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A Regulatory Role for IL-10 Receptor Signaling in Development and B Cell Help of T Follicular Helper Cells in Mice

Gang Cai,*1,† Xiaomeng Nie,*†,1 Weiwei Zhang,‡ Beiyi Wang,‡ Jiafei Lin,* Huazhou Wang,‡ Cen Jiang,* and Qian Shen‡

IL-10 is widely accepted as a survival, proliferation, and differentiation factor for B cells. However, IL-10 deficiency accelerates disease progression as the result of autoantibody production in many autoimmune disease models. It was demonstrated that T follicular helper cells (T\(\text{FH}\) cells) play a key role in helping B cells that are secreting Abs. In this study, we demonstrated a regulatory role for IL-10R signaling on the development and B cell help function of T\(\text{FH}\) cells in vitro and in vivo. IL-1R subunit β-deficient (Il10rb\(^{-/-}\)) Th cells were able to differentiate more readily into T\(\text{FH}\) cells, as well as secrete more IL-21 and IL-17 compared with wild-type Th cell-derived T\(\text{FH}\) cells. Increased IL-21 and IL-17 contributed to the enhanced B cell help functions of T\(\text{FH}\) cells. Further experiments demonstrated that IL-6 and IL-23 from dendritic cells in Il10rb\(^{-/-}\) mice contributed to the differentiation of naive Th cells into T\(\text{FH}\) cells, as well as the generation of IL-21– and IL-17–producing T\(\text{FH}\) cells. Our results provide useful information for clarifying the immunoregulatory mechanisms associated with IL-10 deficiency in certain autoimmune disease models. This information could also be of benefit for the development of vaccines. *The Journal of Immunology, 2012, 189: 000–000.

Interleukin-10 is a pleiotropic cytokine with multiple biological effects on various types of blood cells, including roles as a survival, proliferation, and differentiation factor for B cells (1, 2). This was demonstrated most convincingly using human B cells, stimulated either via the IgRs or by a combination of CD40 Abs and IL-4. Under these conditions, IL-10 enhances DNA synthesis and expansion of activated B cells, as well as induces their differentiation into Ab-producing cells that secrete large amounts of IgM, IgG, and IgA Abs (3–5). Patients with lupus, a disease closely linked to abnormal autoantibodies, have a high concentration of serum IL-10, with its level correlating with disease activity (6–9).

Despite the evidence suggesting that IL-10 promotes Ab production in humans, its complex role was first described in studies using rodent models. Its suppressive role is supported by data from IL-10–deficient mice. IL-10 deficiency accelerated lupus in MRL-Faslpr mice (10) and arthritis induced by collagen (11) through a mechanism of increasing autoreactive IgG2a. Additionally, in an Il10rb\(^{-/-}\) model of inflammatory bowel disease (IBD), increased levels of IgG and IgA were found in blood or target organs (12). The data collectively reflect the opposing roles of IL-10 in humoral immunity in rodents, with the precise mechanism(s) of action largely unknown.

Naive B cell differentiation into high-affinity memory B cells and long-lived plasma cells requires the help of CD4+ T cells. T follicular helper cells (T\(\text{FH}\) cells) that express chemokine CXCR5, which facilitates their entry into germinal centers (GCs), have recently been considered a key Th subset for B cell proliferation, somatic hypermutation of Ig variable-region gene segments, and class-switch recombination of Igs (13). Furthermore, these T\(\text{FH}\) cell populations serve as an important reservoir of memory cells for secondary responses to Ags (14). T\(\text{FH}\) cells provide help to B cells through several factors, including CD154 and ICOS (13). In particular, T\(\text{FH}\) cells secrete the cytokine IL-21, which drives the growth, differentiation, and isotype switching of B cells (13). Furthermore, IL-21 provides a positive-feedback loop to induce CD4+ T cells to differentiate into T\(\text{FH}\) cells (15), a process demonstrated in both human and mouse CD4+ T cells. However, the possible influence of other cytokines on T\(\text{FH}\) cells is unknown. Because IL-10 inhibits effective T cell function in many autoimmune models, we wondered whether IL-10 regulated Ab production through a mechanism that affects T\(\text{FH}\) cells. To test this hypothesis, we studied the action of IL-10 on T\(\text{FH}\) cell function in vitro and in vivo.

