

Down-regulation of Gfi-1 expression by TGF- β is important for differentiation of Th17 and CD103⁺ inducible regulatory T cells

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Growth factor independent 1 (Gfi-1), a transcriptional repressor, is transiently induced during T cell activation. Interleukin (IL) 4 further induces Gfi-1, resulting in optimal Th2 cell expansion. We report a second important function of Gfi-1 in CD4 T cells: prevention of alternative differentiation by Th2 cells, and inhibition of differentiation of naive CD4 T cells to either Th17 or inducible regulatory T (iTreg) cells. In *Gfi1*^{-/-} Th2 cells, the *Rorc*, *Il23r*, and *Cd103* loci showed histone 3 lysine 4 trimethylation modifications that were lacking in wild-type Th2 cells, implying that Gfi-1 is critical for epigenetic regulation of Th17 and iTreg cell-related genes in Th2 cells. Enforced Gfi-1 expression inhibited IL-17 production and iTreg cell differentiation. Furthermore, a key inducer of both Th17 and iTreg cell differentiation, transforming growth factor β , repressed Gfi-1 expression, implying a reciprocal negative regulation of CD4 T cell fate determination. Chromatin immunoprecipitation showed direct binding of the Gfi-1–lysine-specific demethylase 1 repressive complex to the intergenic region of *Il17a/Il17f* loci and to intron 1 of *Cd103*. T cell-specific *Gfi1* conditional knockout mice displayed a striking delay in the onset of experimental allergic encephalitis correlated with a dramatic increase of Foxp3⁺CD103⁺ CD4 T cells. Thus, Gfi-1 plays a critical role both in enhancing Th2 cell expansion and in repressing induction of Th17 and CD103⁺ iTreg cells.

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Abbreviations used: ChIP, chromatin immunoprecipitation; cKO, conditional KO; CNS, central nervous system; EAE, experimental allergic encephalitis; Gfi-1, growth factor independent 1; H3K4, histone 3 lysine 4; iTreg, inducible Treg; LSD1, lysine-specific demethylase 1; RV, retroviral vector.

Upon T cell activation, naive CD4⁺ T cells can differentiate into distinct cell lineages, including the classical Th1 and Th2 cells (1, 2), as well as the recently defined Th cells producing IL-17 (Th17 cells) (3) and inducible Treg (iTreg) cells (4). These cells play important roles in adaptive immunity (5). Th1 cells mainly produce IFN- γ and are involved in immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13, are involved in immunity against helminths and other extracellular parasites, and enhance antibody responses. Th17 cells produce IL-17a, IL-17f, IL-21, and IL-22, and are critical for immunity against extracellular bacteria and fungi. Th1, Th2, and Th17 cells are also involved in the induction or maintenance of autoimmune and immunopathologic diseases. Thus, Th1 and Th17 cells are linked to many organ-specific autoimmune diseases, and Th2 cells play critical

roles in asthma and other allergic diseases. iTreg cells, functionally similar to naturally occurring Treg cells (6), can regulate immune responses. Among naturally occurring Treg cells, a subset expressing CD103 has been reported to contain the highest suppressive activity and to be present in the mucosal tissues, such as in the gut, as well as at sites of inflammation (7, 8). Interestingly, a large number of CD4 cells differentiated in the presence of TGF- β in vitro express CD103 (9), and TGF- β has been shown to induce Treg cell differentiation (4).

The cytokine milieu during CD4 T cell activation is one of the most important factors in determining T cell fate. IL-12 and IFN- γ induce

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Th1 cell differentiation (10–12); IL-4 and IL-2 induce Th2 cell differentiation (13–16), TGF- β and IL-6/IL-21 induce Th17 cell differentiation (17–19), and TGF- β and IL-2 induce iTreg cell generation (4, 20). Although several transcription factors are involved in the differentiation and maintenance of each Th cell type, Th cell differentiation is mainly controlled by specific master transcription factors and Stat family members: T-bet and Stat4 for Th1 cells (21–23), GATA-3 and Stat5 for Th2 cells (14, 24–27), ROR γ t and Stat3 for Th17 cells (28, 29), and Foxp3 and Stat5 for Treg cells (4, 20, 30, 31).

Growth factor independent 1 (Gfi-1), a transcriptional repressor, was initially reported as a protooncogene that allows an IL-2-dependent cell line to be IL-2 independent (32). Gfi-1 was also found as a common insertion site in Moloney murine leukemia virus-induced T lymphomas (33, 34). During Th2 cell differentiation, the expression levels of GATA-3 are enhanced as a result of TCR-mediated signaling and/or activation of the IL-4–Stat6 pathway (35, 36). In collaboration with Stat5, activated by IL-2, GATA-3 induces remodeling of the *Il4* locus and the capacity to produce IL-4 when cells are stimulated through TCRs. Gfi-1 is transiently induced by TCR stimulation (37, 38). Its expression is further enhanced and prolonged by IL-4, resulting in optimal Th2 cell proliferation (37). Gfi-1 selects GATA-3^{hi} cells to grow in IL-2, suggesting that there is a selective component involving Gfi-1 in Th2 cell differentiation (37).

Three groups, including ours, have generated germline *Gfi1* KO mice (39–41). Many abnormalities have been observed, including neutropenia (39, 40), a T cell development defect (41, 42), a hematopoietic stem cell defect (43, 44), and defects in dendritic cell development and function (45). The profound defect in T cell development in *Gfi1*^{-/-} mice is associated with a large proportion of their peripheral CD4 T cells displaying memory-like phenotypes (40), presumably as a result of lymphopenia-driven cell proliferation and differentiation.

To analyze the role played by Gfi-1 in the differentiation and/or growth of normal CD4 T cells, we generated *Gfi1* conditional KO (*Gfi1* cKO) mice (41) in which Gfi-1 is deleted only in T cells. Th2 cells differentiated from *Gfi1* cKO mice have a severe defect in cell expansion. In vivo, the Th2 response is dramatically impaired in these KO mice. On the other hand, IFN- γ production is enhanced in the absence of Gfi-1, raising the possibility that Gfi-1 may negatively regulate the differentiation of other Th cell lineages. We report that Gfi-1 suppresses both Th17 and CD103⁺ iTreg cell differentiation, whereas TGF- β , critical for Th17 and iTreg cell induction, down-regulates Gfi-1 expression. In the absence of Gfi-1, Foxp3⁺CD103⁺ Treg cells are preferentially expanded in vivo upon immunization.

RESULTS

Th2 cells lacking *Gfi1* display an active genomic configuration at the *Cd103*, *Rorc*, and *Il23r* loci

Th2 cells differentiated from *Gfi1* cKO (*Gfi1*^{fl/fl}-CD4Cre) mice have a severe defect in cell expansion and also produce

some IFN- γ , suggesting that Gfi-1 is important for sustaining the Th2 cell phenotype. A recent report suggests that Gfi-1, as a transcriptional repressor, is associated with the histone lysine-specific demethylase 1 (LSD1), which results in demethylation of histone 3 lysine 4 (H3K4) at target promoters (46). LSD1 demethylates mono- and dimethylation but not trimethylation of H3K4. However, because of the balance of different methylation states, active gene promoter regions usually contain higher levels of H3K4 trimethylation than of mono- and dimethylation (47). To search for Gfi-1 target genes in T cells, the chromatin immunoprecipitation (ChIP)-Seq technology was applied. We used H3K4 trimethylation, the end product of H3K4 methylation, as an indicator, reasoning that such modification would not accumulate in *Gfi1*^{-/-} cells at Gfi-1 target sites because of a lack of the mono- or dimethylated forms of H3K4. As expected, both wild-type and *Gfi1* cKO Th2 cells displayed a similar pattern of H3K4 trimethylation at the *Gata3* locus (Fig. 1 A). Because H3K4 methylation is associated with active chromatin, this indicates that Gfi-1, although important for Th2 expansion, is not essential for the Th2 polarization process. Interestingly, at the *Cd103* locus, whose expression is associated with a subset of Treg cells, *Gfi1* cKO Th2 cells showed high levels of H3K4 modification that was absent in wild-type Th2 or Th17 cells (Fig. 1 B). At the *Rorc* locus, which encodes ROR γ t, critical for Th17 cell differentiation, *Gfi1* cKO Th2 cells displayed levels of H3K4 modification similar to Th17 cells (Fig. 1 C). H3K4 modification was also detected at the *Il23r* locus in *Gfi1* cKO Th2 cells (Fig. 1 D). Such a modification was absent in wild-type Th2 cells. *Gfi1* cKO Th2 cells did not appear to be contaminated with Th17 cells, because H3K4 trimethylation at the *Il21* and *Il17f* loci was not observed in the *Gfi1* cKO Th2 cells, although it was prominent in Th17 cells (Fig. 1, E and F). These results indicate that Gfi-1 plays an important role in chromatin remodeling of Th17 and iTreg cell-related genes, including *Rorc*, *Il23r*, and *Cd103*, in Th2 cells. Although *Gfi1* cKO Th2 cells displayed an active *Rorc* locus, expression of ROR γ t in *Gfi1* cKO Th2 cells is much lower than that in Th17 cells (unpublished data), indicating that additional factors, possibly induced by TGF- β and IL-6, are required for *Rorc* expression as well as for the induction of IL-17.

