+ and GFP− cells were reseeded in complete medium with IL-2 (1 ng/ml) for 2 days, washed, and restimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml), and Intracellular staining was performed at 48 h after stimulation.

**Intracellular staining and flow cytometry**

Cells were stimulated with PMA (100 ng/ml) plus ionomycin (500 ng/ml) in the presence of monesin (1/1000 dilution) for 4 h, and surface CD4 or CD8 was stained with allophycocyanin-anti-CD4 or allophycocyanin-anti-CD8, fixed, permeabilized, and stained with mouse PE anti-IL-17, coupled with FITC-anti-IFN-γ or FITC-anti-IL-10. Intracellular staining for mouse FoxP3 expression followed the manufacturer’s protocol (eBioscience). Analytical flow cytometry was performed with a BD FACS LSR II (BD Biosciences).

**Colitis induction**

Spleen CD4 T cells were enriched by a CD4-negative isolation kit (Invitrogen/Dynal) and stained with allophycocyanin-anti-CD4, PE-anti-CD25, and FITC-anti-CD45RB mAbs. CD4+CD25−CD45RBhi cells were sorted by MoFlo (Dako). Cells (5 × 10^5) were injected i.p. into each SCID mouse, and control mice were injected with an equal volume of PBS.

**Lamina propria lymphocyte isolation**

Colons were removed, washed in cold HBSS, dissected longitudinally, cut to ~3 cm long, and washed again in cold HBSS three times. Tissue pieces were incubated in 20 ml HBSS/EDTA (1 mM) in 37°C water bath with shaking for 30 min, further cut into 1-mm or smaller pieces, and placed into 10 ml digestion buffer (4% bovine serum, 0.5 mg/ml collagenase and DNase I, and 50 U/ml dispase) and incubated for 20 min in 37°C water bath with shaking. After a second incubation, mixtures were passed through a 40-μm cell strainer. Tissue pieces were collected and digested with fresh buffer three times. Supernatants were then combined and centrifuged in 5% BSA/PBS. Cells were collected and resuspended in 10 ml of 40% Percoll and layered on 5 ml 80% Percoll in a 15-ml tube. Gradient separation was performed by centrifuging at 2500 rpm for 30 min, and lamina propria lymphocytes were collected from layers between 40% and 80% Percoll.

**Histology and immunohistology**

Colons were harvested and fixed in the 10% formalin PBS (Fisher Scientific) and embedded in paraffin wax. H&E staining was performed following the manufacturer’s protocol. Normal goat serum, FITC-goat anti-rat IgG (H+L), and Cy3-goat anti-Armenian hamster IgG (H+L) were purchased from Jackson Immunoresearch Laboratories. DAPI (4′,6-diamidino-2-phenylindole) was purchased from Vector Laboratories. Rat anti-mouse CD4 and hamster anti-mouse CD11c were purchased from BD PharMingen. Fresh tissues were embedded in Tissue-Tek OCT compound from Sakura Finetek and stored in ~80°C. Tissue blocks were cut with a Leica CM1900 (Cryostar Industries) and stained with first and second Abs in the dark at room temperature. All slides were mounted with Vectashield (Vector Laboratories) to preserve fluorescence. Images were acquired with a Leica DM2500 fluorescence microscope with a digital Hamamatsu charge-coupled device camera. Images were collected and analyzed by Openlab software (Improvision).

**Real-time RT-PCR**

Total RNA was isolated using RNeasy Protect Mini kit (Qiagen), treated with DNase I (Invitrogen) to avoid DNA contamination, and cDNA was reverse transcribed using Omniscript RT (Qiagen) with random primers. The primers for PCR are cyclophilin A, AGG GTG GTG GTG ACT TTA CCA GCC AGT CAG CCA TCC AGC GAT ATC AGT CTA CCA GAA GAA; GATA-3, GAA GAA GCC ATC CAG ACC GAAC and ACC CAT GCC GGT GAC CAT GC; RORγT, ACC TCT TTT CAG GGT GAG A and TCC CAC ATC TCC CAC ATT G; IL-17A, CTC CAG AAG GCC CTC AGA CTA and AGG TTT CCC TCC GCA GTG ATC CTA CCA G and CAG CCA CTC AGG CTT ATC A; and IL-12Rβ2, TCC ATA GTT CGT GTT ACT GCC and TCA TCT TCC CAC CAG ATG GTA. Each reaction was performed with the LightCycler system (Roche) and SYBER Green PCR kit (Qiagen). All experiments were performed at least three separate times. Amplification of the cyclophilin A gene was used as a housekeeping gene.

