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IL-12 Converts Foxp3⁺ Regulatory T Cells to Foxp3⁺IFN- γ ⁺ T Cells with Inhibitory Functions During Induction of Colitis

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Abstract

Regulatory T (Treg) cells are plastic, but the *in vivo* mechanisms by which they are converted into Foxp3⁺interferon (IFN)- γ ⁺ T cells, and whether these converted cells retain the ability to inhibit colitis, are not clear.

Methods—Foxp3⁺ Treg cells were generated by culture of naïve CD4⁺ T cells from Foxp3^{GFP} CBir1 T-cell receptor (TCR) transgenic (CBir1-Tg) mice, which are specific for CBir1 flagellin (an immunodominant microbiota antigen), with transforming growth factor (TGF)- β . Foxp3^{GFP} CBir1-Tg Treg cells were isolated by fluorescence-activated cell sorting and transferred into *TCR β δ ^{-/-}* mice. Colitis was induced by transfer of naïve CBir1-Tg CD4⁺ T cells into immunodeficient mice.

Results—Microbiota antigen-specific Foxp3⁺ Treg cells were converted, in the intestine, to IFN- γ ⁺ T-helper (Th)1 cells, interleukin (IL)-17⁺ Th17 cells, and Foxp3⁺ T cells that coexpress IFN- γ and/or IL-17. Conversion of Treg cells into IFN- γ -producing Th1 cells and Foxp3⁺IFN- γ ⁺ T cells required innate cell production of IL-12 in the intestine; blocking IL-12 with an antibody inhibited their conversion to Th1 and Foxp3⁺IFN- γ ⁺ T cells in the intestines of mice that were recipients of Treg cells. Addition of IL-12, but not IL-23, promoted conversion of Treg cells into Th1 and Foxp3⁺IFN- γ ⁺ T cells, *in vitro*. Foxp3⁺IFN- γ ⁺ T cells had regulatory activity, because they suppressed proliferation of naïve T cells, *in vitro*, and inhibited induction of colitis by microbiota antigen-specific T cells. IFN- γ ⁺ Th1 cells were not converted into Treg cells; Foxp3⁺IFN- γ ⁺ T cells differentiated into IFN- γ ⁺ but not Foxp3⁺ T cells.

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Conclusions—IL-12 promotes conversion of Treg cells into IFN- γ -expressing cells; Foxp3⁺IFN- γ ⁺ T cells retain their regulatory functions and develop during the transition of Foxp3⁺ Treg cells into IFN- γ ⁺ Th1 cells.

Keywords

IBD; immune regulation; inflammation; Crohn's disease

Introduction

The gastrointestinal tract represents a major gateway for potential pathogens, and also contains dietary antigens and an extensive and diverse microbiota that needs to be accommodated.¹ To maintain intestinal homeostasis, regulatory elements are constitutively present with a number of independent mechanisms that partially overlap.^{2, 3} Among these regulatory elements, regulatory T (Treg) cells survey a large array of immune responses to reinforce intestinal immune homeostasis.² Many Treg cells express the signature transcription factor, Foxp3, which is essential for Treg cell development as well as their regulatory activity. Foxp3 deficiency leads to impaired Treg cell development and multi-organ autoimmune diseases.⁴

Foxp3⁺ Treg cells have been thought to be stable *in vivo*,⁵ in that most Treg cells retain high Foxp3 expression after adoptive transfer into a nonpathogenic setting,^{6, 7} and Foxp3 expression is controlled by Foxp3 itself through a positive feedback loop.⁸ However, multiple recent reports indicate that the differentiation program of Foxp3⁺ Treg cells is not fixed. A series of studies have shown that Treg cells can differentiate into T helper (Th) 17 or T follicle helper cells in the intestine.^{9–11} TCR-stimulated thymus-derived Foxp3⁺ T cells were shown to produce interleukin (IL)-17 after exposure to IL-6 in the absence of exogenous TGF- β .¹² A fraction of Foxp3⁺ Treg cells express the Th1-specifying transcription factor T-bet during type I inflammatory response,¹³ and some highly purified natural Treg cells can express IFN- γ and T-bet while maintaining Foxp3 expression after being cultured under Th1 cell-polarizing conditions.¹⁴ Two recent reports further demonstrate that Foxp3⁺ Treg cells convert to IFN- γ -expressing cells *in vivo* in pathogenic or inflammatory settings.^{15, 16} However, it remains unclear what mechanisms underlie Treg cell conversion *in vivo* and whether Foxp3⁺IFN- γ ⁺ T cells retain suppressive activity. It is also unknown whether microbiota-specific Treg cells can convert into IFN- γ -producing T cells in the intestine, and if so, what the roles of these converted Foxp3⁺IFN- γ ⁺ and IFN- γ ⁺ Th1 cells are in intestinal inflammation.

