Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses

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To mount an effective type 1 immune response, type 1 T helper (Th1) cells must produce inflammatory cytokines and simultaneously suppress the expression of antiinflammatory cytokines. How these two processes are coordinately regulated at the molecular level is still unclear. In this paper, we show that the proto-oncogene E26 transformation–specific-1 (Ets-1) is necessary for T-bet to promote interferon-γ production and that Ets-1 is essential for mounting effective Th1 inflammatory responses in vivo. In addition, Ets-1–deficient Th1 cells also produce a very high level of interleukin 10. Thus, Ets-1 plays a crucial and unique role in the reciprocal regulation of inflammatory and antiinflammatory Th responses.

Th cells play a pivotal role in adaptive immunity. Type 1 Th (Th1) cells produce mainly IL-2 and IFN-γ and direct immune responses against intracellular pathogens. Type 2 Th (Th2) cells produce IL-4, IL-5, and IL-13 and are important for elimination of certain extracellular pathogens and parasites (1–3). However, Th cells are also capable of producing antiinflammatory cytokines, such as IL-10 and TGF-β, thereby attenuating Th immune responses (4, 5). Thus, both the production of inflammatory cytokines and the suppression of antiinflammatory cytokines must be coordinately regulated to generate effective Th immune responses.

The molecular events controlling the differentiation and function of Th cells have recently been characterized. The differentiation of common Th precursor cells into Th1 or Th2 cells is determined by the cytokine milieu and two critical transcription factors, T-bet and GATA-3 (6, 7). IL-4/STAT6 signals induce the expression of GATA-3, a Th2 cell–specific transcription factor, which promotes the differentiation of Th2 cells (8–10). IFN-γ induces T-bet via STAT1 phosphorylation (11, 12). T-bet is a very potent transactivator of the IFN-γ gene, thereby reinforcing the expression of IFN-γ (7, 13). In addition to T-bet, stable Th1 commitment also requires signals delivered by IL-12 via STAT4 (13, 14). However, naïve CD4 cells do not possess a functional IL-12 receptor (15). T-bet is also responsible for the up-regulation of the IL-12 receptor β2 (IL-12Rβ2) chain (11), enabling IL-12 signaling and terminal Th1 differentiation. Thus, T-bet has been regarded as the “master switch” of Th1 cells. Yet, despite the fact that forced expression of T-bet is sufficient to convert differentiating Th2 cells into Th1 cells, it is unclear whether T-bet can act alone or requires other transcription factors to drive the differentiation of Th1 cells and simultaneously suppress the expression of antiinflammatory cytokines.

Ets-1 (E26 transformation–specific-1) is the prototype of the ETS family of transcription factors, which are characterized by a conserved ETS domain that is capable of binding to DNA sequences containing a core GGAA/T motif (16, 17). Studies on Ets-1–deficient (Ets-1−/−) mice have demonstrated that Ets-1 is important for T cell development, proliferation, and survival (18–20). In addition, Ets-1−/− has been shown to regulate several cytokine gene promoters. Overexpression of Ets-1 suppressed the activity of an IL-2 promoter and transactivated IL-5 and GM-CSF promoters in vitro, suggesting that Ets-1 might also modulate the effector function of Th cells (21–23). Despite these observations, the regulatory roles of Ets-1 in Th immune responses remain unclear.

Here, we show that Ets-1 deficiency not only severely impairs the differentiation and function of Th1 cells but also leads to overproduction of IL-10. In addition, Ets-1−/− Th cells are unable to induce colitis in SCID mice, an animal model of Th1 cell–mediated disease.

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Abbreviations used: ChIP, chromatin immunoprecipitation; Ets-1, E26 transformation–specific-1; ICS, intracellular cytokine staining; ISRE, IFN-γ–stimulated response element.
Ets-1 is required for the expression of T-bet in Th1 cells and cooperates with T-bet to induce IFN-γ production. Together, our data demonstrate that Ets-1 plays an important and complex role in mounting effective Th1 immune responses.

RESULTS

Ets-1-deficient T cells proliferate normally in the presence of CD28 costimulation

Ets-1<sup>−/−</sup> T cells have been described to proliferate poorly upon cross-linking of CD3 in vitro (19, 20), but it is unclear whether the hypoproliferation of Ets-1<sup>−/−</sup> Th cells can be rescued by IL-2 or costimulatory signals. To address this question, we purified CD62L<sup>+</sup> naive Th cells from Ets-1<sup>−/−</sup> mice or wild-type littermates, which were then stimulated in vitro with anti-CD3 alone or in combination with anti-CD28 and/or IL-2. In agreement with previous papers, we found that Ets-1<sup>−/−</sup> Th cells proliferated less robustly in response to anti-CD3 stimulation than wild-type Th cells. However, addition of anti-CD28, but not exogenous IL-2, fully restored the proliferation of Ets-1<sup>−/−</sup> Th cells (Fig. 1 a). In all subsequent in vitro experiments, wild-type and Ets-1<sup>−/−</sup> Th cells were initially stimulated with 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28 unless indicated otherwise. Under such conditions, Ets-1<sup>−/−</sup> Th cells proliferated in a manner comparable to wild-type cells, thereby excluding any confounding effects caused by differences in proliferation.

