Immunity
Article

An Essential Role of the Transcription Factor GATA-3 for the Function of Regulatory T Cells

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DOI 10.1016/j.immuni.2011.08.012

SUMMARY

Forkhead Box P3 (Foxp3)-expressing regulatory T (Treg) cells are central to maintaining self-tolerance and immune homeostasis. How Treg cell function and Foxp3 expression are regulated is an important question under intensive investigation. Here, we have demonstrated an essential role for the transcription factor GATA-3, a previously recognized Th2 cell master regulator, in controlling Treg cell function. Treg cell-specific GATA-3 deletion led to a spontaneous inflammatory disorder in mice. GATA-3-null Treg cells were defective in peripheral homeostasis and suppressive function, gained Th17 cell phenotypes, and expressed reduced amounts of Foxp3. In addition, GATA-3 controlled Foxp3 expression by binding to and promoting the activity of *cis*-acting elements of *Foxp3*. Furthermore, the combined function of GATA-3 and Foxp3 was essential for Foxp3 expression. These findings provide insights into immune regulatory mechanisms and uncover a critical function of GATA-3 in Treg cells and immune tolerance.

INTRODUCTION

Regulatory T (Treg) cells are a $CD4^+$ T cell subset possessing potent immune-suppressive activities critical for maintaining self-tolerance and immune homeostasis. Defects in Treg cell function invariably result in autoimmunity and inflammatory disease in mammals (Sakaguchi et al., 2008; Shevach, 2000). Foxp3, an X-linked transcription factor, is highly and specifically expressed in Treg cells. Ectopic expression of Foxp3 endows non-Treg cells with immune-suppressive function (Brunkow et al., 2001; Khattri et al., 2003), and Foxp3 deficiency leads to systemic autoimmune syndrome in human *IPEX* patients and *Scurfy* mice because of lack of functional Treg cells (Brunkow et al., 2001; Fontenot et al., 2003; Hori et al., 2003; Wildin et al., 2001). Therefore, Foxp3 is recognized as the central regulator of Treg cells. To understand immune tolerance and immune homeostasis, how Treg cell and Foxp3 function are controlled is a critical question yet to be fully addressed. Increasing numbers of transcription factors are shown to be important for Treg cell function (Chaudhry et al., 2009; Kerdiles et al., 2010; Kitoh et al., 2009; Koch et al., 2009; Ouyang et al., 2010; Rudra et al., 2009; Zheng et al., 2009). Curiously, factors controlling the differentiation of Th1 and Th2 cells regulate distinct and specific functions of Treg cells (Kitoh et al., 2009; Koch et al., 2009; Rudra et al., 2009; Szabo et al., 2000; Zheng et al., 2009).

GATA-3 is a transcription factor that is highly expressed in Th2 cells and critical for the differentiation of these cells (Zheng and Flavell, 1997; Zhu et al., 2004). GATA-3 is therefore regarded as the "master regulator" for Th2 cells. Nevertheless, GATA-3 expression and function is not limited to Th2 cells. GATA-3 is expressed in multiple tissues and cell types (Yamamoto et al., 1990) and is required for T cell development and natural killer (NK) cell function (Pai et al., 2003; Samson et al., 2003). Thus, GATA-3 plays multifaceted roles of regulating immune function in a cell-type-specific fashion. Importantly, whether and how GATA-3 is involved in controlling Treg cell function in vivo is unknown.

We found that GATA-3 expression was elevated in Treg cells compared to conventional T cells and was suppressed in Treg cells under Th1 cell polarizing condition, where Treg cell function is often found tempered (Caretto et al., 2010; Oldenhove et al., 2009). We therefore hypothesized that GATA-3 expression in Treg cells is important for their optimal function and that defective GATA-3 expression will alter the properties of Treg cells. To test this hypothesis, we deleted GATA-3 specifically in Foxp3-expressing Treg cells and found that GATA-3 deletion in Treg cells led to the development of an inflammatory disorder in mice. GATA-3-deficient Treg cells were intrinsically defective in their homeostasis and showed compromised immune-suppressive function in vitro and in vivo. In agreement with these observations, GATA-3-deficient Treg cells expressed decreased amounts of Foxp3 and Treg cell "signature genes" and increased amounts of effector cytokines. In addition, we demonstrated that GATA-3 bound to the regulatory region of *Foxp3* locus and promoted the activity of a *cis*-acting element of *Foxp3* gene. Moreover, we showed that the combined function of GATA-3 and Foxp3 was vital for Foxp3 expression, because virtually no Foxp3-expressing cells could be detected when both GATA-3 and Foxp3 were defective. Collectively, this study reveals an essential function of GATA-3 in controlling Treg cell function and Foxp3 expression. It provides further insight into immune regulatory mechanisms and sheds light on GATA-3 function and how immune responses are controlled.

RESULTS

Mice with Treg Cell-Specific GATA-3 Deletion Develop an Inflammatory Disorder

Transcription factors critically involved in directing the differentiation of Th1 and Th2 cells were recently shown to play unique and important roles in controlling Treg cell function (Kitoh et al., 2009; Koch et al., 2009; Rudra et al., 2009; Zheng et al., 2009). However, whether the Th2 cell master regulator GATA-3 is involved in Treg cell function remains unknown. To address this question, we first asked whether GATA-3 is expressed by Treg cells. GATA-3 expression was assessed in purified Treg cells and non-Treg $CD4^+$ T (Tn) cells isolated from *FIR* mice, where Foxp3-expressing cells were marked by the expression of a red fluorescence protein, as described previously (Wan and Flavell, 2005). Treg cells expressed more GATA-3 than Tn cells at both mRNA and protein levels. In addition, GATA-3 expression was downregulated in Treg cells under Th1 cell polarizing condition associating with reduced Treg cell recovery and Foxp3 expression (see Figure S1 available online), agreeing with the finding that Treg cell function is tempered during Th1 cell response (Caretto et al., 2010; Oldenhove et al., 2009). These observations suggest a potential role of GATA-3 in regulating Treg cell function. We therefore investigated how GATA-3 is involved in Treg cell function. To do this, we generated Treg cell-specific GATA-3-deficient mice by crossing *Gata3*$^{fl/fl}$ mice (Amsen et al., 2007) with mice bearing a BAC transgene encoding both enhanced green fluorescence protein (EGFP) and Cre recombinase under the control of Foxp3 promoter (*Foxp3-EGFP-cre* mice [Zhou et al., 2008], hereafter referred to as *FGC* mice). In *FGC* mice, EGFP expression faithfully marks Foxp3-expressing Treg cells, and Cre-mediated gene deletion occurs specifically in Treg cells (Zhou et al., 2008).

Although *Gata3*$^{fl/fl}$*:FGC* mice were born normally and matured to adulthood, they spontaneously developed lymphadenopathy and splenomegaly by 16 weeks of age (data now shown). In agreement with this observation, the total numbers of lymphocytes recovered from the peripheral lymph nodes (PLN) and spleens of *Gata3*$^{fl/fl}$*:FGC* mice were higher compared to those from *Gata3*$^{fl/+}$*:FGC* littermates (Figure 1A), although the distribution of T cell populations in the thymus, spleen, and peripheral lymph nodes was normal and Treg cells were generated in the thymus in *Gata3*$^{fl/fl}$*:FGC* mice (Figure S1). In addition, histological analysis revealed dramatically increased lymphocytic infiltration in the nonlymphoid organs, such as the lung, pancreas, lacrimal glands, and salivary glands of *Gata3*$^{fl/fl}$*:FGC* mice (Figure 1B), suggesting aberrant immune activation. We thus examined the activation and differentiation status of T cells from *Gata3*$^{fl/fl}$*:FGC* mice. $CD4^+$ Tn and $CD8^+$ T cell populations with an activated phenotype ($CD62L^{lo}CD44^{hi}$) were increased in *Gata3*$^{fl/fl}$*:FGC* mice compared to those in *Gata3*$^{fl/+}$*:FGC* littermates (Figures 1C and S1). In addition, interferon-γ (IFN-γ), interleukin-4 (IL-4), and IL-17A production by $CD4^+$ Tn cells were increased in *Gata3*$^{fl/fl}$*:FGC* mice compared to *Gata3*$^{fl/+}$*:FGC* littermates (Figures 1D and 1E). Similarly, $CD8^+$ T cells from *Gata3*$^{fl/fl}$*:FGC* mice produced increased amounts of IFN-γ (Figure S1). Therefore, Treg cell-specific GATA-3 deletion led to an inflammatory disorder in mice, associated with elevated activation and effector function of T cells.

Treg Cells Lacking GATA-3 Are Defective in Homeostasis

We then investigated how the Treg cell population was affected by GATA-3 deletion. First, efficient and specific deletion of GATA-3 in Treg cells purified from *Gata3*$^{fl/fl}$*:FGC* mice was confirmed (Figure 2A). Initial characterization showed that the numbers of Treg cells were not decreased but moderately increased in *Gata3*$^{fl/fl}$*:FGC* mice (Figure 2B). Nevertheless, this observation could be confounded by the inflammatory conditions existed in these mice because Treg cell numbers may be abnormally upregulated in the hosts with inflammation (Kitoh et al., 2009; Liu et al., 2008; Rudra et al., 2009).