In this report, we generated Foxp3^{GFP}.IFN- γ ^{Thy1.1}.CBir1 TCR transgenic dual reporter mice by crossing IFN- γ ^{Thy1.1} and Foxp3^{GFP} reporter mice with CBir1 TCR transgenic (CBir1-Tg) mice which are specific for CBir1 flagellin, an immunodominant microbiota antigen in animal models of colitis as well as in patients with Crohn's disease.¹⁷ We found that Foxp3⁺ Treg cell conversion to IFN- γ ⁺ T cells required IL-12 production in the intestine, in that blockade of IL-12 by anti-IL-12p40 antibody abrogated conversion to Foxp3⁺IFN- γ ⁺ and IFN- γ ⁺ Th1 cells in the intestines of Foxp3⁺ Treg cell recipient mice. Foxp3⁺IFN- γ ⁺ T cells inhibited colitis development induced by CD45RB^{hi} T cells at a similar level to conventional Foxp3⁺ Treg cells. IFN- γ ⁺ Th1 cells did not convert to Foxp3⁺ Treg cells, and Foxp3⁺IFN- γ ⁺ T cells differentiated only into IFN- γ single positive Th1 cells but not Foxp3 single positive Treg cells, indicating that Foxp3⁺IFN- γ ⁺ T cells represent a transition state of Foxp3⁺ Treg cell conversion into IFN- γ ⁺ Th1 cells.

Materials and Methods

Mice

C57BL/6 (B6), CD45.1, OT II, TCR β $\delta^{-/-}$, RAG1 $^{-/-}$ and Foxp3^{GFP} reporter mice were purchased from Jackson Laboratory. IFN- γ ^{Thy1.1} reporter¹⁸ and CBir1-specific TCR transgenic (CBir1-Tg) mice¹⁹ were generated and maintained in the Animal Facility at University of Alabama at Birmingham. Age-matched mice of 8 to 10 weeks old were used in these experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Antibodies and reagents

Fluorochrome-conjugated anti-mouse CD4 (RM4-5), Thy1.1/CD90.1 (OX-7), IL-17A (TC11-18H10), and IFN- γ (XMG1.2) antibodies were purchased from BD Biosciences. Anti-mouse Foxp3 (FJK-16s) were purchased from eBioscience. Recombinant IL-2, IL-12, IL-23, IFN- γ , and TGF- β were purchased from R&D Systems. Anti-mouse IFN- γ (XMG1.2) and IL-12p40 (C17.8) neutralizing monoclonal antibodies were purchased from BioLegend.

CD4⁺ T cell purification and labeling with CFSE

CD4⁺ T cells were isolated by using anti-mouse CD4-magnetic beads (BD Biosciences). For some experiments, CD4⁺ T cells were labeled with 2.5 μ M CFSE (Invitrogen) following the manufacture's protocol.

Isolation of lamina propria cells

As described previously,¹⁹ intestines were removed, sliced and digested by Collagense IV. The cells were resuspended in 40% Percoll and carefully overlaid onto 70% Percoll. The interface containing the lamina propria lymphocytes was collected.

In vitro polarization and isolation of Treg, Th1, and Foxp3⁺IFN- γ ⁺ T cells

CD4⁺ T cells from Foxp3^{GFP}.CBir1-Tg or IFN- γ ^{Thy1.1}.CBir1-Tg reporter mice were cultured in the presence of CBir1 flagellin peptide-pulsed APC under standard Treg- or Th1-polarizing conditions (5 ng/ml TGF- β or 10 ng/ml IL-12, respectively). Five days later, CD4⁺ T cells were harvested and sorted by FACS based on GFP or Thy1.1 expression.

Histopathologic assessment

At necropsy, the small intestine, cecum, and colon were separated and Swiss rolls of each prepared. Tissues were fixed in 10% buffered formalin and paraffin embedded. The sections (5 mm) were stained with hematoxylin and eosin.

Statistical analysis

The nonparametric Mann-Whitney U-test was used for assessing pathology scores. Levels of significance were determined by Student's *t* test. P values of < 0.05 were considered to be statistically significant.

Results

Naïve CBir1-Tg CD4⁺ T cells induce colitis and develop into Treg and effector T cells in the inflamed intestine

In order to evaluate the fate of microbiota antigen-specific naïve T cells, CD4⁺ T cells from CBir1-Tg mice that are specific for the immunodominant commensal antigen, CBir1 flagellin,^{17, 19} or PBS control were transferred intravenously into TCR β $\delta^{-/-}$ mice, which

lack T cells but have a fully responsive innate immune system, B cell repertoire and NK cells, thus allowing us to study T cell fate and colitis development in hosts with a relatively intact immune system. CD4⁺ T cells from OT II mice which are specific for ovalbumin (OVA) were also transferred as a negative control. Both CBir1-Tg and OT II splenic CD4⁺ T cells expressed similar levels of Foxp3 (Supplementary Figure 1A). Approximately 80–85% of CBir1-Tg CD4⁺ T cells were positive for I-Ab-CBir1p tetramer (Supplementary Figure 1B). The recipients were sacrificed 4 or 8 weeks later, and histopathology of the small intestine, cecum, and proximal, middle and distal portions of the colon was examined. Similar to previous report in RAG^{-/-} recipients,²⁰ no control TCRβxδ^{-/-} mice received PBS or OT II T cells developed colitis, however, recipients of CBir1-Tg T cells developed severe colitis 8 weeks after cell transfer (Figure 1A). The lesions in the recipients were focal and prominent in the cecum and proximal colon, as previously described.²¹ The small intestines of these recipients did not show any inflammation (data not shown). To determine the differentiation of CBir1-Tg T cells in the intestinal lamina propria, CD4⁺ T cells were isolated and stained with antibodies against IFN-γ, IL-17, and Foxp3 intracellularly, and analyzed by flow cytometry. CBir1-Tg T cells differentiated into IFN-γ⁺ Th1 cells, IL-17⁺ Th17 cells, IFN-γ⁺IL-17⁺ “Th1+17” cells, as well as Foxp3⁺ Treg cells, Foxp3⁺IFN-γ⁺ and Foxp3⁺IL-17⁺ T cells in the lamina propria (Figure 1B-C, and Supplementary Figure 2). Interestingly, approximately 2% (4 weeks) and 3% (8 weeks) of CBir1-Tg T cells coexpressed Foxp3, IFN-γ, and IL-17 in the lamina propria. Collectively, these data demonstrate that CD4⁺ T cells that are reactive to a single dominant microbiota antigen can induce colitis and differentiate into various T cell subsets, including Th1, Th17, “Th1+17”, Treg cells, as well as Foxp3⁺ T cells coexpressing IFN-γ and/or IL-17 in the intestine. Considering the previous observations that both Th1 and Th17 cells can mediate experimental colitis, the large numbers of IFN-γ⁺IL-17⁺ “Th1+17” cells that emerged in the lamina propria of colitic mice are likely causing the intestinal inflammation.