Expression of Gfi-1 induced by IL-4 inhibits IL-17 production

To test the significance of the chromatin modification change at the *Rorc* locus, wild-type or *Gfi1* cKO CD4 T cells were primed under Th2 conditions for one round, and cells were then either restimulated under Th2 conditions or switched to Th17 priming conditions. *Gfi1* cKO Th2 cells expanded less well than wild-type Th2 cells, as previously reported (41); the expansion defect of *Gfi1* cKO Th2 cells was smaller when they were cultured under Th17 conditions (Fig. 2 A). In the wild-type group, 24.5% of the cells from the culture restimulated under Th2 conditions

produced IL-4, whereas, only 8% of the cells that were switched from Th2 to Th17 conditions produced IL-4, implying that TGF- β suppresses IL-4 production in differentiated Th2 cells (Fig. 2 A). Nevertheless, neither cell population was capable of producing IL-17, suggesting that TGF- β and/or IL-6 is not effective in inducing IL-17 production in differentiated Th2 cells. In contrast, in the *Gfi1* cKO group, although IL-4 production was also reduced (from 16.3 to 5.6%) when cells were switched from Th2 to Th17 conditions, 8.2% of the switched cells were capable of making IL-17. These data indicate that *Gfi1* expression in Th2 cells, induced by IL-4, prevents the induction of IL-17 by TGF- β and IL-6.

To exclude the possibility that the induction of IL-17 in *Gfi1* cKO cells is from a small fraction of non-Th2 cells, we bred our *Gfi1* cKO mice to heterozygous G4 mice, in which one *Il4* allele was replaced by *eGfp* (48). After one round of Th2 priming, GFP⁺ cells (Th2 cells) were sorted and re-stimulated under Th2 or Th17 conditions. Similar to the results shown in the previous paragraph, 6.5% of the GFP⁺ cells from the *Gfi1* cKO group were able to produce IL-17 after reculturing under Th17 conditions. Again, the effect of *Gfi1* in cell expansion under Th17 culture conditions was minimal (Fig. 2 B).

IL-4 has been reported to suppress the induction of IL-17 production (49, 50). The detailed mechanism is not clear. To

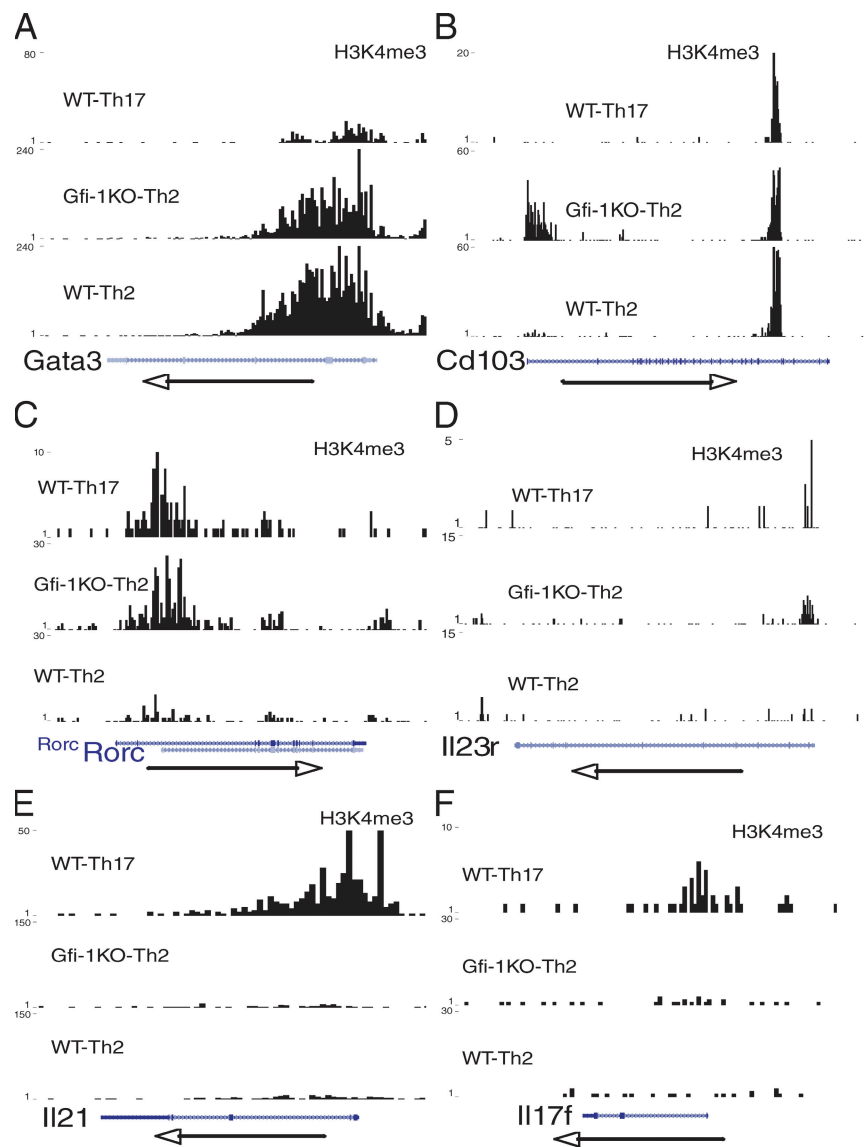


Figure 1. Abnormal histone H3K4 trimethylation at the *Cd103*, *Rorc*, and *Il23r* loci in *Gfi1* cKO Th2 cells. CD4⁺ T cells from naive wild-type or *Gfi1* cKO mice were activated under Th2 or Th17 conditions for 4 d. Cells were harvested and fixed. ChIP-Seq analysis was performed using a specific antibody against H3K4 trimethylation (H3K4me3). The sequence tags mapped to the locus of (A) *Gata3*, (B) *Cd103*, (C) *Rorc*, (D) *Il23r*, (E) *Il21*, and (F) *Il17f* are shown. The total tag numbers mapped to the genome are 2, 6, and 7.1 million for WT-Th17, *Gfi1* cKO-Th2, and WT-Th2, respectively. The scale for the WT-Th17 sample was adjusted separately because of approximately threefold fewer total tag numbers.

test whether Gfi-1 mediates the suppression of IL-17 by IL-4, we performed a Th17 to Th2 switching experiment. Indeed, IL-4 suppressed IL-17 production (from 3.7 to 0.5%) and induced IL-4 production (from 0.1 to 6.4%) in wild-type cells (Fig. 2 C), but IL-4 failed to suppress IL-17 production in *Gfi1* cKO cells even though it still induced its own production. *Gfi1* cKO cells showed an approximately twofold decrease in cell expansion, compared with wild-type cells, when switched from Th17 to Th2 conditions. Strikingly, ~1% of the cells can produce both IL-17 and IL-4 when *Gfi1* cKO Th17 cells were switched to Th2 conditions, suggesting that Gfi-1 plays an important role in IL-4-mediated suppression of IL-17 production.