Materials and Methods

Mice and adoptive transfers

Male 6–8-wk-old wild-type (WT) C57BL/6 (H-2\(^{b}\)) and Rag1\(^{-/-}\) mice (C57BL/6) were purchased from the Nanjing Model Animal Center (Nanjing, China). Il10rb\(^{-/-}\) mice (C57BL/6) were obtained from The Jackson Laboratory. Mice were maintained in a specific pathogen-free animal facility and handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

For adoptive transfers, Rag1\(^{-/-}\) mice were given 2 \times 10\(^6\) purified CD4+ T cells (isolated from naive WT or Il10rb\(^{-/-}\) [knockout (KO)] mice) i.v., together with 10\(^5\) purified WT or KO B cells, 24 h before immuni-

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*Department of Laboratory Medicine, Shanghai Jiaotong University Medical School, Shanghai 200025, China; †Department of Respiratory Diseases, Shanghai Jiaotong Hospital, Second Military Medical University, Shanghai 200433, China; and ‡Department of Experimental Diagnosis, Shanghai Jiaotong Hospital, Second Military Medical University, Shanghai 200433, China

1G.C. and X.N. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Qian Shen, Department of Laboratory Medicine, Shanghai Jiaotong University Medical School, Shanghai 200025, China; or to Gang Cai, Department of Respiratory Diseases, Shanghai Jiaotong Hospital, Second Military Medical University, Shanghai 200433, China. E-mail address: laoshenqch@yahoo.cn.

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Abbreviations used in this article: BFA, brefeldin A; DC, dendritic cell; GC, germinal center; IBD, inflammatory bowel disease; Ig, immunoglobulin; KO, knockout; PNA, pea agglutinin; T\(\text{FH}\) cell, T follicular helper cell; WT, wild-type.

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zation. In some experiments, 100 μg control IgG or neutralizing Ab to IL-17 or soluble IL-21R was transferred into Rag1−/− mice at the time of immunization.

**Immunization of animals**

SRBCs were obtained from Yaohua Biotech Company (Shanghai, China) and prepared as described previously (15). Mice were injected with 1 × 10^9 SRBCs through the vena caudalis. After 7 d, splenocytes were collected for surface and intracellular Ag analysis, and serum was collected for evaluation of Ag-specific Ab production. The control mice were injected with the same volume of saline.

**Cell purification**

CD4+ T cells were purified from naive animals by magnetic depletion of B cells, macrophages, dendritic cells (DCs), NK cells, granulocytes, erythrocyte precursors, and CD8+ T cells (Miltenyi Biotec, Auburn, CA). CD62L+CD4+ and CCR5+CD4+ T cells were further purified by magnetic separation using biotin-coupled CD62L (MEL-14; eBioscience, San Diego, CA) or CCR5 (2G8; BD Biosciences, San Jose, CA) mAbs, respectively. Separation was performed on an autoMACS column using the Posseid program. Splenic B cells were isolated by positive selection with anti-CD19–coupled magnetic beads. DCs were matured from bone marrow following stimulation with 10 ng/ml GM-CSF and 5 ng/ml IL-4. After 6 d, cells were purified with anti-CD11c–coupled magnetic beads and stimulated with 500 ng/ml LPS (Sigma-Aldrich) for another 2 d. The purity of cell fractions in all experiments ranged between 90–98%, as estimated by flow cytometry using FACS (BD Biosciences, San Jose, CA).