Ets-1 is expressed in peripheral T cells (27). To determine whether Ets-1 is differentially expressed in subsets of Th cells, we compared the protein level of Ets-1 in differentiating Th1 and Th2 cells. We found that freshly isolated Th cells already expressed a significant level of Ets-1 protein, which was temporarily down-regulated after stimulation under both Th1- and Th2-skewing conditions but was restored ~3 d later. This expression pattern of Ets-1 is similar to what has been observed in human T cells (28). Nevertheless, the level and kinetics of down-regulation/reexpression of Ets-1 were comparable between differentiating Th1 and Th2 cells (Fig. 1 b).

Deficiency of Ets-1 results in profound defects in Th cytokine production

We examined the cytokine profiles of differentiated Ets-1<sup>−/−</sup> Th1 and Th2 cells by intracellular cytokine staining (ICS). At the time of restimulation, >98% of the differentiated wild-type and Ets-1<sup>−/−</sup> cells were CD4<sup>+</sup>TCR<sup>+</sup>Th cells (Fig. S1 a, available at http://www.jem.org/cgi/content/full/jem.20041330/DC1). As shown in Fig. 2 (a and b), ~70 and 50% of wild-type Th1 cells were stained positive for IL-2 and IFN-γ, respectively, whereas 50 and 65% of wild-type Th2 cells expressed IL-4 and IL-10, respectively. Ets-1-deficiency resulted in profound defects in the production of IL-2 and IFN-γ. Surprisingly, a high percentage (on average 38%) of Ets-1<sup>−/−</sup> Th1 cells started to produce IL-10, an antiinflammatory cytokine that is normally not expressed by Th1 cells. Ets-1<sup>−/−</sup> Th2 cells also displayed a statistically significant reduction in the production of IL-4, and Ets-1 deficiency further enhanced IL-10 production in Th2 cells, which already express a high level of IL-10. Of note, the production of TGF-β, another antiinflammatory cytokine, was not altered by Ets-1 deficiency (unpublished data). We obtained similar results irrespective of whether naive or bulk CD4<sup>+</sup> T cells were used as starting population.

Stimulation with anti-CD3 and anti-CD28 provides a rather strong signal to naive Th cells compared with antigen-specific stimulation. To examine the effect of Ets-1 deficiency on Th cell differentiation and function under more physiologic conditions, we backcrossed Ets-1-deficient mice to DO11.10 mice, which carry a TCR transgene specific for an OVA-derived peptide (OVA<sub>323-339</sub>) presented by I-A<sup>d</sup>. Purified CD4<sup>+</sup> T cells from Ets-1<sup>−/−</sup> or control DO11.10 mice were stimulated with wild-type splenic APCs and OVA<sub>323-339</sub> under Th1- or Th2-skewing conditions. The phenotype of the Th cells was examined by ICS 1 wk later. At this time, >98% of the live cells were CD3<sup>+</sup> cells expressing the DO11.10 TCR transgene (Fig. S1 b). As shown in Fig. 2 c, Ets-1<sup>−/−</sup> DO11.10 Th1 cells still produced considerably less IL-2 and IFN-γ than wild-type cells. In some experiments, a significant portion (up to 16%) of Th1 cells started to produce IL-4, a signature cytokine of Th2 cells. Again, a large number of the Ets-1<sup>−/−</sup> Th1 cells produced
IL-10. The abnormal Th1 cytokine profile was also confirmed by ELISA when the differentiated Ets-1−/− Th1 cells were restimulated with OVA323-339/APCs for 24 h (Fig. 2d). Thus, the aberrant differentiation and function of Ets-1−/− Th1 cells is independent of genetic background or mode of stimulation. The cytokine profile of Ets-1−/− Th2 cells as determined by ICS (i.e., reduced IL-4 and enhanced IL-10 production) was comparable to our earlier findings. However, the impairment in the production of IL-4 by Ets-1−/− Th2 cells was much more significant upon restimulation with OVA/APCs (Fig. 2d), indicating the effect of Ets-1 deficiency on Th2 cells may quantitatively depend on mode of stimulation.

The level of IL-2 in Ets-1−/− Th cultures ranged between 1–4 ng/ml. (b) Freshly isolated wild-type and Ets-1−/− Th cells were stimulated as described in Fig. 2a except that 10 μg/ml anti–IL-10R antibody (open symbols) or control IgG (closed symbols) was added. The differentiated Th cells were restimulated with PMA/ionomycin on day 7, and the production of cytokines was measured by ICS. The horizontal bars represent the average values of three independent experiments.

IL-10. The abnormal Th1 cytokine profile was also confirmed by ELISA when the differentiated Ets-1−/− DO11.10 Th1 cells were restimulated with OVA323-339/APCs for 24 h (Fig. 2d). Thus, the aberrant differentiation and function of Ets-1−/− Th1 cells is independent of genetic background or mode of stimulation. The cytokine profile of Ets-1−/− DO11.10 Th2 cells as determined by ICS (i.e., reduced IL-4 and enhanced IL-10 production) was comparable to our earlier findings. However, the impairment in the production of IL-4 by Ets-1−/− Th2 cells was much more significant upon restimulation with OVA/APCs (Fig. 2d), indicating the effect of Ets-1 deficiency on Th2 cells may quantitatively depend on mode of stimulation.
Because IFN-γ promotes the differentiation of Th1 cells via a positive feedback mechanism, it is possible that the impaired cytokine production by Ets-1−/− Th1 cells is caused by a defect in the expression of IFN-γ during early stages of Th1 differentiation. Therefore, we examined the cytokine production by differentiating Ets-1−/− Th1 cells 48 h after initial stimulation. We found that the levels of IFN-γ were comparable between wild-type and Ets-1−/− Th1 cells at this early time point, whereas the aberrant production of IL-2 and IL-10 was already apparent (Fig. 3 a). Thus, Ets-1 is dispensable for the early secretion of IFN-γ by differentiating Th1 cells, but controls IFN-γ production in terminally differentiated Th1 cells. These data also indicate that the aberrant cytokine production by Ets-1−/− Th1 cells is not caused by an early defect in IFN-γ production.