We therefore compared the ability of GATA-3-deficient and GATA-3-sufficient Treg cells to repopulate the periphery in the same host. To this end, we generated mixed bone marrow chimera by transferring a mixture of bone marrow cells from *Gata3*$^{fl/fl}$*:FGC* mice bearing congenic marker CD45.2 and *Gata3*$^{fl/+}$*:FGC* mice bearing congenic marker CD45.1 into irradiated recipient mice deficient in recombination-activating gene 1 (*Rag1*$^{-/-}$). All chimeric mice were grossly normal and showed no signs of inflammatory disorder (data not shown). Accordingly, in the chimeric mice, $CD4^+$ Tn and $CD8^+$ T cells of both donor origins showed naive phenotype with minimal production of effector cytokines (Figure S2). Although total $CD4^+$ T cells were derived equally from both donors, the majority (70%–80%) of $Foxp3^+$ Treg cells were derived from GATA-3-sufficient donor. And only approximately 20% of Treg cells were derived from GATA-3-deficient donor (Figure 2C), suggesting an intrinsic defect in the homeostasis of GATA-3-deficient Treg cells. To confirm that GATA-3-deficient Treg cells were indeed defective in homeostasis in the periphery, we purified Treg cells from *Gata3*$^{fl/fl}$*:FGC* ($CD45.2^+$) and *Gata3*$^{fl/+}$*:FGC* ($CD45.1^+$) mice by fluorescence-activated cell sorting (FACS). Sorted cells were mixed at 1:1 ratio and then transferred into *Rag1*$^{-/-}$ mice. Eight weeks after transfer, the contributions of different donor cells to $Foxp3^+$ Treg cell populations in the hosts were assessed. GATA-3-deficient Treg cells were much less abundant than GATA-3-sufficient counterparts (Figure 2D). Collectively, these findings suggest that GATA-3-deficient Treg cells are intrinsically defective in the homeostasis.

Immune-Suppressive Activity Is Impaired in Treg Cells Lacking GATA-3

We investigated what functions of Treg cells were affected by GATA-3 deletion. The defining properties of Treg cells are being unresponsive to T cell receptor (TCR) stimulation (anergy) in vitro and being able to suppress $CD4^+$ Tn cell function in vivo and in vitro (Sakaguchi, 2004). We found that GATA-3-deficient Treg cells remained anergic to TCR stimulation, because Treg cells from *Gata3*$^{fl/fl}$*:FGC* mice did not proliferate in response to TCR stimulation in culture (Figure 3A). We then studied whether the immune-suppressive activity of GATA-3-deficient Treg cells was affected in vivo by using an inflammatory bowel disease (IBD) model induced by transferring naive $CD4^+$ cells into *SCID* mice (Powrie et al., 1993). Transferring wild-type naive ($CD25^-CD45RB^{hi}$) $CD4^+$ T cells alone elicited IBD in *Rag1*$^{-/-}$ recipient mice manifested by progressive weight loss (Figure 3B). Cotransfer with wild-type Treg cells was able to prevent the weight loss (Figure 3B). In contrast, cotransfer with GATA-3-deficient

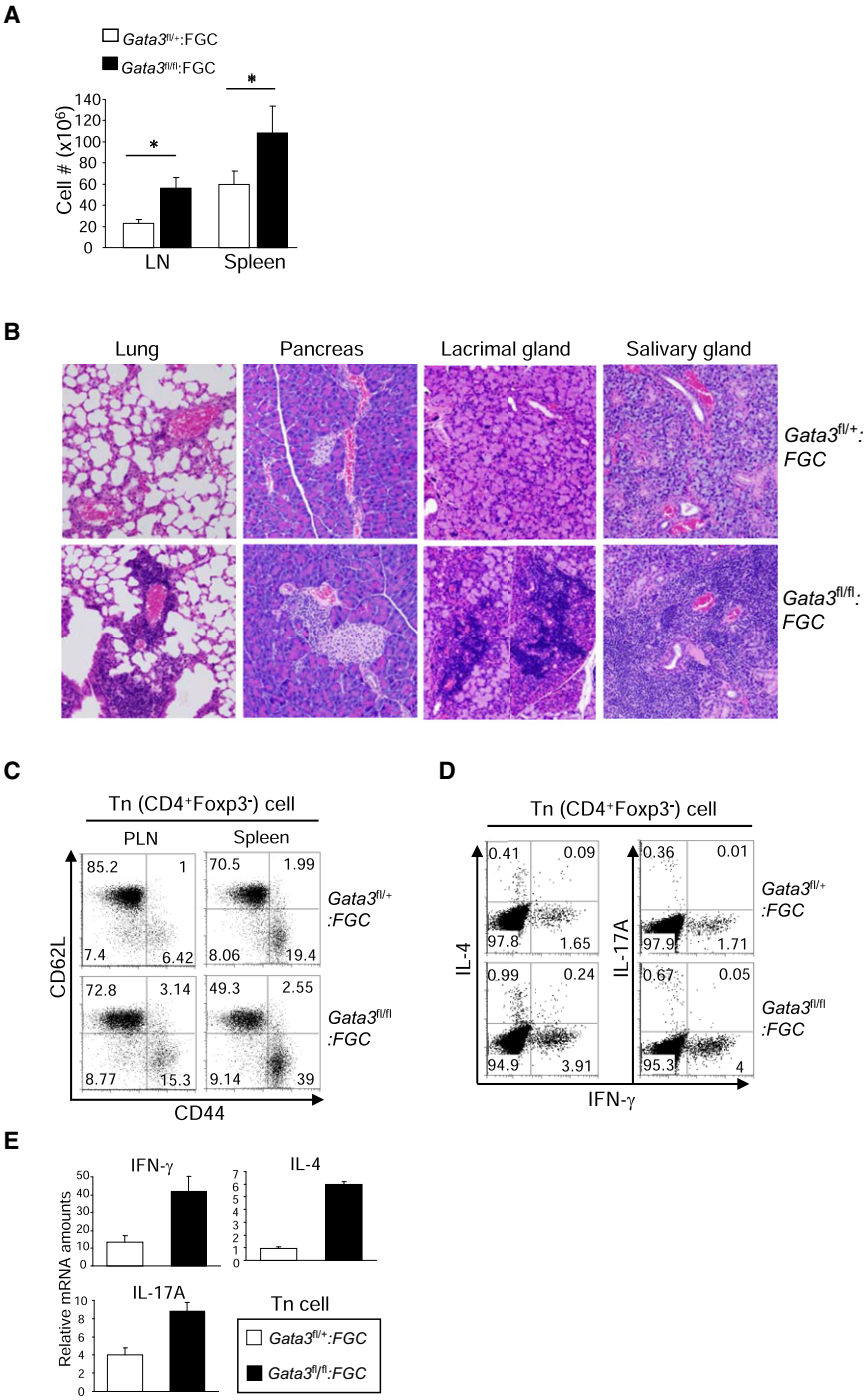

Figure 1. GATA-3 Deletion in Treg Cells Leads to an Inflammatory Disorder in Mice

(A) The numbers of lymphocytes in the PLN and spleens from *Gata3*$^{fl/fl}$*:FGC* and *Gata3*$^{fl/+}$*:FGC* littermates. Data are means ± SD of five mice. *p < 0.05.

(B) Histological analysis of lymphocytic infiltration in the lung, pancreas, lacrimal gland, and salivary gland of indicated mice by H&E staining.

(C) Expression of CD44 and CD62L on CD4$^+$ Foxp3$^-$ (Tn) cells from the lymph nodes and spleens of *Gata3*$^{fl/fl}$*:FGC* mice and *Gata3*$^{fl/+}$*:FGC* littermates. Number in each quadrant showed the percentage of each population. Data represent at least four independent experiments.

(D) IFN-γ, IL-4, and IL-17 expression by CD4$^+$ Foxp3$^-$ (Tn) cells from the PLNs of *Gata3*$^{fl/fl}$*:FGC* mice and *Gata3*$^{fl/+}$*:FGC* littermates. Number in each quadrant showed the percentage of each population. Results are representative of at least four experiments.

(E) Relative mRNA expression amounts of IFN-γ, IL-4, and IL-17A in sorted CD4$^+$GFP$^-$ Tn cells from *Gata3*$^{fl/fl}$*:FGC* mice and *Gata3*$^{fl/+}$*:FGC* littermates. Means ± SD of triplicates done in one experiments representative of three are shown.

See also Figure S1.

Treg cells failed to stop naive T cell-elicited weight loss in the recipient mice (Figure 3B). This observation could be due to reduced numbers of GATA-3-deficient Treg cells in the recipient mice and/or defective suppressive activity of these cells. Further examination revealed that the numbers of Foxp3$^+$ GATA-3-deficient Treg cells recovered from the recipient mice were much lower than those of wild-type Treg cells (Figure 3C). To directly assess immune-suppressive activity of GATA-3-deficient Treg cells, we used an in vitro suppression assay and found that GATA-3-deficient Treg cells showed impaired ability to suppress TCR-stimulated proliferation of coexisting CD4$^+$ Tn cells (Figure 3D). Therefore, GATA-3 was required for intact immune-suppressive activities of Treg cells in vivo and in vitro.