**Priming of naive T cells**

Naive CD4+CD62L− T cells (106 cells/well in 24-well plates) were pre-activated for 72 h with plastic-coated CD3 mAb (145-2C11; 5 μg/ml) and CD28 mAb (37.51; 1 μg/ml). For differentiation of naive Th cells into Th1 cells, naive cells were stimulated with CD3 (5 μg/ml) and CD28 (1 μg/ml) in the presence of IL-6 (5 ng/ml), IFN-γ mAb (XMG1.2; 10 μg/ml), and IL-4 mAb (11B11; 10 μg/ml). T cells were recovered, washed extensively, and rested for 24 h in medium before being tested for cytokine production and B cell help capacity. In some experiments, cultured DC supernatants and/or 10 μg/ml neutralizing Abs to IL-12p40 (C17.8; eBioscience), IL-23p19 (G23.8; eBioscience), and IL-6 (MP5-20F3; eBioscience) were added to the culture. To evaluate T cell priming by DCs, activated DCs (1.5 × 10^6 cells/well) from WT or Il10−/− mice were cultured for 7 d with naive CD4+ T cells (4 × 10^5 cells/well) in RPMI 1640 complete medium. Th1 cell differentiation in vitro was induced by the addition of IL-6 (10 ng/ml) in the presence of IL-4 (10 ng/ml) and IFN-γ (10 ng/ml) mAbs. In some experiments, IL-10 was added to the culture to evaluate its role in inhibiting the differentiation of Th1 cells. Polarization efficacy was verified by intracellular cytokine staining on secondary stimulations.

**Flow cytometry**

Anti-mouse mAbs marked with fluorescein were all purchased from eBioscience. Cells were washed with PBS containing 2% BSA (Sigma-Aldrich). FcγRs were blocked by incubation with CD16/CD32 mAb for 30 min at 4°C. Cells were washed twice before being stained with FITC- and PerCP/Cy5.5-conjugated anti-mouse cell surface molecule or intracellular Abs for 30 min at 4°C in the dark. After staining, cells were washed with PBS/BSA before flow cytometry. Intracellular cytokines and transcription factors were stained with the BD Cytofix/Cytoperm Kit (BD Pharmingen) and Mouse Regulatory T CellStaining Kit (eBioscience), respectively. Flow cytometric analysis was performed on a FACS Canto II (BD Biosciences) and analyzed with Diva software.

**B cell help**

Serial dilutions of CD4+ T cells were cultured for 7 d with purified syngeneic B cells (5 × 10^5 cells/well) in the presence of CD3 mAb or extracted SRBC membrane proteins. T cells were irradiated (2000 cGy) before the beginning of the coculture to prevent their outgrowth during the 7-d culture. IgM, IgG1, and IgG2a Abs in the supernatants were determined by ELISA, as previously described (16). In some experiments, neutralizing Abs to IL-7 (TC11-8H4; BioLegend) and soluble IL-21R or isotype-matched controls were added (10 μg/ml) to the culture.

**Quantitative RT-PCR**

Total cellular RNA was extracted using TRizol reagent, and reverse transcription of mRNA was carried out using moloney-murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantitation, with β-actin as the endogenous reference gene, was performed using the 2−ΔΔCt method. Primer sequences can be obtained on request.

**ELISA for SRBC-specific Abs and cytokines**

SRBC Abs in the serum were measured on day 21 by isotype-specific ELISA. Soluble SRBC Ags were prepared, as described previously (15), and protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Soluble SRBC Ags (5 μg/ml in carbonate buffer [pH 9.6]) were coated onto 96-well Immunol 2HB plates (Thermo Labsystems, Franklin, MA). After blocking with 1% (v/v) BSA in PBS, SRBC-specific IgM and IgG isotypes were determined by incubating serially diluted serum samples for 2 h at 37°C. After washing with 0.05% (v/v) Tween 20 in PBS, wells were incubated with biotin-conjugated isotype-specific mAbs, including anti-mouse IgG1 (Serotec, Raleigh, NC) or anti-mouse IgG2b (BD Pharmingen, San Diego, CA), washed, and then developed with a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) and o-phenylenediamine (Wako, Osaka, Japan). After terminating the reaction with 2 N H2SO4, the optical densities at 490 and 595 nm were measured on a microplate reader (Bio-Rad). Supernatants from DC cultures were collected for ELISA detection of IL-6 (eBioscience), IL-12 (eBioscience), and IL-23 (eBioscience), according to the manufacturer’s instructions.

