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An Intrinsic Mechanism Predisposes Foxp3-Expressing Regulatory T Cells to Th2 Conversion In Vivo

Yunqi Wang,* Abdallah Souabni,† Richard A. Flavell,§§ and Yisong Y. Wan*

Naturally occurring regulatory T (nTreg) cells express Foxp3 and were originally discovered as immune suppressors critical for self-tolerance and immune homeostasis. Through yet-to-be-defined mechanisms, nTreg cells were recently shown to convert into proinflammatory cells. Particularly, attenuation of Foxp3 expression led to Th2 conversion of nTreg cells in vivo. In this paper, we demonstrated an nTreg-specific mechanism controlling their Th2 conversion. We found that wild-type nTreg cells expressing reduced levels of Foxp3 but not those expressing no Foxp3 produced the Th2 cytokine IL-4. Intriguingly, IL-4 production by converted nTreg cells is required for Th2 differentiation of coexisting naive CD4 T cells in vivo, suggesting that Th2 conversion of nTreg cells might be critical for directing Th2 immune responses. Th2 conversion of nTreg cells was not due to their inability to become Th1 cells, because IFN-γ was produced by Foxp3-low–expressing cells when IL-4/STAT-6 signaling was abrogated. Surprisingly, however, unlike naive CD4 T cells whose IL-4 production is dependent on STAT-6, Foxp3-low–expressing cells generated IL-4 independent of STAT-6, indicating an intrinsic mechanism that favors nTreg-to-Th2 differentiation. Indeed, compared with naive CD4 T cells, nTreg expressed elevated levels of GATA-3 independent of STAT-6. And GATA-3 was required for nTreg-to-Th2 conversion. Foxp3 may account for this GATA-3 upregulation in nTreg cells, because ectopic expression of Foxp3 preferentially promoted GATA-3 but not T-bet expression. Thus, we have identified an intrinsic mechanism that imposes a Th2/Th1 imbalance and predisposes Foxp3-expressing cells to IL-4 production independent of STAT-6 signaling. The Journal of Immunology, 2010, 185: 5983–5992.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: DKO, double knockout; IRES, internal ribosome entry site; MFI, mean fluorescence intensity; MIG, MSCV-IRES-enhanced GFP; MSCV, mouse stem cell virus; nTreg, naturally occurring regulatory T; Tfh, T follicular helper; Treg, regulatory T; UTR, untranslated region; WT, wild-type.

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sion of Foxp3 preferentially promoted GATA-3 but not T-bet expression. Collectively, we have identified an IL-4/STAT6-independent and GATA-3-dependent mechanism intrinsic to nTreg cells for the Th2 conversion.

Materials and Methods

Mice and adoptive transfer assays

FIR, FILG, 4GET, IL-4−/−, IL-4−/−IFN-γ−/− [IL−/−], STAT6−/−, CD4Cre, GATA-3bZip−/−, Rag1−/−, and CD45.1 mice are on C57BL/6 background and were kept under specific pathogen-free conditions in the animal care facility at the University of North Carolina (Chapel Hill, NC). Special mouse strains used in this study and their properties are described in Table I. All mouse experiments were approved by Institutional Animal Care and Use Committee of the University of North Carolina or Yale University (New Haven, CT). For adoptive transfer assays, FACS-sorted cells were either transferred alone or were mixed at different ratios as elaborated in the texts or figure legends. A total of 2 × 10^5 cells were transferred into Rag1−/− mice via retro-orbital injection. Eight weeks posttransfer, T cells were recovered from recipient mice for further analysis.

Flow cytometry

Lymphocytes were isolated from the lymph nodes and spleen from 8- to 12-wk-old mice. Abs for FACS analysis were purchased from eBioscience (San Diego, CA). Surface and intracellular staining was performed per manufacturer’s protocols (eBioscience). Stained cells were analyzed on a LSRII station (BD Biosciences, San Jose, CA) and CyAn (DakoCytomation, Carpenteria, CA; Beckman Coulter, Fullerton, CA) or sorted on a MoFlow cell sorter (DakoCytomation; Beckman Coulter).

