Inhibitory role of the transcription repressor Gfi1 in the generation of thymus-derived regulatory T cells

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Foxp3+ regulatory T (Treg) cells are essential for the maintenance of self-tolerance and immune homeostasis. The majority of Treg cells is generated in the thymus as a specific subset of CD4+ T cells, known as thymus-derived or natural Treg (nTreg) cells, in response to signals from T-cell receptors, costimulatory molecules, and cytokines. Recent studies have identified intracellular signaling and transcriptional pathways that link these signals to Foxp3 induction, but how the production of these extrinsic factors is controlled remains poorly understood. Here, we report that the transcription repressor growth factor independent 1 (Gfi1) has a key inhibitory role in the generation of nTreg cells by a noncell-autonomous mechanism. T cell-specific deletion of Gfi1 results in aberrant expansion of thymic nTreg cells and increased production of cytokines. In particular, IL-2 overproduction plays an important role in driving the expansion of nTreg cells. In contrast, although Gfi1 deficiency elevated thymocyte apoptosis, Gfi1 repressed nTreg generation independently of its prosurvival effect. Consistent with an inhibitory role of Gfi1 in this process, loss of Gfi1 dampens antitumor immunity. These data point to a previously unrecognized extrinsic control mechanism that negatively shapes thymic generation of nTreg cells.

Normal development of Foxp3+ regulatory T (Treg) cells is critical for maintaining self-tolerance and preventing exuberant immune responses (1). Treg cells are produced mainly in the thymus, known as thymus-derived or natural Treg (nTreg) cells, and they require expression of the transcription factor Foxp3. T-cell receptor (TCR) specificity to self-antigens seems to be a primary determinant for nTreg lineage commitment in the thymus, with c-Rel being an important factor that links TCR engagement and Foxp3 expression (2, 3). Costimulatory factors (such as CD28) and cytokines, predominantly IL-2, also play crucial roles for the induction of Foxp3 and thymic development of nTreg cells (2, 3). In a two-step model of nTreg development, TCR engagement leads to the expression of the high-affinity IL-2Rα that subsequently responds to IL-2 stimulation for the induction of Foxp3 expression and nTreg lineage commitment (4, 5). However, the cellular source of IL-2 is unclear (6). Moreover, whereas much emphasis has been placed on T cell-intrinsic control of nTreg development, how the production of these extrinsic factors is controlled to shape the nTreg pool remains poorly understood.

Growth factor independent 1 (Gfi1), a transcription repressor, has emerged as an important regulator of hematopoietic and immune system cells. Gfi1 is required for the normal development and homeostasis of hematopoietic stem cells and both myeloid and lymphoid progenitors (7, 8). Specifically, loss of Gfi1 impairs the development of neutrophils and B cells while expanding the monocyte and myeloid populations (9–11). In the T-cell lineage, Gfi1 expression is dynamically regulated (12), and its deficiency diminishes double-negative (DN) cell generation but increases the differentiation of CD8+ T cells in the thymus (13). In the periphery, Gfi1 has been implicated in the differentiation and in vivo function of CD4+ effector and regulatory T-cell subsets (14–18), but it is dispensable for CD8+ T cell-mediated immune responses in vivo (16). These results indicate an important but cell context-dependent function for Gfi1 in the immune system.

Whereas a role for Gfi1 in early thymocytes and peripheral T cells has been described, its function in the development of nTreg cells is unclear. We have previously found that thymic development of nTreg cells is orchestrated by S1P1 (19), which is under the control of Kit2 (20) that can be further regulated by Gfi1 (13), but the roles of Gfi1 in nTreg cells are poorly understood. Therefore, we generated T cell-specific Gfi1-deficient mice and had a surprising finding that Gfi1 deletion enhanced nTreg development through a noncell-autonomous mechanism. Additional analysis revealed an exuberant production of IL-2 by Gfi1-deficient thymocytes as the main mechanism, thereby highlighting a previously unrecognized mechanism in which IL-2 produced by conventional T cells shapes thymic microenvironment to direct nTreg development. Furthermore, Gfi1 function in T cells was required for optimal antitumor immunity, consistent with its effects at inhibiting nTreg generation and function. Finally, although Gfi1 deficiency increased thymocyte apoptosis, Gfi1 repressed generation of nTreg cells independently of its prosurvival effect. These data point to an extrinsic control mechanism that negatively shapes thymic generation of nTreg cells.