GATA-3-Deficient Treg Cells Gain the Ability to Produce Th17 Cytokines

Treg cells normally do not produce effector cytokines. However, perturbation of Treg cell function often leads to aberrant cytokine production by these cells (Gavin et al., 2007; Kitoh et al., 2009; Oldenhove et al., 2009; Ouyang et al., 2010; Wan and Flavell, 2007). Our finding that GATA-3 deletion resulted in defective Treg cell function prompted us to ask whether GATA3-deficient Treg cells produced proinflammatory cytokines. Although freshly isolated GATA-3-deficient Treg cells produced minimal amounts of IFN-γ and IL-4, more than 3% of these cells produced IL-17A, a defining cytokine for Th17 cells (Harrington et al., 2005; Park et al., 2005). At least 3-fold more IL-17A was produced by GATA-3-deficient than -sufficient Treg cells at both mRNA and protein levels (Figures 4A and 4B). This aberrant acquisition of a Th17 cell phenotype from GATA-3-deficient Treg cells was even more pronounced in mice afflicted with IBD (Figures 4C and 4D). Because the inflammatory cytokine IL-6 promotes IL-17A production in activated Foxp3$^+$ Treg cells

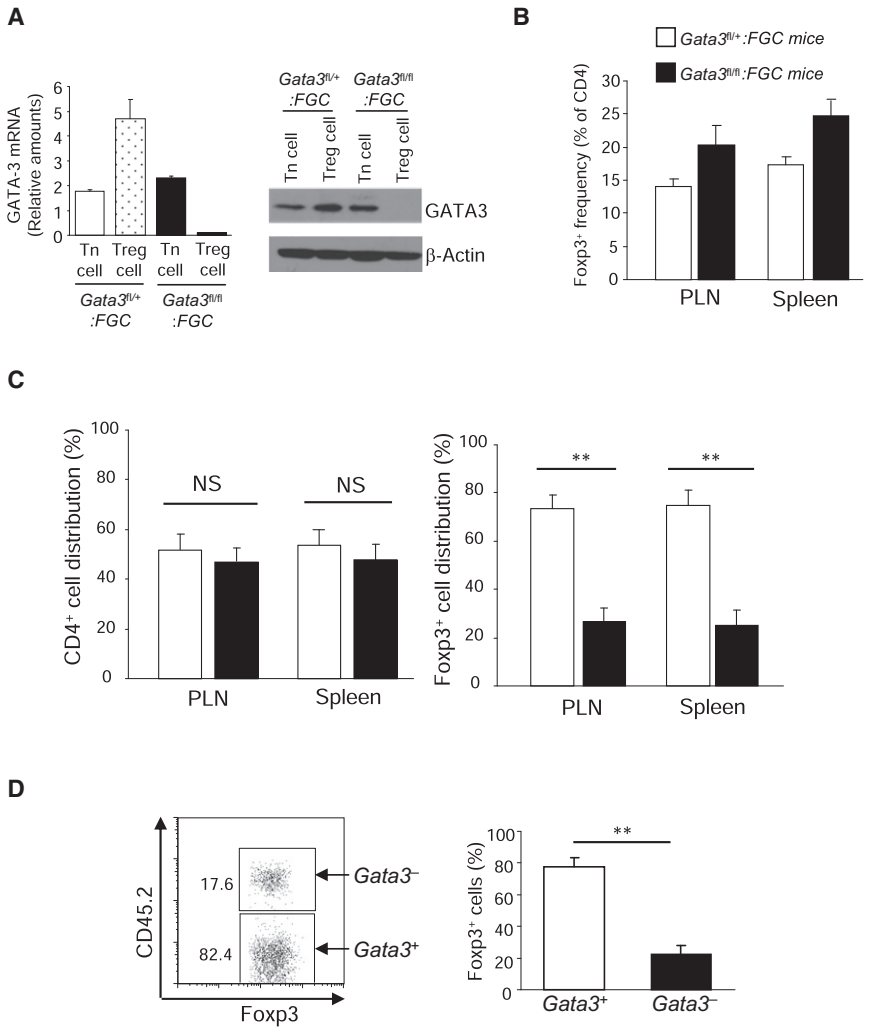

Figure 2. Intrinsic Defects of GATA-3-Deficient Treg Cells in the Peripheral Homeostasis

(A) Efficient GATA-3 deletion specifically in Treg cells from *Gata3*$^{fl/fl}$*:FGC* mice. CD4$^+$ Tn cells (CD4$^+$GFP$^-$) and Treg cells (CD4$^+$GFP$^+$) were sorted from *Gata3*$^{fl/+}$*:FGC* and *Gata3*$^{fl/fl}$*:FGC* mice. mRNA and protein expression of GATA-3 in each population was assessed by qRT-PCR and immunoblotting, respectively. Results for qRT-PCR were means ± SD of triplicates done in one experiment representative of three. Results for immunoblotting were representative of at least three experiments.

(B) The percentages of Foxp3$^+$ Treg cells in CD4 T cells from PLNs and spleens of *Gata3*$^{fl/+}$*:FGC* (open bars) and *Gata3*$^{fl/fl}$*:FGC* (solid bars) mice. Means ± SD of four experiments are shown.

(C) Mixed bone marrow chimera were created by transferring equal numbers of bone marrow cells from *Gata3*$^{fl/+}$*:FGC* (CD45.1$^+$) and *Gata3*$^{fl/fl}$*:FGC* (CD45.2$^+$) mice into sublethally irradiated *Rag1*$^{-/-}$ mice. The contributions of cells originated from *Gata3*$^{fl/+}$*:FGC* (open bars) and *Gata3*$^{fl/fl}$*:FGC* (solid bars) bone marrow cells to CD4$^+$ T cell and Foxp3$^+$ Treg cell populations in reconstituted hosts were determined. Means ± SD of six mice from one experiment representative of two are shown (NS, nonsignificant; **$p < 0.01$).

(D) GFP$^+$ Treg cells were sorted from *Gata3*$^{fl/+}$*:FGC* (CD45.1$^+$) and *Gata3*$^{fl/fl}$*:FGC* (CD45.2$^+$) mice, mixed at a ratio of 1:1, and then transferred into *Rag1*$^{-/-}$ mice. Eight weeks after transfer, Foxp3$^+$ Treg cells from different donors were detected by Foxp3 and CD45 costaining as shown in left plot. The contributions of each donor origin to Treg cell population in the recipients were determined. Means ± SD of five mice from one experiment representative of two are shown (**$p < 0.01$).

See also Figure S2.

in vitro (Xu et al., 2007; Yang et al., 2008), we speculated that IL-6-driven IL-17A expression would be enhanced in Treg cells with GATA-3 deletion. Indeed, upon TCR stimulation in the presence of exogenous IL-6 alone or IL-6 plus transforming growth factor-β (TGF-β), a notably higher percentage of GATA-3-deficient Foxp3$^+$ Treg cells produced IL-17A than that of GATA-3-sufficient Treg cells (Figure 4E). Therefore, GATA-3-deficient Treg cells acquired enhanced ability to produce Th17 cell cytokines.

The Expression of Foxp3 and Treg Cell Signature Genes Is Decreased in GATA-3-Deficient Treg Cells

Aforementioned findings suggested that GATA-3 was essential for Treg cell function and prompted us to investigate which factor critical for Treg cell function was affected by GATA-3 deletion. We first noticed that EGFP expression, which reflected Foxp3 expression at the transcription level in *FGC* mice, was consistently lower in Treg cells from *Gata3*$^{fl/fl}$*:FGC* mice than from *Gata3*$^{fl/+}$*:FGC* mice (Figure 5A). To directly assess Foxp3 mRNA expression, we sorted GATA-3-deficient and -sufficient Treg cells from *Gata3*$^{fl/fl}$*:FGC* and *Gata3*$^{fl/+}$*:FGC* mice, respectively, and detected Foxp3 expression by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Indeed, the amount of Foxp3 mRNA was decreased by approximately 50% upon GATA-3 deletion (Figure 5B). In addition, the amount of Foxp3 protein was reduced to a similar extent in GATA-3-deficient Treg cells (Figure 5C). Treg cell signature genes, such as CD25, CTLA-4, and GITR, are important for Treg cell function (Sakaguchi, 2004). We then examined whether the expression of these genes was affected by GATA-3 deletion. Compared to Treg cells from *Gata3*$^{fl/+}$*:FGC* mice, Treg cells from *Gata3*$^{fl/fl}$*:FGC* mice consistently expressed less CD25, CTLA-4, and GITR at both mRNA and protein levels (Figures 5D and 5E). Similar observation were made in mixed bone marrow chimera that was reconstituted with bone marrow cells from *Gata3*$^{fl/fl}$*:FGC* mice (CD45.2$^+$) and *Gata3*$^{fl/+}$*:FGC* mice (CD45.1$^+$) (Figure 5F); GATA-3-deficient Treg cells expressed less amounts of Foxp3, CD25, CTLA-4, and GITR than those of GATA-3-sufficient counterparts in the same host. Therefore, GATA-3 deletion led to reduced expression of Foxp3 and Treg cell signature genes in Treg cells.