Gfi-1 expression is down-regulated by TGF- β

Because Gfi-1, induced by IL-4, has an effect on both ROR γ t and IL-17, we tested the normal expression levels of Gfi-1 during Th17 cell differentiation. Gfi-1 expression was assessed by quantitative PCR after naive CD4 T cells were primed under Th1, Th2, Th17, and iTreg cell conditions for 4 d. As expected, cells primed under different conditions specifically expressed their hallmark genes (IFN- γ for Th1 cells, IL-4 for Th2 cells, IL-17a for Th17 cells, and Foxp3 for iTreg cells; Fig. 3 A). Th2 cells expressed approximately twice as much Gfi-1 as Th1 cells, as previously reported (37). Th17 and iTreg cells expressed very low levels of Gfi-1. To test when the difference in Gfi-1 expression

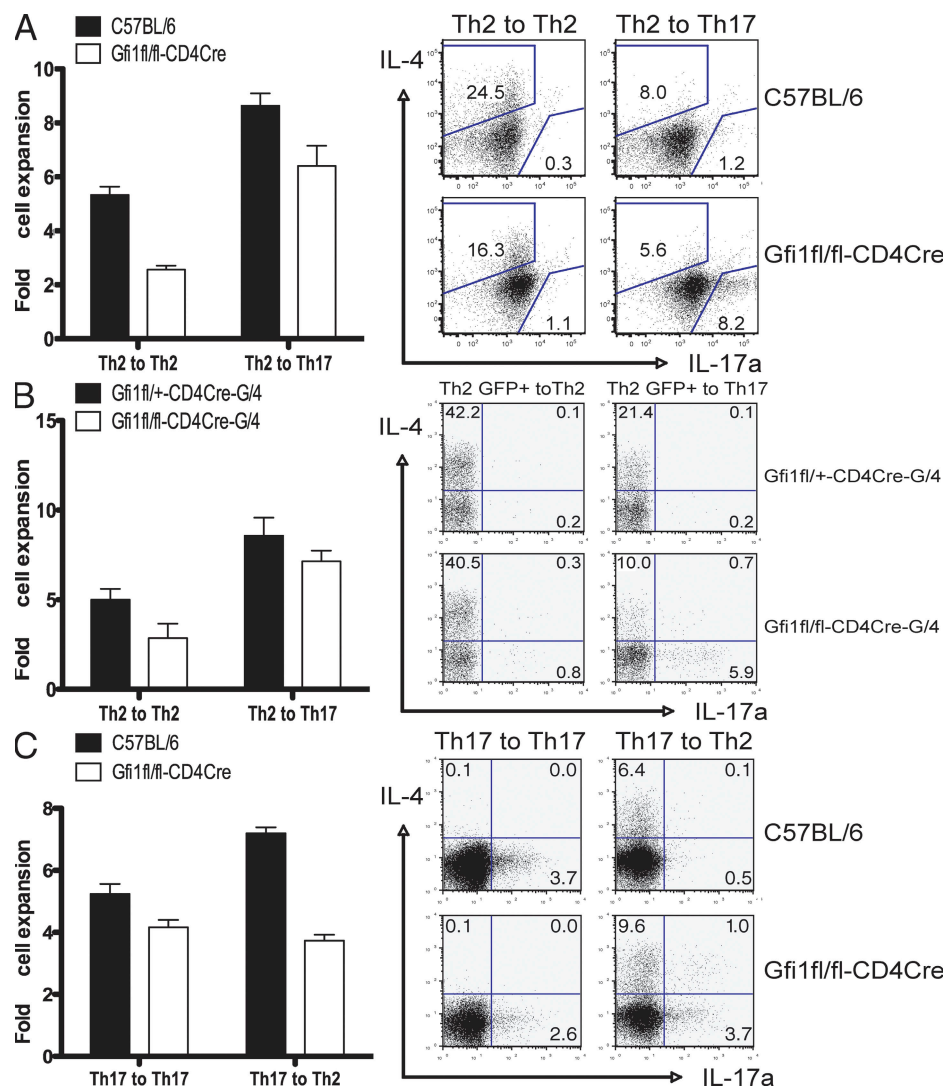


Figure 2. Endogenous Gfi-1 induced by IL-4 suppresses IL-17 production. (A and B) CD4⁺ T cells from naive mice were activated under Th2 conditions for 4 d. After 2 d, cultured in complete RPMI 1640 with IL-2, cells were either (A) unsorted or (B) sorted for GFP⁺ (IL-4-producing) cells and were further stimulated under Th2 or Th17 conditions for another 4 d. (C) CD4⁺ T cells were first activated under Th17 conditions before they were stimulated under Th2 or Th17 conditions for the second round. Cells were counted before and after the second round of stimulation. Fold cell expansion was calculated. Cytokine production was assessed by intracellular staining after PMA plus ionomycin stimulation. Numbers indicate the percentage of cells in each quadrant or gate. Error bars represent means \pm SD, and data are representative of two independent experiments.

occurred during the differentiation process, cells were harvested at various time points after priming under different conditions. As early as 24 h after stimulation, Gfi-1 was strikingly underexpressed in cells primed under Th17 and iTreg cell conditions (Fig. 3 B).

IL-4 rapidly induces Gfi-1 expression in activated but not in naive CD4 T cells. As reported earlier (37), cells previously activated in the presence of anti-IL-4 for 24 h expressed Gfi-1 mRNA in response to IL-4 stimulation within 2 h (Fig. 3 C). In the presence of TGF- β , IL-4 failed to up-regulate Gfi-1. In addition, treatment with TGF- β suppressed the basal level of Gfi-1 expression, suggesting that TGF- β is directly involved in suppressing Gfi-1 expression. In contrast, TGF- β did not block the induction of suppressor of cytokine signaling 1 by IL-4, suggesting the specificity of Gfi-1 down-regulation by TGF- β . It did not appear that the TGF- β treatment mediated its effects by increasing cell death. No major differences in cell yields were observed, and there were no differences in the ex-

pression levels of Bcl-2 and Bim. The suppression of Gfi-1 expression by TGF- β may explain the low levels of Gfi-1 expression in cells that have undergone Th17 and iTreg cell differentiation.

Expression of Gfi-1 inhibits IL-17 production but not induction of ROR γ t in cells primed with TGF- β plus IL-6

To test the importance of Gfi-1 down-regulation by TGF- β , Gfi-1 was introduced by retroviral infection into activated CD4 T cells primed under Th17 conditions. 4 d after infection, IL-17 production was assessed upon restimulation with PMA plus ionomycin. A similar proportion of the cells infected by control GFP-retroviral vector (RV) and uninfected cells, in the same culture, produced IL-17 (17.8 vs. 15%; Fig. 4 A, left). However, only 3.9% of the cells infected by the Gfi-1-GFP-RV, compared with 14.1% of the uninfected cells in the same culture, produced IL-17. Three independent experiments showed significant suppression of IL-17 production by Gfi-1 (Fig. 4 A, right).

Because ROR γ t has been reported to be the master regulator for Th17 cells and TGF- β plus IL-6 are required for Th17 cell differentiation (17–19, 28), we tested the expression of ROR γ t, TGF- β Rs, and IL-6R α among Th1, Th2, and Th17 cells. ROR γ t was selectively expressed in Th17 cells, consistent with previous reports (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20081666/DC1>). Interestingly, both TGF- β R1 and TGF- β R2 were expressed at higher levels in Th17 cells than in Th1 and Th2 cells. IL-6R α was also found to be highest in Th17 cells (Fig. S1 A). 3 d after infection with Gfi-1-GFP-RV, although expression of ROR γ t, TGF- β R1, and TGF- β R2 in infected cells was not affected (Fig. 4 B and Fig. S1 B), expression of IL-6R α was suppressed (Fig. S1 B). Consistent with the intracellular staining results shown in Fig. 4 A, both IL-17a and IL-17f mRNA levels were reduced by enforced expression of Gfi-1 (Fig. 4 B). Enforced Gfi-1 expression in *Gfi1* cKO Th17 cells also suppressed IL-17a but not ROR γ t expression (Fig. S2). Suppression of IL-17 requires the Gfi-1 suppressive domain, because Gfi-1 bearing a single mutation in this domain (Gfi-1 (P2A)) failed to suppress IL-17 production (Fig. 4 C).

The failure of Gfi-1 to suppress ROR γ t transcription in this setting is apparently at odds with the genomic data shown in Fig. 1, in which the *Rorc* locus is not H3K4 trimethylated in wild-type Th2 cells but is trimethylated in *Gfi1* cKO Th2 cells. However, this discrepancy may be explained by the fact that retroviral-mediated expression of Gfi-1 does not occur until 24–48 h after infection, whereas ROR γ t up-regulation by TGF- β occurred within 24 h (unpublished data). Alternatively, Gfi-1 may require another Th2 factor as a cosuppressor of ROR γ t transcription. Nevertheless, the data indicate that Gfi-1 suppresses IL-17 even when ROR γ t is normally expressed.