Results
Gfi1 Deficiency Promotes the Generation of nTreg Cells. To investigate the function of Gfi1 in T-cell development, we first analyzed mice with germ-line deletion of Gfi1 (Gfi1−/− mice) (12). Loss of Gfi1 altered thymic populations, most notably a higher frequency of CD8 single-positive (CD8SP) cells, consistent with...
a previous report using Gfi1−/− mice (13). Detailed analysis of T-cell subsets indicated that the Gfi1−/− CD4 single-positive (CD4SP) compartment contained a markedly increased frequency of thymic Foxp3+ nTreg population (Fig. S1A). Given a prominent role of Gfi1 in the development of multiple hematopoietic lineages (8, 21), it remained possible that the expansion of Gfi1−/− nTreg cells was secondary to defective early development and/or influences by nonlymphoid cells. Hence, we crossed lacZ-flanked Gfi1 alleles (Gfi1fl/fl) with two Cre lines, the hCD2-iCre and CD4-Cre transgenic mice, in which Cre expression was initiated at the early and late DN stages, respectively (called Gfi1iCd2 and Gfi1CD4) (22, 23). Gfi1 was efficiently deleted in double-positive (DP) and single-positive (SP) cells from both lines (Fig. S1B). More efficient recombination was achieved in Gfi1iCd2 mice, possibly because of the codon improvement of the Cre recombinase in hCD2-iCre mice (22). As expected, deletion of Gfi1 in both systems resulted in an elevated ratio of CD8SP/CD4SP T cells (Fig. 1 A and B). More importantly, loss of Gfi1 considerably up-regulated the frequency of nTreg cells among CD4SP T cells (Fig. 1 A and B), although the absolute number of nTreg cells remained largely unaltered because of the reduction of total thymocytes and especially, CD4SP cells (Fig. S1C). Greater proportions of Foxp3+ cells among CD4 T cells were also observed in the spleen (Fig. 1C and Fig. S1D), liver, blood, and even DP thymocytes from Gfi1CD4 mice (Fig. S1E). Comparable results derived from these different deletion systems indicate a specific role for Gfi1 in restraining nTreg generation. For the purpose of clarity and consistency, we mainly used Gfi1CD4 mice in our analyses.

We determined the cellular mechanisms by which Gfi1 restrains nTreg development. We reasoned that the increased frequency of nTreg cells in Gfi1-deficient mice could be caused by differential regulation of cell expansion or survival between Foxp3+ and Foxp3− CD4SP T cells. To assess the effects of Gfi1 deficiency on cell proliferation, we first used BrdU incorporation assays, which label cells that have synthesized new DNA and progressed into the S phase of the cell cycle. Whereas Foxp3+ CD4SP T cells from wild-type (WT) and Gfi1CD4 mice incorporated BrdU to a comparable extent, a greater percentage of Foxp3+ T cells from Gfi1CD4 mice incorporated BrdU (Fig. 1D). Similar results were obtained from splenic Foxp3+ T cells from Gfi1CD4 mice (Fig. S1F). Moreover, KI67 staining, which denotes proliferating cells at all active phases of the cell cycle, also revealed an increased frequency of proliferating Foxp3+ cells in the thymus and spleen of Gfi1CD4 mice (Fig. S1G). We next analyzed apoptosis of different populations of CD4SP cells. Freshly isolated CD4SP T cells from WT and Gfi1CD4 mice showed a similar degree of Annexin V binding and caspase activity (two hallmarks of apoptotic cell death) in both the CD25− and CD25+ compartments (Fig. 1E). Therefore, the increased nTreg population in Gfi1CD4 mice is associated with an enhanced rate of cell proliferation but not survival.