IL-10 is known to inhibit Th1 differentiation, mostly indirectly by affecting APC function, although direct effects on T cells have been reported (29, 30). To test whether the early IL-10 overproduction by Ets-1−/− Th cells was responsible for their altered cytokine profile, we repeated the in vitro differentiation of Th cells in the presence of an anti-IL-10 receptor antibody. As shown in Fig. 3 b, addition of anti–IL-10R antibody increased the production of IL-4 and IL-5 by Ets-1−/− Th2 cells to a level comparable to that of wild-type Th2 cells in the absence of anti–IL-10R antibody, although still lower than that of wild-type Th2 cells in the presence of anti–IL-10R antibody. Addition of anti–IL-10R antibody also modestly reduced the level of IL-10 in Ets-1−/− Th1 cells. In contrast, IL-10R blockade only had negligible effects on the levels of IL-2 and IFN-γ. Thus, deficiency of Ets-1 impairs the production of IL-2 and IFN-γ by an IL-10–independent mechanism.

Homologous recombination at the Ets-1 locus might also disturb the expression of other genes, thereby causing the aberrant cytokine profile of Ets-1−/− Th1 cells. In addition, it is unclear whether reconstitution of Ets-1 is sufficient to normalize the production of cytokines by Ets-1−/− Th1 cells once the process of differentiation has been set into motion. To address these questions, we infected differentiating Ets-1−/− Th1 cells with a bicistronic retrovirus encoding either GFP alone or together with Ets-1 36 h after the initial stimulation. As shown in Fig. 4 a, retroviral Ets-1 completely or nearly completely restored the production of IFN-γ and IL-2 upon secondary stimulation, and concurrently attenuated the production of IL-10. Therefore, the aberrant cytokine production by Ets-1−/− Th1 cells is reversible and is indeed caused by the deficiency of Ets-1. In contrast, overexpression of Ets-1 did not substantially influence the expression of cytokines by wild-type Th1 cells (Fig. 4 b), which already express a high level of endogenous Ets-1.

**Ets-1−/− Th cells do not induce colitis in SCID mice**

Our in vitro findings suggest that Ets-1 is required for optimal production of inflammatory cytokines by Th1 cells while suppressing antiinflammatory IL-10. To investigate the in vivo effect of Ets-1 deficiency on Th1-driven inflammatory responses, we chose a mouse model of inflammatory bowel disease that is induced by transferring CD45RBhigh Th cells into SCID mice. The development of colitis in this model depends on IFN-γ and is inhibited by IL-10 (31). More importantly, this model allowed us to specifically examine the function of Ets-1−/− Th cells, in a “wild-type” environment.

Wild-type or Ets-1−/− CD45RBhigh Th cells were purified by a MoFlo sorter and were injected into SCID mice, which were weighed weekly to monitor the activity of the disease. As shown in Fig. 5 a and Table I, all of the animals continued to gain weight at comparable rates up to 5 wk after Th cell transfer. Thereafter, the weight of mice that had received wild-type cells dropped substantially, eventually below the starting weight. In contrast, uninjected mice and mice that had received Ets-1−/− Th cells continued to gain weight at comparable rates until 10 wk after transfer when the mice were killed. Histological examination revealed marked inflammation in the mucosal and submucosal layers

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**Figure 4.** Overexpression of Ets-1 normalizes the production of cytokines by Ets-1−/− Th1 cells. (a) Freshly isolated Ets-1−/− or wild-type Th cells were stimulated in vitro under Th1-skewing conditions. 36 h after the initial stimulation, the differentiating Th1 cells were transduced with GFP-RV/Ets-1 or empty GFP-RV retroviruses. The cells were restimulated with PMA/ionomycin on the seventh day, and the production of cytokines was determined by ICS. The cells were separated into transduced (GFP+ ) and nontransduced (GFP− ) populations, and the staining of indicated cytokines was displayed as histograms. The numbers represent the percentages of cells that are stained positive for indicated cytokines. (b) Wild-type Th cells were stimulated as described before and infected with GFP-RV/Ets-1. The production of indicated cytokines by transduced (GFP+ ) and nontransduced (GFP− ) Th1 cells was determined by ICS as described before.
of the colons of mice that had received wild-type Th cells. In contrast, we observed no such inflammation in uninjected mice or in mice that had been injected with Ets-1−/− Th cells (Fig. 5 b and not depicted).

To examine the cytokine profile of the donor cells, we recovered CD4+ T cells from the mesenteric lymph nodes of the recipient animals. The recovered Ets-1−/− and wild-type donor Th cells expressed comparable levels of several memory and activation markers, such as CD69 and CD44 (unpublished data), indicating antigen encounter in vivo. The recovered Th cells were restimulated with anti-CD3 in vitro and the production of cytokines was measured by ELISA. Similar to our earlier findings, Ets-1−/− Th cells produced virtually no IFN-γ or IL-2 and a significantly elevated level of IL-10, whereas the production of IL-4 was comparable between Ets-1−/− and wild-type cells (Fig. 5 c). These results firmly demonstrate that deficiency of Ets-1 also leads to defective Th1 cell–mediated inflammation in vivo.