To test the direct binding of Gfi-1 to its putative target genes, ChIP using anti-Gfi-1 antibody was performed. The Gfi-1 DNA binding sites contain AATC, and sequences containing AAATCANNNG are represented at the highest frequency in a random oligonucleotide selection assay, suggesting

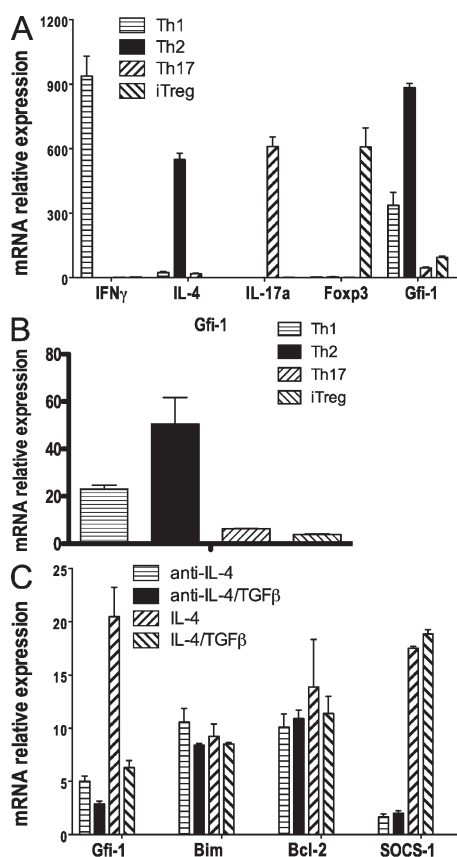


Figure 3. Gfi-1 expression is down-regulated by TGF- β . CD4⁺ T cells from naive mice were activated under Th1, Th2, Th17, or iTreg conditions for (A) 4 d or (B) 24 h, or (C) under ThN (nonpolarization) conditions for 24 h followed by cytokine stimulation for 2 h. Cells were harvested and total RNAs were isolated. The relative expression levels of different genes normalized to GAPDH were assessed by real-time PCR after reverse transcription. Error bars represent means \pm SD, and data are representative of two independent experiments.

that such sequences may have particularly high binding affinity for Gfi-1 (51). Eight putative Gfi-1 binding sites containing AAATCANNNG or its complement CANNNGATTT, including the *Rorc* promoter (*Rorc*p) from the *Rorc* locus and nine sites from the *Il17a/Il17f* loci along with *Gfi1* promoter (*Gfi1*p) as a positive control (52), were tested for Gfi-1 binding. Only the *Gfi1*p region near the GAAAATCGAAGTA

sequence and an intergenic region of *Il17a/Il17f* near the AGAAATCTCAGGG sequence but not the other sites tested were enriched by the anti-Gfi-1 antibody in this assay (Fig. 4 D, left). Consistent with Gfi-1 binding, this *Il17a/Il17f* intergenic region also recruited LSD1 in wild-type cells, and the binding of LSD1 to this site was not found in the absence of Gfi-1 (Fig. 4 D, right).

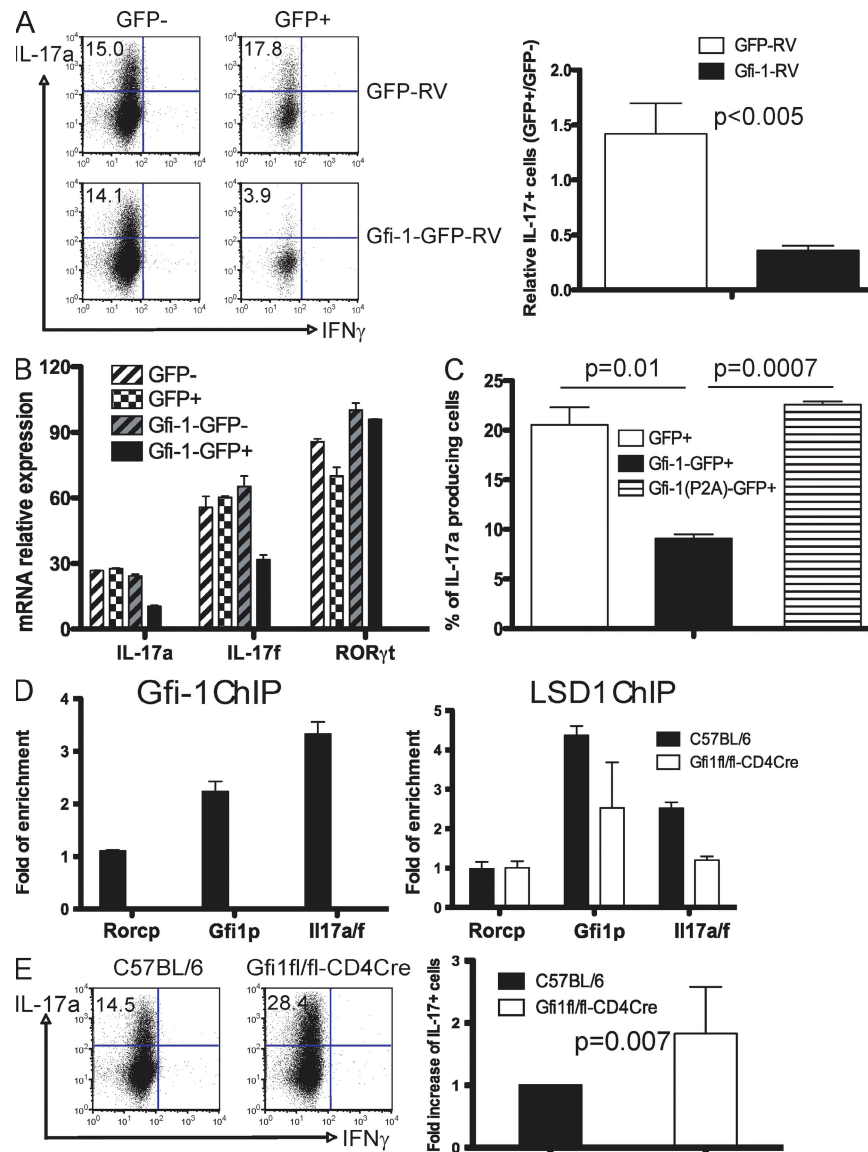


Figure 4. Gfi-1 binds to the *Il17a/Il17f* intergenic region and inhibits IL-17a and IL-17f expression. CD4⁺ T cells from naive mice were activated under neutral conditions for 24 h and infected with (A–C) GFP-RV, (A–C) Gfi-1-GFP-RV, (C) or Gfi-1(P2A)-GFP-RV. (A and C) Infected cells were further primed under Th17 conditions for 4 d. Cytokine production was assessed by intracellular staining after PMA plus ionomycin stimulation. (A) Numbers indicate the percentage of cells in each quadrant (left). Relative IL-17-producing cells (GFP⁺/GFP[−]) pooled from three independent experiments are shown (right). (B) GFP⁺ and GFP[−] cells were sorted 24 h after infection. Sorted cells were maintained under Th17 conditions for another 2 d. Cells were harvested and total RNAs were isolated. The expression levels of different genes normalized to GAPDH were assessed by real-time PCR after RT. (C) The percentage of IL-17-producing cells from each GFP⁺ group was plotted. (D) Gfi-1-GFP-RV-infected Th17 cells were used for Gfi-1 ChIP (left). Wild-type and *Gfi1* cKO Th2 cells were used for LSD1 ChIP (right). The enrichment of individual sites was normalized to a negative control from *Gata3*-intron3. (E) CD4 T cells from naive wild-type or *Gfi1* cKO mice were primed under Th17 conditions for 4 d. Cytokine production was assessed by intracellular staining after PMA plus ionomycin stimulation. Numbers indicate the percentage of cells in each quadrant (left). Data from nine independent experiments are shown (right). Error bars represent means \pm SD.