**Results**

Stat6 deficiency enables T-bet-deficient CD4 T cells to produce IFN-γ

CD4+CD25− T cells from wild-type, T-bet KO, and Stat6 and T-bet DKO mice were purified and stimulated with anti-CD3 mAbs in the presence of T cell-depleted splenocytes as accessory cells (APC), plus low concentrations of IL-2, along with IL-12, IFN-γ, or IL-4. After 4 days of culture, intracellular staining was performed to measure IFN-γ and IL-4 expression. In agreement with a previous report (10), blocking the IL-4/Th2 pathway enabled T-bet-deficient CD4 T cells to respond to IL-12 for IFN-γ induction (Fig. 1A), confirming that IL-12 could induce T-bet independent of IFN-γ expression. Stat6 deficiency did not fully restore the IL-12-driven IFN-γ response, with 15.4% IFN-γ-expressing cells in DKO compared with 48.1% in wild-type cells (Fig. 1A). Similar results were observed with an APC-independent culture system in which CD4−CD25− T cells were stimulated with plate-bound anti-CD3 plus soluble anti-CD28 mAbs (data not shown). Dose-response analysis further confirmed that IL-12-induced, T-bet-independent, IFN-γ production was reduced (Fig. 1B). Exogenous IFN-γ-driven IFN-γ production is dependent on T-bet (Fig. 1A). There was very limited IL-4 expression in DKO CD4 T cells (Fig. 1, A and B), with or without exogenous IL-4, suggesting that decreased IFN-γ induction by IL-12 was not due to inhibition from IL-4/Th2 signals.

Real-time RT-PCR showed that there was no IL-12Rβ2 expression in T-bet-deficient CD4 T cells, while Stat6 deficiency restored IL-12Rβ2 expression, and IL-12Rβ2 expression was comparable in wild-type and DKO cells (Fig. 1C), indicating that reduced IFN-γ induction by IL-12 was not due to differential IL-12Rβ2 transcription. There was very little GATA-3 expressed in Stat6/T-bet DKO CD4 T
Fig. 1. Stat6 deficiency enables T-bet-deficient CD4 T cells to respond to IL-12 and TCR for IFN-γ production. A, Flow cytometry of intracellular IFN-γ and IL-4 expression in CD4^+CD25^- T cells from wild-type, T-bet-deficient, and Stat6/T-bet DKO mice stimulated with anti-CD3 (1 μg/ml), IL-2 (1 ng/ml), and T-depleted wild-type splenocytes with or without IL-12 (20 ng/ml), IFN-γ (20 ng/ml), or IL-4 (20 ng/ml) for 4 days. B, Dose response of IL-12-mediated IFN-γ production. CD4^+CD25^- T cells were stimulated with plate-bound anti-CD3 plus soluble anti-CD28, with various concentrations of IL-12 as indicated. C, IL-12Rβ2 and GATA-3 expression were measured by real-time RT-PCR in CD4^+CD25^- T cells from wild-type, T-bet-deficient, Stat6/T-bet DKO mice 2 days after anti-CD3 plus anti-CD28 mAb stimulation with or without IL-12 (20 ng/ml).
cells, and it was suppressed by IL-12 to similarly low levels in wild-type CD4 T cells (Fig. 1C), indicating that reduced IFN-γ induction by IL-12 was also not due to increased GATA-3 expression. TCR activation alone in APC-independent cultures that lack IL-12 induced low but significant IFN-γ (Fig. 1B). This indicated that TCR activation itself drives IFN-γ expression, independent of either T-bet or IL-12/Stat4. The finding that TCR stimulation and IL-12 both induced less IFN-γ in DKO compared with wild-type CD4 T cells suggests that although the requirement for T-bet may not be absolute, T-bet plays a major regulatory role for IFN-γ production independent of its effects on IL-12β2 or GATA-3.

**Eomes compensates for T-bet in IFN-γ expression in CD4 T cells**

Since TCR activation induced IFN-γ expression independent of both T-bet and IL-12/Stat4, this suggested another pathway for IFN-γ regulation. Eomes plays a complementary role for T-bet in IFN-γ regulation in CD8 T cells (11–13). We examined if Eomes also plays a role for CD4 IFN-γ regulation. Real-time RT-PCR for Eomes expression was performed on wild-type, T-bet-deficient, and Stat6/T-bet DKO CD4 T cells after 2 days of stimulation. As shown in Fig. 2A, there were much higher levels of Eomes expression in stimulated DKO CD4 T cells compared with wild-type or T-bet-deficient CD4 T cells, suggesting that similar to T-bet, Eomes was also inhibited by IL-4/Stat6/GATA-3. Examination of Eomes expression in naive CD4⁺CD25⁻ and CD8 T cells showed that its expression in CD4 T cells is very limited compared with CD8 T cells, and Stat6 and T-bet double deficiency did not affect expression (Fig. 2A), indicating that higher levels in stimulated CD4 T cells were not due to abnormal basal expression.

