

# T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on *IFNG* gene acetylation and transcription

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T helper type 1 (Th1) development is facilitated by interrelated changes in key intracellular factors, particularly signal transducer and activator of transcription (STAT)4, T-bet, and GATA-3. Here we show that CD4<sup>+</sup> cells from T-bet<sup>-/-</sup> mice are skewed toward Th2 differentiation by high endogenous GATA-3 levels but exhibit virtually normal Th1 differentiation provided that GATA-3 levels are regulated at an early stage by anti-interleukin (IL)-4 blockade of IL-4 receptor (R) signaling. In addition, under these conditions, Th1 cells from T-bet<sup>-/-</sup> mice manifest *IFNG* promoter accessibility as detected by histone acetylation and deoxyribonuclease I hypersensitivity. In related studies, we show that the negative effect of GATA-3 on Th1 differentiation in T-bet<sup>-/-</sup> cells arises from its ability to suppress STAT4 levels, because if this is prevented by a STAT4-expressing retrovirus, normal Th1 differentiation is observed. Finally, we show that retroviral T-bet expression in developing and established Th2 cells leads to down-regulation of GATA-3 levels. These findings lead to a model of T cell differentiation that holds that naive T cells tend toward Th2 differentiation through induction of GATA-3 and subsequent down-regulation of STAT4/IL-12Rβ2 chain unless GATA-3 levels or function is regulated by T-bet. Thus, the principal function of T-bet in developing Th1 cells is to negatively regulate GATA-3 rather than to positively regulate the *IFNG* gene.

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Abbreviations used: ChIP, chromatin immunoprecipitation; MFI, mean fluorescence intensity.

Over the past several years, the molecular mechanisms governing Th1 differentiation have led to the identification of two major Th1-related signaling pathways, one involving IL-12/STAT4 (1–11) and the other involving IFN-γ/STAT1/T-bet (8–11). Of these, the latter or T-bet pathway has been assumed to be the most important because T-bet-deficient mice (T-bet<sup>-/-</sup> mice) cannot mount Th1 responses, and retroviral expression of T-bet in developing and/or developed Th2 cells not only induces IFN-γ production, but also suppresses IL-4 and IL-5 production (10, 11). Moreover, such effects of T-bet have been argued to be at least partially independent of STAT4 because under certain stimulation conditions retroviral expression of T-bet in developing Th1 cells derived from STAT4-deficient mice has been shown to support IFN-γ synthesis (9, 12). Finally, evidence has been adduced with the use of in vitro reporter assays

and retroviral constructs expressing T-bet or a dominant-negative form of T-bet that this transcription factor is essential for early acquisition of accessibility at the *IFNG* promoter (9, 13). Thus, a symmetrical model has been put forth in which T-bet and GATA-3 function early in Th development to regulate *IFNG* and *IL-4/IL-5* gene expression, respectively. The cytokines *IL-12* and *IL-4/IL-5* function secondarily, acting via STATs to promote cell growth and extinguish expression of either GATA-3 or T-bet (14).

However, recent data argue against this model. First, Afkarian et al. (12) reported that retroviral T-bet expression in Th2 cells induced only low amounts of IFN-γ, failed to suppress Th2 cytokines when the cells were stimulated under more “physiological conditions” (antigen plus APCs), and induced only relatively small amounts of IFN-γ when the cells were stimulated with pharmacologic agents (PMA/ionomycin). Second, we and Afkarian et al. reconfirmed that STAT4<sup>-/-</sup> Th cells mount a defective Th1

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response when they are stimulated by Con A/APCs or antigen (OVA)/APCs (12, 15). Finally, we recently reported that developing Th2 cells from T-bet<sup>-/-</sup> mice could differentiate into Th1 cells when STAT4 and IL-12Rβ2 chain expression are maintained (16). These data supporting the importance of STAT4 signaling in Th1 differentiation are thus in general agreement with our previous studies showing that GATA-3 suppresses Th1 development through down-regulation of STAT4 rather than through inhibition of T-bet or the IL-12Rβ2 chain, and that the maintenance of STAT4 expression overcomes the effect of GATA-3 in developing Th2 cells.

Based on the uncertainties described above concerning Th differentiation, we conducted studies of cells from T-bet<sup>-/-</sup> mice to better define the relation of T-bet to GATA-3, STAT4, and other key factors. These studies show that an essential, nonredundant function of T-bet in developing Th1 cells is to antagonize GATA-3 expression and/or function that would otherwise abort Th1 differentiation, and that T-bet does not have an early, obligate role in chromatin remodeling and/or transcription of the *IFNG* gene.

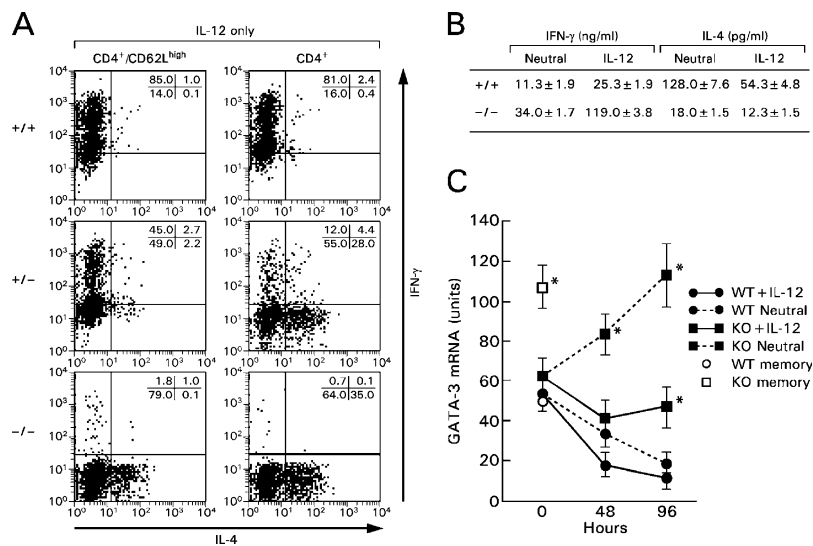
**RESULTS**

**Elevated IL-4 and GATA-3 in committed Th2 cells of T-bet<sup>-/-</sup> mice**

In initial studies, we compared the ability of whole CD4<sup>+</sup> T cells and naive (CD4/CD62L<sup>high</sup>) T cells from T-bet<sup>+/-</sup>, T-bet<sup>-/-</sup>, and wild-type mice to undergo Th1/Th2 dif-

ferentiation in vitro under the Th1 conditions ordinarily encountered in vivo (IL-12 only and no anti-IL-4 antibody). As shown in Fig. 1 A, the percentages of Th1 cells were high and Th2 cells were low in cell cultures of both whole CD4<sup>+</sup> T cells and naive T cells of wild-type mice, whereas comparable T cell populations from T-bet<sup>-/-</sup> mice exhibited marked Th2 differentiation, particularly when whole CD4<sup>+</sup> T cells were studied. These data suggest that many CD4<sup>+</sup> T cells in T-bet<sup>-/-</sup> as well as in T-bet<sup>+/-</sup> mice are precommitted to Th2 cell differentiation in vivo.

We next determined the capacity of naive CD4<sup>+</sup> T cells from wild-type and T-bet<sup>-/-</sup> mice to produce IFN-γ and IL-4 after primary stimulation. As shown in Fig. 1 B, we found that naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> mice secreted seven and four and one half times more IL-4 than similar cells from wild-type mice when cultured under neutral and sub-optimal Th1 conditions (defined here and below as IL-12 in the absence of anti-IL-4 antibody), respectively. In addition, as shown also in Fig. 1 B, although T-bet<sup>+/-</sup> cells produced considerably more IFN-γ than T-bet<sup>-/-</sup> cells, particularly in the presence of IL-12 signaling, T-bet<sup>-/-</sup> cells were nonetheless able to produce substantial amounts of this cytokine. One interpretation of these data are that naive T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells can be characterized as cells with low responsiveness to IL-12 rather than as cells that are intrinsically unable to produce IFN-γ.