IL-12 and TGF-β are required for generation of Foxp3⁺IFN-γ⁺ T cells

It has been reported that lamina propria dendritic cells (DC) are able to induce Treg cells to acquire IFN-γ expression during lethal infection, which is enhanced by the addition of IL-12.¹⁶ To determine the cytokines involved in generation of CBir1-specific Foxp3⁺IFN-γ⁺ T cells, naïve CD4⁺Foxp3⁻ CBir1-Tg T cells were isolated from Foxp3^{GFP}.CBir1-Tg reporter mice and cultured with CBir1 flagellin peptide-pulsed splenic antigen presenting cells (APC) in the presence of various cytokines alone or in different combinations. Five days later, Foxp3 and IFN-γ expression of T cells was determined by flow cytometry. Because IL-2 has been shown to inhibit Treg cell production of IFN-γ *in vivo*,¹⁶ we also included IL-2 in these experiments to define the role of IL-2 in the induction of Foxp3⁺IFN-γ⁺ T cells. Addition of IL-12 or TGF-β alone stimulated IFN-γ or Foxp3 expression, respectively, but there were essentially no Foxp3⁺IFN-γ⁺ T cells under either condition (Figure 2A and C). Notably, addition of both IL-12 and TGF-β induced an apparent population of Foxp3⁺IFN-γ⁺ T cells. To determine whether IL-12 promotes Foxp3⁺IFN-γ⁺ T cells through induction of IFN-γ, recombinant IFN-γ or anti-IFN-γ monoclonal antibody (mAb) was added to the T cell cultures under various conditions. IFN-γ inhibited TGF-β-induced Foxp3 expression to a similar extent as IL-12, however, unlike IL-12, it did not promote Foxp3⁺IFN-γ⁺ T cells. Addition of anti-IFN-γ mAb to IL-12 and TGF-β T cell culture greatly inhibited generation of Foxp3⁺IFN-γ⁺ T cells as well as IFN-γ single positive T cells, and total Foxp3 frequency remained at a level comparable to T cells cultured with TGF-β alone (Figure 2A and C). Notably, IL-17 was not detected in any of these culture conditions (data not shown). These data indicate that both IL-12 and TGF-β are indispensable for the generation of Foxp3⁺IFN-γ⁺ T cells. In the presence of TGF-β, IL-12-induced IFN-γ is necessary but not sufficient to induce Foxp3⁺IFN-γ⁺ T cells, as some other IL-12-induced factors are also required for optimal generation of Foxp3⁺IFN-γ⁺ T cells.

Addition of IL-2 enhanced IL-12-induced IFN- γ production, but did not affect TGF- β -induced Foxp3 expression (Figure 2B and C). However, in the presence of IL-2, IL-12- and TGF- β -induced Foxp3⁺IFN- γ ⁺ T cells were increased substantially (from 5.3% to 19%). While blockade of IFN- γ inhibited Foxp3⁺IFN- γ ⁺ T cells, there were still considerable numbers of T cells coexpressing Foxp3 and IFN- γ , rescued by IL-2. Furthermore, in the presence of IL-2, IFN- γ and TGF- β promoted generation of Foxp3⁺IFN- γ ⁺ T cells at a level comparable to that induced by IL-12 and TGF- β in the absence of IL-2. Together, these data indicate that in addition to TGF- β , IL-12-stimulated T cell production of IL-2 and IFN- γ are required for generation of Foxp3⁺IFN- γ ⁺ T cells.