Expression levels of IFN-γ, Eomes, IL-12β2, and GATA-3 were compared after 24 and 48 h of activation. As shown in Fig. 2B, TCR and IL-12 induced rapid Eomes and IFN-γ expression by 24 h of activation, significant IL-12β2 expression was observed 48 h after stimulation, and there was no difference in GATA-3 expression between 24 and 48 h in DKO CD4 T cells. This suggested that Eomes, but not IL-12β2 or GATA-3, played a role in the early IFN-γ response. Bicistronic GFP retroviral vectors expressing Eomes or T-bet were used to transduce Stat6/T-bet DKO CD4 T cells to determine whether T-bet or Eomes directly regulated IFN-γ. GFP⁺ and GFP⁻ cells were purified by flow cytometric sorting. Real-time RT-PCR confirmed that retroviral infection resulted in a 100-fold increase in T-bet or Eomes expression (data not shown). After resting for 2 days, cells were restimulated with anti-CD3 plus anti-CD28 mAbs for another 2 days. Intracellular staining showed that both GFP-Eomes⁺ and GFP⁻ cells had marked IFN-γ production (Fig. 2C). These data indicated that both T-bet and Eomes directly up-regulated IFN-γ expression, and the failure to observe Eomes for CD4 IFN-γ responses in T-bet-deficient cells was likely due to its repression by elevated IL-4/Stat6/GATA-3 Th2 signals.

**FIGURE 2.** Eomes compensates T-bet for IFN-γ expression. A, Real-time RT-PCR for Eomes expression in CD4⁺CD25⁻ stimulated with anti-CD3 plus anti-CD28 mAbs for 2 days, and naive CD4⁺CD25⁻ and CD8 T cells. B, Real-time RT-PCR for Eomes, IFN-γ, GATA-3, and IL-12β2 expression in Stat6/T-bet DKO CD4 T cells 24 or 48 h after anti-CD3 plus anti-CD28 mAb stimulation with or without IL-12 (20 ng/ml). C, Intracellular staining for IFN-γ expression in Stat6/T-bet DKO CD4 T cells infected with T-bet-GFP or Eomes-GFP retrovirus. After sorting, GFP⁺ and GFP⁻ cells were rested for 2 days and restimulated with anti-CD3 plus anti-CD28 mAbs for an additional 2 days.
T-bet-independent IFN-γ and Th1 development are very susceptible to suppression by IL-6 and TGFβ}

Th17 development is driven by the combination of IL-6 plus TGFβ signals. We examined if Stat6/T-bet DKO T cells respond differently to Th17 polarizing signals. CD4^+CD25^- wild-type and DKO cells were cultured in APC-dependent or -independent conditions, with combinations of IL-6, TGFβ, or IL-12. As shown in Fig. 3A, IFN-γ expression is more susceptible to inhibition by IL-6 and TGFβ in DKO compared with wild-type cells. IL-6 or TGFβ alone completely abolished IFN-γ production, and exogenous

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**TABLE 1.** Summary of results

<table>
<thead>
<tr>
<th>Condition</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-17 (pg/ml)</th>
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<td>1.2</td>
</tr>
<tr>
<td>DKO</td>
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<td>0.6</td>
</tr>
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<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>+ TGFβ</td>
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<tr>
<td>+ IL-6 + TGFβ</td>
<td>0.01</td>
<td>0.001</td>
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</table>