T cell culture, activation, and retroviral transduction

T cells were cultured in Bruff’s medium supplemented with 10% FBS and 1% penicillin/streptomycin. T cells were activated with soluble 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for long-term culturing or with 40 ng/ml PMA and 1 μM monomycin to stimulate cytokine production. To ectopically express genes of interest in T cells, mouse stem cell virus (MSCV)-mediated gene transfer was performed. recombinant MSCV viral constructs encoding EGFP (MSCV-IRES–enhanced GFP [MIG]) and EGFP/FIR (MIG-Foxp3) were provided by Dr. A. Rudensky (Memorial Sloan–Kettering Cancer Center, New York, NY) and Dr. S. Ziegler (Benaroya Research Institute, Seattle, WA). These constructs were transfected into Phoenix-Eco packaging cells using Lipofectamine 2000 per manufacturer’s protocols (Invitrogen, Carlsbad, CA), and recombinant retroviruses were harvested 48 and 72 h after transfection. Foxp3−/− CD4 T cells were sorted from FIR mice (Fig. 6) or from FIR mice deficient in STAT6 (Supplemental Fig. 6). Purified cells were activated for 48 h under culturing conditions and then spin-inoculated with MIG or MIG-Foxp3 viruses. The expression of the genes of interest was assessed by flow cytometry analysis 72 h posttransduction.

Quantitative RT-PCR and immunoblotting

RNA was extracted using TRIzol reagent and cDNA was synthesized by Superscript II reverse transcriptase per the manufacturer’s protocols (Invitrogen). Quantitative PCR was performed on the ABI 7900HT Real-time PCR system using primer/probe sets purchased from Applied Biosystems (Foster City, CA). Abs against GATA-3, T-bet, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoblotting per manufacturer’s protocols.

Statistical analysis

Data from at least three sets of samples were used for statistical analysis. Statistical significance was calculated by Student t test. A p value <0.05 was considered significant.

Results

Downregulation of Foxp3 expression in nTreg cells in vivo

Foxp3-expressing cells have been reported to convert into Th2 cells either in FILG mice where the Foxp3 expression was substantially attenuated (8) or in mice deficient in Runx1-Cbfβ transcription complex, where Foxp3 expression was reduced modestly (14). It, however, remains unknown as whether wild-type nTreg cells can attenuate Foxp3 expression and convert into IL-4-producing Th2 cells in vivo.

The existence of small numbers of T cells in immune-deficient lymphopenic hosts may lead to Th2 immune diseases (15, 16). We therefore hypothesized that transferring nTreg cells into lymphopenic hosts could result in the downregulation of Foxp3 expression and nTreg-to-Th2 conversion. To test this hypothesis, we purified RFP+ cells, wild-type nTreg equivalent from FIR mice where an IRES-mRFP expression cassette was knocked into 3′-UTR of endogenous Foxp3 gene (17). These purified nTreg cells were then transferred into Rag1−/− mice. Eight weeks post-transfer, the Foxp3 expression in recovered T cells was assessed by intracellular staining. More than 50% of recovered cells showed no Foxp3 expression (Foxp3−), whereas ~40% of them maintained Foxp3 expression (Foxp3+) (Fig. 1A). However, the expression levels of Foxp3 in recovered Foxp3− cells were modestly reduced when compared with freshly isolated nTreg cells (Fig. 1B). The degree of Foxp3 expression reduction was similar to that found in mice deficient in Runx1-Cbfβ transcription complex (14, 18).

Although a small fraction of nTreg cells lost Foxp3 expression under homeostatic conditions (7), a great number of nTreg cells lost Foxp3 expression when they were transferred into T cell-deficient CD3ε−/− mice (6). The high percentage of Foxp3− regulatory T (Treg) cells found in the lymphopenic CD3ε−/− and Rag1−/− recipient mice could be due to the absence of coexisting naive CD4 T cells. We therefore examined whether the presence of naive CD4 T cells might affect Foxp3 expression in transferred nTreg cells. CD4 nTreg cells (RFP+) sorted from FIR mice bearing CD45.2 congenic marker were mixed at a ratio of 1:1 with naive CD4 T cells (CD45.2CD4+CD45RBsh−IFN-γ−/−) sorted from wild-type mice bearing the CD45.1 congenic marker. The cell mixtures were then transferred into Rag1−/− mice. Eight weeks posttransfer, the cells...
recovered from different donor origins were distinguished by congenic markers, and the Foxp3 expression was assessed by intracellular staining. The coexistence of naive CD4 T cells largely prevented the generation of Foxp3− Treg cells; on average, 5% of Foxp3− Treg cells was recovered (Fig. 1C), a percentage similar to what had been reported under homeostatic conditions (7). Nevertheless, the expression levels of Foxp3 in recovered Foxp3+ cells remained modestly reduced when they were compared with freshly isolated nTreg cells (Fig. 1D). Thus, wild-type nTreg cells can indeed attenuate Foxp3 expression under lymphopenic condition, regardless the presence or absence of naive CD4 T cells.