GATA-3 Binds to CNS2 of *Foxp3* Locus and Promotes Its Activity

Foxp3 is central to controlling various aspects of Treg cell function. The observation that GATA-3 deletion caused a decrease of

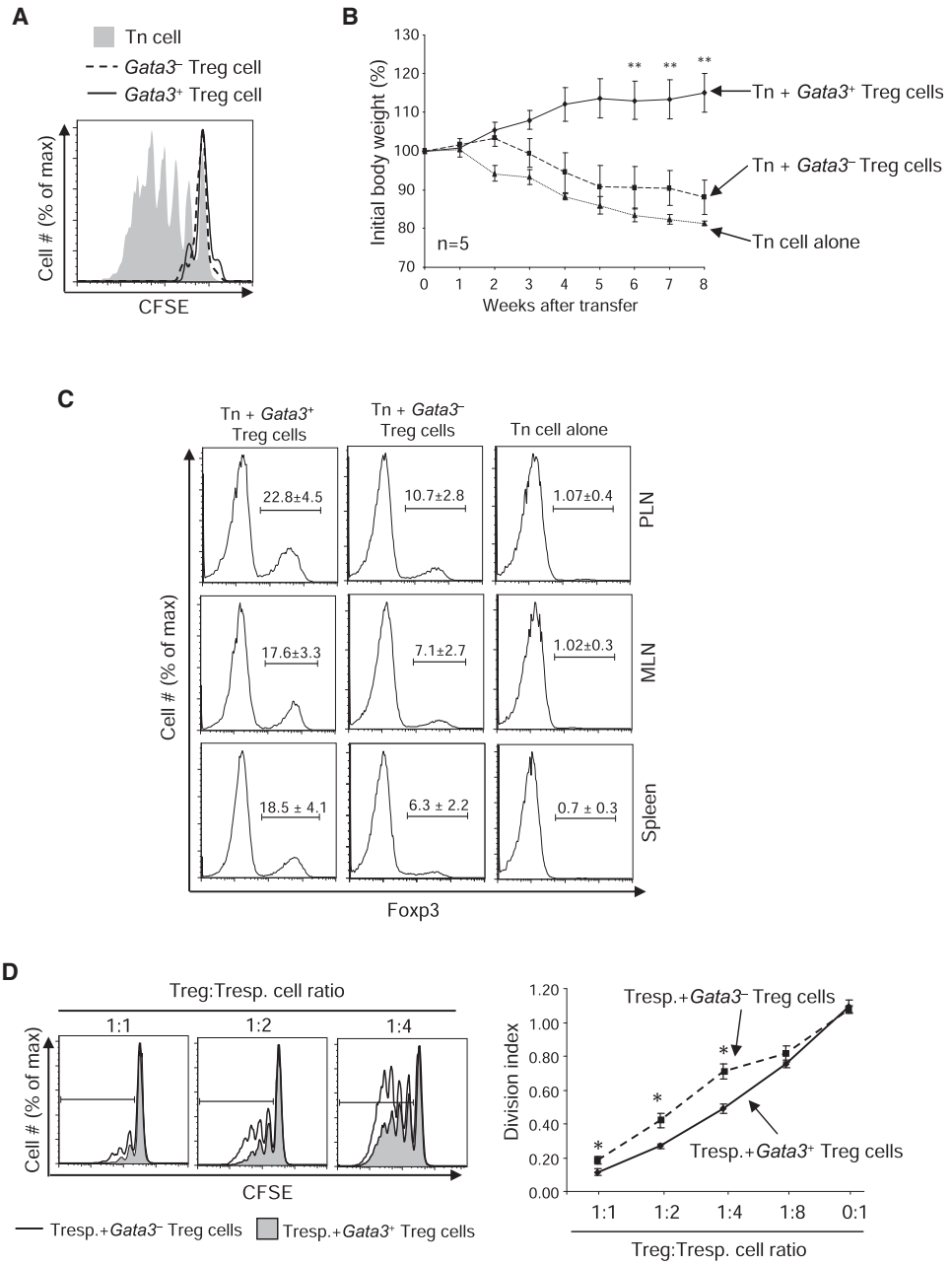

Figure 3. Immune-Suppressive Activity of Treg Cells Is Impaired in the Absence of GATA-3

(A) GATA-3-deficient Treg cells remained anergic in vitro. GFP$^+$ Treg cells were sorted from *Gata3*$^{fl/fl}$*:FGC* (*Gata3*$^-$, dashed line) and *Gata3*$^{fl/+}$*:FGC* (*Gata3*$^+$, solid line) mice. As a control, GFP$^-$CD4$^+$ Tn (shaded) cells were sorted from *Gata3*$^{fl/+}$*:FGC* mice. Sorted cells were labeled with CFSE and stimulated with soluble anti-CD3 in the presence of irradiated APC. Cell proliferation was assessed by CFSE dilution 72 hr after TCR stimulation in vitro.

(B) GATA-3-deficient Treg cells failed to suppress T cell function in vivo. *Rag1*$^{-/-}$ mice were transferred with 2 × 10^5 CD4$^+$CD25$^-$CD45RB^{hi} T cells sorted from wild-type C57BL/6 mice alone (dotted line) or together with 1 × 10^5 of GFP$^+$ Treg cells sorted from *Gata3*$^{fl/fl}$*:FGC* mice (dashed line) or from *Gata3*$^{fl/+}$*:FGC* mice (solid line). Body weight of recipient mice was monitored weekly for 8 weeks after transfer. The percentage of body weight change was calculated and plotted. Means ± SEM of five mice in one experiment representative of two are shown (**$p < 0.01$).

(C) The percentages (indicated by the numbers above the brackets) of Foxp3$^+$ Treg cells in CD4$^+$ T cells recovered from the peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), and spleens of recipient mice were determined at the end of experiments described in (B). Means ± SD of five mice in one experiment representative of two are shown.

(D) GATA-3-deficient Treg cells were defective in suppressing T cell function in vitro. CD4$^+$CD25$^-$CD45RB^{hi} responder T (Tresp.) cells were sorted from wild-type C57BL/6 mice and labeled with CFSE. Labeled responder T cells were then either cultured alone or mixed with varying amounts (as indicated) of GFP$^+$ Treg cells sorted from *Gata3*$^{fl/fl}$*:FGC* or *Gata3*$^{fl/+}$*:FGC* mice. Cell mixtures were stimulated with soluble anti-CD3 in the presence of irradiated APC. The proliferation of responder T cells was assessed by CFSE dilution as shown in left plots. The division-index of responder T cells was determined by FlowJo software and plotted (right). Means ± SD of triplicate done in one experiments representative of two are shown (*$p < 0.05$).