Gfi1 Deficiency Enhances nTreg Function and Impairs Antitumor Immunity. Having shown a role of Gfi1 in nTreg development, we next examined whether Gfi1 affected the immunosuppressive function of nTreg cells. Gfi1-deficient nTreg cells exhibited a stronger inhibitory capacity against the proliferation of conventional

![Fig. 1](image_url)
T cells in standard in vitro Treg suppression assays based on carboxyfluorescein succinimidyl ester (CFSE) dilution and thymidine incorporation (Fig. 2A and Fig. 2B). We then examined the expression of the integrin molecule CD103 and the ectonucleotidase CD73, effector molecules associated with nTreg cell suppressive activity (24, 25). Whereas Gfi1 has been shown to regulate these molecules in discrete contexts (17, 26), the extent to which this regulation is operative in nTreg cells is poorly defined. Compared with the WT counterparts, deletion of Gfi1 resulted in prominent up-regulation of CD103 (Fig. 2B) and CD73 (Fig. 2C) on nTreg cells from both the thymus and periphery. Thus, Gfi1 negatively controls nTreg function, which is associated with its effects to down-modulate expression of selective suppressive molecules (24, 25).

Immune tolerance mediated by nTreg cells is a major component that prevents productive immune responses against tumors (27–29). Given the inhibitory effects of Gfi1 on the nTreg pool and suppressive activity, we tested whether lack of Gfi1 affected antitumor immunity using a well-established B16 melanoma model. Gfi1CD4 mice failed to restrain the late phase of tumor growth (Fig. 2D), despite slightly increased proportions of IFN-γ+ cells among tumor-infiltrating CD4+ and CD8+ cells (Fig. S2B). Associated with impaired antitumor immunity in Gfi1CD4 mice was an increased number of Foxp3+ cells in tumor-infiltrating cells (Fig. 2E) and a greater Foxp3+/Foxp3− ratio (Fig. 2F). Furthermore, CD103 and CD73 expression levels on tumor-infiltrating nTreg cells were elevated in Gfi1CD4 mice (Fig. 2G). These results indicate that Gfi1 is required for optimal antitumor immunity, likely through its inhibitory effects on Treg generation and function.

**Noncell-Autonomous Effects of Gfi1 Deficiency on nTreg Development.**

We next investigated whether the increased thymic nTreg population in Gfi1-deficient mice was a cell-autonomous defect. To this end, we generated mixed bone marrow (BM) chimeras by reconstituting lymphoid Rag1−/− mice with a 1:1 mixture of WT or Gfi1CD4 (CD45.2+; donor) and WT (CD45.1+; spike) BM cells. As a comparison, we also generated chimeras that contained WT or Gfi1CD4 BM cells exclusively and observed the expected increase of the thymic and splenic nTreg population in reconstituted chimeras derived from Gfi1-deficient BM cells (Fig. S3A). In the mixed chimeras, Gfi1CD4-derived thymocytes retained the higher CD8SP/CD4SP ratio compared with WT- or spike-derived cells in the mixed chimeras (Fig. 3A), indicative of a cell-autonomous effect of Gfi1 deficiency on CD8+ differentiation.

In contrast, the frequency of thymic and splenic Foxp3+ cells derived from Gfi1CD4 donor cells in the mixed BM chimeras was indistinguishable from the frequency of Foxp3+ cells from WT donors or the cotransferred CD45.1+ spike cells (Fig. 3B and Fig. S3B). Thus, the increased nTreg population in the unmanipulated Gfi1CD4 mice seemed to be a noncell-autonomous or bystander defect. However, the up-regulation of CD103 expression among nTreg cells persisted in Gfi1-deficient donor cells (Fig. S3C), and this cell-intrinsic effect on CD103 expression is in agreement with previous studies identifying CD103 as a direct Gfi1 target gene (17). To further investigate the possibility of a bystander effect on the nTreg population caused by Gfi1-deficient cells, we set up mixed BM chimeras, in which WT or Gfi1CD4 (CD45.2+) progenitors were a majority and WT spike (CD45.1.2+) cells were a minority (at a 20:1 ratio). As expected, the Foxp3+ population was increased in Gfi1-deficient cells, but more strikingly, the nTreg percentage derived from spike thymocytes was also elevated in the predominant Gfi1-deficient thymus, even with a greater degree that probably reflected a better survival fitness of these cells (see below) (Fig. S3D). Similar observations were obtained in the spleen (Fig. S3D). The up-regulation of Foxp3 in WT spike cells indicates that Gfi1 deficiency in T cells exerts a cell-extrinsic effect on the nTreg population.