**Th2 conversion of Foxp3-expressing cells and its impact on Th2 differentiation of coexisting naive CD4 T cells**

We next examined whether nTreg cells with reduced Foxp3 expression could convert into IL-4-producing Th2 cells or other types of effector T cells. To this end, we generated FIR-4GET mice where Foxp3-expressing cells were marked by mRFP expression from FIR allele (17), and IL-4-expressing cells were marked by EGFP expression from 4GET alleles (19). RFP+ cells were purified from FIR-4GET mice and transferred into lymphopenic Rag1−/− hosts. Foxp3 and IL-4 expression in CD4 T cells recovered from the recipients was assessed by RFP and GFP expression, respectively. Although little IL-4 was expressed by Foxp3− CD4 T cells, ~10% of Foxp3+ cells expressed IL-4 (Fig. 2A). Further analysis revealed that Foxp3+ cells produced minimum amounts of IFN-γ or IL-17A (Supplemental Fig. 1). In contrast to Foxp3+ cells, Foxp3− cells recovered from the recipients adopted a Th1 phenotype with IFN-γ but not IL-17A being produced (Supplemental Fig. 1), agreeing with a previous report (7). The presence of naive CD4 T cells did not prevent Th2 conversion of nTreg cells, because ~4% of Foxp3+ cells expressed IL-4 when naive CD4 T cells were cotransferred (Fig. 2B). These findings suggest that, with reduced Foxp3 expression, wild-type nTreg cells could indeed convert into IL-4–producing Th2 cells.

Intrigued by above findings, we speculated that Th conversion of nTreg cells may impact the differentiation of coexisting naive CD4 T cells. To investigate whether IL-4 and IFN-γ production by converted nTreg cells could affect the differentiation of coexisting naive CD4 T cells, we transferred wild-type naive CD4 T cells together with either wild-type nTreg cells or nTreg cells lacking both IL-4 and IFN-γ genes (4−γ DKO). Naive CD4 T and nTreg cells were distinguished by the congenic markers CD45.1 and CD45.2, respectively. Although IL-4 and IL-13 were produced by wild-type naive CD4 T cells when wild-type nTreg cells were cotransferred, such a production was virtually abolished when 4−γ DKO nTreg cells were cotransferred (Fig. 2C). Large numbers of naive CD4 T cells produced IFN-γ when wild-type nTreg cells were cotransferred; such a production was modestly decreased when 4−γ DKO Treg cells were cotransferred (Supplemental Fig. 2). Thus, IL-4 and, to a lesser extent, IFN-γ production by converted nTreg cells was important for the differentiation of coexisting naive CD4 T cells, underscoring a potential role of dysregulated nTreg cells in directing differentiation of coexisting naive CD4 T cells.

**Foxp3-expressing cells were permissive for Th1 conversion upon abrogation of IL-4/STAT-6 signaling**

High level of Foxp3 expression suppresses the production of effector cytokines in T cells (3). Th2 conversion of nTreg cells upon Foxp3 attenuation could be attributed to at least two explanations. One is that high levels of Foxp3 expression are required to suppress IL-4 expression, but low levels of Foxp3 are sufficient to suppress IFN-γ expression. Thus only IL-4 but not IFN-γ could be produced by nTreg cells when Foxp3 expression is reduced. The other is that high levels of Foxp3 expression are required to suppress both IL-4 and IFN-γ expression; IL-4 and IFN-γ can be expressed in nTreg cells when Foxp3 expression is decreased. Nevertheless, mechanisms exist in nTreg cells to preferentially express IL-4 but not IFN-γ to promote Th2 conversion. To investigate the mechanisms underlying the Th2 bias of Foxp3-expressing cells, we tested these two possibilities.