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**FIGURE 3.** IL-6 and TGFβ signals induce higher levels of IL-17 expression in vitro. Intracellular staining for IFN-γ and IL-17 in wild-type or Stat6/T-bet DKO CD4^+CD25^- T cells stimulated with (A) anti-CD3 (1 μg/ml), IL-2 (1 ng/ml), and T-depleted wild-type splenocytes with or without various cytokines as indicated for 3 days. B: Eomes expression was measured by real-time RT-PCR in CD4^+CD25^- cells 2 days after anti-CD3 plus anti-CD28 mAb stimulation with or without IL-6, TGFβ, or IL-6 plus TGFβ. C: Flow cytometry of intracellular IFN-γ and IL-17 expression in CD8 T cells stimulated with coated anti-CD3 (5 μg/ml) plus soluble anti-CD28 (1 μg/ml) mAbs, with indicated cytokines for 3 days. D: Real-time RT-PCR for Eomes expression in wild-type and Stat6/T-bet DKO CD8 T cells 48 h after stimulation. E: Intracellular staining for IL-17 and IFN-γ expression in Stat6/T-bet DKO CD4 T cells infected with Eomes-GFP or GFP retrovirus. After sorting, GFP^+ cells were rested for 2 days and restimulated with anti-CD3 plus anti-CD28 mAbs in combination with IL-6 plus TGFβ for an additional 2 days.
IL-12 failed to restore IFN-γ production. IL-6 plus TGFβ also induced much higher levels of IL-17 in DKO than in wild-type cells. IL-12 only partially inhibited IL-17 expression. These data indicated that without T-bet, Th1 differentiation was susceptible to suppression from Th17 polarizing signals, and Th17 development was strongly favored. Since Eomes compensated for T-bet for IFN-γ transcription (Fig. 2), we examined if susceptibility to suppression of IFN-γ production was related to Eomes inhibition. IL-6 and TGFβ both inhibited Eomes expression in both DKO and wild-type cells (Fig. 3B). In CD8 T cells, which express higher levels of Eomes, IL-6 or IL-6 combined with TGFβ inhibited IFN-γ, induced higher levels of IL-17, and inhibited Eomes to a greater degree in DKO compared with wild-type T cells (Fig. 3, C and D). Forced Eomes expression in DKO CD4 T cells suppresses IL-17 expression while it prevents IL-6- and TGFβ-mediated IFN-γ inhibition (Fig. 3E). Taken together, these data indicated

**FIGURE 4.** IL-6 induces IL-17 expression. A, Real-time RT-PCR for IL-17A, IL-17F, RORγt, and TGFβ expression in wild-type and Stat6/T-bet DKO CD4+CD25− T cells 48 h after anti-CD3 plus anti-CD28 mAbs stimulation with or without IL-6. B, Flow cytometry of FoxP3 expression in wild-type or DKO CD4+CD25− T cells stimulated with coated anti-CD3 (5 μg/ml) plus soluble anti-CD28 (1 μg/ml) mAbs with or without IL-6 for 3 days. C, Flow cytometry of intracellular IL-17 and IFN-γ expression in Stat6/T-bet DKO CD4+CD25− T cells stimulated with plate-bound anti-CD3 (5 μg/ml) plus soluble anti-CD28 (1 μg/ml) mAbs, IL-6, and graded doses of anti-TGFβ Abs for 3 days. D, Real-time RT-PCR for IL-17A and IL-17F expression of Stat6/T-bet DKO CD4+CD25− T cells stimulated with plate-bound anti-CD3 (5 μg/ml) plus soluble anti-CD28 (1 μg/ml) mAbs, IL-6, and graded doses of anti-TGFβ Abs for 48 h.
that both T-bet and Eomes protected Th1 from Th17 polarization, and inhibition of Eomes by Th17 polarizing signals leads to preferential Th17 development in the absence of T-bet.

Stat6 and T-bet double deficiencies enable IL-6 to initiate Th17 cell differentiation

IL-6 treatment induced IL-17 production in DKO CD4 and CD8 T cells (Fig. 3, A, B, and D). Real-time RT-PCR showed that IL-6 induced IL-17A and IL-17F transcripts, as well as RORγT (Fig. 4A). IL-17 polarization is dependent on TGFβ (25–30); however, IL-6 alone induced IL-17 expression in APC-independent conditions, and there was no difference in TGFβ expression in wild-type compared with DKO CD4 T cells (Fig. 4A). FoxP3-expressing regulatory CD4 T cells are suggested to be a source for TGFβ in Th17 and regulatory T cell differentiation (34); however, intracellular staining showed no FoxP3-expressing cells in either wild-type or DKO CD4^CD25^ T cells after IL-6 stimulation (Fig. 4B). To further investigate if
IL-6 mediated IL-17 expression, neutralizing anti-TGFβ Abs were added to cultures. As shown in Fig. 4C, 0.05 μg/ml anti-TGFβ significantly inhibited IL-6-driven IL-17 expression, but there was still low but consistent IL-17 expression even in the presence of the highest concentrations of anti-TGFβ Abs (5 μg/ml). Real-time RT-PCR also showed that at a dose as low as 0.05 μg/ml, anti-TGFβ reduced expression of IL-17A and IL-17F, yet the highest concentrations of anti-TGFβ Ab did not fully extinguish IL-17 expression (Fig. 4D). Collectively, these data indicated that CD4 T cells deficient in both Stat6 and T-bet are much more sensitive to IL-6 for Th17 development.