**Figure 1. Elevated IL-4 and GATA-3 and committed Th2 cells in T-bet<sup>-/-</sup> mice.** (A) Naive CD4<sup>+</sup> T cells and whole CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup>, T-bet<sup>+/-</sup>, and wild-type mice were stimulated with anti-CD3/anti-CD28 and maintained under Th1 conditions (IL-12 only) and expanded with IL-2. On day 6, the cells were subjected to intracellular cytokine staining after restimulation with PMA/ionomycin. These data are representative of results obtained from three independent experiments. (B) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with anti-CD3/anti-CD28 under Th1 conditions (IL-12 only) or neutral (no cytokines or antibodies added) conditions for 48 h, and then culture supernatants were harvested

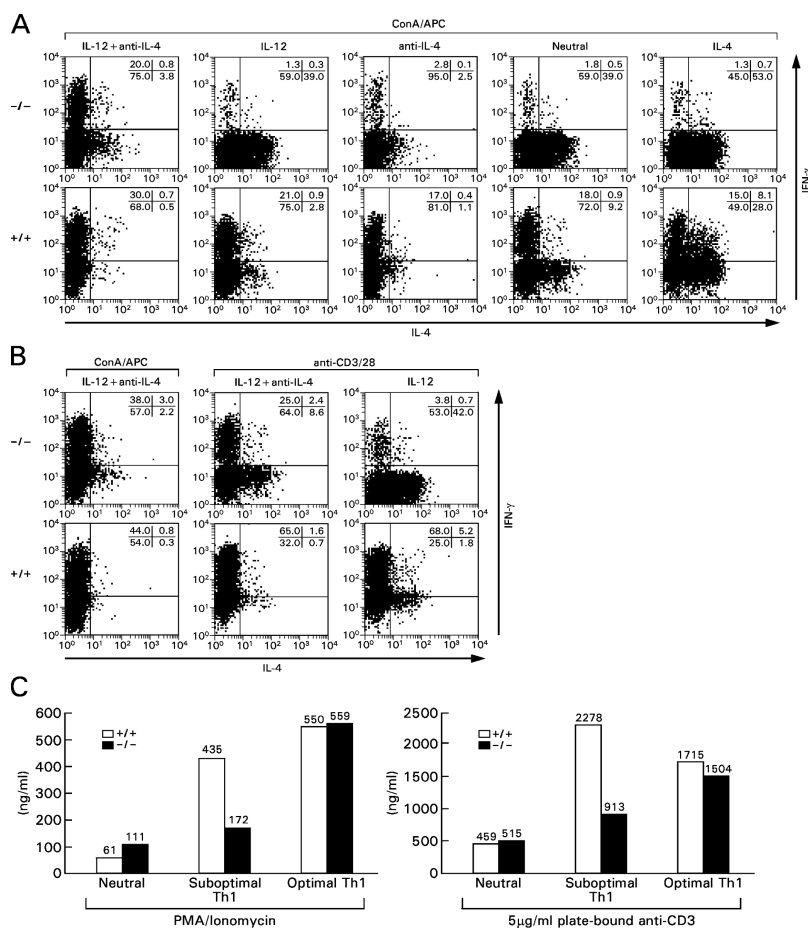
for IFN-γ and IL-4 determination by ELISA. The data shown are averages ± SE derived from three independent experiments. (C) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with anti-CD3/anti-CD28 under Th1 conditions (IL-12 only) or neutral conditions and maintained for the time periods shown in the figure. At each time point, cells were harvested and total RNAs were isolated and subjected to real-time PCR analysis for GATA-3 mRNA quantification. Independently, total RNAs from freshly isolated naive and memory CD4<sup>+</sup> T cells were prepared for day 9 samples. The data shown are averages ± SE derived from three independent experiments.

In related studies, we measured the kinetics of GATA-3 mRNA production in CD4<sup>+</sup> T cells stimulated with anti-CD3/anti-CD28 under neutral or suboptimal Th1 conditions as defined above. As shown in Fig. 1 C, T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells initially displayed a slightly increased level of GATA-3 mRNA level (measured by real-time RT-PCR) as compared with wild-type cells; however, under either neutral or suboptimal Th1 conditions, GATA-3 mRNA levels decreased over time in wild-type cells, whereas these levels steadily increased under neutral conditions and only slightly declined under suboptimal Th1 conditions in T-bet<sup>-/-</sup> cells. Furthermore, T-bet<sup>-/-</sup> effector-memory CD4<sup>+</sup> T cells (cells stimulated under neutral conditions and then studied after 9 d of culture) displayed markedly elevated GATA-3 mRNA levels as compared with wild-type cells.

These data indicate that naive T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells under neutral or suboptimal Th1 conditions do not suppress GATA-3 as do wild-type cells.

### T-bet-independent Th1 development and IL-12Rβ2 chain expression

The data described above suggest that the defective Th1 response in T-bet<sup>-/-</sup> mice would be due to a constitutive increase of GATA-3 levels. Because a major regulator of GATA-3 is IL-4, we next considered whether the defective Th1 response in T-bet<sup>-/-</sup> mice could be altered by neutralization of endogenous IL-4 by anti-IL-4 antibody; i.e., when cells are stimulated under optimal Th1 conditions (defined here and below as IL-12 plus anti-IL-4 antibody). To test this possibility, naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type



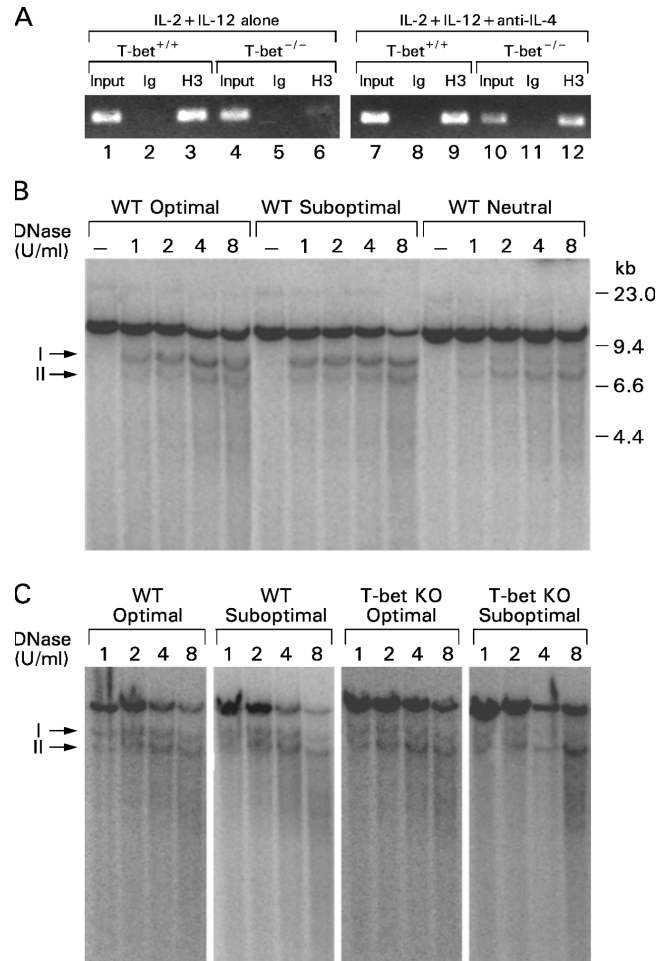
**Figure 2. T-bet-independent Th1 development and IL-12Rβ2 chain expression.** (A) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice (purified by flow cytometric sorting) were stimulated with Con A/APC (30 Gy-irradiated splenocytes from wild-type mice) under optimal Th1 conditions (IL-12 + anti-IL-4), suboptimal Th1 conditions (IL-12 only or anti-IL-4 only), neutral conditions, or suboptimal Th2 (IL-4 only) conditions. The cells were then expanded with IL-2 as well as cytokines and antibodies on days 2 and 4. On day 8, the cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IL-4 and IFN-γ. See Materials and methods for further details. (B) Experimental design was the same as in A, except that

cells subject to two kinds of stimulation (Con A/APC and anti-CD3/anti-CD28) were compared. These data are representative of results obtained from three independent experiments. (C) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice (purified by flow cytometric sorting) were stimulated with anti-CD3/anti-CD28 in the presence of IL-12 and 20 μg/ml anti-IL-4 (for optimal Th1 conditions) or with 5 ng/ml IL-12 (for suboptimal Th1 conditions). The cells were expanded by the addition of IL-2 on day 4 of culture and then washed and restimulated with PMA/ionomycin or plate-bound (pb) anti-CD3 on day 6, and culture supernatants were collected after 6 and 24 h, respectively, for assay of IFN-γ by ELISA (See Materials and methods).