As previously described, when an IRES-luciferase-IRES-EGFP expression cassette was knocked into the 3′-UTR of endogenous Foxp3 gene, Foxp3 expression was decreased 5- to 10-fold in FILIG nTreg cells when compared with wild-type nTreg cells (8). The suppressive activity of Foxp3-expressing cells from FILIG mice was greatly impaired, leading to the activation of T cells and the development of an aggressive autoimmune syndrome in FILIG/mice (8). In addition, both Foxp3+ and Foxp3− CD4 T cells from FILIG/mice predominantly produced Th2 signature cytokine IL-4 (Supplemental Fig. 3) (8). IL-4/STAT-6 signaling axis is critical for Th2 differentiation of T cells (20–22). To test whether Foxp3+ cells are able to convert into effector cells other than Th2 cells in FILIG/Y mice, we abrogated the IL-4/STAT-6 signaling pathways in T cells in FILIG/Y mice by deleting IL-4 or STAT6 genes. Deletion of IL-4 or STAT6 gene in control F1 hybrid mice did not perturb the normal T cell phenotype (data not shown). Compared with wild-type nTreg cells, Foxp3+/− cells from IL-4−/−FILIG/Y and STAT6−/−FILIG/Y mice expressed 5- to 10-fold less Foxp3. Similar to what had been observed in FILIG/Y mice, Foxp3−/− CD4 T cells displayed activated T cell phenotypes in IL-4−/−FILIG/Y and STAT6−/−FILIG/Y (Supplemental Fig. 4).
We assessed cytokines production in Foxp3-expressing T cells from IL-4−/−·FILIG/Y and STAT-6−/−·FILIG/Y mice by intracellular staining. nTreg cells from control F/J mice deficient in IL-4 or STAT-6 produced minimal amounts of IFN-γ or IL-17A (data not shown). However, IFN-γ but not IL-17A was produced by a large percentage of Foxp3+ T cells in IL-4−/−·FILIG/Y mice (Fig. 3A). To gain more information on the Th conversion of Foxp3-expressing cells in these mice, we purified Foxp3+ CD4 T cells from FILIG/Y and IL-4−/−·FILIG/Y mice, based on GFP expression and assessed mRNA levels of cytokines and related transcription factors. In agreement with the results of intracellular staining and flow cytometry analysis, compared with Foxp3+ CD4 T cells from FILIG/Y mice, Foxp3+ CD4 T cells from IL-4−/−·FILIG/Y mice expressed higher mRNA levels of the Th1 signature cytokine IFN-γ and specifying transcription factor T-bet (23), lower levels of the Th2-specifying factor GATA-3 (Fig. 3B) (24, 25), and comparable levels of the Th17 signature cytokine IL-17A and specifying factor ROR-γt (Fig. 3B) (26).

Similarly, IFN-γ but not IL-17A was produced by a large percentage of Foxp3+ CD4 T cells from STAT-6−/−·FILIG/Y mice (Fig. 3C). Compared with Foxp3+ CD4 T cells from FILIG/Y mice, Foxp3+ CD4 T cells from STAT-6−/−·FILIG/Y mice expressed higher levels of IFN-γ and T-bet, lower levels of GATA-3, and similar levels of IL-17A and ROR-γt (Fig. 3D). Thus, Treg cells are permissive for converting into Th1 cells in the absence of IL-4/STAT-6 signaling.

Persistent Th2 conversion of Foxp3-expressing cells in the absence of IL-4/STAT-6 signaling

Th1 and Th2 differentiation programs antagonize each other in a reciprocal fashion. Once Th1 differentiation is initiated, Th1 cells reinforce their differentiation by both promoting Th1 program and shutting down Th2 program. Thus, Th1-Th2 differentiation is generally thought to be mutually exclusive (27). Unexpectedly, however, we found that Th2 differentiation of Treg cells persisted even when IL-4/STAT-6 signaling was abrogated.