CBir1-specific Foxp3⁺ Treg cells convert to IL-17⁺ Th17 cells and IFN- γ ⁺ Th1 cells in the intestine

To determine the stability of CBir1-specific Foxp3⁺ Treg cells in the intestine, Treg cells were generated by culturing naïve CD4⁺ T cells from Foxp3^{GFP}.CBir1-Tg reporter mice in the presence of TGF- β for 5 days, sorted by flow cytometry based on GFP expression, and transferred into TCR β δ ^{-/-} mice. Four weeks after cell transfer, most transferred Treg cells in the spleen, mesenteric lymph nodes (MLN), and intestinal lamina propria lost Foxp3 expression (Figure 3A). A large fraction of Treg cells that lost Foxp3 expression produced IL-17, IFN- γ , or both IL-17 and IFN- γ . Remarkably, a small but significant fraction of transferred Treg cells were Foxp3⁺IL-17⁺IFN- γ ⁺. The generation of different populations from Treg cells was not caused by Foxp3 negative contaminants but reflected the ones truly converted from Foxp3⁺ Treg cells, as small numbers of CD45.1 Foxp3 negative naïve T cells that were mixed with sorted CD45.2 Foxp3⁺ Treg cells at a percentage (3%) similar to that of Foxp3 negative cells among the sorted Foxp3⁺ T cells failed to dominate production of IFN- γ and IL-17 in the recipient mice (Supplementary Figure 3). Despite the substantial conversion of Treg cells to effector T cells in the intestine, there were no signs of colitis at 4 weeks (data not shown) or even 8 weeks post-transfer (Figure 3B).

To investigate where CBir1-specific Foxp3⁺ Treg cell conversion to IFN- γ - and IL-17-expressing T cells takes place, FACS-sorted CBir1-specific Treg cells were transferred into TCR β δ ^{-/-} mice, and the recipients were sacrificed after 2 or 6 weeks. As shown in Figure 4A-B, most transferred Treg cells lost Foxp3 expression and acquired IFN- γ and IL-17 expression in the intestinal lamina propria 2 weeks after transfer, in that only about 6.6% of transferred Treg cells maintained Foxp3 expression, whereas 36.3% expressed IL-17 and 46.8% expressed IFN- γ . Furthermore, there were significantly fewer unconverted Foxp3⁺ Treg cells and more converted IFN- γ - and/or IL-17-expressing T cells in the lamina propria compared to spleen and MLN (Supplementary Figure 4). With the time progression, IFN- γ ⁺ T cells gradually increased in the spleen and MLN to levels comparable to those in the lamina propria after six weeks (Figure 4CD, and Supplementary Figure 4). Although there were still significantly more IL-17⁺ T cells in the lamina propria than in the spleen and MLN, the numbers of both IL-17 single positive and IFN- γ ⁺IL-17⁺ T cells also steadily increased. Collectively, these data indicate that CBir1-specific Foxp3⁺ Treg cell conversion to IFN- γ - as well as IL-17-expressing T cells could be driven by endogenous CBir1 flagellin antigen stimulation and predominantly takes place in the intestine.

IL-12 promotes Foxp3⁺ Treg cell conversion to IFN- γ ⁺ T cells

IL-6 has been implicated as a key mediator for Treg cell conversion to Th17 cells. We next assessed the cues that mediate Treg cell conversion to IFN- γ -producing T cells. Previous reports have demonstrated that IL-12 not only promotes Th1 cell development,²² but also facilitates Th17 cell conversion to Th1 cells.²³ To investigate the factors driving Treg cell conversion to IFN- γ ⁺ T cells, FACS-sorted CBir1-Tg Foxp3⁺ Treg cells were cultured with CBir1 flagellin-pulsed APC in the absence or presence of different cytokines. As shown in

Figure 5A, Treg cells lost Foxp3 expression after 4 days in culture with medium alone, in that only 57.4% of T cells were Foxp3⁺ but they did not express IFN- γ . The presence of TGF- β not only maintained Foxp3 expression, which is consistent with a previous report,²⁴ but also moderately induced IFN- γ expression by T cells. Addition of IL-12 substantially enhanced Treg cell conversion to IFN- γ ⁺ T cells by decreasing Foxp3 expression and promoting IFN- γ production. Remarkably, IL-12 promoted both Foxp3⁺IFN- γ ⁺ (from 0.5% to 17.1%) and IFN- γ single positive T cells (from 1.2% to 45.7%). Although IL-12 failed to downregulate Foxp3 expression in the presence of TGF- β , it still promoted IFN- γ production, mainly by Foxp3⁺IFN- γ ⁺ T cells. This acquisition of IFN- γ expression by Treg cells paralleled IFN- γ secretion in the T cell cultures (Figure 5B). The role of IFN- γ , a major cytokine stimulated by IL-12, in Treg cell conversion to IFN- γ ⁺ T cells was next investigated. Similarly, addition of IFN- γ downregulated Foxp3 expression, and induced IFN- γ single positive as well as Foxp3⁺IFN- γ ⁺ T cells, but to a much lesser extent than T cells with IL-12 (Figure 5C). Because IL-23 has also been shown to promote Th17 cell conversion to Th1 cells,²³ we evaluated the role of IL-23 in Treg cell conversion to IFN- γ ⁺ T cells. Unexpectedly, IL-23 did not promote Treg cell acquisition of IFN- γ expression (data not shown).

To determine if IL-12 also mediates Treg cell conversion *in vivo*, CBir1-specific Treg cells were adoptively transferred into TCR β x δ ^{-/-} mice, and the recipients were given anti-IL-12p40 or control antibody intraperitoneally at the time of cell transfer and weekly thereafter. Four weeks later, the CD4⁺ T cell cytokine profile was determined by flow cytometry. As shown in Figure 5D, blockade of IL-12 significantly reduced Treg cell conversion to IFN- γ ⁺ T cells in the intestinal lamina propria and spleen. Treatment with anti-IL-12 limited Treg cell conversion to not only IFN- γ single positive but also Foxp3⁺IFN- γ ⁺ T cells. Taken together, these data reveal a crucial role of IL-12 in promoting Treg cell conversion to IFN- γ ⁺ T cells both *in vitro* and *in vivo*.