**Statistical analysis**

The results are expressed as mean ± SD from four mice in each group. Data were analyzed with SPSS software to calculate an unpaired, two-way Student t test, including an F test to compare variances. The p values < 0.05 were considered significant.

**Results**

Blockade of IL-10 promotes the B cell help function of activated Th cells

IL-10 is known to induce activated B cell expansion and differentiation into Ab-producing cells that secrete a large amount of IgM, IgG, and IgA Abs (4, 5). However, it is not clear whether IL-10 imparts its effects on Ab responses through regulation of Th cells. In these experiments, we first differentiated naive T cells into effector cells and then cocultured these effector cells with B cells. After several days in culture, the effects of these cells on B cell help were evaluated. Naive CD4+CD62L+ T cells were first stimulated in vitro by a combination of CD3 and CD28 mAbs, with or without IL-10. After a brief resting period, these cells were cocultured with purified CD19+ B cells from WT mice in the presence of anti-IgM for another 7 d. The ability of Th cells to provide B cell help was evaluated by examining the accumulation of IgG1 and IgG2a secretion in the supernatant at the end of the culture period. To our surprise, activated WT CD4+ T cells displayed inferior B cell help capacity compared with Il10−/− T cells, and their effects were inhibited by added IL-10 (Fig. 1A). Because IL-6 was reported to drive the naive Th cell acquisition of B cell help functions and is dependent on Th1 differentiation, we stimulated naive Th cells under Th1-polarizing conditions, with or without IL-10, and cocultured them with B cells, as described above. After 7 d, Th cells cultured under polarizing conditions produced more IgG than did those cultured under neutral conditions, and Il10rb−/− CD4+ T cells consistently displayed superior B cell help capacity compared with WT controls. As anticipated, IL-10 inhibited B cell help of Th cells, which were differentiated under Th1-polarizing conditions (Fig. 1B).

Th cell IL-10 deprivation promotes thymus-dependent Ab production in vivo

The results described above suggested an inhibitory role for IL-10 in naive Th cell acquisition of B cell help functions, but it was still unknown whether this would affect Ab production in vivo.
We analyzed serum-specific Abs after immunization with SRBCs, a strong polyvalent Ag stimulus for T cell-dependent Ab responses. At 21 d postimmunization, mice that were transferred with both T and B cells from Rag1−/− mice that were previously pretreated with IL-10, were immunized with SRBCs; 7 d later, the generation and B cell help function of CD4+ CXCR5+ cells in secondary lymphoid organs were analyzed. As anticipated, Il10rb−/− mice exhibited more CD4+ CXCR5+ cells in their spleens than did WT mice after immunization, and these cells within WT mice decreased when mice were pretreated with IL-10 (Fig. 3A). Staining for Bcl-6, the key transcription factor of TFH cells (17), also confirmed greater TFH cell differentiation in immunized Il10rb−/− mice than in WT mice, and even fewer CD4+ Bcl-6+ cells accumulated in mice pretreated with IL-10 (Fig. 3A). Next, WT B cell proliferation and Ig secretion in the presence of CD4+ CXCR5+ cells from immunized WT and Il10rb−/− mice were compared after 7 d of T–B cell coculture. CD4+ CXCR5+ cells from immunized Il10rb−/− mice had a greater capacity to promote B cell proliferation (Fig. 3B) and specific Ab secretion (Fig. 3C). We also found that TFH cells from IL-10–treated WT mice showed inferior B cell help capacities (Fig. 3C). These data suggested that IL-10 controlled both the quantity and quality of TFH cells.