We noted that an analogous bystander up-regulation of thymic chemokine receptor expression in Kll2-deficient mice results in an age-related accumulative effect (30). We, therefore, examined whether Gfi1 deficiency affected the kinetics of nTreg generation by analyzing thymic nTreg cells in mice of different ages. Linear regression analysis indicated that the enhancing effect of Gfi1 deficiency on CD103 generation was in agreement with previous studies identifying CD103 as a direct Gfi1 target gene (17). To further investigate the possibility of a cell-extrinsic effect on the thymic nTreg population. See below.

Data are representative of (A and D–G) two and (B and C) five independent experiments. Error bars indicate SEM. *P < 0.05.

**Fig. 2.** Gfi1 restrains nTreg function and is required for antitumor immunity. (A) CFSE levels in CFSE-labeled effector T (Teff) cells activated in the presence of nTreg cells for 3 d. (B and C) Expression of (B) CD103 or (C) CD73 among Foxp3+ cells. (D–G) WT and Gfi1CD4 mice were inoculated with B16 melanoma cells, and (D) tumor size was measured. Tumor-infiltrating lymphocytes were isolated at day 16 and analyzed for Foxp3 staining; shown are (E) Foxp3+ numbers, (F) the ratio of Foxp3+/Foxp3− among CD4+ cells, and (G) mean fluorescent intensity (MFI) of CD103 and CD73 among Foxp3+ tumor-infiltrating lymphocytes. Data are representative of (A and D–G) two and (B and C) five independent experiments. Error bars indicate SEM. *P < 0.05.
to nTreg-stimulating factors that are differentially produced by Gfi1-deficient T cells.

To further dissect the noncell-autonomous effects of Gfi1 on nTreg development, we crossed Gfi1fl/fl mice with Foxp3YFP-Cre mice, in which the Cre recombinase was knocked into the endogenous Foxp3 locus (31), to delete Gfi1 selectively in nTreg cells (called Gfi1YFP3 mice). Gfi1 expression was considerably diminished in Foxp3+ nTreg cells in the thymus, and complete deletion was achieved in the periphery (Fig. 3D), which was in line with the timing of Cre expression in Foxp3YFP-Cre mice (31). Consistent with a direct role of Gfi1 in CD103 expression, CD103 was up-regulated in Gfi1-deficient nTreg cells, especially in splenic nTreg cells that had more efficient Gfi1 deletion (Fig. S3E). Accordingly, nTreg cells from Gfi1YFP3 mice had a small increase of suppressive activity (Fig. S3F). However, the specific deletion of Gfi1 in nTreg cells did not affect the frequency of the Foxp3+ nTreg population in the thymus or spleen (Fig. S3E). Taken together, we conclude that the nTreg cell expansion induced by Gfi1 deficiency was caused by a noncell-autonomous mechanism. This expansion was a unique effect, because altered CD8SP/CD4SP ratios and CD103 regulation in nTreg cells were intrinsic to the loss of Gfi1 function. These findings further highlight that deletion of Gfi1 in total T cells altered the thymic environment important for nTreg generation, and the underlying mechanism was further explored in the following studies.

