Interleukin-12 Converts Foxp3$^+$ Regulatory T Cells to Interferon–γ-Producing Foxp3$^+$ T Cells That Inhibit Colitis

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BACKGROUND & AIMS: Regulatory T (Treg) cells are plastic, but the in vivo mechanisms by which they are converted into foxhead box p3 (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 (Tg) (CBir1-Tg) mice, which are specific for CBir1 flagellin (an immunodominant microbiota antigen), with transforming growth factor-β. Foxp3^{GFP+} CBir1-Tg Treg cells were isolated by fluorescence-activated cell sorting and transferred into TCRβx$\delta^{-/-}$ 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. **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: Inflammatory Bowel Disease; IBD; Immune Regulation; Inflammation; Treg.

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 multiorgan 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] T-cell receptor (TCR)-stimulated thymus-derived Foxp3$^+$ T cells were shown to produce interleukin (IL)-17 after exposure to IL-6 in the absence of exogenous transforming growth factor (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 interferon (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

Abbreviations used in this paper: **APC, antigen-presenting cells; CBir1-Tg, CBir1 T-cell receptor transgenic; CFSE, carboxyfluoroscein succinimidyl ester; FACS, fluorescence-activated cell sorter; IFN, interferon; IL, interleukin; MLN, mesenteric lymph nodes; TCR, T-cell receptor; TGF, transforming growth factor; Tg, transgenic; Treg, regulatory T cells.**

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converted Foxp3$^+$IFN-γ^+ and IFN-γ^+ Th1 cells are in intestinal inflammation.

In this report, we generated Foxp3^{GFP}.IFN-$\gamma^{Thy1.1}$.CBir1 TCR Tg dual reporter mice by crossing IFN-$\gamma^{Thy1.1}$ and Foxp3^{GFP} reporter mice with CBir1 TCR Tg (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 because 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, TCRβx$\delta^{-/-}$, RAG1$^{-/-}$, and Foxp3^{GFP} reporter mice were purchased from Jackson Laboratory (Bar Harbor, ME). IFN-$\gamma^{Thy1.1}$ reporter[18] and CBir1-specific TCR Tg (CBir1-Tg) mice[19] 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 (San Diego, CA). Anti-mouse Foxp3 (FJK-16s) were purchased from eBioscience (San Diego, CA). Recombinant IL-2, IL-12, IL-23, IFN-γ, and TGF-β were purchased from R&D Systems (Minneapolis, MN). Anti-mouse IFN-γ (XMG1.2) and IL-12p40 (C17.8) neutralizing monoclonal antibodies were purchased from BioLegend (San Diego, CA).

CD4$^+$ T-Cell Purification and Labeling With Carboxyfluoroscein Succinimidyl Ester

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 μmol/L carboxyfluoroscein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA) 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-$\gamma^{Thy1.1}$.CBir1-Tg reporter mice were cultured in the presence of CBir1 flagellin peptide-pulsed antigen-presenting cells (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 fluorescence-activated cell sorter (FACS) based on green fluorescence protein 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 H&E.

Statistical Analysis

The nonparametric Mann–Whitney *U* test was used for assessing pathology scores. Levels of significance were determined by Student *t* test. *P* values of $< .05$ 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

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 phosphate-buffered saline control were transferred intravenously into TCRβx$\delta^{-/-}$ mice, which lack T cells but have a fully responsive innate immune system, B-cell repertoire, and natural killer 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, were also transferred as a negative control. Both CBir1-Tg and OT II splenic CD4$^+$ T cells expressed similar levels of Foxp3 (Supplementary Figure 1*A*). Approximately 80%–85% of CBir1-Tg CD4$^+$ T cells were positive for I-Ab-CBir1p tetramer (Supplementary Figure 1*B*). The recipients were killed 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