Ets-1 promotes the differentiation and function of Th1 cells via multiple mechanisms

Thus far, our data indicate that the differentiation and function of Th1 cells are severely impaired in the absence of Ets-1. The molecular events regulating the differentiation and function of Th1 cells have been characterized recently. Naive Th cells produce small amounts of both IL-4 and IFN-γ upon primary stimulation (32). IFN-γ/STAT1 signals induce the expression of T-bet, which in turn strengthens IFN-γ expression and drives Th1 differentiation (13). T-bet also induces the expression of the IL-12Rβ2 chain, thus enabling
IL-12 to further promote Th1 differentiation via STAT4 signaling (11). Ets-1 deficiency may interfere with either one or both pathways. Alternatively, Ets-1 may control the differentiation and IFN-γ production via a novel mechanism independent of IL-12/STAT4 and IFN-γ/STAT1/T-bet.

Because we used IL-12 in our culture system to drive Th1 differentiation, we first examined the expression and phosphorylation of STAT4. As naive Th cells express very little IL-12Rβ2 chain, which is induced by TCR signals in a T-bet–dependent manner, we cultured Th cells under Th1- or Th2-skewing conditions for 72 h before treating the cells with IL-12. Very little phosphorylated STAT4 (p-STAT4) was detected in the absence of IL-12 (Fig. 6 a, lanes 1–4). Addition of IL-12 quickly induced phosphorylation of STAT4 in wild-type Th cells and the level of p-STAT4 was much higher in Th1 than in Th2 cells (Fig. 6 a, lanes 5 and 7). Interestingly, we found that the level of p-STAT4 was modestly reduced in Ets-1−/− Th1 cells (Fig. 6 a, lanes 5 and 6). The difference in the level of p-STAT4 between differentiating wild-type and Ets-1−/− Th cells was detected as early as 24 h, but most prominent at 48–96 h after primary stimulation (Fig. S2 a, available at http://www.jem.org/cgi/content/full/jem.20041330/DC1). The reduction in the level of p-STAT4 could be attributed to either a lower level of total STAT4, IL-12R, or both. We found that total STAT4 levels were indeed reduced in Ets-1−/− Th1 cells at 48–96 h after

Figure 6. Regulation of IFN-γ production in Ets-1−/− Th cells.
(a) Analysis of the IL-12–STAT4 signaling pathway. Wild-type and Ets-1−/− Th cells were stimulated in vitro with anti-CD3/anti-CD28 under Th1- or Th2-skewing conditions for 72 h and treated with 10 ng/ml IL-12 for 20 min or left untreated (n.s.). Whole cell lysates were prepared and subjected to Western analyses with the indicated antibodies. (b) Wild-type or Ets-1−/− Th cells were stimulated using either IL-12 or IFN-γ as Th1-skewing cytokine. After 7 d of culture, the cells were restimulated with PMA/ionomycin and the cytokine production was measured by ICS. (c-e) Analyses of IFN-γ–STAT1–T-bet pathway. (c) Freshly isolated wild-type and Ets-1−/− Th cells were treated with 10 ng/ml IFN-γ for 20 min or left untreated (n.s.). Whole cell lysates were subjected to Western analysis with the indicated antibodies. (d) Wild-type or Ets-1−/− Th cells were stimulated under Th1-skewing conditions. At different time points, total cell lysates were prepared, and T-bet expression was analyzed by Western blot. (e) Wild-type and Ets-1−/− Th cells were subjected to in vitro differentiation under Th1-skewing conditions, either with IL-12 or IFN-γ, or Th2-skewing conditions for 72 h. Nuclear extracts were prepared and the levels of T-bet were determined by Western analysis. (f) Th1 cells obtained by in vitro differentiation were left untreated (n.s.) or treated with PMA/ionomycin. At the indicated time points, the T-bet expression was determined by Western analysis in total cell lysates. (g) Cells of the indicated genotypes were stimulated in vitro under Th1-skewing conditions and infected with GFP-RV/T-bet retroviruses 36 h after initial stimulation. The infected Th1 cells were restimulated on day 7 and the production of IFN-γ was examined by ICS. GFP-negative (nontransduced) and GFP-positive (transduced) populations were separately gated, and the staining of IFN-γ was displayed as histograms. The numbers represent the percentage of cells stained positive for IFN-γ.
stimulation (Fig. 6 a and Fig. S2 a). In addition, these cells also expressed lower levels of IL-12R\(\beta_2\) transcript (Fig. S2 b). Together, these data suggest that Ets-1 is required for the up-regulation of both STAT4 and IL-12R\(\beta_2\) during Th1 differentiation, and thus for proper IL-12 signaling.

If the impaired IL-12 signaling is the main cause of defective Th1 differentiation in the absence of Ets-1, then Ets-1 deficiency should not affect the differentiation of Th1 cells in an IL-12–free system. However, when we used IFN-\(\gamma\) instead of IL-12 to drive Th1 differentiation (thus eliminating any role of IL-12/STAT4 in our system), we still observed the same defect in cytokine production by Ets-1\(^{-/-}\) Th1 cells upon secondary stimulation (Fig. 6 b). This result argues strongly for the presence of IL-12/STAT4-independent mechanisms mediating the effects of Ets-1 deficiency.