Although IL-13 was minimally produced by CD4 T cells from F/J, IL-4−/−·FILIG/Y, and STAT-6−/−·FILIG/Y mice (data not shown), it was substantially upregulated in Foxp3+ and Foxp3− CD4 T cells in FILIG/Y mice (Fig. 4A). Interestingly, in IL-4−/−·FILIG/Y mice, although IL-4 deficiency led to a dramatic increase of IFN-γ production by Foxp3+ CD4 T cells (Fig. 3A), IL-13 production by these cells was virtually unchanged (Fig. 4A), suggesting that Th2 differentiation occurred when IL-4 production was absent, and IFN-γ production was dominant. Consistent with this, CD4 T cells (Foxp3+ or Foxp3−) purified from FILIG/Y and

**FIGURE 3.** Th1 but not Th17 conversion of Foxp3-expressing cells upon ablation of IL-4/STAT-6 signaling. A, Foxp3+ CD4 T cells from FILIG/Y mice (FILIG/Y) and IL-4−/−·FILIG/Y mice (IL-4−/−·FILIG/Y) were identified by intracellular staining. The percentages of IFN-γ- and IL-17A-producing cells in Foxp3+ T cells were determined by intracellular staining and are shown with mean value ± SD from three experiments. B, Relative mRNA levels of IFN-γ, IL-17A, T-bet, Rorc, and GATA-3 in Foxp3+ (GFP+) CD4 T cells sorted from FILIG/Y mice and IL-4−/−·FILIG/Y mice were determined by quantitative RT-PCR. Data are mean value ± SD of triplicates done in one experiment representative of three. The differences observed in IFN-γ, T-bet, and GATA-3 expression were statistically significant. C, Foxp3+ CD4 T cells from FILIG/Y mice (FILIG/Y) and STAT-6−/−·FILIG/Y mice (STAT-6−/−·FILIG/Y) were identified by intracellular staining and are shown with mean value ± SD from three experiments. D, Relative mRNA levels of IFN-γ, IL-17A, T-bet, Rorc, and GATA-3 in Foxp3+ (GFP+) CD4 T cells sorted from FILIG/Y mice and STAT-6−/−·FILIG/Y mice were assessed by quantitative RT-PCR. Data are mean value ± SD of triplicates done in one experiment representative of three. The differences observed in IFN-γ, T-bet, and GATA-3 expression were statistically significant.
**FIGURE 4.** Persistent Th2 conversion of Foxp3-expressing cells in the absence of IL-4/STAT-6 signaling. *A*, IL-4 and IL-13 production by Foxp3+ (GFP+) and Foxp3− (GFP−) CD4 T cells sorted from FILIG/Y mice (FILIG/Y) and IL-4−deficient FILIG/Y mice (IL-4−/−-FILIG/Y) were assessed by intracellular staining. Results representative of two experiments are shown. *B*, Relative mRNA levels of IL-13 and IL-5 in Foxp3+ (GFP+) and Foxp3− (GFP−) CD4 T cells sorted from FILIG/Y mice and IL-4−deficient FILIG/Y mice were determined by quantitative RT-PCR. Data are mean value ± SD of triplicates done in one experiment representative of three. *C*, IL-4 and IL-13 production by Foxp3+ (GFP+) and Foxp3− (GFP−) CD4 T cells sorted from FILIG/Y mice (FILIG/Y) and STAT-6−deficient FILIG/Y mice (STAT-6−/−-FILIG/Y) were assessed by intracellular staining. Results representative of two experiments are shown. *D*, Relative mRNA levels of IL-4 and IL-13 in Foxp3+ (GFP+) CD4 T cells sorted from FILIG/Y mice and STAT-6−deficient FILIG/Y mice and RFP+ (Foxp3+) CD4 T cells sorted from FIR/Y mice were determined by quantitative RT-PCR. Data are mean value ± SD of triplicates done in one experiment representative of three. *E*, STAT-6−/− nTreg cells (RFP+) sorted from STAT-6−deficient FIR mice were transferred into Rag1−/− mice. Eight weeks later, the expression of IL-4, IFN-γ, and IL-13 in Foxp3+ and Foxp3− cells recovered from the recipient mice was assessed. Representative results of three experiments are shown.