**IL-10 deprivation induces the generation of IL-21+IL-17+ TFH cells**

Other than CXCR5, which located TFH cells in the B cell region of GCs, other TFH cell surface molecules, such as ICOS (18–20), CD154 (21), OX40 (22), and CD84 (23), mediated B cell propagation and help, GC B cell survival, differentiation, Ig class switching, somatic hypermutation, and/or memory B cell responses. We examined whether TFH cells from Il10rb−/− mice shared these properties. To our surprise, there was no significant difference in the expression of those molecules on TFH cells between immunized WT and Il10rb−/− mice, either at the level of RNA (Supplemental Fig. 1A) or protein (Supplemental Fig. 1B). Consisting with this finding, IL-10–treated WT mice did not show downregulation of those molecules on TFH cells compared with untreated WT mice (Supplemental Fig. 1A, 1B).

The observation that membrane molecules were not involved in enhancing the B cell help capacity of TFH cells from Il10rb−/− mice prompted us to investigate whether soluble mediators were involved. Therefore, we analyzed intracellular cytokine expression, including IL-21, IFN-γ, IL-17, and IL-4, of Th cells from immunized mice by flow cytometry. Splenocytes from SRBC-immunized WT mice injected with IL-10 or PBS or Il10rb−/− mice were stimulated in vitro with SRBC membrane Ag for 24 h and then activated with PMA and ionomycin (IO) for another 6 h in the presence of brefeldin A (BFA). As described previously (24), IFN-γ- and IL-4–producing CD4+ cells were mainly CXCR5+ cells, and few IFN-γ−CD4+ CXCR5+ or IL-4−CD4+ CXCR5+ splenocytes were found in either WT or Il10rb−/− mice. In contrast, IL-21 was produced by both CXCR5+ and CXCR5+ Th cells, with larger numbers of IL-21+ CXCR5+ cells than IL-21+ CXCR5− cells. Additionally, we found that IL-10 signaling played a vital role in the generation of CXCR5+ IL-21+ Th cells, because Il10rb−/− mice generated more of these cells than did WT mice, and exogenous IL-10 further downregulated their production. We also observed a number of IL-17+ CD4+ CXCR5+ cells in the Il10rb−/− group but few among WT controls, with or without IL-10 injection (Fig. 4). The results of flow cytometry were also confirmed by immunofluorescence of histological sections that IL-17–producing CD4+ cells existed in or out of B cell follicle (Supplemental Fig. 1C). In addition to IL-17 and IL-21, IL-10 has effects on IFN-γ production, but the effects were mainly on CD4+ CXCR5− cells rather than on CD4+ CXCR5+ cells. It was in line with the report

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**FIGURE 1.** Activated Il10rb−/− Th cells display a superior capacity for B cell help compared with WT controls. (A) Naïve CD62L−CD4+ T cells purified from the spleen of Il10rb−/− mice (KO) and WT mice were stimulated for 72 h with CD3 and CD28 mAbs in the presence or absence of IL-10 and then rested for 24 h in fresh medium. Serial dilutions of recovered Th cells were incubated with purified WT B cells (5 × 106 cells/well) and anti-IgM mAbs (1 μg/ml). Inactive CD62L−CD4+ T cells were purified at the time of T and B cell coculture testing. (B) CD62L+CD4+ T cells were stimulated in neutral culture medium, T FH-polarizing culture medium, or IL-10–pulsed medium for 72 h, and cells were rested and cocultured with WT B cells, as in (A). Culture supernatants were tested on day seven for the presence of IgG1 and IgG2a. Results are expressed as mean ± SD of triplicate. Similar results were obtained in two additional experiments. *p < 0.01, †p < 0.05, versus WT-active; ‡p < 0.01, versus WT-active + IL-10.