Th1 cells do not convert to Foxp3⁺ T cells

To further probe the origin of Foxp3⁺IFN- γ ⁺ T cells, we investigated whether Th1 cells could convert to Foxp3⁺IFN- γ ⁺ T cells. Th1 cells generated from IFN- γ ^{Thy1.1}.CBir1-Tg reporter mice under standard Th1-polarizing conditions were sorted by FACS based on Thy1.1 expression. Purified IFN- γ ^{Thy1.1+} Th1 cells were then cultured with CBir1 flagellin-pulsed APC. In contrast to Treg cells which lost Foxp3 expression, Th1 cells were stable after restimulation, in that about 96% of T cells were IFN- γ ⁺ after 4 days in culture (Figure 6A). As we have shown in Figure 2 that both IL-12 and TGF- β were required for generation of Foxp3⁺IFN- γ ⁺ T cells, we then investigated whether IL-12 and/or TGF- β would affect Th1 cell stability. Addition of IL-12, TGF- β , or both IL-12 and TGF- β did not affect IFN- γ expression, or induce Foxp3 expression even in the presence of high amounts of TGF- β (Figure 6A). Retinoic acid (RA) has been demonstrated to promote T cell Foxp3 expression.^{25, 26} Interestingly, although addition of RA to Th1 cell cultures reduced IFN- γ production, it did not induce Foxp3 expression, either alone or in synergy with TGF- β (Figure 6A).

To determine whether Th1 cells are stable or capable of converting to Foxp3-expressing T cells *in vivo*, CBir1-specific IFN- γ ^{Thy1.1+} Th1 cells were transferred into TCR β x δ ^{-/-} mice. Ten weeks after transfer, the recipient mice developed moderate colitis (Figure 6B). As shown in Figure 6C, more than 65% of Th1 cells lost IFN- γ expression in the intestine. There was, however, almost no Foxp3 expression by those adoptively transferred Th1 cells. Similarly, about 60% and 48% of T cells maintained IFN- γ expression in the spleen and MLN, respectively, but no T cells expressed Foxp3 at either site. Altogether, these data indicate that IFN- γ ⁺ Th1 cells are stable and do not acquire Foxp3 expression even in inflammatory settings.

Foxp3⁺IFN- γ ⁺ T cells differentiate into IFN- γ ⁺ but not Foxp3⁺ T cells

We thus conclude that Foxp3⁺IFN- γ ⁺ T cells arise from Foxp3⁺ Treg cells rather than IFN- γ ⁺ Th1 cells. In order to study the fate of Foxp3⁺IFN- γ ⁺ T cells, we generated Foxp3^{GFP}.IFN- γ ^{Thy1.1}.CBir1-Tg dual reporter mice by crossing IFN- γ ^{Thy1.1} and Foxp3^{GFP} reporter mice with CBir1-Tg mice. CD4⁺ T cells from Foxp3^{GFP}.IFN- γ ^{Thy1.1}.CBir1-Tg mice were cultured with CBir1-pulsed APC in the presence of IL-12 and TGF- β for 5 days. CBir1-specific Foxp3^{GFP}.IFN- γ ^{Thy1.1} T cells, as well as Foxp3^{GFP} and IFN- γ ^{Thy1.1} single positive T cells, were then sorted by FACS, and transferred into RAG^{-/-} mice. Consistent with previous data in Figures 6C and 3A, eight weeks after cell transfer, T cells from IFN- γ ^{Thy1.1} recipients partially lost IFN- γ expression and did not acquire Foxp3 expression, whereas adoptively transferred Foxp3^{GFP} T cells dramatically lost Foxp3 expression and converted into IFN- γ -expressing T cells in the intestinal lamina propria, MLN and spleen. In contrast, Foxp3⁺IFN- γ ⁺ T cells converted only into IFN- γ ⁺ Th1 cells but not into Foxp3⁺ Treg cells at all sites (Figure 6D). Thus, these data indicate that Foxp3⁺IFN- γ ⁺ T cells represent a transition state of Foxp3⁺ Treg cell conversion into IFN- γ ⁺ Th1 cells.

Foxp3⁺IFN- γ ⁺ T cells retain regulatory function and suppress colitis development

To investigate the functions of Foxp3⁺IFN- γ ⁺ T cells *in vivo*, we transferred CBir1-specific Foxp3^{GFP}.IFN- γ ^{Thy1.1} T cells and Foxp3^{GFP} single positive Treg cells into RAG^{-/-} recipients separately. Eight weeks after cell transfer, recipients of neither Foxp3⁺IFN- γ ⁺ T cells nor Foxp3 single positive T cells exhibited any signs of intestinal inflammation, indicating that despite the ability to produce IFN- γ , Foxp3⁺IFN- γ ⁺ T cells did not acquire effector T cell function (Figure 7A).