IL-4−/−-FILIG/Y mice expressed similar levels of IL-13 and IL-5 mRNA (Fig. 4B). More strikingly, in STAT6−/−-FILIG/Y mice, although IL-4 and IL-13 production in Foxp3+ CD4 T cells was nearly abolished, a great number of Foxp3+ CD4 T cells produced IL-4 and IL-13 (Fig. 4C), albeit at reduced levels when compared with IL-4 and IL-13 produced by Foxp3+ CD4 T cells from FILIG/Y mice (Fig. 4C). In addition, we noticed that the frequencies of IL-4−producing cells among Foxp3+ CD4 cells did not decline with age in STAT6−/−-FILIG/Y mice. In fact, similar percentages of Foxp3+ cells produced IL-4 in 3- and 10-wk-old STAT6−/−-FILIG/Y mice (Supplemental Fig. 5). Consistent with these findings, we detected substantial amounts of IL-4 and IL-13 mRNA produced by Foxp3+ T cells isolated from STAT6−/−-FILIG/Y (Fig. 4D). However, compared with Foxp3+ isolated from FILIG/Y mice, Foxp3+ T cells isolated from STAT6−/−-FILIG/Y produced fewer amounts of IL-4 and IL-13 mRNA. Collectively, these findings suggest that Foxp3 reduction leads to Th2 conversion of nTreg cells independent of STAT-6, and STAT-6 is nevertheless
FIGURE 5. GATA-3 expression is elevated in nTreg cells and required for nTreg-to-Th2 conversion. A, GATA-3 and T-bet expression in Foxp3+ and Foxp3− CD4 T cells in wild-type mice were assessed by intracellular staining and flow cytometry analysis. MFIs of GATA-3 and T-bet intracellular staining are also shown. Results are representative of at least five experiments. B, GATA-3 and T-bet expression in purified RFP+ (Foxp3+) and RFP− (Foxp3−) CD4 T cells from FIR mice were assessed by immunoblotting and quantitative RT-PCR. Data for quantitative RT-PCR are mean value ± SD of triplicates done in one experiment. Representative results from three experiments are shown. The differences observed in GATA-3 and T-bet mRNA expression levels were statistically significant. C, GATA-3 and T-bet expression in Foxp3+ and Foxp3− CD4 T cells in STAT-6−/− mice assessed by flow cytometry. MFIs of GATA-3 and T-bet staining are also shown. Results are representative of three experiments. D, GATA-3 and T-bet expression in sorted RFP+ (Foxp3+) and
important for the optimal/maximal IL-4 production by converted T cells.

We next investigated whether nTreg cells without genetically forced attenuation of Foxp3 can indeed convert into IL-4-producing cells independent of STAT-6 in vivo. To this end, nTreg cells were purified from STAT6−/−/FIR/Y mice and transferred into Rag1−/− mice. Eight weeks after transfer, the expression of Foxp3, IL-4, IL-13, and IFN-γ in recovered T cells was assessed by intracellular staining. In accordance with the abovementioned findings, Foxp3+ but not Foxp3+ cells produced IL-4 and IL-13 in the absence of STAT-6 (Fig. 4E). Our findings demonstrated that, rather surprisingly, Th2 conversion of Treg cells is independent of IL-4/STAT-6. These results suggested that, unlike naive CD4 T cells whose Th2 differentiation is dependent on IL-4/STAT-6 signaling (20–22), IL-4/STAT-6-independent signaling pathway(s) exists in nTreg cells to program their Th2 conversion.

**GATA-3 is upregulated in Foxp3-expressing cells and required for Th2 conversion of nTreg cells**

Foxp3 and T-bet are recognized as the master regulators to program Th2 conversion of nTreg cells (30). Upon deletion of Foxp3 specifically in T cells by crossing mice bearing floxed Foxp3 alleles with mice transgenic for the gene encoding Cre recombinase driven by the Cd4 promoter (32) and then with FIR mice (17) to obtain CD4cre-GATA-3lox/lox, FIR/Y mice, GATA-3 was efficiently deleted in CD4 T cells from these mice (data not shown) and led to reduced numbers of CD4 T cells in the periphery as reported previously (33). Nevertheless, sufficient numbers of Foxp3+ (RFP+) cells were purified from these mice and transferred into IL-4−/−/FIR/Y mice, the further increase of GATA-3 in both Foxp3+ and Foxp3+ CD4 T cells were blunted (Fig. 5E, middle and right panels). Nevertheless, we found that Foxp3+ cells consistently expressed higher levels of GATA-3 than coexisting Foxp3− CD4 T cells in these mice. Collectively, these observations suggest that, although IL-4/STAT-6-dependent signaling pathway remains functional in converted Foxp3+ cells to further promote GATA-3 expression, IL-4/STAT-6–independent mechanisms exist in Foxp3+ cells to elevate GATA-3 expression.