mice were isolated as described in Materials and methods and stimulated with Con A plus APCs under optimal or suboptimal Th1 conditions, as well as under neutral or Th2 conditions (the latter consisting of cultures containing IL-4 only). On day 4, surface expression of IL-12R $\beta$ 2 chain was measured by flow cytometry, and on day 7, cytokine production was measured by intracellular cytokine staining. As shown in Fig. 2 (A and B), the percentage of cells displaying IFN- $\gamma$  production obtained in cultures of naive T-bet $^{-/-}$  CD4 $^{+}$  cells stimulated under optimal Th1 conditions was quite comparable to that seen in wild-type cells, whereas, under suboptimal Th1 conditions, very few Th1 cells appeared. Similarly, as shown in Fig. 2 B, comparable results were obtained in cell cultures in which plate-bound anti-CD3/soluble anti-CD28 was the initial stimulus. Furthermore, as shown in Fig. 2 C, secretion of IFN- $\gamma$  into the culture supernatant by naive T-bet $^{-/-}$  CD4 $^{+}$  T cells (in this case, CD44 $^{lo}$ /CD62L $^{hi}$  cells obtained by cell sorting) was equivalent to that of wild-type cells under optimal Th1 conditions but not under suboptimal conditions. In this case, IFN- $\gamma$  secretion was measured after initial stimulation of cells by anti-CD3/soluble anti-CD28 for 6 d followed by restimulation with either plate-bound anti-CD3/soluble anti-CD28 for 48 h or PMA and ionomycin for 24 h. These studies indicate that T-bet $^{-/-}$  cells and wild-type cells stimulated under optimum Th1 conditions are equivalent with respect to IFN- $\gamma$  production when evaluated either by the number of cells expressing IFN- $\gamma$  or by the secretion of IFN- $\gamma$  over a period of time. Finally, as shown in Fig. S1 (available at <http://www.jem.org/cgi/content/full/jem.20052165/DC1>), although T-bet $^{-/-}$  cells cultured under optimal conditions expressed virtually normal levels of IL-12R $\beta$ 2, those cultured under suboptimal Th1, neutral, or Th2 conditions expressed low levels of IL-12R $\beta$ 2. Overall, the results described above lead to the conclusion that naive T-bet $^{-/-}$  cells differentiate into Th1 cells and express IL-12R $\beta$ 2 chain quite normally under optimal Th1 conditions in which GATA-3 induction is limited by blockade of IL-4 activity.

#### Accessibility of the *IFNG* promoter in T-bet $^{-/-}$ cells

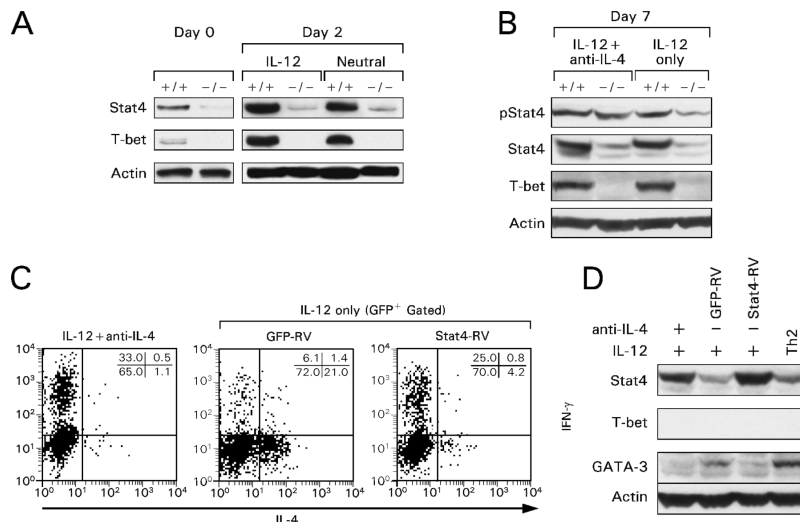
Previous studies based on the use of T-bet-expressing retroviruses have led to the view that T-bet is an obligate regulator of *IFNG* promoter remodeling (9, 13, 14). In light of the results obtained above showing that T-bet $^{-/-}$  cells can produce IFN- $\gamma$  provided that GATA-3 levels are controlled by blockade of IL-4R/STAT6 signaling with anti-IL-4, we next reexamined the relation of T-bet to *IFNG* promoter remodeling (accessibility) by assessing *IFNG* promoter histone acetylation using the chromatin immunoprecipitation (ChIP) assay. In these studies, naive spleen cells were obtained from T-bet $^{-/-}$  and wild-type mice, stimulated with anti-CD3/anti-CD28 under suboptimal (IL-12 in the absence of anti-IL-4) or optimal (IL-12 in the presence of anti-IL-4) Th1 conditions for 3 d, and then processed for ChIP assay (see Materials and methods). As shown in Fig. 3 A, T-bet $^{-/-}$  cells stimulated under suboptimal Th1 conditions and, as



**Figure 3.** T-bet $^{-/-}$  cells cultured under optimal Th1 conditions (in the presence of anti-IL-4) display normal *IFNG* promoter accessibility (histone acetylation). (A) Naive CD4 $^{+}$  T cells from T-bet $^{+/+}$  and T-bet $^{-/-}$  mice were stimulated with anti-CD3/anti-CD28 under suboptimal (IL-12) or optimal (IL-12 plus anti-IL-4) Th1 conditions. After 3 d, the cells were subjected to ChIP assay as described in Materials and methods. Ig, isotype control; H3,  $\alpha$ -acetyl-histone 3. (B) DNase I hypersensitivity pattern of the *IFNG* locus of wild-type naive T cells stimulated with anti-CD3/anti-CD28 and cultured under optimal, suboptimal, or neutral conditions for 6 d. Hypersensitivity sites I and II are indicated by arrows. (C) Comparison of DNase I hypersensitivity patterns of the *IFNG* locus in wild-type and T-bet $^{-/-}$  naive CD4 $^{+}$  T cells cultured under optimal or optimal conditions for 6 d. Hypersensitivity sites I and II are indicated by arrows.

shown above, exhibiting little IFN- $\gamma$  production displayed little or no histone acetylation at the H3 site, whereas T-bet $^{+/+}$  cells displayed a strong acetylation signal. In contrast, T-bet $^{-/-}$  cells stimulated under optimal Th1 conditions and exhibiting virtually normal IFN- $\gamma$  production also displayed a strong acetylation signal.

We confirmed these results by analyzing DNase hypersensitivity of the *IFNG* locus. As shown in Fig. 3 B, the locus is readily accessible to digestion when wild-type cells are incubated under optimal or suboptimal conditions, but not under neutral conditions. In contrast, the *IFNG* locus is



**Figure 4. STAT4 signaling is impaired in T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells.** (A) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with anti-CD3/anti-CD28 under either suboptimal Th1 conditions (IL-12 only) or neutral conditions. After maintenance for the time periods shown in the figure, whole cell lysates were subjected to Western blot analysis. Independently, lysates from freshly isolated naive CD4<sup>+</sup> T cells were prepared for day 0 samples. Similar results were obtained in three other independent experiments. (B) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with anti-CD3/anti-CD28 under either optimal Th1 conditions (IL-12 + anti-IL-4) or suboptimal Th1 conditions (IL-12 only), and then expanded with IL-12 as well as cytokines and antibodies on days 2 and 4. On day 7, the cells were restimulated with fresh IL-12 for 30 min and lysed

for Western blot analysis. Recombinant GATA-3 protein was prepared from GATA-3-expressing retrovirus-transfected Phoenix-Eco packaging cell line. Similar results were obtained in three other independent experiments. (C) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> mice were stimulated and maintained as described in B. On day 2, the cells were infected with either control retrovirus (GFP-RV) or STAT4-expressing retrovirus (STAT4-RV). On day 7, the cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IL-4 and IFN- $\gamma$ . (D) The cells used in C were sorted by a flow cytometer according to GFP expression on day 6 and then lysed for Western blot analysis. The lysate of the Th2 line from T-bet<sup>-/-</sup> mice was also prepared as a positive control for GATA-3 protein. These data are representative of those obtained in three independent experiments.

sensitive to digestion when T-bet-deficient cells are cultured under optimal conditions but is less evident under suboptimal conditions. Collectively, the ChIP assay and the DNase I analysis provide strong evidence that *IFNG* promoter accessibility can proceed in the absence of T-bet under optimal Th1 conditions. As such, these data are consistent with the data reported above relating to IFN- $\gamma$  production under these conditions.