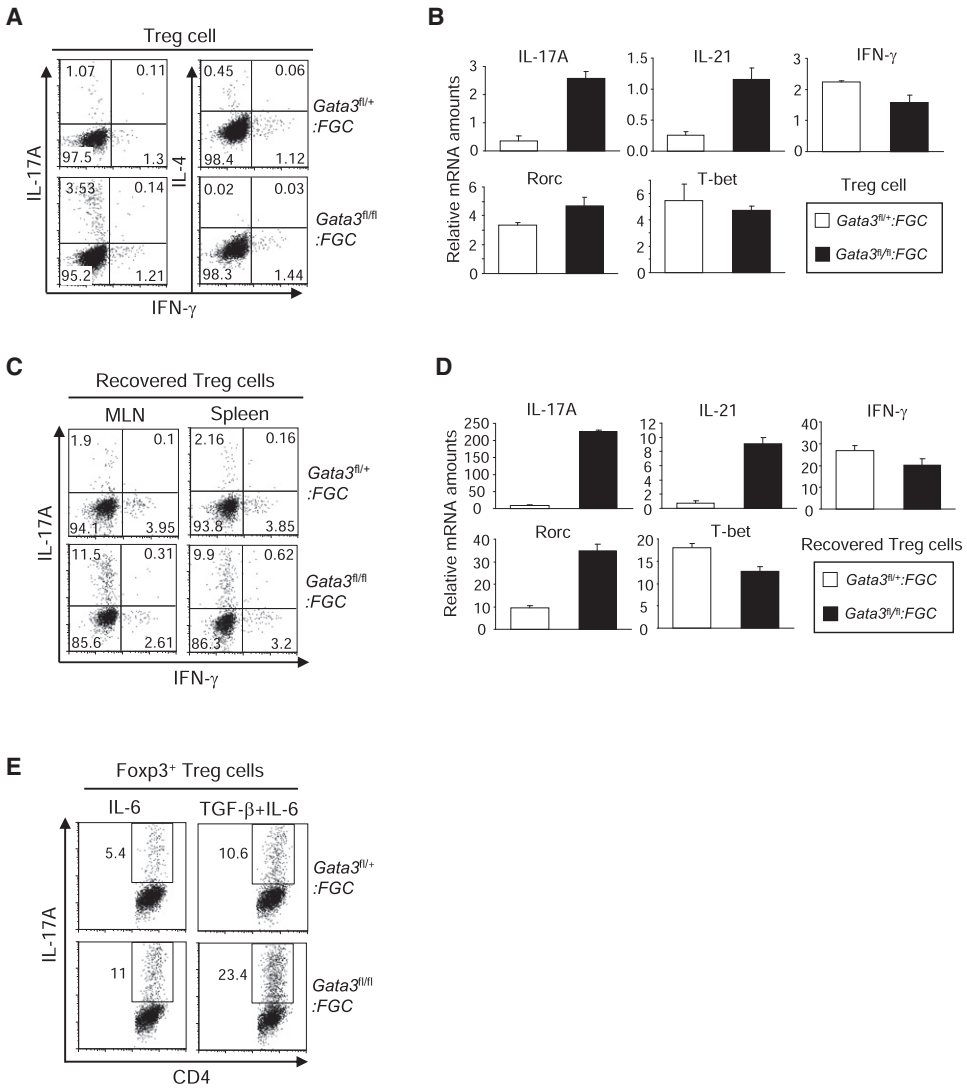

Figure 4. Th17 Cytokine Production by GATA-3-Deficient Treg Cells

(A and B) GFP$^+$ Treg cells were purified from *Gata3*$^{fl/fl}$*:FGC* and *Gata3*$^{fl/+}$*:FGC* mice. Protein expression of IFN-γ, IL-4, and IL-17A in sorted Treg cells was assessed by intracellular staining. The numbers in each quadrant indicate the percentage of relevant population. Representative results of at least five experiments are shown (A). Relative mRNA amounts of IL-17A, IL-21, IFN-γ, Rorc, and T-bet in sorted Treg cells were determined by qRT-PCR. Means ± SD of triplicates done in one experiment representative of three are shown (B).

(C and D) At the end of IBD experiment described in Figure 3B, CD4$^+$ T cells were recovered from mesenteric lymph nodes (MLN) and spleens of the recipient mice. Protein expression of IL-17A and IFN-γ in recovered Foxp3$^+$ Treg cells was determined by intracellular staining. Representative results of three experiments are shown (C). Relative mRNA amounts of IL-17A, IL-21, IFN-γ, Rorc, and T-bet in sorted GFP$^+$ Treg cells from the spleens were determined by qRT-PCR. Means ± SD of triplicates done in one experiment representative of three are shown (D).

(E) GFP$^+$ Treg cells were sorted from *Gata3*$^{fl/fl}$*:FGC* and *Gata3*$^{fl/+}$*:FGC* mice and then stimulated in vitro with anti-CD3 and anti-CD28 in the presence of IL-6 (40 ng/ml) or IL-6 (40 ng/ml) + TGF-β (2 ng/ml). Four days after stimulation, IL-17A production in Foxp3$^+$ cells was assessed. Results representative of three experiments are shown.

Foxp3 expression (Figure 5) prompted us to study the mechanisms by which GATA-3 regulates Foxp3 expression. Although reduced CD25 expression in GATA-3-deficient Treg cells can contribute to decreased Foxp3 expression as indicated by the fact that IL-2 signaling is critical for Foxp3 expression (Fontenot et al., 2005; Rubtsov et al., 2010), we nonetheless hypothesized that GATA-3 may also directly regulate Foxp3 expression. With PROMO, a transcription factor binding site prediction program, we identified putative GATA-3 binding sites in previously defined Foxp3 regulatory regions (Figure 6A). Four highly conserved noncoding DNA sequences (CNS) including promoter, CNS1, CNS2, and CNS3 in *Foxp3* locus have been identified (Long et al., 2009; Zheng et al., 2010). CNS1 is critical for TGF-β-induced Foxp3 expression, CNS2 is important to maintain Foxp3 expression in thymus-derived Treg cells, and CNS3 acts as a pioneer element for Foxp3 expression. We first investigated whether GATA-3 bound to these putative sites in Treg cells by performing chromatin immunoprecipitation (ChIP) assay. As expected, we found

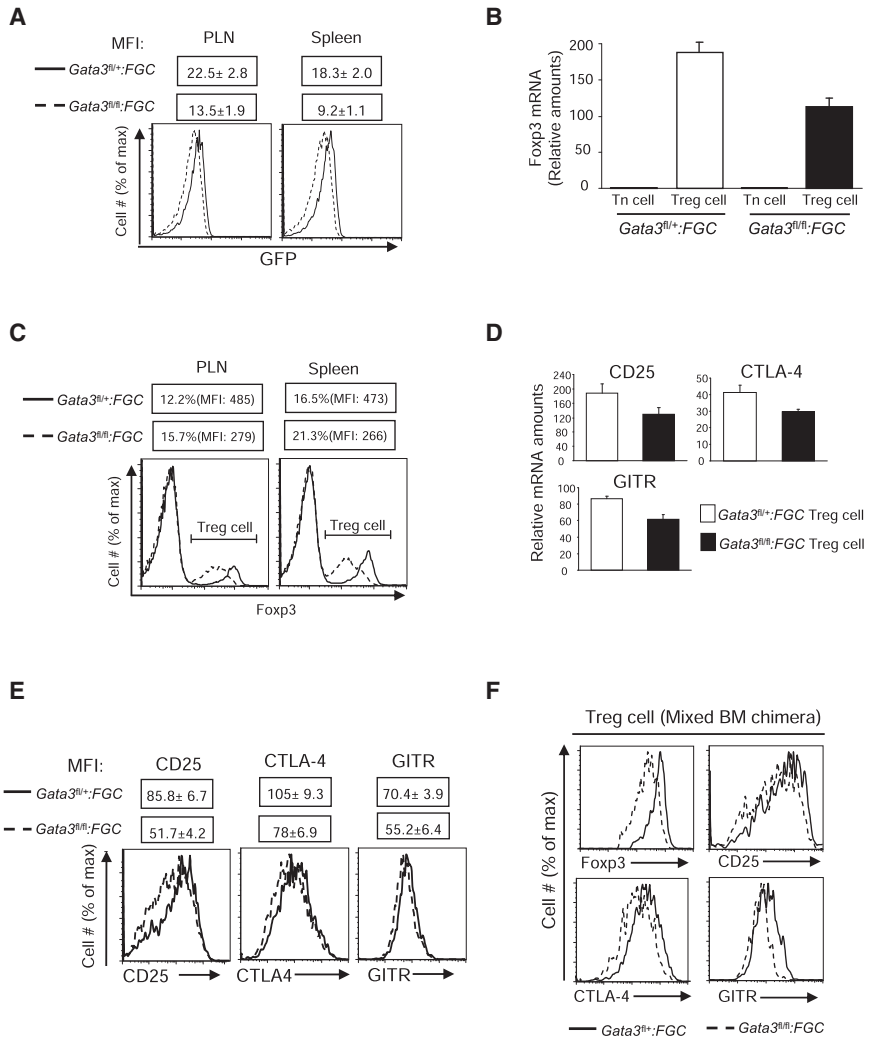

Figure 5. Decreased Expression of Foxp3 and Treg Cell Signature Genes in GATA-3-Deficient Treg Cells

(A) EGFP expression of gated $CD4^+GFP^+$ Treg cells from PLNs and spleens of *Gata3*$^{fl/fl}$*:FGC* (dashed lines) and *Gata3*$^{fl/+}$*:FGC* (solid lines) mice. Mean fluorescence intensity (MFI) of EGFP is also shown as means ± SD of the results from three mice.

(B) Quantitative RT-PCR analysis of Foxp3 mRNA in $CD4^+GFP^-$ (Tn) and $CD4^+GFP^+$ (Treg) cells sorted from *Gata3*$^{fl/fl}$*:FGC* (solid bars) and *Gata3*$^{fl/+}$*:FGC* (open bars) mice. Means ± SD of triplicates done in one experiment representative of at least four experiments are shown.

(C) Flow cytometric analysis of Foxp3 expression in $CD4^+$ T cells in PLNs and spleens of *Gata3*$^{fl/fl}$*:FGC* (dashed lines) and *Gata3*$^{fl/+}$*:FGC* (solid lines) mice. The percentages of $Foxp3^+$ cells and MFI of Foxp3 staining in $Foxp3^+$ cells are also shown.

(D) Relative mRNA amounts of CD25, CTLA-4, and GITR in GFP^+ Treg cells sorted from *Gata3*$^{fl/fl}$*:FGC* (solid bars) and *Gata3*$^{fl/+}$*:FGC* (open bars) mice. Means ± SD of triplicates done in one experiments representative of four are shown.

(E) The expression of CD25, CTLA-4, and GITR on GFP^+ Treg cells from *Gata3*$^{fl/fl}$*:FGC* (dashed lines) and *Gata3*$^{fl/+}$*:FGC* (solid lines) mice were assessed by flow cytometric analysis. MFIs are shown as means ± SD of three mice.