To determine whether Foxp3⁺IFN- γ ⁺ T cells retain regulatory cell function, CD45.2 Foxp3^{GFP}.IFN- γ ^{Thy1.1} CBir1-Tg T cells were cultured with CFSE-labeled CD45.1 CBir1-Tg CD4⁺ T cells in the presence of CBir1-pulsed APC. T cell proliferation was determined by CFSE intensity of gated CD45.1⁺ population. CBir1-Tg naïve CD4⁺ T cells proliferated effectively in response to CBir1 antigen stimulation (Figure 7B). Similar to classic Foxp3 single positive Treg cells, Foxp3⁺IFN- γ ⁺ T cells efficiently suppressed naïve T cell proliferation. This was confirmed by assessment of T cell proliferation using tritiated thymidine incorporation (Figure 7C).

To further define the regulatory role of Foxp3⁺IFN- γ ⁺ T cells *in vivo*, we investigated whether these double positive T cells could inhibit colitis development by using a well-established adoptive transfer model of CD45RB^{hi} CD4⁺ T cells. CD45RB^{hi} CD4⁺ T cells from CBir1-Tg mice were transferred into RAG^{-/-} mice alone, together with CBir1-Tg Foxp3⁺IFN- γ ⁺ T cells, or with CBir1-Tg Foxp3 single positive T cells. Four weeks later, the recipients of CD4⁺CD45RB^{hi} T cells developed severe colitis, which was abolished by Foxp3⁺IFN- γ ⁺ T cells as efficiently as conventional Foxp3⁺ Treg cells (Figures 7D-E). Taken together, these data indicate that Foxp3⁺IFN- γ ⁺ T cells retain regulatory functions both *in vitro* and *in vivo*.

Discussion

Accumulating evidence suggests that Treg cells are a dynamic population that can convert to IL-17- or IFN- γ -expressing T cells under certain conditions.^{12, 15, 16} However, the physiological relevance of Treg cell conversion to IFN- γ -expressing T cells remains unclear, as are the mechanisms involved in such conversion *in vivo*. The functions of such converted IFN- γ -expressing Treg cells, namely whether they function as effector T cells contributing to chronic inflammation or retain regulatory function suppressing progression of inflammation remain to be defined. We report here that microbiota antigen stimulation

drives Foxp3⁺ Treg cell conversion to IFN- γ -expressing T cells in the intestinal lamina propria, which is dependent on local innate cell-produced IL-12. However, these Foxp3⁺IFN- γ ⁺ T cells functioned as regulatory T cells to inhibit effector T cell-induced intestinal inflammation.

Foxp3⁺ Treg, IFN- γ ⁺ Th1, as well as IL-17⁺ Th17 cells are abundant in the intestinal lamina propria. Specific commensal microbiota is required for differentiation or migration of Th17 cells and/or Th1 cells to the gut lamina propria,^{27, 28} with these potentially proinflammatory T cells presumably held in check by local Treg cells. Our data showed that during the progression of intestinal inflammation, not only effector T cells (Th1, Th17 and “Th1+17” cells) but also Foxp3-expressing T cells (including classic Foxp3⁺ Treg cells, as well as Foxp3⁺IL-17⁺, Foxp3⁺IFN- γ ⁺ and Foxp3⁺IL-17⁺IFN- γ ⁺ T cells) developed in the inflamed intestine, which is consistent with a previous observation that during the progression of experimental autoimmune encephalomyelitis, both effector T cells and Treg cells accumulated in the brain lesions.²⁹ This raises the questions that how these T cell subsets develop during chronic intestinal inflammation, and how Treg cells function under this condition.

Consistent with previous reports, our data demonstrate that Treg cells can convert into Th1 and Th17 cells in the intestine, particularly under inflammatory conditions, which is dependent on local innate cell production of IL-12 and IL-6,^{9, 12} respectively. Treg cell conversion to IFN- γ ⁺ T cells has been reported in an autoimmune diabetic model¹⁵ as well as in a lethal infection model.¹⁶ However, based on the small frequency of Treg cells in these settings, the overall contribution of Treg cells to the pathogenesis of autoimmune diseases and infection remains difficult to assess. In the gut, commensal bacteria stimulate DC to produce proinflammatory cytokines through signaling of Toll-like receptors.³⁰ It is plausible that these proinflammatory cytokines might promote conversion of Treg cells into effector T cells that could have detrimental consequences. However, this does not happen, at least under normal exposure to commensal flora. For instance, the symbiont *Bacteroides fragilis*, via production of polysaccharide A, influences intestinal immune homeostasis and protects animals from experimental colitis by inhibiting Th17 cell differentiation and inducing IL-10 production.³¹ Our data demonstrate that IFN- γ ⁺Foxp3⁺ T cells retain regulatory functions to suppress colitis induced by effector T cells. Furthermore, adoptively transferred CBir1-Tg Foxp3⁺ Treg cells did not induce colitis in immunodeficient mice, even though most Treg cells converted into Th1 or Th17 cells in the lamina propria. This suggests that under physiological conditions, although Treg cells adopt an effector phenotype, the converted IFN- γ ⁺Foxp3⁺ T cells and unconverted Treg cells still maintain regulatory functions to inhibit inflammation. The balance between the converted effector T cells and remaining regulatory Foxp3⁺ T cells could be the tipping point in maintaining homeostasis or promoting inflammation. To initiate inflammation, Th1 and Th17 cells converted from Treg cells probably have to reach a threshold locally. Under steady-state conditions, such conversion does not reach that threshold, and thus Treg cells keep effector T cells in check. However, under highly inflammatory conditions, the strong proinflammatory cytokine milieu promotes Treg cell conversion into effector cells to overcome that threshold, and thereby favors the progression of chronic intestinal inflammation. Nevertheless, since the specific Treg cell to effector T cell ratio representing the tipping point between homeostasis and rampant chronic inflammation is unknown, it is also possible that the lack of colitis represents a deficiency in the proinflammatory effector function in converted Treg cell descendants. Both IL-12 and IL-23 are enriched in the intestine, and promote Th17 cell conversion to Th1 cells.²³ Although blockade of IL-12p40, which is shared by both IL-12 and IL-23, inhibited Foxp3⁺ Treg cell conversion to IFN- γ -expressing cells *in vivo*, addition of IL-12 but not IL-23 promoted Foxp3⁺ Treg cell conversion to IFN- γ -expressing cells, suggesting a crucial role for IL-12 but not IL-23 in