GATA-3 plays critical roles for Th2 differentiation (25) through STAT-6–independent autoactivation mechanisms (30). Thus, elevated GATA-3 expression in nTreg cells may explain their tendency toward Th2 conversion. Nevertheless, in light of above finding that nTreg cells may use mechanisms distinct from naive CD4 T cells to engage in IL-4 production and Th2 conversion, we further investigated whether GATA-3 is required for Th2 conversion of nTreg cells. We deleted GATA-3 specifically in T cells by crossing mice bearing floxed GATA-3 alleles with mice transgenic for the gene encoding Cre recombinase driven by the Cd4 promoter (32) and then with FIR mice (17) to obtain CD4cre-GATA-3lox/lox, FIR/Y mice. GATA-3 was efficiently deleted in CD4 T cells from these mice (data not shown) and led to reduced numbers of CD4 T cells in the periphery as reported previously (33). Nevertheless, sufficient numbers of Foxp3− (RFP−) cells were purified from these mice and transferred into Rag1−/− mice, resulting in the downregulation of Foxp3 expression in these cells (Fig. 5F). Although IL-4 and IFN-γ was minimally produced by freshly isolated GATA-3–deficient nTreg cells before the transfer (data not shown), large numbers of CD4 T cells recovered from the recipient mice produced IFN-γ but not IL-4 or IL-13 (Fig. 5F). Therefore, GATA-3 is essential for Th2 conversion of nTreg cells in vivo.

**Ectopic expression of Foxp3 specifically promoted the expression of GATA-3 but not T-bet**

In a STAT-6–independent manner, GATA-3 but not T-bet expression was found elevated specifically in Foxp3+ but not Foxp3+ CD4 T cells (Fig. 5C, SD), suggesting that intrinsic mechanisms exist in Treg cells to promote GATA-3 expression. We then sought to address whether Foxp3 expression may differentially affect the expression of GATA-3 and T-bet in T cells. To this end, we ectopically expressed Foxp3 in activated T cells and assessed its impact on GATA-3 and T-bet expression. Retrovirus-mediated
expression of Foxp3 increased GATA-3 but not T-bet expression in activated CD4 T cells, which was confirmed by intracellular staining, immunoblotting, and quantitative RT-PCR assays (Fig. 6). This Foxp3-driven GATA-3 upregulation was independent of STAT-6 signaling as ectopic expression of Foxp3 elevated GATA-3 but not T-bet expression in STAT-6–deficient CD4 T cells (Supplemental Fig. 6).

Collectively, we have demonstrated that, without genetic manipulation, wild-type nTreg cells could indeed attenuate Foxp3 expression to result in Th2 conversion of Foxp3-expressing cells and to promote Th2-differentiation of coexisting naïve CD4 T cells in vivo. In addition, we identified an intrinsic mechanism in nTreg cells that controls Th2 conversion in a STAT-6–independent manner. GATA-3 was found to be the central component of this mechanism, because its expression was found elevated in nTreg cells, and it was required for nTreg-to-Th2 conversion in vivo (Table I). Moreover, we found that Foxp3 expression could account for the specific upregulation of GATA-3 expression in nTreg cells as

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Ectopic expression of Foxp3 promoted GATA-3 but not T-bet expression. Foxp3– (RFP–) CD4 T cells were sorted from FIR mice. Purified cells were then stimulated and subsequently transduced with MIG and MIG-Foxp3 retroviruses. A, The expression of Foxp3, GATA-3, and T-bet was assessed by intracellular staining and flow cytometric analysis. After MIG-Foxp3 transduction, expression levels of GATA-3 and T-bet were compared between Foxp3+ (solid lines) and Foxp3– (dashed lines) CD4 T cells that coexisted in the same culture. MFI of GATA-3 and T-bet staining are also shown. Results are representative of at least three experiments. B, The protein and mRNA levels of GATA-3 and T-bet in MIG- or MIG-Foxp3–transduced CD4 T cells were compared by immunoblotting and quantitative RT-PCR. Results are representative of three experiments. The differences observed in Foxp3 and GATA-3 expression were statistically significant.

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<th>Table I. Special mouse strains used in this study and their properties</th>
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ectopic expression of Foxp3 promoted GATA-3 but not T-bet expression in T cells. Thus, this study revealed an intrinsic mechanism predisposing Foxp3-expressing nTreg cells to Th2 conversion in vivo.