#### STAT4 signaling is impaired in T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells

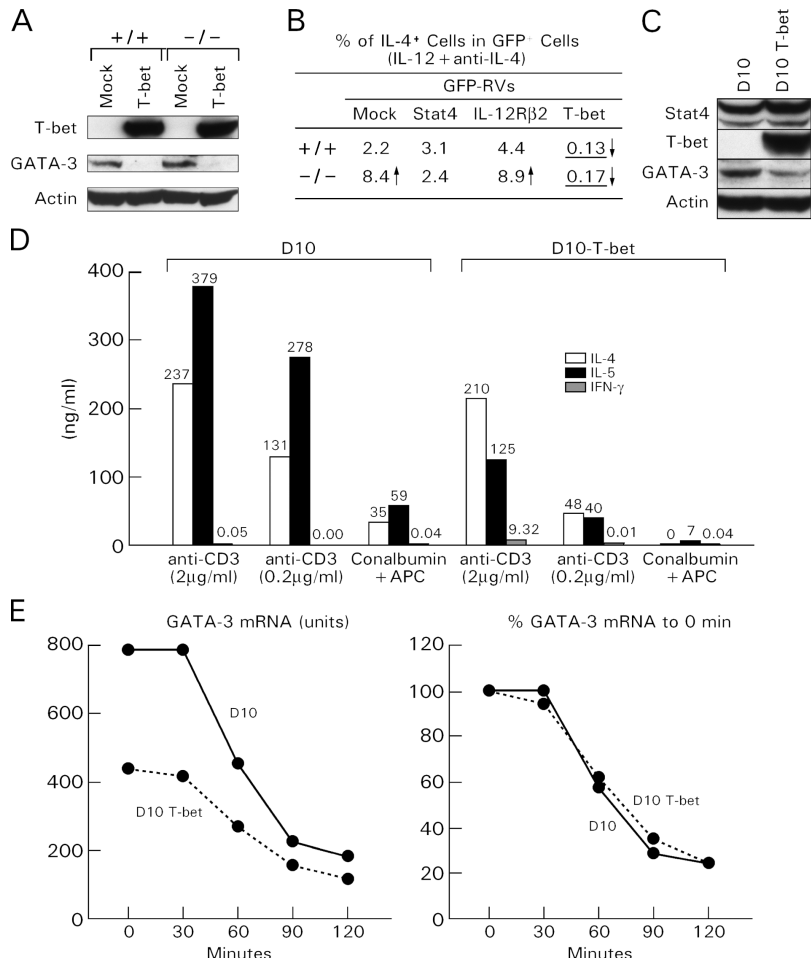
Because in previous studies we have shown that elevated GATA-3 levels are associated with decreased STAT4 expression and unresponsiveness to IL-12 in wild-type cells (16), we next examined if the same were true in T-bet<sup>-/-</sup> cells. Accordingly, we determined STAT4 protein levels by Western blot in wild-type and T-bet<sup>-/-</sup> cells at various times after stimulation. As shown in Fig. 4 (A and B), on day 0, naive T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells expressed slightly lower amounts of STAT4 protein than wild-type cells, and on day 2, this difference was more pronounced and was observed under both suboptimal Th1 and neutral conditions. Finally, on day 7, both total STAT4 and phosphorylated STAT4 protein were decreased in T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells, especially under suboptimal Th1 conditions. Similar findings have recently been reported by Underhill et al. (17). On the other hand, phosphorylated STAT4 was present in normal amounts in

T-bet<sup>-/-</sup> cells stimulated in the presence of anti-IL-4 antibody, probably because IL-12R $\beta$ 2 chain expression is maintained under these conditions (Fig. S1). Finally, we detected GATA-3 protein only in T-bet<sup>-/-</sup> T cells that were maintained under suboptimal Th1 conditions (not depicted).

The findings described above led to studies of naive CD4<sup>+</sup> T-bet<sup>-/-</sup> cells in which STAT4 levels were maintained by a STAT4-expressing retrovirus. As shown in Fig. 4 (C and D), maintenance of STAT4 expression using this approach restored Th1 differentiation very effectively, even under suboptimal Th1 conditions, and this effect was associated with the down-regulation of GATA-3. These data indicate that the maintenance of STAT4 expression is enough to rescue Th1 development in T-bet<sup>-/-</sup> T cells (despite the reduction in IL-12R $\beta$ 2 expression; Fig. 2 B). As such, they complement the results shown in Fig. 2 A, in that we again saw a virtual normalization of IFN- $\gamma$  production in T-bet<sup>-/-</sup> cells under conditions in which GATA-3 effects are obviated.

#### Retroviral T-bet expression suppresses GATA-3 and Th2 cytokines

Szabo et al. (10) suggested earlier that T-bet effects on Th2 cytokine production may in part result from its ability to suppress GATA-3 expression. However, this was not directly examined. To examine this question, we first determined the effect of retroviral T-bet expression on GATA-3 expression



**Figure 5. T-bet overexpression suppresses GATA-3 and Th2 cytokines.** (A) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with anti-CD3/anti-CD28 under strict Th2 conditions (IL-4 + anti-IFN-γ + anti-IL-12). On day 2, the cells were infected with control (mock) or T-bet-expressing retrovirus (T-bet-RV). Finally, on day 7, GFP<sup>+</sup> cells were sorted by flow cytometry and lysed for Western blot studies. These data are representative of those obtained in three independent experiments. (B) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with anti-CD3/anti-CD28 under optimal Th1 conditions. On day 2, the cells were infected with control (mock) or STAT4-, IL-12Rβ2 chain-, or T-bet-expressing retroviruses. Finally, on day 7, cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IL-4. The values shown were the percentages of IL-4<sup>+</sup> cells in the GFP<sup>+</sup> population. These data are representative of those obtained from two independent experiments. (C) An established murine Th2 cell line (D10 cells) was stimulated with conalbumin/APCs and then infected with a T-bet-expressing retrovirus on days 2, 3, 4, and 5.

On day 14, GFP<sup>+</sup> cells were enriched by flow cytometric sorting and restimulated with antigen/APC. Finally, on day 21, GFP<sup>+</sup> and GFP<sup>-</sup> cells were separated by sorting (purities were >92%) and lysed for Western blot analysis. (D) T-bet-expressing and nonexpressing D10 cells were obtained and cultured as described in C. On day 21, the cells were restimulated with either high (2 μg/ml) or low (0.2 μg/ml) concentrations of plate-bound anti-CD3 and/or conalbumin/APCs. 48 h after such restimulation, culture supernatants were collected and IL-4, IL-5, and IFN-γ were measured by ELISA. Similar results were obtained in three independent experiments. (E) T-bet-expressing and nonexpressing D10 cells were obtained and cultured as described in C. On day 21, 2 μg/ml actinomycin D was added to the cultures, and cells were harvested every 30 min for extraction of total RNA and subsequent real-time PCR analysis for GATA-3 mRNA. The left panel depicts the relative amount of GATA-3 mRNA normalized by 18S rRNA, and the right panel depicts the percent decreases from the nontreated sample. Similar results were obtained in two independent experiments.