(F) At the end of mixed bone marrow chimera experiments described in Figure 2C, the expression of Foxp3, CD25, CTLA-4, and GITR in GFP^+ Treg cells originated from *Gata3*$^{fl/fl}$*:FGC* (dashed lines) or *Gata3*$^{fl/+}$*:FGC* (solid lines) donors were determined by flow cytometric analysis and compared. Results described in this figure are representative of at least three experiments unless stated otherwise.

enrichment of GATA-3 binding to the Th2 cell locus (positive control) (Lee et al., 2006) but not the promoter region of *Gmpr* gene (negative control). Nuclear lysates prepared from Treg and $CD4^+$ Tn cells sorted from wild-type mice were subjected to ChIP analysis. Enrichment of GATA-3 binding to different sites was determined by quantitative PCR. GATA-3 showed minimal or no binding to the promoter, CNS1, and CNS3 regions of *Foxp3* locus in both Treg and $CD4^+$ Tn cells (Figure 6B). In contrast, a dramatic increase of GATA-3 binding to CNS2 was observed only in Treg cells but not in $CD4^+$ Tn cells, suggesting that GATA-3 binds to and regulates CNS2 activity to control Foxp3 expression specifically in Treg cells (Figure 6B). To investigate whether GATA-3 is able to regulate CNS2 activity, we utilized luciferase reporter assays. Luciferase reporter constructs containing Foxp3 promoter alone or promoter plus CNS2 (Long et al., 2009) were transfected into Jurkat T cells together with either a GATA-3-expressing construct or an empty construct (control). Although GATA-3 expression slightly enhanced the activity of Foxp3 promoter in the absence of CNS2, it greatly promoted the activity of Foxp3 promoter in the presence of CNS2 (Figure 6C). This activity appeared to require direct binding of GATA-3, because GATA-3-promoted activity was virtually abolished when a substitution mutation of the GATA-3 binding site in CNS2 was created (Figure 6D). Taken together, these findings suggest that GATA-3 controls Foxp3 expression through binding to CNS2 to regulate the activity of *cis*-acting elements of the *Foxp3* gene.

The Combined Function of GATA-3 and Foxp3 Is Essential for Foxp3 Expression

Multiple transcription factors, including Foxp3 itself, were shown to be important for Foxp3 expression and Treg cell function (Gavin et al., 2007; Kerdiles et al., 2010; Kitoh et al., 2009; Ouyang et al., 2010; Rudra et al., 2009; Zheng et al., 2010). Nevertheless, defects in a single factor rendered incomplete abrogation of Treg cell population and/or function, suggesting that transcription factors may act in combination and redundancy to control Foxp3 expression and Treg cell function. Genome-wide studies to predict transcription factor binding sites showed that Treg cell-specific genes and Foxp3-dependent genes tend to have binding sites for both GATA-3 and Foxp3 (Lee et al., 2010), indicating a functional relationship between GATA-3 and Foxp3 in Treg cells. Because Foxp3 is critical for its own expression (Gavin et al., 2007; Zheng et al., 2010)

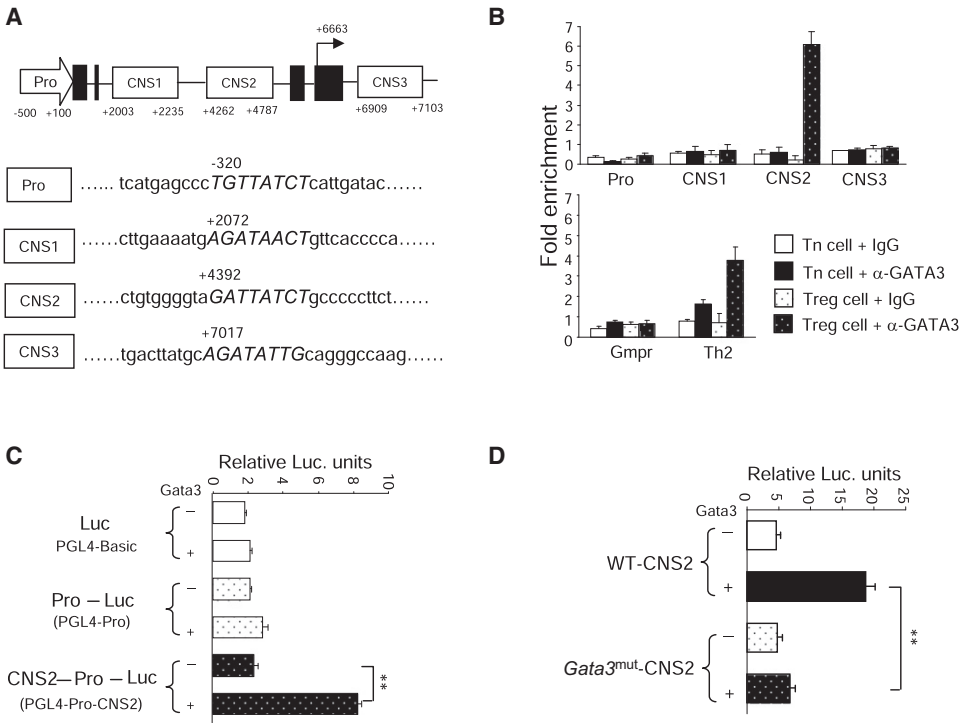

Figure 6. GATA-3 Bound to CNS2 of *Foxp3* Locus and Promoted Its Activity

(A) An illustration of putative GATA-3 binding sites in the Foxp3 promoter (Pro), CNS1, CNS2, and CNS3. The numbers denote relative positions to the transcription start site. Exons are marked by filled boxes.

(B) ChIP-coupled quantitative PCR analysis of GATA-3 binding to Foxp3 regulatory regions in sorted GFP$^-$CD4$^+$ Tn cells and GFP$^+$ Treg cells purified from *FGC* mice. Normal IgG and anti-GATA-3 were used for immunoprecipitation assays. *Gmpr* locus was used as a negative control and Th2 cell locus was used as a positive control. Enrichment of GATA-3 binding to each locus was determined. Means ± SD of triplicates done in one experiment representative of at least three are shown.

(C and D) Luciferase reporter assays. Reporter constructs of PGL4-basic, PGL4 linked with Foxp3 promoter (PGL4-Pro), or PGL4 linked with Foxp3 promoter and CNS2 (PGL4-Pro-CNS2) were transfected into Jurkat T cells together with a GATA-3-expressing plasmid (+) or an empty plasmid (–) (C). PGL4-Pro-CNS2 reporter constructs containing wild-type CNS2 (WT-CNS2) or containing CNS2 with a substitute mutation of GATA-3 binding site (*Gata3*^{mut}-CNS2) were transfected into Jurkat T cells together with a GATA-3-expressing plasmid (+) or an empty plasmid (–) (D). In (C) and (D), a Renilla luciferase vector (pRL-TK) was cotransfected as internal controls. Transfected cells were cultured for 36 hr before relative luciferase units were determined. Means ± SD of triplicates done in one experiment representative of at least three are shown. **$p < 0.01$.

See also Table S1.

and we found that GATA-3 was important for Foxp3 expression, we hypothesized that the combined function of GATA-3 and Foxp3 is essential to control Foxp3 expression and that defects in both GATA-3 and Foxp3 would lead to drastic reduction of Treg cells.

To test this hypothesis, we first investigated whether GATA-3 remained bound to CNS2 of *Foxp3* locus in Treg cells isolated from *FILIG* mouse, a hypomorphic Foxp3 strain in which Treg cells express greatly reduced Foxp3 and are marked by EGFP (Wan and Flavell, 2007). A strong association of GATA-3 to CNS2 was detected only in Foxp3-expressing Treg cells but not in CD4$^+$ Tn cells purified from *FILIG* mice (Figure 7A), suggesting that GATA-3 is involved in Foxp3 expression in *FILIG* Treg cells. Indeed, by crossing *Gata3*$^{fl/fl}$*:FGC* mice with *FILIG* mice, we found that the numbers of Foxp3-expressing Treg cells detected in *Gata3*$^{fl/fl}$*:FGC:FILIG* mice were lower than in *Gata3*$^{fl/+}$*:FGC:FILIG* mice (Figure 7B). A closer examination revealed an incomplete deletion of GATA-3 in Treg cells purified from *Gata3*$^{fl/fl}$*:FGC:FILIG* mice (Figure 7C). Thus, Treg cells that remained in *Gata3*$^{fl/fl}$*:FGC:FILIG* mice were "escapees" that expressed GATA-3. Because the Cre expression reflects endogenous Foxp3 expression in *FGC* mice (Zhou et al., 2008), the great reduction of Foxp3 expression in *FILIG* mice (Wan and Flavell, 2007) could result in insufficient Cre expression and thus incomplete deletion of GATA-3 in *Gata3*$^{fl/fl}$*:FGC:FILIG* mice. To circumvent this pitfall and to achieve efficient deletion of GATA-3 in *FILIG* Treg cells, we bred *Gata3*$^{fl/fl}$*:FILIG* mice with *CD4-Cre* transgenic mice (Lee et al., 2001) to generate *Gata3*$^{fl/fl}$*:CD4cre:FILIG* mice. Sufficient numbers of CD4$^+$ T cells were recovered from *Gata3*$^{fl/fl}$*:CD4cre:FILIG* mice. In addition, efficient deletion of GATA-3 in these cells was confirmed (Figure S3). Although more than 20% of GATA-3-sufficient CD4$^+$ *FILIG* cells expressed Foxp3, virtually no GATA-3-deficient CD4$^+$ *FILIG* cells expressed Foxp3 (Figure 7D). Therefore, defective function of both GATA-3 and Foxp3 led to ablation of Treg cells, suggesting that the combined function of GATA-3 and Foxp3 is essential for Foxp3 expression.