Next, we investigated whether the IFN-\(\gamma\)–STAT1–T-bet pathway was intact in Ets-1\(^{-/-}\) Th cells. Freshly isolated Ets-1\(^{-/-}\) Th cells contained a normal amount of STAT1 proteins and were capable of phosphorylating STAT1 upon exposure to IFN-\(\gamma\) (Fig. 6 c), indicating intact IFN-\(\gamma\)/STAT1 signaling in Ets-1\(^{-/-}\) Th cells. Next, we examined the levels of T-bet in developing Ets-1\(^{-/-}\) Th1 cells at different time points after primary stimulation with anti-CD3 and anti-CD28. In agreement with the normal IFN-\(\gamma\)/STAT1 signaling and normal early IFN-\(\gamma\) production (Fig. 3 a), differentiating Ets-1\(^{-/-}\) Th1 cells only had a very negligible reduction in the level of T-bet (Fig. 6 d). This was independent of whether IL-12 or IFN-\(\gamma\) were used as Th1-skewing cytokines (Fig. 6 e). Resting differentiated Th1 cells expressed very little T-bet, which was induced by stimulation with PMA/ionomycin in wild-type Th1 cells. Interestingly, the expression of T-bet was markedly impaired in Ets-1\(^{-/-}\) Th1 cells (Fig. 6 f). The defect in T-bet expression upon secondary stimulation may well explain the impaired function of Ets-1\(^{-/-}\) Th1 cells. Therefore, we tested whether restoring the expression of T-bet could overcome the effects of Ets-1 deficiency. Wild-type or Ets-1\(^{-/-}\) Th cells were stimulated under Th1-skewing conditions and infected with a bicistronic retrovirus encoding both T-bet and GFP 36 h later. After 6 d of culturing, cytokine production was analyzed by ICS. Overexpression of T-bet in wild-type Th1 cells, which already expressed a high level of T-bet, further raised the percentage of IFN-\(\gamma\)-producing cells from 33 to 52%. But to our surprise, retroviral T-bet had absolutely no effect on the production of IFN-\(\gamma\) by Ets-1\(^{-/-}\) Th1 cells (Fig. 6 g). This observation indicates that the function of T-bet is severely compromised in the absence of Ets-1 and led us to hypothesize either that Ets-1 may directly or indirectly transactivate the IFN-\(\gamma\) promoter independently of T-bet or that Ets-1 may function as a cofactor enhancing the activity of T-bet.

**Ets-1 is recruited to the IFN-\(\gamma\) promoter and enhances the function of T-bet**

To distinguish between the aforementioned scenarios, we transfected 293T cells with a reporter construct containing the levels of T-bet in developing Ets-1\(^{-/-}\) Th1 cells (Fig. 6 c), indicating intact IFN-\(\gamma\)/STAT1 signaling in Ets-1\(^{-/-}\) Th cells. Next, we examined the levels of T-bet in developing Ets-1\(^{-/-}\) Th1 cells at different time points after primary stimulation with anti-CD3 and anti-CD28. In agreement with the normal IFN-\(\gamma\)/STAT1 signaling and normal early IFN-\(\gamma\) production (Fig. 3 a), differentiating Ets-1\(^{-/-}\) Th1 cells only had a very negligible reduction in the level of T-bet (Fig. 6 d). This was independent of whether IL-12 or IFN-\(\gamma\) were used as Th1-skewing cytokines (Fig. 6 e). Resting differentiated Th1 cells expressed very little T-bet, which was induced by stimulation with PMA/ionomycin in wild-type Th1 cells. Interestingly, the expression of T-bet was markedly impaired in Ets-1\(^{-/-}\) Th1 cells (Fig. 6 f). The defect in T-bet expression upon secondary stimulation may well explain the impaired function of Ets-1\(^{-/-}\) Th1 cells. Therefore, we tested whether restoring the expression of T-bet could overcome the effects of Ets-1 deficiency. Wild-type or Ets-1\(^{-/-}\) Th cells were stimulated under Th1-skewing conditions and infected with a bicistronic retrovirus encoding both T-bet and GFP 36 h later. After 6 d of culturing, cytokine production was analyzed by ICS. Overexpression of T-bet in wild-type Th1 cells, which already expressed a high level of T-bet, further raised the percentage of IFN-\(\gamma\)-producing cells from 33 to 52%. But to our surprise, retroviral T-bet had absolutely no effect on the production of IFN-\(\gamma\) by Ets-1\(^{-/-}\) Th1 cells (Fig. 6 g). This observation indicates that the function of T-bet is severely compromised in the absence of Ets-1 and led us to hypothesize either that Ets-1 may directly or indirectly transactivate the IFN-\(\gamma\) promoter independently of T-bet or that Ets-1 may function as a cofactor enhancing the activity of T-bet.

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The functional synergy between T-bet and Ets-1 may be dependent on their physical interaction. However, we have thus far been unable to demonstrate the existence of an Ets-1/T-bet complex in Th cells by communoprecipitation (unpublished data). Alternatively, Ets-1 may be recruited to the vicinity of the Brachiury site in the IFN-γ promoter without direct physical interaction with T-bet. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) on wild-type Th1 cells using an Ets-1-specific antibody. The precipitated DNA was amplified using primers flanking the Brachiury site (Fig. 7 b, arrows). As shown in Fig. 7 c, both Ets-1 and T-bet were recruited to the proximal region of the endogenous IFN-γ promoter. As a negative control, no recruitment of Ets-1 or T-bet to the second intron/third exon of the Ets-1 gene was detected by ChIP analysis (Fig. 7 c).