Rag1−/− mice that were pretreated with T and B cells from a different source, we analyzed serum-specific Abs after immunization with SRBCs, a strong polyclonal Ag stimulus for T cell-dependent Ab responses. At 21 d postimmunization, mice that were transferred with Il10rb−/− T cells and WT B cells exhibited significantly higher anti-SRBC membrane Abs, including IgM, IgG1, and IgG2a, than did their controls that were transferred with WT T cells and WT B cells (Fig. 2A). In another parallel control experiment, mice transferred with both T and B cells from Il10rb−/− mice showed significantly higher Ag-specific Abs than did corresponding controls transferred with WT T cells and Il10rb−/− B cells (Fig. 2A). We also observed that the level of serum Abs in mice with WT B cells was slightly higher than in mice with Il10rb−/− B cells, but their difference did not reach significance. These results provided evidence for a B cell co-stimulatory function of IL-10 and suggested a complex role for IL-10 in Ab responses. To verify our Ab findings, peanut agglutinin (PNA)-positive B cells, which reflected the generation of a GC, were analyzed by flow cytometry. More PNA+B220+ splenocytes were evident in mice transferred with Il10rb−/− T cells and WT B cells than in their controls transferred with WT T cells and WT B cells. Additionally, compared with those transferred with WT T cells and Il10rb−/− B cells, more PNA+B220+ splenocytes could be found in mice transferred with Il10rb−/− T cells and Il10rb−/− B cells (Fig. 2B).

**IL-10 regulates the quantity and quality of CD4+ CXCR5+ cells in secondary lymphoid organs of SRBC-immunized mice**

If IL-10 could inhibit Ab production through TFH cells, we wondered whether their quantity or quality was regulated by IL-10. To answer the question, Il10rb−/− mice and WT mice, treated or not with IL-10, were immunized with SRBCs; 7 d later, the generation and B cell help function of CD4+ CXCR5+ cells in secondary lymphoid organs were analyzed. As anticipated, Il10rb−/− mice exhibited more CD4+ CXCR5+ cells in their spleens than did WT mice after immunization, and these cells within WT mice decreased when mice were pretreated with IL-10 (Fig. 3A). Staining for Bcl-6, the key transcription factor of TFH cells (17), also confirmed greater TFH cell differentiation in immunized Il10rb−/− mice than in WT mice, and even fewer CD4+ Bcl-6+ cells accumulated in mice pretreated with IL-10 (Fig. 3A).
that IL-10 inhibits IFN-γ production (25). Because II10rb<sup>−/−</sup>-<br>TFH cells expressed both IL-21 and IL-17, we wanted to deter-<br>mine the relationship between IL-21<sup>+</sup> and IL-17<sup>+</sup> TFH cells.<br>Costaining showed that most IL-17–producing TFH cells also<br>produced IL-21 (Fig. 4C). To further characterize the IL-17–<br>producing TFH cells, we analyzed the expression of RORγt, IL-<br>23R, CCR6, and IL-22 and compared those of CD4<sup>+</sup>CXCR5<sup>+</sup> cells with<br>CXCR5<sup>+</sup> cells produced higher levels of CCR6 and<br>IL-22 but comparable RORγt and IL-23R than CXCR5<sup>+</sup> cells<br>(Supplemental Fig. 1D).

**FIGURE 2.** Effects of IL-10 signaling deficiency on Ab production and B cell help capacity after T cell-dependent immunization. (A) Rag1<sup>−/−</sup> mice pretransferred with naive Th cells and B cells from either II10rb<sup>−/−</sup> (KO) or WT mice were immunized with SRBCs (1 × 10<sup>9</sup> cells i.v.). Sera were collected on day 21 and tested with SRBC surface Ag-specific Abs. (B) Spleens were collected on day 21 after SRBC immunization, and B cells (CD19<sup>+</sup>) from the spleen were analyzed for PNA receptor expression by flow cytometry. Similar results were obtained in two additional experiments.