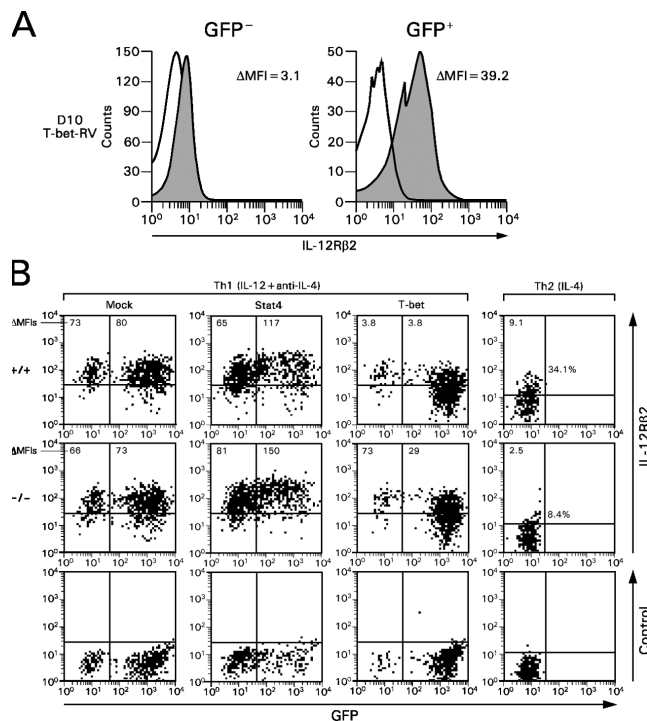
in developing Th2 cells; i.e., in cells naturally expressing GATA-3. Naive CD4<sup>+</sup> T cells from either T-bet<sup>-/-</sup> or wild-type mice were stimulated and maintained under optimal Th2 conditions (IL-4 plus anti-IL-12) and then infected with a mock or T-bet-expressing retrovirus on day 2. On day 7, GFP<sup>+</sup> cells were obtained by flow cytometric sorting and subjected to Western blot analysis. As shown in Fig. 5 A, it

was clear that GATA-3 protein expression was shut down almost completely in cells that expressed retroviral T-bet. We next examined the effect of retroviral infection on developing Th1 cells from T-bet<sup>-/-</sup> and wild-type mice with retroviruses expressing STAT4, IL-12Rβ2 chain, or T-bet on the percentages of IL-4-producing cells. As shown in Fig. 5 B (as well as Fig. 2, A and C), low but clearly increased percentages

of IL-4-producing cells (5–10%) were seen even in T-bet<sup>-/-</sup> cell populations that were maintained under optimal Th1 conditions (IL-12 plus anti-IL-4 antibody). It is therefore very difficult to completely block Th2 development in T-bet<sup>-/-</sup> Th1 cells. However, in the presence of IL-12, retroviral T-bet expression could dramatically suppress the appearance of IL-4-producing cells (50- and 20-fold reduction in T-bet<sup>-/-</sup> and wild-type Th1 cells, respectively), but under the same conditions, retroviral IL-12Rβ2 chain expression failed to do so and retroviral STAT4 expression had only a modest effect. These data support our previous report in which we showed that retroviral expression of IL-12Rβ2 chain cannot change the fate of Th1/Th2 differentiation, whereas retroviral expression of STAT4 can promote Th1 differentiation and block Th2 differentiation (15, 16).

In related studies, we examined the effect of retroviral expression of T-bet on GATA-3 expression in a fully differentiated D10 Th2 cell line. In the present study, we infected D10 cells with a T-bet-expressing retrovirus, sorted the cells according to GFP expression, and subjected the positive cells obtained to Western blot analysis. As shown in Fig. 5 C, GATA-3 protein levels were clearly decreased in T-bet-expressing D10 cells compared with noninfected, wild-type D10 cells. Then, using the same cells, we measured IL-4, IL-5, and IFN-γ secretion by ELISA under various conditions of restimulation. As shown in Fig. 5 D, retroviral expression of T-bet in D10 cells did not suppress IL-4 secretion when these cells were stimulated with high concentrations of anti-CD3 antibody (2 μg/ml), partially suppressed IL-4 when they were stimulated with low concentrations of anti-CD3 antibody (0.2 μg/ml), and completely suppressed IL-4 when they were stimulated under suboptimal Th1 conditions by antigen (conalbumin plus APC). IL-5, a cytokine whose synthesis is controlled by GATA-3 more directly than IL-4 (18), was suppressed to an even greater extent. Of interest, retroviral expression of T-bet in D10 cells led to only low-level production in IFN-γ, even when they were stimulated with high concentrations of anti-CD3. These data again suggest that the main function of T-bet is to suppress GATA-3 expression and is not sufficient to directly regulate the *IFNG* gene, at least at this stage of T cell differentiation.

Finally, we examined the mechanism of GATA-3 suppression by T-bet by measuring GATA-3 mRNA stability after treatment of the D10 cells expressing retroviral T-bet with actinomycin D. As shown in Fig. 5 E, although GATA-3 mRNA levels in untreated T-bet-expressing D10 cells were suppressed to about half of the level found in the wild-type cells, the kinetics of degradation of GATA-3 mRNA was similar in T-bet-expressing and nonexpressing D10 cells. These results suggest that T-bet suppresses GATA-3 at a transcriptional level rather than at a posttranscriptional level. However, it should be noted that some posttranslational control may also exist because the down-regulation of GATA-3 by T-bet is more evident at the protein level than it is at the mRNA level.



**Figure 6. T-bet is not a direct inducer of IL-12Rβ2 chain.** (A) T-bet-expressing and nonexpressing D10 cells were obtained and cultured as described in Fig. 4 C and harvested for IL-12Rβ2 chain staining on day 21. The values shown in each panel depict the  $\Delta$  mean fluorescence intensities (MFIs) for the IL-12Rβ2 chain stain after subtraction of the MFIs of a control stain. Open histograms depict isotype controls, and filled histograms depict IL-12Rβ2 chain-specific staining. (B) Naive CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> and wild-type mice were stimulated with Con A/APCs under optimal Th1 (IL-12 + anti-IL-4) or Th2 conditions. On day 2, the cells were infected with control (mock), STAT4-, or T-bet-expressing retroviruses, and on day 5, surface IL-12Rβ2 chain staining was performed. The values shown in each panel represent the  $\Delta$ MFIs of IL-12Rβ2 chain expression in either GFP<sup>-</sup> or GFP<sup>+</sup> populations after subtraction of the MFIs of control stainings.

### T-bet is not an essential factor for IL-12Rβ2 chain expression

Another possible role of T-bet in T cell differentiation is the induction of IL-12Rβ2 chain. Although Afkarian et al. (12) suggested that T-bet is a sufficient factor for IL-12Rβ2 chain expression, our present data shown in Fig. S1 suggested that this is not in fact the case. To address this discrepancy further, we first measured IL-12Rβ2 chain expression in retroviral T-bet-expressing D10 cells that were used in the studies described above. As shown in Fig. 6 A, IL-12Rβ2 chain expression was induced even in fully differentiated Th2 cells expressing retroviral T-bet, consistent with the data previously provided by Afkarian et al. However, when we measured IL-12Rβ2 chain expression in developing Th1 cells, i.e., naive CD4<sup>+</sup> T cells from either T-bet<sup>-/-</sup> or wild-type mice stimulated under optimal Th1 conditions, a different picture emerged. As shown in Fig. 6 B, we observed normal IL-12Rβ2 chain expression in mock retrovirus-infected

T-bet<sup>-/-</sup> Th1 cells, but neither retroviral T-bet expression in wild-type T cells nor retroviral T-bet maintenance in T-bet<sup>-/-</sup> Th1 cells led to IL-12Rβ2 chain expression. On the contrary, under these conditions, IL-12Rβ2 expression was suppressed compared with mock retrovirus-infected cells. Furthermore, we found that cells expressing retroviral STAT4 exhibited a higher level of IL-12Rβ2 chain expression in both Th1 cells from T-bet<sup>+/+</sup> and T-bet<sup>-/-</sup> mice, with only slightly greater expression of the IL-12Rβ2 chain in the T-bet<sup>-/-</sup> cells. Finally, IL-12Rβ2 chain expression was severely reduced in developing Th2 cells from T-bet<sup>-/-</sup> mice. These data strongly suggest that the initial expression of IL-12Rβ2 chain is induced by TCR stimulation alone independently of T-bet and is negatively regulated by GATA-3 (and positively regulated by STAT4). The findings of Afkarian et al. are likely explained by an indirect effect of T-bet on GATA-3 rather than a direct effect of T-bet on the IL-12Rβ2.