Sequence analysis of the mouse IFN-γ promoter did not uncover any conventional Ets-1 binding sites, but revealed a putative binding site for Ets proteins at −66 bp has been shown to be critical for transactivation of this promoter by T-bet (34). As shown in Fig. 7 a (top left, WT), the IFN-γ reporter construct could be transactivated by T-bet more than fivefold compared with the background activity. Overexpression of Ets-1 alone had a negligible effect on the promoter, but significantly enhanced the promoter activity in the presence of T-bet. These results not only demonstrate the functional synergy between T-bet and Ets-1 but also argue that Ets-1 can serve as a cofactor of T-bet. Similar results were obtained when we used a human IFN-γ reporter construct containing the entire human IFN-γ gene (7) in both 293T and EL4 cells (unpublished data).

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Sequence analysis of the mouse IFN-γ promoter did not uncover any conventional Ets-1 binding sites, but revealed a putative binding site for Ets proteins at −23 bp. Similar binding sites were also found in the promoters of the rat and human IFN-γ gene (Fig. 7 b). In addition, a putative IFN-stimulated response element (ISRE) is located at −175 bp. Consensus ISRE sites also contain the GGAA motif and have been reported to bind Ets-1 (35). To determine whether the EBS and/or ISRE were needed to mediate the functional synergy between Ets-1 and T-bet, we generated several IFN-γ promoter/reporters bearing a mutation in the −23 EBS, −175 ISRE, or both sites and repeated the luciferase assays (Fig. 7 a). None of the mutations affected the T-bet–induced transactivation but mutation in the −175 ISRE or both sites nearly completely abrogated the functional synergy between Ets-1 and T-bet. In contrast, mutation in the −23 EBS alone had no effect on the promoter activity. Together, these results depict that Ets-1 is recruited to the proximal region of the IFN-γ promoter and enhances the activity of T-bet via the −175 ISRE.

**DISCUSSION**

The differentiation of Th1 cells and the coordination of in vivo Th1 responses requires a number of important transcription factors, both T cell–specific and more widely expressed (14, 36–40). Our data demonstrate that Ets-1 promotes the differentiation and function of Th1 cells by several nonmutually exclusive mechanisms. Ets-1 is required for proper up-regulation of IL-12Rβ2 and STAT4 during the differentiation of Th1 cells. However, the differentiation and function of Ets−1−/− Th1 cells is still markedly compromised in an IL-12/STAT4-free system, arguing against a decisive role of IL-12/STAT4, at least in vitro. Ets-1 also controls the level of T-bet in Th1 cells in a differentiation stage-dependent manner. Deficiency of Ets-1 only negligibly affects the expression of T-bet in differentiating Th1 cells, but significantly impairs the induction of T-bet in fully differentiated Th1 cells. However, forced expression of T-bet through retroviral transduction did not rescue the differentiation and function of Ets−1−/− Th1 cells. These observations led us to discover that Ets-1 is required for the function of T-bet. In the absence of Ets-1, the IFN-γ–promoting effect of T-bet is severely impaired. Thus, Ets-1 can be considered as a functional cofactor of T-bet.

IL-2/STAT5 was recently shown to influence the differentiation of Th cells (41). Ets−1−/− Th cells produced a low level of IL-2 even during primary stimulation, raising the possibility that the aberrant differentiation of Ets−1−/− Th cells may be at least partly attributed to a low level of IL-2. To rule out this possibility, we repeated the in vitro differentiation, adding recombinant IL-2 at the very beginning instead of 24 h after stimulation. We still observed the same defect in cytokine production by Ets−1−/− Th cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20041330/DC1). Thus, Ets-1 does not influence the differentiation of Th cells indirectly through IL-2/STAT5. As retroviral T-bet could not rescue the production of IFN-γ by Ets−1−/− Th1 cells, we believe that the lack of T-bet/Ets-1 synergy is the main, but not the only, reason for the defective IFN-γ production by Ets−1−/− Th1 cells. To the best of our knowledge, Ets-1 is thus far the only transcription factor that is necessary for T-bet to exert its function.