Treg cell conversion to IFN- γ -expressing cells. These data demonstrate that IL-12 and IL-23 differentially regulate plasticity of different T cell subsets in the intestine. Although it is still unclear whether IL-12 acts directly on T cells or via co-cultured APC, a previous report demonstrated that exposure of Treg cells from *Toxoplasma gondii*-infected mice to IL-12 induced significantly higher Stat4 phosphorylation, a hallmark of IL-12 signaling, compared to Treg cells from naive mice¹⁶, suggesting a direct effect of IL-12 on T cells.

Foxp3⁺IFN- γ ⁺ T cells have been reported to be present in mice infected with *Toxoplasma gondii* and in nonobese diabetic mice,^{15, 16} as well as in humans. However, their origin and function have been unclear. Adoptively transferred CBir1-Tg CD4⁺ T cells gave rise to Foxp3⁺IFN- γ ⁺ T cells as well as Th1 and Treg cells in TCR β x δ ^{-/-} recipient mice with severe intestinal inflammation. Thus Foxp3⁺IFN- γ ⁺ T cells could derive from IFN- γ ⁺ Th1 cells or Foxp3⁺ Treg cells. We showed that Treg cells converted to Foxp3⁺IFN- γ ⁺ and IFN- γ single positive T cells both *in vitro* and *in vivo*, whereas Th1 cells did not acquire Foxp3 expression, even under highly polarized conditions. Furthermore, Foxp3⁺IFN- γ ⁺ T cells developed only into IFN- γ ⁺ Th1 cells but not into Foxp3⁺ Treg cells *in vivo*, indicating that Foxp3⁺IFN- γ ⁺ T cells represent a transition state of Foxp3⁺ Treg cell conversion into IFN- γ ⁺ effector T cells.

In summary, our data demonstrate that Treg cells convert to IFN- γ - and IL-17-expressing T cells in the lamina propria, which is driven by cognate microbiota antigen stimulation. Foxp3⁺IFN- γ ⁺ T cells represent a transition state of Foxp3⁺ Treg cell conversion to IFN- γ ⁺ effector T cells, but retain suppressive functions. Treg cell conversion to effector T cells likely contributes to progression of inflammation, but we postulate that chronic inflammation occurs only after conversion reaches a threshold or tipping point. Even though our data demonstrate that Treg cell transfer in general does not cause inflammation due to the strong regulatory functions of converted Foxp3⁺IFN- γ ⁺ T cells and remaining Foxp3⁺ Treg cells, more factors need be taken into consideration for Treg therapy such as suppression of inflammatory cytokines, such as blocking IL-12p40, which could significantly block Treg cell conversion to IFN- γ -producing cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

IL	interleukin
Tg	transgenic
Treg	regulatory T cells

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Figure 1. Naïve CBir1-Tg T cells induce colitis and differentiate into various subsets in $\text{TCR}\beta\text{x}\delta^{-/-}$ mice

$\text{TCR}\beta\text{x}\delta^{-/-}$ mice were injected intravenously with PBS, or 1×10^6 naïve CD4^+ T cells from CBir1-Tg or OT II mice. (A) Colonic histopathology of the recipients 8 weeks after cell transfer. (B,C) Spleen, MLN, and LP CD4^+ T cell cytokine was determined by flow cytometry 4 weeks (B) and 8 weeks (C) after cell transfer. Plot numbers represent the percentage of CD4^+ T cells in the respective quadrants (Left). Foxp3 negative or positive T cells were overlaid on CD4^+ T cells, and the plot numbers represent the percentage of Foxp3 negative or positive T cells in the respective quadrants (Right). Data are representative of three or more experiments with similar results.

Figure 2. IL-12 and TGF- β are required for induction of Foxp3⁺IFN- γ ⁺ T cells
CBir1-Tg CD4⁺ T cells were cultured with CBir1-peptide pulsed-splenic APC with various cytokines or antibody in the absence (A) or presence (B) of IL-2. Five days later, T cell Foxp3 and IFN- γ expression was determined by flow cytometry and analyzed by gating on CD4⁺ population. (A,B) FACS profiles. One representative of at least three experiments was shown. (C) Bar chart represents aggregate data with mean $\pm$ SEM of three experiments.