**Gfi1 Affects nTreg Development by Modulating IL-2 Production.**

Thymic nTreg development is dependent on TCR, costimulation, and cytokine signaling (2, 3). A noncell-autonomous effect mediated by Gfi1 on nTreg development prompted us to test whether Gfi1 deficiency altered cytokine production. We first focused on IL-2, a key cytokine capable of driving nTreg development and expansion (32–34). However, the cellular source responsible for IL-2 production in this process remains poorly defined (6), because IL-2 can be produced by dendritic cells and additional cells other than T cells (35). Compared with WT cells, CD4SP and CD8SP T cells from Gfi1CD mice expressed a higher level of Il2 mRNA under resting conditions as well as after phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation (Fig. 4A). The increased Il2 was restricted to the non-nTreg population of Gfi1-deficient CD4SP cells, because IL-2 expression in the mutant nTreg cells was unaltered (Fig. S4A). Also, splenic CD4+ cells or thymic dendritic cells of Gfi1CD mice showed normal expression of IL-2 (Fig. S4 B and C). Consistent with mRNA analysis, intracellular cytokine staining revealed an increased proportion of Gfi1-deficient CD4SP T cells capable of producing IL-2 (Fig. 4B). A similar trend of increase was noticed in Gfi1-deficient CD8SP cells, although this increase did not reach statistical significance in the limited number of mice analyzed (Fig. 4B).

We then determined whether augmented IL-2 production from Gfi1-deficient thymocytes contributed to the nTreg development phenotypes. Specifically, we used two approaches to block IL-2 function. First, we crossed Gfi1CD mice with Il2−/− mice to generate Gfi1CD, Il2−/− double KO mice. To avoid the confounding effects caused by an autoimmune syndrome manifested in adult Il2−/− mice (34), we analyzed these mice at a young age (5–6 wk). Deletion of IL-2 blocked the higher frequency of Foxp3+ cells in the thymus of Gfi1CD mice (Fig. 4C). Second, we treated WT and Gfi1CD mice with blocking antibodies for IL-2 or IL-2R (36). After administration of either neutralizing antibody, the frequency of Foxp3+ cells in the thymus between WT and Gfi1CD mice became comparable, whereas the difference remained evident in PBS-treated control groups.
Thus, an important mechanism by which Gfi1 deficiency promotes nTreg development is to induce excessive IL-2 production. Aside from IL-2, the immunosuppressive cytokines TGF-β1 and IL-10 have been implicated in promoting Foxp3+ cell generation under different conditions (37–40). Whereas TGF-β1 expression was comparable between WT and Gfi1 CD4 cells (Fig. 4E), increased IL-10 expression was observed in Gfi1 CD4 CD4SP cells (Fig. 4F). To test the contribution of the elevated IL-10 expression to the phenotypic alteration of nTreg cells, we generated Gfi1 CD4 Il10−/− mice. Deletion of IL-10 had minimal effects on the altered Foxp3+ percentage or CD103 expression among nTreg cells in Gfi1 CD4 mice (Fig. S4D and E), indicating that the enhanced IL-10 expression by Gfi1-deficient T cells does not play an important role in nTreg cell generation. Altogether, our results highlight an IL-2–dependent, cell-extrinsic mechanism responsible for the expansion of nTreg cells in Gfi1 CD4 mice.

Survival-Independent Functions of Gfi1 in Thymic nTreg Generation. Gfi1 mutation is frequently associated with elevated apoptosis of multiple cell types (8, 21). Undoubtedly, regulation of apoptosis is a major component of thymocyte developmental processes, including nTreg generation (2, 3). Although Gfi1 germ-line deletion promoted apoptosis of early DN thymocytes (13), Gfi1 did not seem to be essential for the survival of DP and SP thymocytes under steady state conditions (Fig. 1E). However, because apoptotic cells can be rapidly cleared in vivo, we performed additional analysis to closely examine the effects of Gfi1 loss on thymocyte apoptosis.
survival. When cultured for 2 d in vitro, Gfi1CD4 CD4SP thymocytes showed modestly impaired survival (Fig. 5A). Moreover, in a competitive environment introduced by the mixed chimeras, Gfi1CD4-derived donor cells had impaired capacity to populate the thymic CD4SP compartment (Fig. 5B). These results reveal a requirement of Gfi1 for the survival fitness of mature thymocytes.

To directly address whether this prosurvival effect of Gfi1 is a contributing factor to nTreg development, we crossed Gfi1CD4 mice with lymphocyte-specific transgenic mice for Bcl-2 that potently inhibits thymocyte death (41). As expected, the introduction of the Bcl-2 transgene nearly completely rectified the survival defects of Gfi1-deficient thymocytes in vitro (Fig. 5C). However, Gfi1CD4 Bcl2-TG mice still contained an increased thymic nTreg population (Fig. 5D). Similarly, depletion of Bim (encoded by Bcl2/11), a proapoptotic molecule important for thymic-negative selection (42), did not affect the increased nTreg cells in Gfi1CD4 mice (Fig. S5). Collectively, Gfi1 controls thymic generation of nTreg cells through a mechanism largely independently of cell survival.