Although our data indicate that the functional synergy between T-bet and Ets-1 is mediated by the −175 ISRE, we have not completely ruled out the possibility that Ets-1 directly interacts with T-bet. Ets-1 has been found to physically interact with several nuclear proteins, including NFAT, AP1, and STAT5 (23, 42, 43), which can modulate the activity of Ets-1. The physical interaction between Ets-1 and T-bet may be too weak to be detected by communoprecipitation. We would also like to point out that the interplays between T-bet and Ets-1 may exceed the IFN-γ gene and Th1 differentiation. Both Ets-1 and T-bet are essential for the development of NKT cells (44, 45). This observation suggests the presence of other common target genes. Alternatively, Ets-1 could also regulate the expression of T-bet in NKT cells. Unfortunately, the tissue-specific and/or functionally critical cis-acting elements of the T-bet gene have not been identified, precluding further analyses on how Ets-1 may regulate the expression of T-bet.
Why are Ets-1−/− Th cells unable to induce colitis in SCID mice? The development of colitis in SCID mice depends on the production of IFN-γ and TNF-α by transferred Th cells and can be inhibited by IL-10 (31, 46). The cytokine profile of Ets-1−/− Th cells recovered from SCID mice is obviously counterproductive for the development of colitis. However, it is still unclear whether it is the reduction in IFN-γ, overproduction of IL-10, or a combination of both that protects the SCID mice from developing colitis. It has been shown that Th cells engineered to produce IL-10 do not induce colitis in a model similar to the one we used (46). One approach to addressing this question is the generation of Ets-1/IL-10 double-deficient mice. If the overproduction of IL-10 is responsible for the inability of Ets-1−/− Th cells to induce colitis, CD45RB<sup>high</sup> Th cells from these double knockout mice should be able to induce colitis. More recently, it has been demonstrated that regulatory T cells are capable of inhibiting colitis induced by CD45RB<sup>high</sup> Th cells in SCID mice (47–49). Ets-1−/− Th cells bear several features of the type 1 regulatory T cells, including overproduction of IL-10 and poor production of IL-2, raising an intriguing possibility that Ets-1−/− Th cells possess the activity of regulatory T cells. This possibility is currently under investigation.

We have yet to determine how Ets-1 suppresses the expression of IL-10. The regulation of IL-10 expression in Th cells is not well understood (30). It has been shown that the expression of IL-10 can be controlled both at the transcriptional level by Sp1 and Sp3, and at the posttranscriptional level by mRNA stability (50, 51). Our unpublished data indicate that Ets-1 suppresses the expression of IL-10 at least partly at the transcriptional level. It is known that IFN-γ can suppress the expression of Th2 cytokines, including IL-10, raising the possibility that the overproduction of IL-10 by Ets-1−/− Th cells may be secondary to a low level of IFN-γ. However, the following observations argue strongly against this scenario. First, in Fig. 3 a, we show that differentiating Ets-1−/− Th1 cells already produced much more IL-10 after primary stimulation, when the production of IFN-γ was normal. Second, Ets-1−/− Th1 still produced more IL-10 than wild-type cells when differentiated in the presence of IFN-γ instead of IL-12 (Fig. S4 a, available at http://www.jem.org/cgi/content/full/jem.20041330/DC1) or when restimulated with PMA/ionomycin plus IFN-γ (Fig. S4 b).

Although Ets-1−/− Th2 cells obtained by in vitro differentiation also produced less IL-4, Ets-1−/− Th cells recovered from the SCID mice were capable of expressing IL-4 at a level comparable to that of wild-type Th cells. This discrepancy may arise from the differences in microenvironment between in vitro (as in the in vitro differentiation) and in vivo (as in the SCID colitis model) priming or in mode of stimulation. Thus, the effect of Ets-1 deficiency on the function of Th2 cells in vivo is still unclear and warrants further investigation.

In summary, we found that Ets-1 has a very broad and profound influence on Th immune responses. Ets-1 not only is essential for mounting Th1 inflammatory responses but also suppresses the expression of the antiinflammatory cytokine IL-10. The ability of Ets-1 to influence the decision between inflammatory and antiinflammatory Th immune responses makes it a potential therapeutic target for various Th1-mediated autoimmune diseases, such as Crohn’s disease or multiple sclerosis.

**MATERIAL AND METHODS**

**Mice.** Ets-1−/− mice have been described previously (18). Heterozygous mice on a mixed C57BL/6 and 129SV background were intercrossed to generate Ets-1−/− and wild-type littermate control mice. For antigen-specific stimulation, the mice were backcrossed to DO11.10 mice (24) for four generations before being intercrossed. The animals were housed under specific pathogen-free conditions, and experiments were performed in accordance with the institutional guidelines for animal care at Dana-Farber Cancer Institute under approved protocols. Male or female mice aged 6–8 wk were used. In all experiments, wild-type littersmates were used as controls. For the colitis model, female, 6–8-wk-old B6-CB17-Prkd<sup>−/−</sup> mice were obtained from The Jackson Laboratory as were DO11.10 mice.

**Cell purification and in vitro differentiation of Th cells.** CD4<sup>+</sup> T cells were purified from spleens and lymph nodes by magnetic cell sorting (Miltenyi Biotech). For most experiments, total CD4<sup>+</sup> T cells were purified using anti-CD4–coupled magnetic beads. For some experiments, naive CD4<sup>+</sup> T cells were purified in two steps. First, total CD4<sup>+</sup> T cells were isolated using FITC-labeled anti-CD4 (BD Biosciences) and anti-FITC–coupled beads (Miltenyi Biotech). The beads were enzymatically released, and naive CD4<sup>+</sup> T cells were enriched using anti-CD62L–coated magnetic beads (Miltenyi Biotech). The purity of the isolated CD4<sup>+</sup>CD62L<sup>high</sup> cells was >90%. The cells (2 × 10<sup>5</sup>/ml) were stimulated with 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 under Th1-skewing (3 ng/ml IL-12 plus 10 μg/ml anti–IL-4) or Th2-skewing (10 ng/ml IL-4 plus 10 μg/ml anti–IFN-γ) conditions. In some experiments, IL-12 was replaced with 10 ng/ml IFN-γ. For IL-10–blocking experiments, 10 μg/ml anti–IL-10R was added on day 0 and again on day 2. 100 U/ml recombinant human IL-2 (rhIL-2) was added after 24 h, and the cells were expanded in complete medium containing IL-2 for 7 d. On day 7, the cells were restimulated with 50 ng/ml PMA and 1 μM ionomycin. The production of cytokines was examined by ICS. For antigen-specific stimulation, 2.5 × 10<sup>5</sup> CD4<sup>+</sup> T cells from wild-type or Ets-1-deficient DO11.10 mice were stimulated with 5 × 10<sup>4</sup> irradiated (3,000 rad) splenic APCs obtained from BALB/c mice and 0.3 μM OVA<sub>323–339</sub> under Th1- or Th2-skewing conditions. Skewing conditions were the same as with the anti-CD3–stimulated cultures, except for the use of anti–IL-12 instead of anti–IFN-γ for Th2 cells. IL-2 was added after 72 h, and the cells were cultured for a total of 7 d before their cytokine profile was tested. Recombinant human IL-2 and anti–IL-4 (11B11) were provided by the National Cancer Institute, Preclinical Repository. IL-4 and IL-12 were purchased from PeproTech, NA/LE-grade anti–IFN-γ (XMG1.2), anti–CD3 (2C11), anti–CD28 (37.51), anti–IL-12 (C17.8), and anti–IL-10R (1B1.3a) were obtained from BD Biosciences.