#### T-bet is induced in cells expressing retroviral STAT4 even in the absence of STAT1 and IFN-γ signaling

The last question we considered was the relation of STAT4 signaling to T-bet expression in Th1/Th2 differentiation. In our initial studies along these lines, we determined T-bet expression during the conversion of developing Th2 cells into Th1 cells under the influence of retrovirally expressed STAT4, using cells from both wild-type (BALB/c) and STAT1<sup>-/-</sup> mice (the latter because STAT1 has been reported to be a critical factor for T-bet induction; references 8 and 12). Accordingly, naive CD4<sup>+</sup> T cells were isolated from wild-type and STAT1<sup>-/-</sup> mice and stimulated with anti-CD3/anti-CD28 under optimal Th2 conditions (IL-4 plus anti-IL-12 plus anti-IFN-γ). On day 2, the cells were infected with mock or STAT4-expressing retroviruses, and on day 4, after extensive washing, the Th2 cells thus obtained were cultured under Th1 conditions (IL-12 plus anti-IL-4 antibody) for another 3 d. Finally on day 7, intra-cellular cytokine staining and Western blot analysis of the sorted cell populations were performed. As we reported previously (16) and as shown in Fig. 7 A, the switch to Th1 conditions on day 4 did not result in the appearance of many IFN-γ-producing cells at day 7 in cells infected with a mock retrovirus, indicating that only 4 d of polarization under Th2 conditions were enough to establish a Th2 phenotype. In contrast, when cells (from both wild-type and STAT1<sup>-/-</sup> mice) were switched to Th1 conditions and infected with a retrovirus expressing STAT4, a large fraction of the developing Th2 cells was converted into IFN-γ-producing cells. Furthermore, as shown in Fig. 7 B, the wild-type and STAT1<sup>-/-</sup> cells undergoing Th2 to Th1 conversion and expressing retroviral STAT4 contained normal levels of T-bet protein, and as shown in Fig. 7 C, the level of T-bet protein expression in cells expressing retroviral STAT4 increases with the strength of the IL-12/STAT4 signal. These data demonstrate that IL-12/STAT4 signaling can induce T-bet expression by a STAT1-independent mechanism.

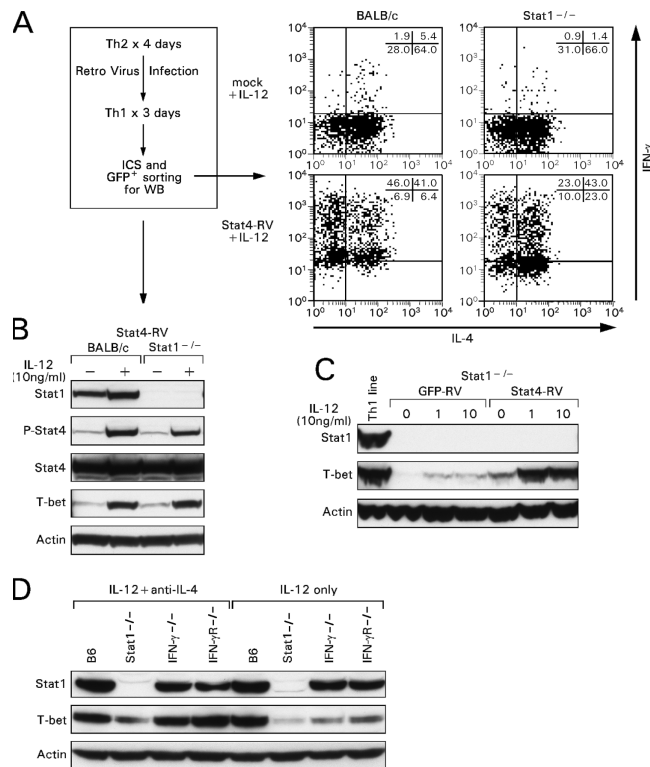
In further studies addressing T-bet expression in cells not infected with a retrovirus expressing STAT4 (but expressing endogenous STAT4), we showed that in cells from STAT1<sup>-/-</sup>, IFN-γ<sup>-/-</sup>, and IFN-γR<sup>-/-</sup> mice, T-bet expression is increased in cells maintained under optimal Th1 conditions as compared with cells maintained under suboptimal Th1 conditions, suggesting that STAT4 does indeed induce some T-bet expression independently of STAT1. It should be noted, however, that such signaling may not be quantitatively important because T-bet expression was decreased in STAT1<sup>-/-</sup> cells, especially if the latter are maintained in the absence of anti-IL-4 antibody (i.e., under optimal Th1 conditions), and STAT1 deficiency also leads to elevated IL-4/GATA-3 levels and decreased T-bet expression (not depicted). Therefore, we conclude that although at least some T-bet induction can occur in the absence of STAT1 signaling, the latter is probably necessary for the optimal T-bet induction in normal cells and thus for the regulation of GATA-3 in such cells.

#### DISCUSSION

The key finding in this study was that T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells are in fact capable of producing virtually normal amounts of IFN-γ provided that one of two conditions are met: GATA-3 accumulation is prevented by blockade of IL-4R signaling or GATA-3 negative regulation of STAT4 levels is bypassed by retroviral STAT4 expression. These results are seemingly in conflict with previous studies by Szabo et al. (11) that showed that CD4<sup>+</sup> T cells from T-bet<sup>-/-</sup> mice produce very low amounts of IFN-γ. However, this discrepancy can be explained by the fact that in this study only naive CD4<sup>+</sup> T cells were studied, whereas in the previous study such preselection was not used. Naive cells must be used in these studies because as cells mature in vivo before being harvested for study they accumulate relatively high levels of GATA-3, especially in the absence of T-bet and, as shown in a previous study by Usui et al. (16), such GATA-3 can shut down STAT4 production and make it difficult to induce Th1 polarization. Thus, to show that T-bet is not necessary for Th1 polarization, one must use highly purified naive T cells. An additional reason for the discrepancy is that T-bet<sup>-/-</sup> cells grow less well in culture and produce less IFN-γ than wild-type cells unless cultured at a relatively high density. This condition was met in the studies of IFN-γ secretion in the present study, but not in previous studies.

IFN-γ production in the absence of T-bet was accompanied at the molecular level by histone acetylation and the establishment of a DNase I hypersensitivity site in the *IFNG* promoter. These findings, along with observations showing that T-bet has only indirect effects on IL-12Rβ2 chain expression through its effects on GATA-3, led us to the view that the main effect of T-bet in developing Th1 cells is the suppression of GATA-3 levels or GATA-3 function. This view was supported by the additional finding that retroviral T-bet expression down-regulates GATA-3 levels in developing Th2 cells and partially down-regulates GATA-3 protein





**Figure 7. STAT4 signal induces T-bet in the absence of STAT1.**

(A) Naive CD4<sup>+</sup> T cells from BALB/c and STAT1<sup>-/-</sup> mice were stimulated with anti-CD3/anti-CD28 under Th2 conditions for 4 d. 24 h after stimulation, the cells were infected with mock-RV or STAT4-RV. On day 4, the cells were washed extensively and maintained under Th1 conditions (IL-12 and anti-IL-4 antibody) for another 3 d. Finally, the cells were restimulated by PMA/ionomycin and subjected to intracellular cytokine staining for IL-4 and IFN- $\gamma$ . Similar results were obtained in three independent experiments. (B) The same cells used in A were initially cultured under Th2 conditions for 4 d and then either cultured under Th1 conditions or continued under Th2 conditions for 3 d. The cells were then sorted on day 7 according to GFP expression to obtain cells expressing STAT4-RV and then lysed for Western blot analysis. Similar results were obtained in three independent experiments. (C) The same cells used in A were initially cultured under Th2 conditions for 4 d and then either cultured under Th1 conditions with two concentrations of IL-12 or continued under Th2 conditions for 3 d. The cells were then sorted on day 7 according to GFP expression to obtain cells expressing STAT4-RV or GFP-RV (mock) and then lysed for Western blot analysis. Similar results were obtained in three independent experiments. (D) Naive CD4<sup>+</sup> T cells from C57BL/6, STAT1<sup>-/-</sup>, IFN- $\gamma$ <sup>-/-</sup>, and IFN- $\gamma$ R<sup>-/-</sup> mice were stimulated with anti-CD3/anti-CD28 either under strict Th1 conditions (IL-12 + anti-IL-4 antibody) or suboptimal Th1 conditions (IL-12 only) and expanded by hu-IL-2. On day 7, these cell lines were lysed and subjected to Western blot analysis. Similar results were obtained in two independent experiments.