**FIGURE 3.** Effects of IL-10 signaling deficiency on TFH cell generation and capacity for B cell help after T cell-dependent immunization. (A) With or without IL-10–treated WT mice, as well as II10rb<sup>−/−</sup> (KO) mice were immunized with SRBCs as in Fig. 2. Representative dot plots showing the flow cytometric analysis of splenic CD4<sup>+</sup>CXCR5<sup>+</sup> cells and CD4<sup>+</sup>Bcl-6<sup>+</sup> cells gated on total CD4<sup>+</sup> T cells 7 d after SRBC immunization. The numbers in the dot plot quadrants represent the percentages. Data are representative of three experiments. (B) Splenic CD4<sup>+</sup>CXCR5<sup>+</sup> T cells were cocultured with CFSE-stained WT B cells for 7 d. A representative graph shows that TFH cells promoted B cell proliferation. Numbers indicate the percentages of CFSE<sup>low</sup> cells. Data are representative of three experiments. (C) Splenic CD4<sup>+</sup>CXCR5<sup>+</sup> T cells were cocultured with B cells as in (B), and supernatants were collected for testing Ab production. Data are combined individual values from three experiments (n = 5–6). *p < 0.01, **p < 0.05, KO versus WT mice; ***p < 0.01, ***p < 0.05, WT versus IL-10–treated WT mice.
Moreover, it suggested a role for T<sub>FH</sub> cell-derived IL-17 in (13, 15) and IL-17 (26, 27) played roles in B cell help functions. These results confirmed the previous observation that both IL-21 effect on IgM production (Fig. 5A). Additionally, anti–IL-17 im-

shows IFN-<sub>γ</sub> expression by flow cytometry. A representative dot plot

Inasmuch as differentiation of naive CD4<sup>+</sup> T cells was primed with APCs, such as DCs, we next examined whether the presence of IL-17<sup>+</sup> T<sub>FH</sub> cells in IL-21<sup>−/−</sup> mice was associated with the properties of DCs in these mice. Thus, DCs from IL-10 signal-defective DCs promote IL-17<sup>+</sup>IL-21<sup>+</sup> T<sub>FH</sub> cell generation. Inasmuch as differentiation of naive CD4<sup>+</sup> T cells was primed with APCs, such as DCs, we next examined whether the presence of IL-17<sup>+</sup> T<sub>FH</sub> cells in IL-10<sup>−/−</sup> mice was associated with the properties of DCs in these mice. Thus, DCs from IL-10<sup>−/−</sup> mice or WT controls, generated by culturing bone marrow cells with GM-CSF and IL-4 for 6 d, were stimulated or not with LPS for 2 d. These DCs were subsequently cultured with IL-10<sup>−/−</sup>CD4<sup>+</sup> T cells. After 7 d of culturing, CD4<sup>+</sup> T cells were restimulated with PMA and IO for 5 h in the presence of BFA to assess CXCR5 expression. A representative dot plot shows IFN-γ, IL-4, IL-17, and IL-21 expression gated on CD4<sup>+</sup>CXCR5<sup>+</sup> and CD4<sup>+</sup>CXCR5<sup>−</sup> cells. Numbers in dot plot quadrants represent percentages. Data are representative of three experiments.

To test the role of IL-21 and IL-17 in enhancing the B cell help function of T<sub>FH</sub> cells in vivo, we injected IL-17<sup>−/−</sup> mice with WT DCs. Ad-

ditionally, significantly less IL-17 and IL-21 production was found in those mice, even when induced by IL-10<sup>−/−</sup>DCs (Fig. 6B).