When CD4 T cells from *Gfi1* cKO mice were primed under Th17 conditions, a higher percentage of the *Gfi1* cKO cells than of wild-type cells produced IL-17 (28.4 vs. 14.5%, Fig. 4 E, left). In a series of nine independent experiments, there was a highly statistically significant increase in IL-17-producing cells among *Gfi1* cKO Th17 cells compared with wild-type Th17 cells (Fig. 4 E, right). These data indicate that endogenous expression of Gfi-1 in Th17 cells, even at low levels, is sufficient to modulate IL-17 production. Indeed, because Gfi-1 can be induced transiently by TCR stimulation even in Th17 cells (unpublished

data), such induction may serve as a negative regulator of IL-17 production.

Gfi-1 limits TGF- β -mediated differentiation of iTreg cells in vitro

Because Gfi-1 is also underexpressed in iTreg cells, we tested the effect of enforcing Gfi-1 expression during iTreg cell differentiation. Cells primed under iTreg cell conditions were infected with Gfi-1-GFP-RV or control GFP-RV. 4 d after infection, cells were costained for Foxp3 and IL-2 to identify bona fide Treg cells. In the group infected with control

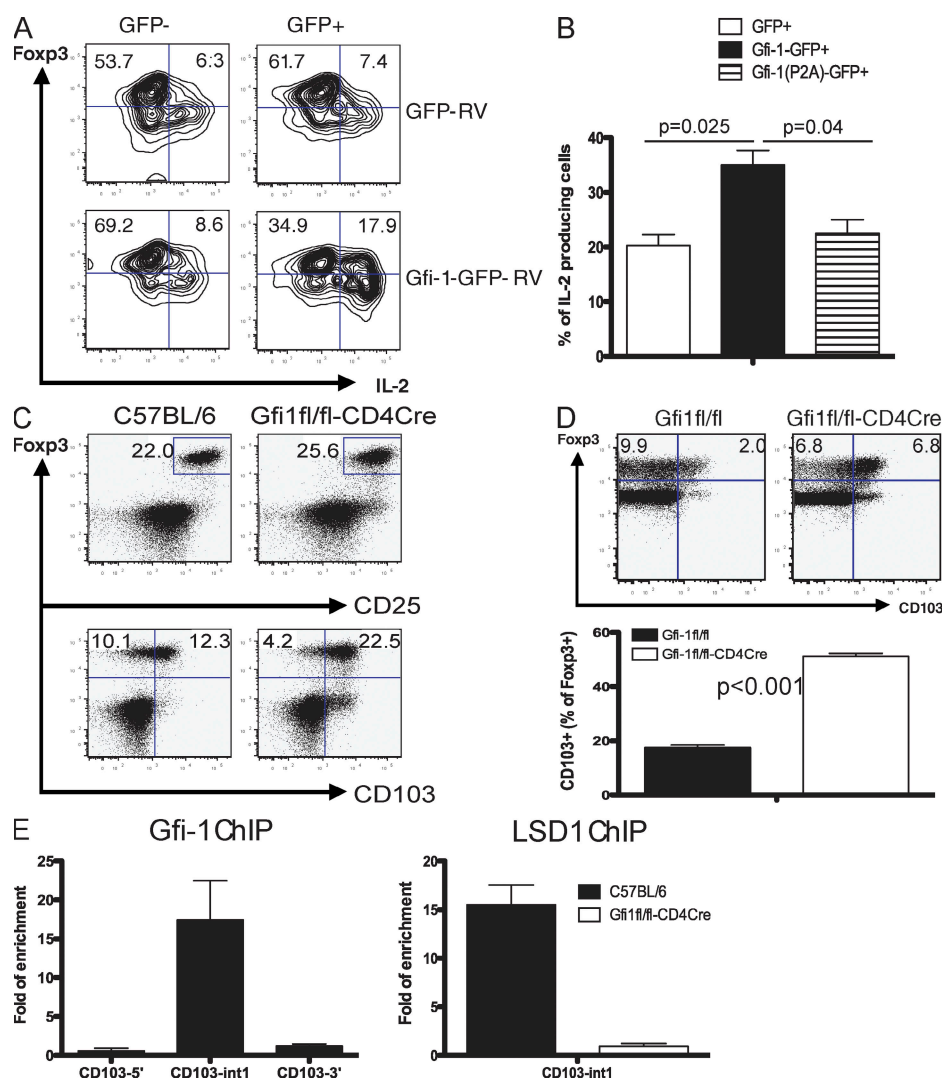


Figure 5. Gfi-1 limits the differentiation of iTreg cells and regulates the CD103⁺Foxp3⁺ subset. (A and B) CD25-depleted CD4⁺ T cells were activated under neutral conditions for 24 h, infected with RV, and further primed under iTreg cell conditions for 4 d. Cells were restimulated with PMA plus ionomycin for 4 h in the presence of monensin during last 2 h. Cytokine production and Foxp3 expression was assessed by intracellular staining. (C) CD25-depleted CD4⁺ T cells were activated under iTreg cell conditions for 4 d. Cell-surface markers were costained with Foxp3. Dot plots were gated on CD4⁺ cells. (D) Lymph node cells were stained directly with CD4, Foxp3, and CD103. Dot plots were gated on CD4⁺ cells. Numbers indicate the percentage of cells in each quadrant (top). Within the CD4⁺Foxp3⁺ population, the percentage of CD103⁺ cells was calculated from the data obtained from four mice per group (bottom). (E) Wild-type CD4 T cells that had been primed under Th2 conditions for 4 d were used for Gfi-1 ChIP (left), and both wild-type Th2 and *Gfi1* cKO Th2 cells were used for LSD1 ChIP (right). Enrichment of individual sites from the *Cd103* locus was normalized to a negative control from *Gata3*-intron3. Error bars in B, D, and E represent means \pm SD.

GFP-RV, the majority of both the GFP⁻ and GFP⁺ cells were Foxp3⁺IL-2⁻ (53.7 and 61.7%; Fig. 5 A). Although 69.2% of GFP⁻ cells from the Gfi-1-GFP-RV-infected group were Foxp3⁺IL-2⁻, only 34.9% of GFP⁺ cells were Foxp3⁺IL-2⁻. Thus, enforced expression of Gfi-1 significantly reduced the generation of iTreg cells. Gfi-1 had a minimal effect on the frequency of Foxp3⁺ cells induced by TGF- β , but among the Foxp3⁺ cells, a higher percentage of cells also produced IL-2. In a separate experiment, enforced Gfi-1 expression during iTreg cell differentiation enhanced the proportion of IL-2-producing cells from ~20 to ~35%, whereas Gfi-1(P2A) had no effect (Fig. 5 B), suggesting that the repressive domain of the Gfi-1 is required for its function in regulating iTreg cell generation.

When naive *Gfi1* cKO CD4 cells were primed under iTreg cell conditions, 25.6% of the cells became Foxp3⁺CD25^{hi} cells, similar to wild-type cells primed under same conditions (22%; Fig. 5 C, top). However, 22.5% of the cells from the *Gfi1* cKO culture were also Foxp3⁺CD103⁺, whereas only 12.3% of the wild-type cells displayed such a phenotype (Fig. 5 C, bottom). In addition, 93.3% of the Foxp3⁺ cells from the wild-type culture were CD62L^{hi}, whereas only 63.5% of the Foxp3⁺ *Gfi1* cKO cells expressed CD62L (unpublished data). The more abundant presence of CD103⁺CD62L^{lo} cells in the *Gfi1* cKO culture suggests that endogenous Gfi-1 limits differentiation of a population of highly activated Treg cells. Indeed, ex vivo staining of lymph node cells from wild-type and *Gfi1* cKO mice showed an apparently different phenotype of Treg cells (Fig. 5 D). Although the total percentage of Foxp3⁺ cells did not differ from the wild type to the KO, there was a substantial difference in Foxp3⁺CD103⁺ cells (6.8% in the *Gfi1* cKO and 2% in the wild type; Fig. 5 D, top). A more careful analysis showed that ~20% of the Foxp3⁺ cells in wild-type mice were CD103⁺, whereas in *Gfi1* cKO mice, such CD103⁺ cells represented ~50% of the Foxp3⁺ Treg cells (Fig. 5 D, bottom). Three putative Gfi-1 binding sites containing AAATCACAG from the *Cd103* locus were tested for Gfi-1 binding. The site from *Cd103* intron 1 (near the GGAAATCACAGAG sequence), but not the other two sites, was enriched by Gfi-1 antibody by 15–20-fold (Fig. 5 E, left). LSD1 was also recruited to this site in wild-type but not *Gfi1* cKO cells (Fig. 5 E, right). These data suggest that CD103 is a direct target of Gfi-1.