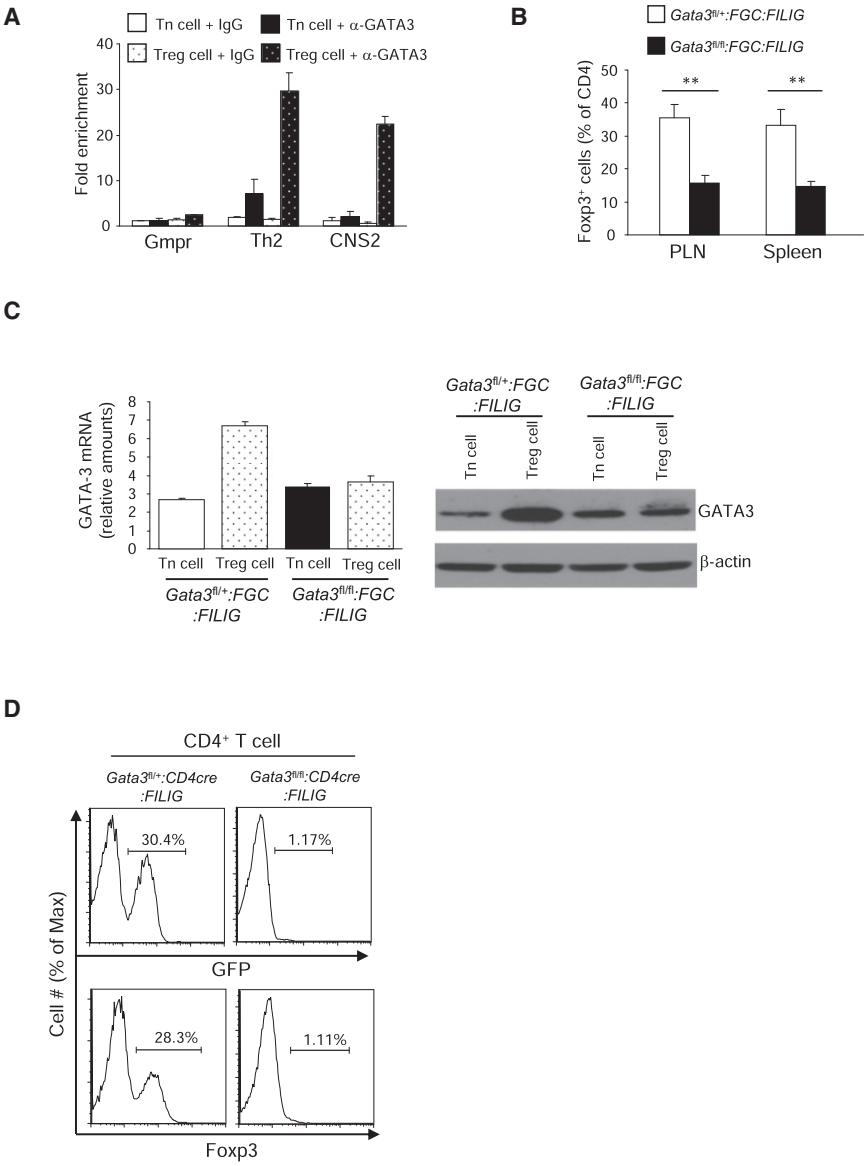

Figure 7. The Combined Function of GATA-3 and Foxp3 Was Essential for Foxp3 Expression

(A) ChIP-coupled quantitative PCR analysis of GATA-3 binding to CNS2 of *Foxp3* locus in sorted GFP$^-$CD4$^+$ Tn cells and GFP$^+$ Treg cells from *FILIG* mice. Normal IgG and anti-GATA-3 were used for immunoprecipitation assays. *Gmpr* locus was used as a negative control and Th2 cell locus was used as a positive control. Enrichment of GATA-3 binding to each locus was determined. Means ± SD of triplicates done in one experiment representative of three are shown.

(B) The percentages of Foxp3$^+$ Treg cells in the CD4$^+$ T cells isolated from PLN and spleens of *Gata3*$^{fl/+}$*:FGC:FILIG* and *Gata3*$^{fl/fl}$*:FGC:FILIG* mice. Means ± SD of four mice are shown. **$p < 0.01$.

(C) GATA-3 expression in GFP$^-$CD4$^+$ Tn and GFP$^+$ Treg cells sorted from *Gata3*$^{fl/+}$*:FGC:FILIG* and *Gata3*$^{fl/fl}$*:FGC:FILIG* mice. mRNA expression of *GATA-3* in sorted cells were assessed by quantitative RT-PCR (left). Means ± SD of triplicates done in one experiment representative of three are shown. In addition, protein expression of *GATA-3* in sorted cells was assessed by immunoblotting (right). Results are representative of three experiments.

(D) Foxp3-expressing Treg cells in the PLNs of *Gata3*$^{fl/+}$*:CD4cre:FILIG* and *Gata3*$^{fl/fl}$*:CD4cre:FILIG* mice were detected based on GFP and Foxp3 expression. The percentages of Foxp3$^+$ cells were determined (shown above the brackets). Representative results from at least three experiments are shown.

See also Figure S3.

DISCUSSION

This study revealed an indispensible role of GATA-3 in regulating Treg cell function and immune tolerance. Deletion of GATA-3 specifically in Treg cells resulted in an inflammatory syndrome in mice that can be ascribed to defective function of Treg cells. In addition, we found that GATA-3 controlled Foxp3 expression. GATA-3-deficient Treg cells expressed reduced amounts of Foxp3. GATA-3 bound to the regulatory regions of *Foxp3* locus and promoted the activity of *cis*-acting elements of *Foxp3* gene. Moreover, we demonstrated that the combined function of GATA-3 and Foxp3 was vital for Foxp3 expression in Treg cells. This study provides insights into the modulation of Treg cell function and sheds light on how autoimmunity and inflammatory diseases may be controlled.

GATA-3 is a member of GATA-binding protein family consisting of six members (GATA-1 through -6) that interact with the GATA DNA sequence. GATA-3 regulates an array of biological processes including development, differentiation, and tumorigenesis (Pandolfi et al., 1995; Pei et al., 2009). In particular, GATA-3 is critically involved in immune regulation. GATA-3 is highly expressed by Th2 cells and is required for Th2 cell differentiation (Zheng and Flavell, 1997; Zhu et al., 2004). In addition, GATA-3 is essential for T cell development (Pai et al., 2003). Moreover, it is important for the function of NK cells (Samson et al., 2003). Therefore, GATA-3 exerts diverse functions to control immune response in a cell type-specific manner. Nevertheless, the understanding of how GATA-3 regulates immune function is far from complete. Indeed, this study unveiled a function of GATA-3 in controlling Treg cell function. Such a function of GATA-3 is vital for self-tolerance and immune homeostasis, because when GATA-3 was deleted in Treg cells, mice spontaneously developed an inflammatory disorder. This finding can have broad implications. Th1 cell response is important for clearing pathogens and also for causing severe autoimmune diseases, situations where Treg cell function is often tempered (Caretto et al., 2010; Oldenhove et al., 2009). Th1 cell polarizing condition antagonizes GATA-3 expression in non-Treg cells (Szabo et al., 2003). Similarly, we found that Th1 cell polarizing condition also triggers

the downregulation of GATA-3 in Treg cells. It is therefore conceivable that the strong Th1 cell response during pathogen clearance and inflammation will lead to GATA-3 downregulation in Treg cells, which may in turn contribute to the reduced function of these cells. In addition, we have demonstrated that GATA-3-deficient Treg cells showed enhanced ability to produce inflammatory cytokines, indicating that GATA-3 downregulation under inflammatory conditions could allow Treg cells to acquire effector functions that contribute to inflammation. Our study therefore suggests that GATA-3 expression in Treg cells is important for the modulation of Treg cell function and immune response, and thus needs to be considered in order to fully understand how protective (to clear pathogen) and pathogenic (to cause autoimmunity and inflammatory disease) immune responses are controlled.

Treg cells are central to controlling immune tolerance and immune homeostasis. Foxp3 is recognized as a single gene determinant essential for Treg cell function. Perturbations of Foxp3 expression, even slight, often lead to impaired Treg cell function and are associated with various inflammatory diseases and autoimmunity. Therefore, in order to understand immune tolerance, how Foxp3 transcription is controlled is a critical question under intensive investigation. Many factors including Runx-CBFβ complex, NF-κB, Foxp3, FOXO1, and FOXO3 were shown to be important for Foxp3 expression (Gavin et al., 2007; Kerdiles et al., 2010; Kitoh et al., 2009; Long et al., 2009; Ouyang et al., 2010; Rudra et al., 2009; Zheng et al., 2010). Nonetheless, deletion of any one of these genes causes incomplete abrogation of Foxp3 expression. These findings not only underscore the vital importance of maintaining certain amounts of Foxp3 expression but also imply that multiple factors are involved in promoting Foxp3 expression in a combinatorial and somewhat redundant manner. Indeed, combined deficiency of certain set of genes, such as FOXO1 and FOXO3, results in much more drastic defect in Treg cells than any one gene deficiency achieved (Kerdiles et al., 2010; Ouyang et al., 2010). Similarly, our results demonstrated that, although impaired function of GATA-3 or Foxp3 alone caused decrease of Foxp3 expression, defects in both GATA-3 and Foxp3 led to ablation of Foxp3 expression, suggesting that GATA-3 and Foxp3 control Foxp3 expression in combination. Therefore, although we are investigating how a single factor is involved in controlling Foxp3 expression, we also need to consider how different factors function together to control Foxp3 expression and Treg cell function.