Figure 3. CBir1-specific Foxp3⁺ Treg cells convert into IFN- γ ⁺ and IL-17⁺ T cells *in vivo*
 0.5×10^6 Foxp3^{GFP} Treg cells were transferred into TCR β $\times\delta^{-/-}$ mice. (A) Four weeks later, cytokine production of transferred Treg cells was analyzed by flow cytometry by gating on CD4⁺ cells (Left). Foxp3 negative or positive T cells were overlaid on CD4⁺ T cells, and the plot numbers represent the percentage of Foxp3 negative or positive T cells in the respective quadrants (Right). (B) Colonic histology of the Treg cell or PBS recipients four weeks after transfer. Data are representative of three experiments.

Figure 4. CBir1-specific Treg cell conversion to effector T cells takes place in the intestine
 0.5×10^6 Foxp3^{GFP+} Treg cells were transferred into TCR β $\delta^{-/-}$ mice. Two (A,B) and six weeks (C,D) later, cytokine production of transferred Treg cells was analyzed by flow cytometry by gating on CD4⁺ population or CD4⁺ Foxp3 negative population (A,C). (B,D) Bar chart represents aggregate data with mean $\pm$ SEM of three experiments (* P < 0.05; ** P < 0.01). Data are representative of two (C,D) or three (A,B) experiments.

Figure 5. IL-12 promotes Foxp3⁺ Treg cell conversion to IFN- γ -expressing T cells
(A-C) Foxp3^{GFP+} Treg cells were cultured with CBir1 peptide-pulsed splenic APC. Five days later, T cells Foxp3 expression and IFN- γ production were determined by flow cytometry. Plot numbers represent the percentage of CD4⁺ population in the respective quadrants **(A,C)**. Supernatant was collected on day 3 of cell culture, and IFN- γ production was determined by ELISA **(B)**. **(D)** 0.5×10^6 Foxp3^{GFP+} Treg cells were transferred into TCR β x $\delta^{-/-}$ mice. The recipients were injected intraperitoneally with 100 μ g of control or anti-IL-12p40 antibodies at the time of cell transfer and weekly thereafter. Four weeks later, cytokine production of transferred Treg cells was analyzed by flow cytometry with CD4⁺ T cells gated. Data represent two **(D)** or three **(A-C)** experiments with similar results.

Figure 6. Th1 cells do not convert to Foxp3⁺ T cells, and Foxp3⁺IFN- γ ⁺ T cells develop into IFN- γ ⁺ but not Foxp3⁺ T cells

(A) IFN- γ ^{Thy1.1+} Th1 cells from IFN- γ ^{Thy1.1}.CBir1-Tg mice were cultured with CBir1 peptide-pulsed splenic APC. Five days later, T cell Foxp3 and IFN- γ expression was determined by flow cytometry by gating on CD4⁺ population. (B,C) 0.5 $\times$ 10⁶ Th1 cells or PBS were transferred into TCR β δ ^{-/-} mice, and the recipients were sacrificed 10 weeks later. Colonic histopathology was assessed (B), and cytokine production of transferred CD4⁺ Th1 cells was analyzed by flow cytometry with CD4⁺ T cells gated (C). (D) 0.25 $\times$ 10⁶ CBir1-specific Foxp3^{GFP}-IFN- γ ^{Thy1.1+}, Foxp3^{GFP}⁺IFN- γ ^{Thy1.1-}, and Foxp3^{GFP}⁺IFN- γ ^{Thy1.1+} T cells were transferred into RAG^{-/-} mice. Eight weeks later, the recipients were

sacrificed and cytokine production of transferred Treg cells was analyzed by flow cytometry. Plot numbers represent the percentage of CD4⁺ T cells in the respective quadrants. One of three experiments with similar results was shown.

Figure 7. Fcpx3⁺IFN-γ⁺ T cells retain regulatory functions

(A) 0.25×10^6 CBir1-Tg Fcpx3^{GFP+}IFN-γ^{Thy1.1-} and Fcpx3^{GFP+}IFN-γ^{Thy1.1+} T cells were transferred into RAG^{-/-} mice. The recipients were sacrificed and colonic histopathology was analyzed 4 weeks later. (B) 0.2×10^6 CFSE-labeled CD4⁺ T cells from CD45.1 CBir1-Tg mice were cultured alone, or with 0.2×10^6 CD45.2 CBir1-Tg Fcpx3^{GFP+}IFN-γ^{Thy1.1-} or Fcpx3^{GFP+}IFN-γ^{Thy1.1+} T cells. T cell proliferation was analyzed by flow cytometry based on the dilution of CFSE intensity by gating on CD4⁺CD45.1⁺ T cells. (C) 5×10^4 CD4⁺ T cells from CBir1-Tg mice were cultured alone, or with 5×10^4 Fcpx3^{GFP+}IFN-γ^{Thy1.1-} or Fcpx3^{GFP+}IFN-γ^{Thy1.1+} T cells. Tritiated thymidine was added for the last 8 h of a 48-h incubation period for T cell proliferation. Mean CPM of triplicates ± SEM are shown.

(D,E) 0.25×10^6 CD45RB^{hi} T cells from CBir1-Tg mice were transferred into RAG^{-/-} mice alone or together with 0.25×10^6 Foxp3^{GFP+}IFN- γ ^{Thy1.1⁻} or Foxp3^{GFP+}IFN- γ ^{Thy1.1⁺} T cells. Four weeks later, the recipients were sacrificed and histopathology was assessed. Combined colon and cecum histological scores **(D)** and colonic histopathology **(E)** are shown. *** $P < 0.001$. Data represent two **(D,E)** or three **(A-C)** experiments with similar results.