Gfi-1 limits the expansion of Foxp3⁺CD103⁺ cells in vivo

To address the physiological effects of Gfi-1 on TGF- β -mediated responses in vivo, we studied the responses of the cKO mice and normal controls to MOG-induced experimental allergic encephalitis (EAE). As expected, 13–14 d after MOG immunization, wild-type mice began to develop clinical symptoms, with a peak in intensity at 3 wk. The onset of disease in *Gfi1* cKO mice was significantly delayed, by approximately 1 wk (Fig. 6 A). IL-17 has been reported as a key player in the induction of the disease (53). IL-17a mRNA in CD4 T cells was measured by real-time PCR before or after various times of immunization without in vitro restimulation. There was an

approximately twofold increase of IL-17a mRNA in *Gfi1* cKO mice at all time points after MOG immunization (Fig. 6 B). Neither IFN- γ nor IL-4 mRNA was found to be consistently different from the levels in wild-type mice, possibly because of the modest Th1 or Th2 responses of these mice to immunization. CD103 mRNA is higher in *Gfi1* cKO CD4 cells before immunization, consistent with the staining data shown in Fig. 5 D, and was further increased after MOG immunization. Ex vivo restimulation of CD4 T cells 1 wk after immunization followed by anti-IL-17 intracellular staining confirmed the enhancement of IL-17 production in *Gfi1* cKO mice (Fig. 6 C). However, intracellular staining of IL-17-producing cells 3 wk after immunization revealed no difference between wild-type and *Gfi1* cKO CD4 T cells (unpublished data). The discrepancy between IL-17 mRNA and IL-17 protein at 3 wk after immunization may result from the very strong stimulus used for restimulation. Nevertheless, the data certainly indicate that there was no defect in the generation and expansion of Th17 cells in *Gfi1* cKO mice. Flow cytometry analysis showed a continuous increase of Foxp3⁺ cells in *Gfi1* cKO mice after immunization, which peaked at 3 wk (39.1 and 38.5% in *Gfi1* cKO mice compared with 22.8 and 20.5% in wild-type mice; Fig. 6 D). Although CD103⁺ Treg cells were already high in *Gfi1* cKO mice before immunization, there was a substantial increase of such cells after immunization (26.1 and 28.9% in *Gfi1* cKO mice vs. 6% and 3.7% in wild-type mice), suggesting that Gfi-1 normally plays a negative role in the expansion of Treg cells in vivo. No apparent lymphocyte infiltration into the central nervous system (CNS) was found in healthy mice from either group after immunization. However, among the sick mice, Foxp3⁺ cells were more abundant in the CNS of the *Gfi1* cKO mice than of the wild-type mice (Fig. S3 A, available at <http://www.jem.org/cgi/content/full/jem.20081666/DC1>). Most Foxp3⁺ cells in the CNS were also CD103⁺ (Fig. S3 B). Thus, Foxp3⁺CD103⁺ Treg cells appear to have a dominant effect over the IL-17-producing cells in *Gfi1* cKO mice resulting in the delay of disease onset.

DISCUSSION

T cell activation as well as IL-4 stimulation transiently induces Gfi-1 expression, resulting in optimal Th2 cell expansion (37). In the absence of Gfi-1, Th2 expansion is affected both in vitro and in vivo (41). Th1 cells from *Gfi1* cKO mice expand normally but make more IFN- γ than WT Th1 cells. In addition, *Gfi1* cKO Th2 cells display a different pattern of chromatin histone modification than wild-type Th2 cells. These observations led us to reassess the function of Gfi-1 during T cell differentiation, particularly because two new Th lineages, Th17 and iTreg cells, have been recently defined. In this paper, we showed that Gfi-1 is down-regulated by TGF- β stimulation during Th17 and iTreg cell-inducing conditions, and enforced expression of Gfi-1 inhibits both Th17 and iTreg cell differentiation. Furthermore, in the absence of Gfi-1, the percentage of Foxp3⁺ cells that express CD103 was substantially higher than in wild-type mice, and this subset expanded in vivo upon immunization.

Gfi-1 is a transcriptional repressor (51). Gfi-1 and its congener Gfi-1b have been recently reported to associate with the histone demethylase LSD1 and CoREST to form a repressive complex (46). Gfi-1 directs the complex to the promoter of target genes where LSD1 can demethylate H3K4 residues and thus repress transcription. Knockdown of LSD1 in erythroid cells leads to increased H3K4 dimethylation and trimethylation of Gfi-1b targets. In this paper, we showed that *Il17a/Il17f* and *Cd103* are the targets for Gfi-1 in T cells. Furthermore, LSD1 is recruited to those sites in a Gfi-1-dependent manner.

The *Rorc* and *Il23r* promoter regions are heavily methylated on H3K4 residues in *Gfi1* cKO Th2 cells but not in WT Th2 cells; methylation levels are comparable to those found in WT Th17 cells. However, we were not able to locate Gfi-1

binding sites within the *Rorc* or *Il23r* loci, suggesting that they may not be the direct targets of Gfi-1. A future goal will be to understand the mechanism through which Gfi-1 regulates the chromatin modification at the *Rorc* and *Il23r* loci. ROR γ t is also expressed in the cells cultured under iTreg cell-inducing conditions, correlating with the H3K4 methylation in such cells, although iTreg cells fail to express IL-17 (unpublished data). These results indicate that ROR γ t can be up-regulated by TGF- β alone (28) and that down-regulation of Gfi-1 by TGF- β is critical for ROR γ t expression during Th17 cell differentiation.

In a model of EAE involving immunization with MOG, the percentage of IL-17-producing cells found in *Gfi1* cKO mice was modestly higher than in wild-type controls. In addition,