Discussion

Thymic development of nTreg cells is orchestrated by signals transduced from TCRs, costimulation, and cytokines (2, 3). Although the involvement of nTreg-intrinsic pathways has been described extensively, how nTreg development is shaped by extrinsic factors remains poorly understood (43). Additionally, although a role for IL-2 to promote thymic nTreg development has been appreciated for some time (32–34), the source of IL-2-producing cells remains undefined (6). In this study, we have identified a noncell-autonomous role of Gfi1 in inhibiting nTreg generation by down-regulating IL-2 expression from conventional thymocytes. Our conclusions are derived from the analysis of mixed BM chimeras and Gfi1Foxp3 mice, the exuberant production of IL-2 from Gfi1-deficient thymocytes, and the functional consequences of IL-2 blocking. This model is further supported by age-dependent accumulation of thymic nTreg cells observed in Gfi1CD4 mice and the increased BrdU incorporation among thymic nTreg cells lacking Gfi1. Altogether, these data establish a previously unrecognized mechanism for the active control of IL-2 production in the thymic microenvironment to shape nTreg development.

Our identification of a noncell-autonomous mode of nTreg cell generation echoes the recent findings describing bystander development of innate CD8+ T cells, another nonconventional T-cell subset derived from the thymus. Specifically, thymocytes deficient in transcription factors such as Klf2, Ikt, or Id3 aberrantly produce IL-4 that mediates the generation of such cells (30, 44–46). These studies collectively highlight the importance and complexity of transcription factor-dependent shaping of thymic cytokine levels to ensure normal development of various thymus-derived populations.

Using a B16 melanoma tumor model, we showed that Gfi1 function is required for efficient antitumor immunity. The uncontrolled tumor growth in Gfi1CD4 mice was associated with increased nTreg cells infiltrating the tumor as well as elevated expression of CD103 and CD73 that endows nTreg cells with strong suppressive activity (24, 25). Furthermore, nTreg cells deficient in Gfi1 had enhanced suppressive activity in vitro. These results highlight the important roles of Gfi1 in negatively affecting both nTreg generation and suppressive activity. Despite the impaired antitumor immunity in Gfi1CD4 mice, we observed increased IFN-γ production from effector cells lacking Gfi1. These results indicate that the increased frequency and function of Gfi1-deficient nTreg cells are important factors for the defective in vivo responses, although we could not exclude additional contribution from conventional T cells.

Consistent with a prosurvival role of Gfi1 observed in other cell types (8, 21), deficiency of Gfi1 modestly elevated apoptosis

Fig. 5. Survival defect of Gfi1-deficient thymocytes does not contribute to the altered nTreg development. (A) CD4SP thymocytes from WT or Gfi1CD4 mice were cultured for 2 d followed by Annexin V and 7-AAD staining. (B) Competitive fitness of CD4SP thymocytes in vivo. Mixed BM chimeras were set up as described in Fig. 3A, and the contributions of WT or Gfi1CD4 donor (CD45.2+) and spike (CD45.1+) cells were analyzed. (Right) Ratios of CD45.2/CD45.1 (after normalization to nonrelevant B220+ cells). (C) CD4SP thymocytes from WT, Gfi1CD4, Bcl2-TG, or Gfi1CD4 Bcl2-TG mice were cultured for 3 d followed by Annexin V and 7-AAD staining. (D) Frequency of Foxp3+ cells among gated CD4SP cells from WT, Gfi1CD4, Bcl2-TG, or Gfi1CD4 Bcl2-TG mice. Data are representative of (A–C) five and (D) three independent experiments. Error bars indicate SEM. **P < 0.01.
of CD4SP thymocytes and rendered them a competitive disadvantage. However, forced expression of the Bcl2 transgene or deletion of Bim did not affect the generation of nTreg cells in Gfi1fl/fl−/− mice. In contrast, in hematopoietic stem cells deficient in Gfi1, the survival defect is a central mechanism for impaired stem cell function, because ectopic Bcl-2 expression allows Gfi1-deficient stem cells to self-renew and initiate multilineage differentiation (43). These results reveal that Gfi1-mediated prosurvival function exerts context-specific physiological effects.