Discussion
Th cells were traditionally thought to be terminally differentiated and fully committed lineages. Emerging evidence suggests that Th cells are “plastic” and can convert into other types of Th cells. Treg cells appear to possess the most diversity as they were reported to convert into Th1, Th2, Th17, and Th9 cells in vivo. The loss of Foxp3 expression led to Th1, Th17, and Th9 conversion of nTreg cells (6, 7). Reduction but not loss of Foxp3 expression led to Th2 conversion of nTreg cells in genetically modified mouse models (8, 14). Nevertheless, the question remains as to whether wild-type nTreg cells can indeed attenuate Foxp3 expression and become Th2 cells in vivo. In this paper, we showed that wild-type nTreg cells downregulated Foxp3 expression in lymphopenic hosts and those Foxp3-expressing cells converted into IL-4-producing Th2 cells. In addition, we found that the IL-4 production by Treg cells was important for the Th2 differentiation of coexisting naive CD4 T cells. Reconstitution with limited T cells into lymphopenic mice induced a Th2-type immune disease (15, 16). Our results thus suggest that Th2 conversion of Treg cells may be involved in these diseases. In addition, drastic attenuation of Foxp3 expression in Treg cells was found in human patients with chronic asthma (34), which may result in the Th2 conversion of Treg cells to contribute to asthma development. Nevertheless, whether and how nTreg-to-Th2 conversion is involved in Th2-type immune disorders needs to be investigated in the future.

nTreg-to-Th conversion has gained much attention recently in immunology research. The underlying mechanism through which Treg cells become other types of Th cells is yet to be defined. Treg is a unique T cell lineage phenotypically distinct from naive CD4 T cells even when Foxp3 expression was absent (35). Thus, nTreg and naive CD4 T cells may use different mechanisms to engage in the Th2 program. Indeed, we have identified an intrinsic mechanism favoring Th2 conversion of nTreg cells. Exogenous IL-4 plays a dominant role in directing Th2 differentiation in naive CD4 T cells by activating STAT-6 and subsequently GATA-3 to initiate and to stabilize Th2 phenotype. STAT-6 and GATA-3 are essential for Th2 differentiation of naive CD4 T cells (22, 25). We found that, in the absence of STAT-6, whereas the Th2 differentiation of naive CD4 T cells was abolished, the Th2 conversion of Treg cells persisted. Thus, Th2 conversion of Treg cells is controlled by STAT-6–independent mechanisms. Nevertheless, STAT-6–dependent pathways were found to be critical for the optimal IL-4 production in converted nTreg cells, because IL-4 and IL-13 production and GATA-3 expression in FilGly mice decreased when STAT-6 was deficient. Therefore, both STAT-6–independent and –dependent mechanisms are operating during Th2 conversion of nTreg cells. So it appeared that, during nTreg-to-Th2 conversion, IL-4 production and Th2 program could be initiated through a STAT-6–independent mechanism and be further enhanced and stabilized through a STAT-6–dependent mechanism via an IL-4/STAT-6/GATA-3 feed-forward loop. GATA-3 may serve as the converging point for the pathways controlling nTreg-to-Th2 conversion. We demonstrated that ectopic expression of Foxp3 upregulated GATA-3 expression at the protein and mRNA level independent of STAT-6. Such an effect of Foxp3 on GATA-3 expression is likely to be through a direct binding of Foxp3 to the GATA-3 promoter or enhancers. Using a genome-wide ChIP-on-Chip tiling array analysis, a previous study has shown that Foxp3 directly binds to the GATA-3 promoter, although the exact binding site(s) was not revealed (40). Such a binding was correlated with the increased rather than decreased GATA-3 expression. Therefore, our results agreed with these findings and furthermore suggest that Foxp3 expression is sufficient to cause the preferential upregulation of GATA-3 expression, providing an explanation for the intrinsic ability of Treg cells to convert into Th2 cells. Numerous mechanisms exist to promote Th2 differentiation of naive CD4 T cells (41); Foxp3-promoted GATA-3 expression may not be the only mechanism involved in Treg-to-Th2 conversion. Further investigation is needed to reveal additional mechanisms controlling Th2 conversion of Treg cells.

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Disclosures
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References
Tregs ARE INTRINSICALLY BIASED TO Th2 CONVERSION