levels in an established Th2 line. In previous studies conducted by Mullen et al. (9, 13), it was shown that retroviral expression of T-bet in STAT4<sup>-/-</sup> cells (stimulated under Th2 conditions) led to the early appearance of a DNase I hypersensitivity site in the *IFNG* gene promoter and retroviral expression of a dominant-negative T-bet construct in cells (stimulated under Th1 conditions) blocked the appearance of

this site. In contrast, in more quantitative studies conducted by Fields et al. (19), it was shown that retroviral expression of T-bet in STAT4<sup>-/-</sup> cells led to only a modest increase in histone acetylation, far lower than that seen in STAT4<sup>+/+</sup> cells not expressing retroviral T-bet. In the present study we showed that histone H3 acetylation and DNase I hypersensitivity sites of the *IFNG* promoter occurred in T-bet<sup>-/-</sup> cells provided that the cells were naive and stimulated under optimum Th1 conditions (in the presence of anti-IL-4). These results indicate that the production of IFN- $\gamma$  in T-bet-deficient cells is, in fact, accompanied by accessibility of the *IFNG* promoter and that such accessibility is not dependent on T-bet.

Despite this latter conclusion it remains possible that although T-bet is not necessary for *IFNG* accessibility and transcription, it nevertheless takes part in this process in normal cells. In previous studies relevant to this possibility we showed that CD4<sup>+</sup> T cells infected with both a retrovirus expressing STAT4 and GATA-3 exhibit normal IFN- $\gamma$  production under appropriate stimulation, whereas the same cells infected with retroviruses expressing T-bet and GATA-3 exhibit greatly reduced IFN- $\gamma$  production (16). These findings are most easily explained on the assumption that under conditions in which GATA-3 down-regulates STAT4, T-bet does not induce *IFNG* accessibility and transcription in and of itself. These previous findings, assuming that they apply to cells containing normal levels of STAT4, suggest that even when a normal level of T-bet is present within a developing Th1 T cell, it does not ordinarily exert an effect on either *IFNG* accessibility or transcription. This conclusion is not necessarily at odds with reports that T-bet interacts with the promoter (or enhancers) of the *IFNG* gene (9, 13, 20–22) because these reports speak only to the potential for such interaction and not to its actuality under normal conditions.

In their initial studies of T-bet, Szabo et al. (10) showed that T-bet suppresses IL-4 and IL-5 production in Th2 cells and suggested that such suppression occurred because T-bet inhibited GATA-3 activity. This view, however, was not supported in subsequent studies by Afkarian et al. (12), who found that cells stimulated under Th2 conditions and expressing retroviral T-bet exhibited normal GATA-3 expression by Western blot. In the present studies we provide evidence that in T cells newly differentiating under optimal Th2 conditions, infection with a T-bet-expressing retrovirus has a profound inhibitory effect on GATA-3 protein expression. Similarly, GATA-3 protein expression was down-regulated in an established Th2 cell line, the D10 cell line. Although data derived from studies in which factors are overexpressed by retroviral infection must be interpreted with caution, it should be noted that in these studies levels of T-bet in Th2 cells infected with a retrovirus expressing T-bet did not greatly exceed that in developing Th1 cells; thus, the effect of the T-bet-expressing retrovirus cannot be ascribed to overexpression per se. In studies using actinomycin D-treated cells, we showed that T-bet at least partially mediates an effect on GATA-3 levels by exerting an effect on

GATA-3 transcription rather than an effect on newly synthesized GATA-3 mRNA. Another possibility not addressed in these studies is that T-bet affects GATA-3 function as well as its level. Evidence for this possibility has recently come from Hwang et al. (23), who have shown that T-bet is phosphorylated by a Tec kinase (mainly ITK) early during Th1 differentiation and the activated T-bet thus generated physically interacts with GATA-3 and prevents its binding to the IL-5 promoter. Furthermore, these authors have shown that a mutation at Y525F that prevents phosphorylation at this site disables T-bet binding to GATA-3 and retroviral expression of the mutated protein in T-bet<sup>-/-</sup> cells was not effective in repressing Th2 cytokine expression. Thus, T-bet might act on GATA-3 via multiple mechanisms.

It should be noted that in the present studies, although T-bet-expressing D10 cells produced less IL-4 and IL-5, such reduction was shown to be at least partially dependent on the strength of the TCR signal, indicating that the latter can override T-bet effects by as yet unknown mechanisms. In addition, T-bet-expressing D10 cells produced only a small amount of IFN- $\gamma$  (compared with normal Th1 cells). This result is consistent with earlier studies that showed that retroviral T-bet expression can only induce large amounts of IFN- $\gamma$  production after stimulation of cells with a nonphysiological stimulus, PMA and ionomycin (12). Perhaps more importantly, it is also consistent with the view that T-bet is not an important transactivator of the *IFNG* promoter.

In additional studies, we addressed the relation between T-bet and IL-12R $\beta$ 2 chain expression. Previously, Afkarian et al. (12) reported that retroviral T-bet expression in developing Th2 cells induces IL-12R $\beta$ 2 chain expression, and, using a similar approach, we confirmed this result using a fully differentiated Th2 clone. However, we found that neither retroviral expression nor endogenous expression of T-bet led to IL-12R $\beta$ 2 expression in developing Th1 cells. This correlated with the fact that T-bet<sup>-/-</sup> cells expressed normal levels of IL-12R $\beta$ 2 chain under optimal Th1 conditions, but expressed relatively low levels of IL-12R $\beta$ 2 chain under suboptimal Th1 conditions (IL-12 in the absence of anti-IL-4 antibody), in which case they produce large amounts of IL-4 and preferentially develop into Th2 cells. Although these data demonstrate that T-bet is not essential for IL-12R $\beta$ 2 chain expression, collectively with the data provided by Afkarian et al., they suggest that T-bet has an indirect influence on such expression through its ability to obviate GATA-3 effects on STAT4. Relevant to this last possibility, we show here that STAT4 signaling can induce IL-12R $\beta$ 2 chain expression in the absence of T-bet and have shown previously that GATA-3 down-regulation of the IL-12R $\beta$ 2 chain occurs through down-regulation of STAT4. The idea that T-bet affects IL-12R $\beta$ 2 chain expression indirectly via its effects on GATA-3 is consonant with previous studies establishing the centrality of GATA-3 in Th2 differentiation (24, 25).

Because GATA-3 is present in normal resting (naive) CD4<sup>+</sup> cells even before activation (26, 27), the findings

described above suggest that STAT4 expression necessary for Th1 development requires early down-regulation of GATA-3 levels or function early on, presumably by induction of T-bet. In the present studies we show that T-bet is expressed in STAT1<sup>-/-</sup> cells undergoing conversion from Th2 to Th1 cells expressing retroviral STAT4, implying that in the presence of high levels of STAT4, IFN- $\gamma$ /STAT1 signaling is not necessary for early expression of T-bet. Furthermore, there is some evidence that even endogenous STAT4 can induce some T-bet in the absence of STAT1 signaling. This said, T-bet expression is not robust in STAT1<sup>-/-</sup> cells expressing only endogenous STAT4, especially under suboptimal Th1 conditions, and STAT1<sup>-/-</sup> T cells are similar to T-bet<sup>-/-</sup> T cells in that they exhibit enhanced GATA-3 expression, presumably because of lack of sufficient T-bet. Collectively, then, the data suggest that in normal CD4<sup>+</sup> T cells, T-bet expression is initiated by TCR signaling acting in concert with IL-12/STAT4 signaling, but only reaches its full extent with subsequent IFN- $\gamma$ /STAT1 signaling.