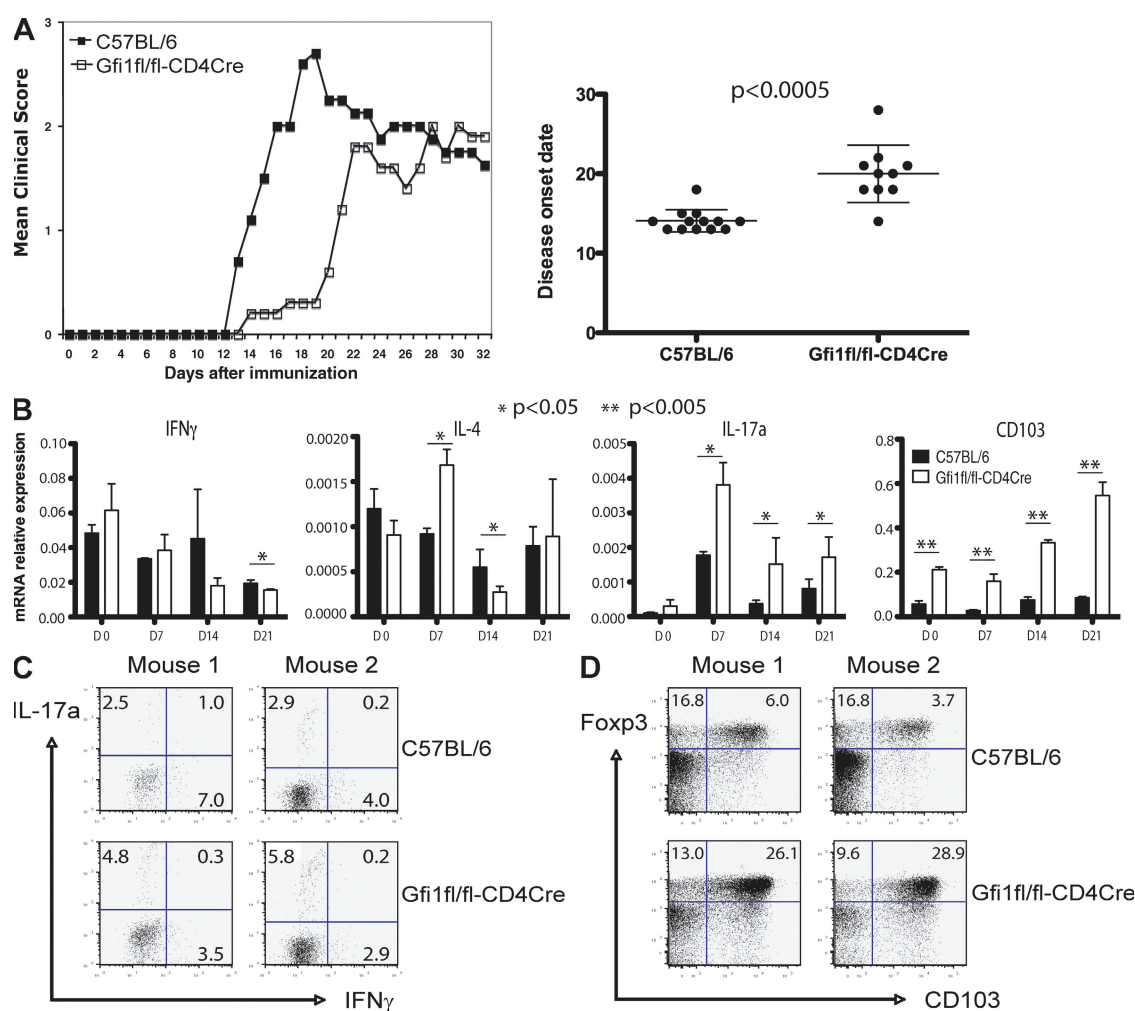


Figure 6. Delayed onset of EAE induction in *Gfi1* cKO mice is correlated with the increased Foxp3⁺CD103⁺ population. (A, left) The time course of the disease induction in wild-type and *Gfi1* cKO mice after MOG immunization. (right) The dates of disease onset after immunization in an individual mouse, pooled from three independent experiments, are shown. (B) Splenocytes were harvested at various times after immunization as indicated. CD4 T cells were purified and RNAs were made without in vitro stimulation. The expression of IFN- γ , IL-4, IL-17a, and CD103 relative to CD4 was assessed by real-time PCR. Error bars in A and B represent means \pm SD. (C) Splenocytes were harvested at day 7 after immunization. Cells were stimulated with platebound anti-CD3/anti-CD28 for 6 h with the presence of monensin during the last 2 h before intracellular staining. The dot plots were gated on the CD4⁺CD44^{hi} population. (D) Splenocytes harvested from mice 3 wk after immunization were directly stained for CD4, CD103, and Foxp3. The dot plots were gated on CD4⁺ splenocytes. Numbers indicate the percentage of cells in each quadrant. Data are representative of at least two independent experiments.

Toxoplasma gondii infection or *Schistosoma mansoni* egg injection induced higher levels of IL-17 production in *Gfi1* cKO than in wild-type mice (unpublished data). Thus, similar to our in vitro results, the optimal appearance of IL-17-producing cells induced by TGF- β in vivo requires down-regulation of Gfi-1 expression, which is transiently induced by T cell activation and/or IL-4 stimulation.

Gfi-1 expression is also down-regulated by TGF- β during iTreg cell differentiation, and enforced expression of Gfi-1 partially suppresses the induction of Foxp3⁺IL-2⁻ Treg cells. By down-regulating Gfi-1, Treg cells become more active and also display a tissue-seeking phenotype. In *Gfi1* cKO mice, the proportion of Foxp3⁺CD103⁺ effector/memory Treg cells is already high, but this population is further increased after MOG immunization. In addition, Foxp3⁺CD103⁺ are also expanded during *T. gondii* infection or in response to *S. mansoni* egg injection (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20081666/DC1>). We have not yet determined whether the increased percentage of Foxp3⁺CD103⁺ cells in the *Gfi1* cKO mice and the expansion of this subset after immunization reflects a unique role for Gfi-1 in the development of Foxp3⁺CD103⁺ thymic-derived Foxp3⁺ T cells, or whether the higher percentage of Foxp3⁺CD103⁺ T cells is secondary to an increased contribution of peripherally generated Foxp3⁺CD103⁺ in the cKO mice that is further potentiated after immunization. In normal mice, the Foxp3⁺CD103⁺ cell population is actively cycling and contains a high proportion of apoptotic cells (7), so it remains possible that Gfi-1 may play a role in the homeostasis of this subset.

A selective effect of Gfi-1 on Treg cells with a modest effect on Th17 cell differentiation in vivo could be explained by the differential requirement of TGF- β in the induction of these two lineages. A low concentration of TGF- β is sufficient for Th17 cell differentiation, whereas iTreg cell differentiation requires a higher concentration of TGF- β (54). In the absence of Gfi-1, Treg cell numbers may increase in response to levels of TGF- β that normally only induce Th17 cell differentiation in wild-type mice after MOG immunization.

The selective expansion of the Foxp3⁺CD103⁺ cells correlates with delayed EAE onset. Treg cells have been suggested to suppress autoimmune diseases by several mechanisms: by suppressing the activation and expansion of the effector cells (6); by suppressing functions of effector cells, such as cytokine production (55); and by limiting recruitment of effector cells to sites of inflammation (56). In our model, the disease onset was delayed in the *Gfi1* cKO mice, yet the number of effector cells that are capable of making IL-17 was not only not reduced but was modestly increased. Thus, it is most likely that the Foxp3⁺CD103⁺ cells suppress disease by affecting recruitment and/or activation of the effector cells at inflammation sites consistent with the tissue-seeking phenotype of the Foxp3⁺CD103⁺ T cells that are increased in frequency in *Gfi1* cKO mice. The majority of Treg cells found in the CNS are Foxp3⁺CD103⁺, even in wild-type mice. In addition, Treg cells are a much larger proportion of CNS CD4 T cells in *Gfi1* cKO than in wild-type mice.

Given the dual effects of Gfi-1 on effector and Treg cells, modulation of its expression and function could have opposite effects depending on the timing, location, and cell type in which it is expressed, just as is the case with the administration of TGF- β or anti-TGF- β (57). However, blockade of Gfi-1 function during early stages of autoimmune diseases may be beneficial through the resultant expansion of Treg cells. Likewise, the specific targeting of Gfi-1 in Treg cells may enhance their migration to sites of inflammation as well as increase their suppressive activity. Thus, the predominant effect of Gfi-1 on Treg cells in vivo suggests Gfi-1 and its pathway of action could be considered as a target for modulating Treg cells.

MATERIALS AND METHODS

Mice. C57BL/6 mice were obtained from Taconic or the Jackson Laboratory. *Gfi1*^{fl/fl}-CD4Cre (41) mice have been backcrossed to C57BL/6 mice for nine generations. G4 mice have been previously described (48). *Gfi1*^{fl/+}-CD4Cre-G/4 and *Gfi1*^{fl/fl}-CD4Cre-G/4 mice were generated by crossing *Gfi1*^{fl/fl}-CD4Cre to G4 mice. All mice were bred and maintained in a National Institute for Allergy and Infectious Diseases (NIAID) specific pathogen-free animal facility, and the experiments were performed when mice were 8–16 wk of age under protocols approved by the NIAID Animal Care and Use Committee.

Cell culture. Lymph node CD4 or CD8 T cells were prepared by MACS purification using CD4 or CD8 microbeads (Miltenyi Biotec), and purity was usually 90–95%. In some experiments, lymph node nonregulatory naive CD4 T cells were prepared by depleting CD8, B220, IAb, CD24, NK1.1, CD16/CD32, CD11b, and CD25-positive cells using FITC-labeled antibodies (BD), followed by anti-FITC microbeads and autoMACS purification (Miltenyi Biotec). T cell-depleted APCs were prepared by incubating spleen cells with anti-Thy1.2 and rabbit complement (Cedarlane Laboratories Limited) at 37°C for 45 min, and then irradiating them at 30 Gy (3,000 rad). T cells were co-cultured with APCs at a 1:5 ratio in the presence of 1 μ g/ml anti-CD3 and 3 μ g/ml anti-CD28, together with different combinations of antibodies and cytokines: for Th1 conditions, 10 μ g/ml anti-IL-4, 10 ng/ml IL-12, and 100 U/ml IL-2; for Th2 conditions, 10 μ g/ml anti-IFN- γ and 10 μ g/ml anti-IL-12, 5,000 U/ml IL-4, and 100 U/ml IL-2; for Th17 conditions, 10 μ g/ml anti-IL-4, 10 μ g/ml anti-IFN- γ , 10 μ g/ml anti-IL-12, 5 ng/ml TGF- β , 10 ng/ml IL-6, and 10 ng/ml IL-1 β ; for iTreg cell conditions, 10 μ g/ml anti-IL-4, 10 μ g/ml anti-IFN- γ , 10 μ g/ml anti-IL-12, 5 ng/ml TGF- β , and 100 U/ml IL-2; for ThN (nonpolarization) conditions, 10 μ g/ml anti-IL-4, 10 μ g/ml anti-IFN- γ , 10 μ g/ml anti-IL-12, and 100 U/ml IL-2; and for neutral conditions, no exogenous cytokines or antibodies were added.