In summary, we report here a nonconventional mechanism for the regulation of nTreg development. The transcription repressor Gfi1 restrains production of IL-2 from conventional T cells, thereby shaping the development of nTreg cells by a noncell-autonomous mechanism. Loss of Gfi1 results in expansion of nTreg cells and enhanced suppressive activity that contribute to impaired antitumor immunity. Our studies highlight that Treg cells are regulated by more than Treg-intrinsic pathways that sense and integrate signals from TCR and cytokines. Rather, thymic generation of these cells is further shaped by active control of cytokine production from conventional T cells.

Materials and Methods

Mice and in Vivo Studies. C57BL/6, CD4−/1, Rag1−/−, II10−/−, and II10−/− mice were purchased from the Jackson Laboratory. CD4-Cre, HCD2-ire, and Foxp3Cre-1TP mice were as described (22, 23, 31). Details for the generation of Gfi1fl/mice will be described elsewhere. The LoxP flanked region included coding exons for zinc aminohydrid Rag1−/− mice with 5 × 106 BM cells depleted of T cells as previously described (49), with the specified ratios of donor cells from various sources distinguished by congenic markers. To label proliferating cells in vivo, mice were injected with 0.8 mg BrdU/mouse i.p., and BrdU incorporation was analyzed 18 h later using the BrdU Staining Kit (BD Biosciences). To block IL-2 signaling, mice were injected i.v. with anti-IL-2 (clone S4B6) or anti-IL-2R (clone PC61) antibody (1 mg/mouse). Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude Children’s Research Hospital.

Flow Cytometry. For flow cytometric analyses of surface markers, cells were stained with antibodies (eBioscience) in PBS containing 2% (wt/vol) BSA.

Supporting Information
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Fig. S1. Increased natural regulatory T (nT<sup>reg</sup>) cells in growth factor independent 1 (Gfi1) -deficient mice. (A) Flow cytometry analysis of thymic populations in wild-type (WT) and mice with germ-line deletion of Gfi1 (Gfi1<sup>−/−</sup>). (B) Deletion efficiency of Gfi1 in Gfi1<sup>hCD2</sup> (left) and Gfi1<sup>CD4</sup> (right) mice as assessed by real-time PCR. (C) Cell numbers of total, Foxp3<sup>+</sup>, CD4 single-positive (CD4SP), and CD8 single-positive (CD8SP) thymocytes. (D) Cell numbers of total, Foxp3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> splenocytes. (E) Frequency of Foxp3<sup>+</sup> cells in double-positive (DP) thymocytes, peripheral blood, and liver of WT or Gfi1<sup>−/−</sup> mice. (F) BrdU staining in splenic Foxp3<sup>+</sup> cells of WT and Gfi1<sup>−/−</sup> mice at 18 h after injection with BrdU. (G) Ki-67 staining in thymic and splenic Foxp3<sup>+</sup> cells of WT and Gfi1<sup>−/−</sup> mice. Data are representative of two to three independent experiments. Error bars indicate SEM. *P < 0.05; **P < 0.01; NS, not significant.
Fig. S2. Role of Gfi1 in T\textsubscript{reg} suppression activity and antitumor immunity. (A) T\textsubscript{reg} cells from WT or Gfi\textsuperscript{CD4} mice were cocultured with effector T (T\textsuperscript{eff}) cells in the presence of anti-CD3 for 72 h with \[^{3}H\]thymidine added during the final 8 h to assess the suppressive activity of T\textsubscript{reg} cells. (B) WT and Gfi\textsuperscript{CD4} mice were inoculated with B16 melanoma cells, and tumor-infiltrating lymphocytes were isolated from tumors at day 16 and analyzed by IFN-\gamma intracellular staining. Data are representative of (A) four and (B) two independent experiments. Error bars indicate SEM. CPM, counts per minute. **P < 0.01.