As a final point, it is worth noting that T cells retain the capacity to produce small amounts of IFN- $\gamma$  in the absence of STAT4 (28), and under these circumstances other factors including T-bet may come into play as major transcription factors. In addition, STAT4-/STAT6-deficient T cells or STAT4-deficient T cells cultured in the presence of anti-IL-4 produce more IFN- $\gamma$  than STAT4-deficient T cells cultured in the absence of anti-IL-4, although still far less than wild-type Th1 T cells (28). The latter observation is consonant with the present findings in that it suggests that even in the absence of STAT4, GATA-3 is a negative regulator of IFN- $\gamma$  production, hence the importance of T-bet regulatory function in this context.

In summary, these findings provide the basis of a modified model of T cell differentiation (see Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20052165/DC1>). In this model, GATA-3 is assumed to be the central factor whose level/activity decides the fate of Th cell differentiation, not only through its capacity to induce Th2 cytokine production, but also through its capacity to block Th1 cytokine production via down-regulation of STAT4 and (indirectly) the IL-12R $\beta$ 2 chain. Thus, in the course of T cell differentiation occurring in the presence of IL-12 and IL-4 (the usual milieu of naive T cells), such Th1 differentiation is attenuated because concomitant IL-4 induction of GATA-3 suppresses STAT4 and IL-12R $\beta$ 2 chain expression. If, however, T-bet is also induced at sufficient levels, such GATA-3 suppression is counteracted, permitting Th1 differentiation to occur. This ability of T-bet to oppose the action of GATA-3 is its most essential function in Th1 differentiation rather than any ability of T-bet to directly affect *IFNG* gene transcription, as has been argued previously.

## MATERIALS AND METHODS

**Reagents and mice.** Human recombinant IL-2 and anti-mouse IL-4 (11B11) antibody were obtained from National Cancer Institute (NCI)/NIH. Murine recombinant IL-12, IL-4, anti-mouse IL-12, and anti-mouse IFN- $\gamma$  antibody were purchased from PeproTech. Anti-mouse IL-12R $\beta$ 2

chain monoclonal antibody (PDL-HAM10B9) was prepared as described previously (15). T-bet<sup>-/-</sup>, T-bet<sup>+/-</sup>, and wild-type mice (C57BL/6 × 129/SvJ) on an F2 background were provided by H. Young (NCI/NIH, Frederick, MD) and were originally from L. Glimcher (Harvard School of Public Health, Boston, MA). STAT1<sup>-/-</sup> and BALB/c mice were purchased from The Jackson Laboratory. All animal experiments were performed under protocols approved by the Animal Care Branch of the NIAID, NIH.

**Cell isolation, culture, and retroviral transduction.** Whole CD4<sup>+</sup> T cells from splenocytes were isolated by positive selection using anti-mouse CD4 microbeads (Miltenyi Biotec). Naive (CD4<sup>+</sup>/CD62L<sup>hi</sup>) and memory (CD4<sup>+</sup>/CD62L<sup>lo</sup>) Th cells were purified by negative selection using the Mouse CD4<sup>+</sup> T Cell Isolation kit followed by positive selection using anti-mouse CD62L microbeads (Miltenyi Biotec). Alternatively, naive CD4<sup>+</sup> T cells in negatively selected CD4<sup>+</sup> T cell populations were also isolated by flow cytometric sorting of CD4<sup>hi</sup> CD62L<sup>hi</sup> cells. The purity of the naive CD4<sup>+</sup> T cells obtained after the latter procedure was >99%. Whole or naive CD4<sup>+</sup> T cells were stimulated with either Con A plus APC (30 Gy-irradiated splenocytes from wild-type mice) or with 5 μg/ml of plate-bound anti-CD3 and 1 μg/ml of soluble anti-CD28 under various T cell-polarizing conditions. For studies of intracellular cytokine production, naive CD4<sup>+</sup> T cells purified by anti-CD62L microbeads were stimulated as described above under various T cell-polarizing conditions and expanded with IL-2 on days 2 and 4 of culture. On day 8, the cells were washed, restimulated with 20 ng/ml PMA and 1 μM ionomycin for 6 h, and then stained as indicated below. For studies of IFN-γ secretion, naive CD4<sup>+</sup> T cells purified by cell sorting were stimulated with 5 μg/ml of plate-bound anti-CD3 and 1 μg/ml of soluble anti-CD28 under various T cell-polarizing conditions. After 2 d, the cells were expanded for 4 d by culture in 50 U/ml rIL-2 in the continued presence of the above cytokines and antibodies. On day 6, cells were washed and restimulated with either 5 μg/ml of plate-bound CD3 for 24 h or with PMA/ionomycin for 6 h. Cells were cultured at a concentration of 5 × 10<sup>5</sup> cells/ml during the initial culture and 10<sup>6</sup> cells/ml during reculture. The amount of cytokines secreted into the culture supernatant was measured by ELISA.

Mouse Th2 clone D10.G4.1 (D10) cells were purchased from American Type Culture Collection and maintained by conalbumin/APC stimulation every 2 wk. cDNAs encoding murine STAT4, T-bet, and IL-12Rβ2 chain were cloned into pBMN-IRES-EGFP (provided by G. Nolan, Stanford University, Palo Alto, CA), and cells were transduced as described previously (16, 29).

**Cytokine measurements.** ELISAs for IL-4, IL-5, and IFN-γ were performed using the OptEIA Mouse kit (BD Biosciences) according to the manufacturer's protocol using a TMB Microwell (BioFX) system. Intracellular cytokine staining was performed as described previously (30).

**mRNA and Western blot analysis.** Protein extraction and Western blot analysis were performed as described previously (16). Total RNA was isolated with a STAT60 RNA isolation kit (Tel-Test, Inc.), treated with RQ1 RNase-free DNase (Promega), and reverse transcribed. Real-time PCR was performed using ABI Prism 7700 Sequence BioDetector (PE Applied Biosystems) using ribosomal RNA as a control. The expression level of GATA-3 mRNA was measured by ΔC<sub>t</sub> method after justifying it by using serially diluted standard sample from D10 cells. The results were normalized to 18S rRNA abundance and then unit was recalculated using standard GATA-3 mRNA level of D10 cells, which was set at 800 units.

**ChIP and DNase hypersensitivity analysis.** ChIP assay was performed as described previously (31) according to the manufacturer's instructions (Upstate Biotechnology). PCR was performed as described previously (29). PCR primers for the IFNγ promoter were 5'-CGTAATCCCCGAGGAGCCTTC-3' and 5'-CTTCAATGACTGTGCCGTGG-3'. DNase I hypersensitivity assay was performed as described by Agarwal et al. (32). In brief, nuclei were isolated from 4 × 10<sup>7</sup> cells, divided into eight aliquots, and incu-

bated with 0–63 U/ml DNase I (Roche) for 20 min at 37°C. DNA was purified and digested with BamHI. Samples were resolved on a 0.8% agarose gel, transferred to Nyron membranes, and hybridized with an exon 4 probe from the IFN-γ locus. Hybridized membranes were exposed to imaging screens and analyzed by a phosphorimager (STORM 860; Molecular Dynamics).

**Densitometry and statistical analysis.** Band densities in the autoradiogram were scanned and measured using an NIH-Image (NIH), and statistical analysis was performed by Student's *t* test.

**Online supplemental material.** Fig. S1 shows the expression of IL-12Rβ2 in cells from T-bet<sup>-/-</sup> mice after stimulation under various Th1 and Th2 conditions, and Fig. S2 shows a schematic diagram of Th1/Th2 differentiation based on the role of T-bet established in this study. Figs. S1 and S2 are available at <http://www.jem.org/cgi/content/full/jem.20052165/DC1>.

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