**ICS.** The protocol for ICS has been described previously (10). The following antibodies were purchased from BD Biosciences: anti–IL-2 FITC (JES6-5H4), anti–IL-4 PE (11B11), anti–IL-5 PE (TRFK5), anti–IL-10 FITC, or PE (JESS-16E3), and anti–IFN-γ FITC (XMG1.2). The stained cells were subjected to flow cytometric analysis on a FACScan (BD Biosciences) and analyzed with CELLQuest software.
ELISA. Freshly isolated or differentiated Th cells were stimulated as indicated. Cytokines were quantified by sandwich ELISA using the following monoclonal antibody pairs (BD Biosciences): anti–IL-2 (JES6-1A12)/biotin–anti–IL-2 (JES6-5H4), anti–IL-4 (11B11)/biotin–anti–IL-4 (BVD6-24G2), anti–IL-10 (JES5-2A5)/biotin–anti–IL-10 (JES5-16E3), and anti–IFN–γ (R4-6A2)/biotin–anti–IFN–γ (XMG1.2).

SCID colitis model. Spleen and lymph node cells from female wild type or Ets-1−/− mice were labeled with anti–CD45+PE (RM4-5) and anti–CD45RB-FITC (16A). CD4+CD45RB+Bw6 Th cells were sorted using a Mo-Flo cell sorter. 8-wk-old female B6-CB17-PkdcScid mice were injected intravenously with 3 × 10^5 sorted cells per mouse. The injected mice were weighed weekly for 10 wk before being killed. The large intestines of the host animals were fixed with 10% formalin and paraffin embedded, and the retroviral transduction of Th cells. The protocol for retroviral transduction of Th cells has been described previously (25). In brief, differentiating Th cells were infected ~36 h after primary stimulation. The infected cells were washed once, replated in fresh complete skewing medium, and cultivated for another 6 d before analysis. The bicistronic vectors GFP-RV and GFP-RV/T-bet were described previously (26) and were gifts from G. Nolan (Stanford University, Stanford, CA) and L. Glimcher (Harvard School of Public Health, Boston, MA), respectively (7). GFP-RV/Ets-1 was constructed by cloning a full-length murine Ets-1 cDNA into the BamHI site of pcDNA3 Flag (provided by J. Leiden, Abbott Laboratories, Chicago, IL), resulting in an NH2 terminally FLAG-tagged Ets-1 cDNA into the BamHI site of pcDNA3 Flag (provided by J. Leiden, Abbott Laboratories). The following antibodies were used for precipitation: anti–Ets-1 (a gift from L. Glimcher). To amplify the IFN–γ promoter region and antisense: 5′-GGAGAGATCGAAATCTTGAGGCC-3′ and 5′-GATATCCACACACCGTGAAGCC-3′. Western blot analysis. Nuclear extracts were obtained from CD4+TCRβ+ cells by nuclei extraction using an ECL cell lysis buffer (PerkinElmer).

Luciferase assays and site-specific mutagenesis. For luciferase assays, the adenovirus-transformed human kidney cell line 293T was cultured in DMEM plus 10% FCS. 1 d before transfection, the cells were seeded into 24-well plates at 10^5 cells/well. The next day, cells were transfected using the Effectene kit (Qiagen). Per transfection, 50 ng of the IFN–γ reporter plasmid pGL3-mIFNγ564-Luc, 2 ng of the Renilla luciferase control vector pRL-TK (Promega), and 50 or 200 ng of the expression vectors encoding T-bet or Ets-1, respectively, or empty control vectors were used. 1 d after transfection, the cells were harvested and the cell lysates were assayed for luciferase activity using the Dual Luciferase Reporter System (Promega) according to the manufacturer’s instructions. The IFN–γ reporter pGL3-mIFNγ564-Luc, a gift from M. Townsend (Harvard School of Public Health, Boston, MA) and L. Glimcher, contains 564 bp upstream of the mouse IFN–γ transcriptional start site and 112 bp downstream up to the ATG. The T-bet expression vector pcDNA3 Flag-Ets1 was constructed by inserting a full-length murine Ets-1 into the BglII site of the GFP-RV vector. The T-bet expression vector pcDNAHis-Tbet was a gift from L. Glimcher and was described previously (7). Site-specific mutagenesis of po-