**IL-23 promotes IL-6-driven Th17 differentiation**

IL-23 promotes Th17 survival, expansion, and phenotype maintenance (12, 23, 24). IL-23 induced very little IL-17 expression, indicating that Stat6 and T-bet double deficiencies do not autoinitiate Th17 development (Fig. 5A). When combined with IL-6, IL-23 significantly enhanced IL-6-driven IL-17 expression, even in the presence of high concentrations of anti-TGFβ neutralizing Abs. IL-21 negatively regulates IFN-γ expression through Eomes inhibition (15), and IL-6 alone inhibited Eomes (Fig. 3, B and D). We investigated if Eomes inhibition by IL-21 may lead to IL-17 induction. IL-21 did inhibit Eomes expression, but it induced far less IL-17 compared with IL-6 in DKO CD4 T cells (Fig. 5, B and C). When IL-21 was combined with IL-23, significant IL-17 expression was observed, further indicating that Eomes inhibition played a role in Th17 differentiation. IL-21 did not potentiate IL-6- or IL-6 plus IL-23-driven IL-17 induction. This suggested that IL-6 and IL-21 may use the same pathways to regulate IL-17, likely through Stat3 induction and Eomes inhibition.

**IL-6 drives pathogenic Th17 cell generation**

In addition to IL-17, recent studies demonstrated that IL-6 combined with TGFβ also induces IL-10, and IL-10 in turn restrains the pathologic effects of IL-17 (31, 32). To determine whether IL-6-driven Th17 cells also expressed IL-10, cells were stimulated with IL-6, TGFβ, and/or IL-23 (Fig. 6A). Only IL-6 plus TGFβ induced both IL-17 and IL-10, while IL-6 alone or IL-6 plus IL-23 induced only IL-17 without IL-10. In the SCID adoptive transfer colitis model, IL-6-cultured Th17 induced more severe colitis than did IL-6 plus TGFβ-cultured Th17 cells. Immunohistochemistry for CD4 and CD11c showed many more infiltrating cells in colons of mice receiving IL-6-cultured Th17 (not shown), and real-time RT-PCR showed significantly more IL-17 expression (Fig. 6B).

**Stat6 and T-bet DKO CD4 T cells induce Th17-dominant colitis development**

To determine whether Th17-dominant responses by DKO CD4 T cells occurred in vivo, wild-type, T-bet-deficient, and Stat6/T-bet DKO CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>high</sup> T cells were purified and transferred into SCID mice, and body weight and the development of colitis were monitored. Recipients of wild-type CD4 T cells had weight loss and development of colitis within 8 wk, whereas T-bet deficiency abolished colitis development, as previously reported (17). Transfer of DKO cells also led to weight loss and colitis. Colitis induced by transfer of wild-type or DKO CD4 T cells was similar in degree (Fig. 7, A and B). Immunohistochemistry for CD4 and CD11c showed similar patterns and numbers of CD4 and DC infiltration (Fig. 7C). These results suggested that relieving IL-4/Stat6 Th2 signals in T-bet-deficient CD4 T cells enabled them to be pathologically active. To determine whether colitis induced by Stat6/T-bet DKO CD4 T cells was due to Th1 or Th17 cell generation, lamina propria lymphocytes were purified and IFN-γ and IL-17 expression examined by real-time RT-PCR. As shown in Fig. 7D, there were striking differences in IFN-γ and IL-17 expression. Stat6/T-bet DKO colitis T cells expressed little IFN-γ but much more IL-17 than did wild-type colitogenic T cells, indicating that Th17 development also occurred in vivo. There was also no IL-10 expression, suggesting the induction of pathogenic IL-10<sup>+</sup>Th17. Administration of anti-IL-17 neutralizing mAbs at days 0 and 7 significantly attenuated Stat6/T-bet DKO cell-induced colitis, but not wild-type CD4 T cell-induced colitis (Fig. 7E). Similarly, administration of anti-IL-6 mAbs on days 0, 7, and 14 after adoptive transfer completely prevented colitis (Fig. 7F). Thus, there is an in vivo process permitting the development of IL-6-dependent colitogenic Th17 without IL-10 production.
Discussion
Although there are questions of whether T-bet directly regulates IFN-γ and Th1 differentiation (10), its essential role in Th1 and Th2 developmental paradigms has been well established (1–3). Herein, we provide evidence that T-bet plays both indirect and direct roles in IFN-γ regulation and Th1 development. It prevents the Th2 transcription factor GATA-3 from suppressing IL-12Rβ2 expression to maintain the IL-12/Stat4 signaling pathway for IFN-γ regulation. T-bet also directly regulates IFN-γ production and Th1 development, independent of its effects on GATA-3 or IL-12/Stat4. Without T-bet, both TCR- and IL-12-mediated IFN-γ production are reduced, and retroviral T-bet induces significant IFN-γ expression in Stat6/T-bet DKO CD4 T cells.