As a transcription factor, GATA-3 exerts its function mainly through DNA binding. It is therefore likely that cell-type-specific function of GATA-3 is mediated through its binding to different sets of loci in different cell types. To support this notion, we found that, in non-Treg cells, GATA-3 bound to Th2 cell locus but not *Foxp3* locus. However, in Treg cells, GATA-3 bound to both Th2 cell locus and CNS2 of *Foxp3* locus. Interchromosomal interaction has been proposed to regulate activities of *cis*-acting elements on different chromosomes (Spilianakis et al., 2005). Such interchromosomal interactions may be mediated through protein complexes containing specific transcription factors. The observation that GATA-3 bound to both *Foxp3* and Th2 cell loci in Treg cells suggests a potential physical interaction and mutual regulation between *Foxp3* and Th2 cell loci in Treg cells, an issue that warrants further investigation to elucidate the mutual regulation between Th2 and Treg cells. Although we have found that GATA-3 bound to CNS2 but not CNS1 (a site modulating Foxp3 expression in TGF-β-induced Treg cells) (Zheng et al., 2010) of *Foxp3* locus in thymic-derived Treg cells, consensus GATA-3 binding sites were identified in CNS1, suggesting that GATA-3 may be also involved in regulating Foxp3 expression in TGF-β-induced Treg cells. Th2 cell polarizing condition was shown to antagonize TGF-β-driven Foxp3 expression and to promote Th9 cell differentiation (Dardalhon et al., 2008; Veldhoen et al., 2008), a process that may involve GATA-3 (Mantel et al., 2007). Further investigation is therefore needed to address whether GATA-3 is involved in such a process by regulating Foxp3 expression through CNS1 activities.

EXPERIMENTAL PROCEDURES

Mice

Foxp3-GFP-Cre (*FGC*) mice were purchased from Jackson laboratory and have been backcrossed into C57BL/6 background for more than five generations. *Gata3*$^{fl/fl}$, *CD4-Cre*, *FILIG*, *Rag1*$^{-/-}$, and *CD45.1* congenic mice were on C57BL/6 background. All mice were housed and bred in specific-pathogen-free conditions in the animal facility at the University of North Carolina at Chapel Hill. All mouse experiments were approved by Institution Animal Care and Use Committee of the University of North Carolina.

Flow Cytometric Analysis and Cell Sorting

Lymphocytes were isolated from the various organs (as described) of age- and sex-matched mice of 8–16 weeks of age. Fluorescence-conjugated antibodies for CD4, CD8, CD25, CD44, CD62L, GITR, CD45.1, CD45.2, IFN-γ, IL-4, IL-17A, Foxp3 (eBioscience), and CTLA-4 (BD Bioscience) were purchased. Surface and intracellular staining was performed per manufacturer's protocols. For intracellular cytokine staining, lymphocytes were stimulated with 50 ng/ml of phorobol 12-myristate 13-acetate (PMA) and 1 μM of ionomycin for 3–4 hr in the presence of Brefeldin A. Stained cells were analyzed on a LSRII station (BD Biosciences) or sorted on Moflow cell sorter (Dako cytomation, Beckman Coulter).

In Vitro Cell Proliferation and Suppression Assay

CD4$^+$GFP$^+$ Treg cells (suppressor) and CD4$^+$GFP$^-$ non-Treg cells (responder) were sorted. To assess Treg cell proliferation in vitro, 2 × 10^4 sorted Treg cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with soluble CD3 antibody (1 μg/ml) in the presence of 1 × 10^5 irradiated (3000 cGy) T cell-depleted splenocytes as antigen-presenting cells (APC). Cell proliferation was assessed by CFSE dilution detected by flow-cytometric analysis 72 hr poststimulation. To assess the efficacy of Treg cell-mediated immune suppression in vitro, 2 × 10^4 sorted responder T cells were labeled with CFSE and mixed with varying amounts (as indicated) of Treg suppressor cells. Cell mixtures were stimulated with soluble CD3 antibody (1 μg/ml) in the presence of 1 × 10^5 irradiated (3000 cGy) T cell-depleted splenocytes as APC. The proliferation of responder cells was assessed by CFSE dilution detected by flow-cytometric analysis 72 hr poststimulation.

Luciferase Reporter Assay

PGL4-Pro and PGL4-Pro-CNS2 luciferase reporter constructs were kindly provided by S. Ghosh (Columbia University). Substitution mutation of GATA-3-binding site at CNS2 was created with Quickchange Mutagenesis kit (Agilent Technologies). Jurkat T cells were transfected by electroporation with 5 μg of reporter plasmid together with 5 μg of GATA-3-expressing plasmids. 1 μg of pRL-TK plasmid was also cotransfected serving as an internal control. Firefly and Renilla Luciferase activities were determined with a dual-reporter assay kit (Promega) 36 hr posttransfection.

Adoptive Transfer Assay, Treg Cell-Mediated Protection of Naive CD4$^+$ T Cell-Elicited IBD Assay, and Bone Marrow Transplantation Assay

To determine the ability of Treg cells to be maintained in the periphery, GATA-3-sufficient and GATA-3-deficient CD4$^+$GFP$^+$ Treg cells bearing different

CD45 congenic markers were sorted. 1 × 10^5 sorted Treg cells were cotransferred into *Rag1*$^{-/-}$ recipients via retro-orbital injection. The contribution of each donor to the Treg cell population recovered in the hosts was determined 8 weeks posttransfer. To assess Treg cell-mediated protection of naive T cell-elicited IBD in vivo, 1 × 10^5 sorted Treg cells were mixed with 2 × 10^5 naive ($CD25^-CD45RB^{hi}$) $CD4^+$ T cells sorted from wild-type C57BL/6 mice. Cell mixture was transferred into *Rag1*$^{-/-}$ via retro-orbital injection. As control, 2 × 10^5 naive $CD4^+$ T cells were also transferred alone into *Rag1*$^{-/-}$ mice. To monitor IBD development, body weight of the recipient mice was monitored weekly after the transfer. Recipient mice were euthanized 8 weeks after transfer. T cells from these mice were harvested and subjected to immunological analysis. To create mixed bone marrow chimera, bone marrow cells were isolated from the femur bones of *Gata3*$^{fl/fl}$*:FGC* or *Gata3*$^{fl/+}$*:FGC* mice bearing different CD45 congenic markers. 1 × 10^6 bone marrow cells from each donor were mixed and transferred into irradiated (500 cGy) *Rag1*$^{-/-}$ mice. The contribution of each donor to Treg cell population in the recipients was determined 8 weeks after transfer.

Chromatin Immunoprecipitation Assay

ChIP assay was performed per Upstate Biotechnology's protocol. In brief, cells were cross-linked by 1% formaldehyde and lysed in lysis buffer. The lysates were sonicated with a Bioruptor sonicator to shear genomic DNA into 200–500 bp fragments. Chromatin prepared from 2 × 10^6 $CD4^+GFP^+$ Treg cells and $CD4^+GFP^-$ Tn cells were subjected to immunoprecipitation overnight at 4°C with goat anti-GATA-3 (D-16, sc-22206, Santa Cruz) or normal goat IgG (sc-2028, Santa Cruz) antibodies. Quantitative real-time PCR was performed to determine the relative abundance of target DNA. Specific primers for analysis of GATA-3 binding to Foxp3 and other target loci are listed in Table S1.

Statistical Analysis

Data from at least three sets of samples were used for statistical analysis. Statistical significance was calculated by Student's t test. A p value of less than 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.immuni.2011.08.012.

ACKNOWLEDGMENTS

We thank S. Ghosh (Columbia University) for Foxp3 luciferase reporter constructs; J. Engel (University of Michigan) for GATA-3 cDNA constructs; Y. Zheng (Salk Institute) for suggestions on ChIP assays; R. Flavell (Yale University) for *FIR* and *FILIG* mice; M. Busslinger and A. Souabni (Research Institute of Molecular Pathology) for *Gata3*$^{fl/fl}$ mice, N. Fisher and J. Kalnitsky (University of North Carolina Flow-cytometry facility) for cell sorting; J. Ting and G. Matsushima (University of North Carolina) for helpful discussions. This study is supported by the NIH (R00AI072956), the Lupus Research Institute, and the University Cancer Research Fund (Y.Y.W.).

Received: March 22, 2011
Revised: July 4, 2011
Accepted: August 29, 2011
Published online: September 15, 2011

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