Quantitative RT-PCR. Total RNA was isolated using TRIzol (Invitrogen). First-strand cDNAs were made using the SuperScript Preamplification System (Invitrogen). Quantitative real-time PCR was performed on a sequence detection system (model 7900HT; Applied Biosystems). The sequences of the primers and minor groove binder (MGB) probe for Gfi-1 are 5'-TGGGCG-GCGGCTCCTACAAAT-3', 5'-TGCGGTGTGGAGAACACCTGA-3', and FAM-5'-TGCAGCAAGGTGTT-3'-MGB, respectively. Inventoried primer/probe sets for detecting IFN- γ (Mm99999071_m1), IL-4 (4312482), IL-17a (Mm00439619_m1), IL-17f (Mm00521423_m1), Foxp3 (Mm00475156_m1), Bcl-2 (Mm00477631_m1), Bim (Mm00437795_m1), SOCS-1 (Mm00782550_s1), CD103 (Mm00434443_m1), CD4 (Mm00442754_m1), TGF- β RI (Mm00436971_m1), TGF- β RII (Mm00436978_m1), IL-6R α (Mm00439653_m1), GAPDH (4308313), and ROR γ t (Mm01261022_m1) were purchased from Applied Biosystems.

Preparation of retroviral constructs and infection. Gfi-1-GFP-RV and Gfi-1(P2A)-GFP-RV were previously constructed (37). Retroviruses

were packaged in the Phoenix-Eco packaging cell line, as previously described (36). Infection was performed at 24 h after the initiation of the culture in which CD4 T cells were activated with anti-CD3/anti-CD28 under neutral conditions in the presence of APCs.

Flow cytometry analysis. The expression of GFP in retrovirally infected cells was measured by flow cytometry. Cell-surface staining was performed in PBS containing 0.1% PBS with different combination of antibodies. Anti-IFN- γ -FITC, anti-IL-4-PE, anti-IL-17a-PE, anti-CD25-PE, anti-CD25-PerCP-Cy5.5, anti-CD4-PE-Cy5, anti-IFN- γ -allophycocyanin, and anti-CD44-allophycocyanin were purchased from BD. Anti-CD103-FITC (2E7), anti-CD103-PE (2E7), anti-Foxp3-PE (FJK-16s), anti-Foxp3-allophycocyanin (FJK-16s), anti-IL-17a-Alexa Fluor 647 (eBio17B7), anti-IL-2-Pacific blue (JES6-5H4), anti-CD62L-Pacific blue (MEL-14), anti-IFN- γ -PerCP-Cy5.5 (XMG1.2), anti-CD44-allophycocyanin-Alexa Fluor 750 (IM7), and anti-CD4-Alexa Fluor 700 (GK1.5) were purchased from eBioscience. For intracellular cytokine staining, cells were restimulated with 10 ng/ml PMA and 1 μ M ionomycin for 4 h or with platebound anti-CD3 and anti-CD28 (3 μ g/ml each) for 6 h in the presence of 2 μ M monensin during last 3 h. Harvested samples were fixed with 4% formaldehyde, washed, and permeabilized in 0.5% Triton X-100–0.1% BSA in PBS before staining for cytokine production. Intracellular staining of Foxp3 was performed according to the instructions of the Foxp3 staining kit (eBiosciences). When GFP is present, Foxp3 was stained in Triton X-100 buffer, which is used for staining cytokines.

ChIP and histone H3K4 modification analysis. For Gfi-1 and LSD1 ChIP, 5×10^6 CD4⁺ T cells that have been cultured under Th2 or Th17 conditions for 4–5 d were cross-linked with formaldehyde treatment, and the chromatin was sonicated into small fragments from ~200 to 1,000 bp. The fragmented chromatin was immunoprecipitated with the specific antibody. Anti-Gfi-1 (N-20) was purchased from Santa Cruz Biotechnology, Inc. Anti-LSD1 was purchased from Abcam. Precipitated DNA fragments were reverse cross-linked, and the enrichment of specific sites was tested by real-time PCR using SYBR green and the relative enrichment was normalized to a negative site from Gata3-intron3. The primers are as follows: Rorc, 5'-ATTCCATGAGGGCTTGCCCT-3' and 5'-ACCTGTCATCAGCCTCCCAT-3'; Gfi1p, 5'-CGAAG-TATTCTTGGCTCCTGGA-3' and 5'-GCCCAAACAGTCGACAG-GATT-3'; Il17a/Il17f, 5'-TGCTGGGCAGGAAGATACTC-3' and 5'-TCCCCACTCTGTCTTCCAG-3'; Cd103-5', 5'-CCCACCCGCT-GTAGACATGT-3' and 5'-CCCAGCCATACCCGAAGAT-3'; Cd103-intron1, 5'-CCCACCCAAACAGTCCTCTAA-3' and 5'-CTCATAT-CTTAATCTAAGCACTCAGCGTT-3'; Cd103-3', 5'-CCCTGCCCT-GGACTACAAAGTG-3' and 5'-CTGCCTCCACCTCCCAAGT-3'; and Gata3-intron3, 5'-TCAGGGCACTAAGGGTTGTTAACTT-3' and 5'-GAATTCATCCATGAGACACACAA-3'.

For ChIP-Seq experiments (47), 10^7 CD4⁺ T cells that have been cultured under Th2 or Th17 conditions for 4 d were cross-linked with formaldehyde treatment and digested with MNase to generate mainly mononucleosomes with a minor fraction of dinucleosomes. ChIP was performed by using anti-H3K4me3 (Abcam). The ChIP DNA ends were repaired using polynucleotide kinase and Klenow enzyme, followed by treatment with Taq polymerase to generate a protruding 3' A base used for adaptor ligation. After ligation of a pair of Solexa adaptors to the repaired ends, the ChIP DNA was amplified using the adaptor primers for 17 cycles, and the fragments around 220 bp (mononucleosome plus adaptors) were isolated from agarose gel. The purified DNA was used directly for cluster generation and sequencing analysis using the Illumina/Solexa 1G Genome Analyzer according to the manufacturer's protocols. Sequence reads were obtained and mapped to the mouse genome with the Illumina GA Pipeline. Read positions were adjusted 75 bp to be centered on the sequenced chromatin fragments, and the number of adjusted reads originating in 200-bp windows were summed and displayed as custom tracks on the University of California Santa Cruz Genome Browser.

EAE induction. EAE was induced via subcutaneous immunization into the hind flank with 200 μ l of an emulsion containing 400 μ g of MOG35–55

peptide (MEVGWYRSPFSRVVHLYRNGK) and 400 μ g of *Mycobacterium tuberculosis* strain H37Ra in complete Freund's adjuvant (Difco). On days 0 and 2, the mice received an intraperitoneal injection of 200 ng pertussis toxin (EMD) dissolved in PBS. Clinical symptoms were assessed by the following criteria: 0, no signs of disease; 1, complete tail paralysis; 2, hind limb paresis; 3, complete hind limb paralysis; 4, unilateral forelimb paralysis; and 5, moribund/death. Mice observed in a moribund state were killed. Data presented are the mean clinical scores of five mice per group.

Online supplemental material. Fig. S1 shows the expression of ROR γ t, TGF- β RI, TGF-R β RII, and IL-6R α in Th1, Th2, and Th17 cells and in Gfi-1-RV-infected Th17 cells. Fig. S2 shows the suppression of IL-17 expression by Gfi-1 in *Gfi1* cKO Th17 cells. Fig. S3 shows the frequency and the phenotype of Treg cells found in the CNS of the WT and *Gfi1* cKO mice after MOG immunization. Fig. S4 shows the expansion of Foxp3⁺CD103⁺ Treg cells in *Gfi1* cKO mice during *T. gondii* infection or in response to *S. mansoni* egg injection. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20081666/DC1>.

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