In addition to IFN-γ/Stat1/T-bet and IL-12/Stat4, we uncovered a significant role for TCR/Eomes as a third pathway to regulate CD4 IFN-γ production and Th1 development. Due to very low levels of expression in naive CD4 T cells, the role of Eomes for IFN-γ production has mostly been examined in CD8 T cells (11–13). High levels of Eomes expression were observed in Stat6/T-bet DKO, but not in T-bet deficient CD4 T cells (Fig. 2), indicating that similar to T-bet, Eomes is also inhibited by IL-4/Stat6/GATA-3 signals. CD8 T cells strongly favor IFN-γ production and Tc1 development (35), and even T-bet deficiency does not result in comparable IL-4/Stat6/GATA-3 signals in CD8 T cells compared with wild-type CD4 T cells (our unpublished data). Therefore, while the role of Eomes in CD8 T cells can be easily identified, its role in CD4 T cells is masked by the Th2 pathway, especially when T-bet is deficient. By using CD4 T cells deficient in both Stat6 and T-bet, we were able to demonstrate that Eomes also plays an important regulatory role for IFN-γ production and Th1 development in CD4 T cells.

FIGURE 7. Stat6/T-bet DKO CD4 T cells induce Th17-dominant colitis in vivo. A, Gross anatomy changes in colon. Similarly thickened and shortened colons from SCID mice 8 wk after injecting wild-type or Stat6/T-bet DKO CD4+CD25−CD45RBhigh T cells (n > 16). B, Representative histology of colons from wild-type or Stat6/T-bet DKO CD4 T cells (n > 16). C, Representative immunohistochemistry for CD4 (Red) and CD11c (Green) of colitic colons from wild-type or Stat6/T-bet DKO CD4 T cell-reconstituted mice (n > 16). D, Real-time RT-PCR for IL-17A, IL-17F, and IFN-γ expression in lamina propria lymphocytes isolated from colitic mice. E, Histology of colons from SCID mice transferred with wild-type or Stat6/T-bet DKO CD4+CD25−CD45RBhigh cells, and anti-IL-17 mAbs (250 μg/each) at days 0, 7, and 14 (n > 10). F, Histology of colons from SCID mice 8 wk after injection of Stat6/T-bet DKO CD4+CD25−CD45RBhigh T cells with or with anti-IL-6 Ab (250 μg/each) at days 0, 7, and 14 (n > 10).
Ectopic T-bet expression promotes IFN-γ secretion and decreases IL-17 production (33), while mice deficient in T-bet have increased numbers of Th17 cells (25, 26). Herein, we showed that in the absence of T-bet, IFN-γ expression and Th1 development were very susceptible to suppression by Th17 polarizing signals, and CD4 T cells strongly favored Th17 differentiation, demonstrating a critical role for T-bet in Th1 and Th17 selection. Increased IL-17 production corresponded to decreased IFN-γ production (Fig. 3A), suggesting that T-bet may suppress Th17 development through an IFN-γ-dependent pathway. However, in T-bet-deficient and DKO CD8 T cells, there were high levels of both IFN-γ and IL-17, indicating that T-bet also negatively regulates IL-17 expression through an IFN-γ-independent pathway. Higher levels of RORγT expression were observed in DKO CD4 T cells (Fig. 4A), and TCR induces higher levels of RORγT expression in T-bet-deficient or DKO than in wild-type CD8 T cells (data not shown), suggesting that T-bet may regulate IL-17 expression in T-bet-deficient or DKO T cells, thereby regulating both Th1 and Th17. Th1 cytokines and cells limit IL-10 expression, permitting Th17 cells to induce autoimmune disease development.

Disclosures
The authors have no financial conflicts of interest.

References