Fig. S3. Gfi1 exerts noncell-autonomous effects on the nT\textsubscript{reg} population. (A) Complete bone marrow chimeras were generated by reconstituting sublethally irradiated Rag1\textsuperscript{−/−} mice with WT or Gfi\textsuperscript{CD4} bone marrow cells, and they were analyzed 6–8 wk later for the frequency of Foxp3\textsuperscript{+} cells among CD4SP. (B) Mixed bone marrow chimeras were generated by reconstituting sublethally irradiated Rag1\textsuperscript{−/−} mice with a 1:1 mixture of spike (CD45.1\textsuperscript{+}) and WT or Gfi\textsuperscript{CD4} (CD45.2\textsuperscript{+}) bone marrow cells, and they were analyzed 6–8 wk later for the frequency of Foxp3\textsuperscript{+} cells among CD4\textsuperscript{SP} splenocytes. (C) CD103 expression among Foxp3\textsuperscript{+}CD4\textsuperscript{SP} thymocytes of mixed bone marrow chimeras. (D) Predominant bone marrow chimeras were generated by reconstituting sublethally irradiated Rag1\textsuperscript{−/−} mice with a 1:20 mixture of spike (CD45.1.2\textsuperscript{+}; minority) and WT or Gfi\textsuperscript{CD4} (CD45.2\textsuperscript{+}; majority) bone marrow cells, and they were analyzed for the frequency of Foxp3\textsuperscript{+} cells among CD4\textsuperscript{SP} thymocytes (Left) or CD4\textsuperscript{SP} splenocytes (Right). (E) CD103 expression among Foxp3\textsuperscript{+} CD45P thymocytes of WT or Gfi\textsuperscript{Foxp3} mice. The percentages in the parentheses indicate the CD103\textsuperscript{−} and CD103\textsuperscript{+} cells among Foxp3\textsuperscript{+} cells. (F) T\textsubscript{reg} cells from WT or Gfi\textsuperscript{Foxp3} mice were cocultured with T\textsubscript{eff} cells in the presence of anti-CD3 for 72 h, with \[^{3}H\]thymidine added during the final 8 h to assess the suppressive activity of T\textsubscript{reg} cells. Data are representative of two to three independent experiments. Error bars indicate SEM. CPM, counts per minute.
Fig. S4. IL-2 but not IL-10 contributes to the expansion of nT<sub>reg</sub> cells in Gfi1<sup>CD4</sup> mice. (A) Il2 mRNA expression in non-T<sub>reg</sub> (GITR<sup>−</sup>CD25<sup>−</sup>) and T<sub>reg</sub> (GITR<sup>+</sup>CD25<sup>+</sup>) populations from WT or Gfi1<sup>CD4</sup> thymocytes analyzed by real-time PCR. (B) Il2 mRNA expression in splenic CD4<sup>+</sup> cells from WT or Gfi1<sup>CD4</sup> mice. (C) Il2 mRNA expression in dendritic cells isolated from WT or Gfi1<sup>CD4</sup> thymus. (D) Frequency of Foxp3<sup>+</sup> cells among CD4SP thymocytes and (E) CD103 expression among Foxp3<sup>+</sup> cells are shown for WT, Gfi1<sup>CD4</sup>, Il10<sup>−/−</sup>, and Gfi1<sup>CD4</sup> Il10<sup>−/−</sup> mice (6–8 wk). Data are representative of two to three independent experiments. Error bars indicate SEM. **P < 0.01; GITR, glucocorticoid-induced TNF receptor family-related protein; NS, not significant.

Fig. S5. Bim deficiency does not alter Gfi1 effects on the nT<sub>reg</sub> population or CD103 expression. Frequency of Foxp3<sup>+</sup> cells among (A) CD4SP thymocytes and (B) CD103 expression among Foxp3<sup>+</sup> cells are shown for Bcl2l11<sup>−/−</sup> and Gfi1<sup>CD4</sup> Bcl2l11<sup>−/−</sup> mice.