Next, we examined whether the induction of IL-17<sup>−/−</sup>IL-21<sup>−/−</sup> T<sub>FH</sub> cells was due to factor(s) secreted by activated IL-10<sup>−/−</sup>DCs. Thus, supernatants were collected from IL-10<sup>−/−</sup>DCs and WT DCs stimulated for 48 h with LPS. Supernatants from activated DC cultures were added to naive CD4<sup>+</sup> T cells cultured on plate-bound CD3 and CD28 mAbs. After 7 d, the cells were restimulated with anti-CD3 in the presence of BFA to assess IL-17<sup>−/−</sup>IL-21<sup>−/−</sup>CD4<sup>+</sup>CXCR5<sup>+</sup> cell generation. Although supernatants from activated WT DCs did not induce differentiation of IL-10<sup>−/−</sup> naive T cells to IL-17<sup>−/−</sup>IL-21<sup>−/−</sup> T<sub>FH</sub> cells, supernatants from activated IL-10<sup>−/−</sup>DCs did (Fig. 6C). However, IL-10<sup>−/−</sup> DC supernatants still failed to induce WT naive T cells to differentiate into IL-17<sup>−/−</sup>IL-

21<sup>−/−</sup> T<sub>FH</sub> cells (Fig. 6D). Therefore, IL-10 signal-defective DCs secrete soluble factors that control the differentiation of naive CD4<sup>+</sup> T cells into IL-17<sup>−/−</sup> and IL-21<sup>−/−</sup>producing T<sub>FH</sub> cells.

To identify the cytokine(s) that regulate generation of IL-17<sup>−/−</sup>IL-21<sup>−/−</sup> CD4<sup>+</sup>CXCR5<sup>+</sup> cells, we first compared the RNA levels of some cytokines known to be expressed by DCs between IL-10<sup>−/−</sup> and WT DCs after LPS activation. Quantitative RT-PCR showed a marked increase in IL-6, IL-1β, Tgf-β, IL-12p40, IL-12p35, and IL-10, whereas little change was observed in IL-4, IL-27, and TGF-β in both LPS-activated KO and WT DCs compared with resting cells. Next, we evaluated the levels of IL-6, IL-23p19, IL-10, and IL-12p70 in the supernatants of cultured DCs. Accordingly, IL-10<sup>−/−</sup> DCs produced significantly higher levels of these cytokines than did WT controls after activation (Fig. 7A). The elevated IL-10 in IL-10<sup>−/−</sup> DC culture supernatants explained why a comparable frequency of IL-17<sup>−/−</sup>IL-21<sup>−/−</sup>CD4<sup>+</sup>CXCR5<sup>+</sup> cells was induced from naive WT CD4<sup>+</sup> T cells. The increased IL-10 counteracted the effects of those proinflammatory cytokines.

The negative role of IL-10 was demonstrated above, and then we wanted to clarify the relative contribution of other increased cytokines (IL-6, IL-23, and IL-12) to the generation of IL-17<sup>−/−</sup>IL-

21<sup>−/−</sup> T<sub>FH</sub> cells. To answer this question, two experiments were designed. First, anti-CD3/CD28–primed naive CD4<sup>+</sup> T cells were cultured with supernatant((s) secreted by activated IL-10<sup>−/−</sup>DCs in the presence of neutralizing Abs against IL-12p40, IL-23p19, and IL-
Second, we primed naive cells with anti-CD3 and anti-CD28 in the presence of recombinant protein IL-6, IL-23, and/or IL-12 (Fig. 7C, lower panels). After 7 d, the cells were restimulated with anti-CD3 in the presence of BFA for analysis of intracellular cytokines in CD4+CXCR5+ cells. The results clearly showed that IL-6 and IL-23 played important roles in the differentiation of IL-17+IL-21+ TFH cells, because exogenous cytokines promoted IL-17 and IL-21 secretion, and blockade of their signaling by MP5-20F3 and G23-8 mAbs inhibited IL-17+IL-21+ TFH cell expansion. Furthermore, IL-6 and IL-23 showed
additive effects on IL-17+IL-21+ TFH cell production. Although neutralization of IL-12p40 inhibited the production of IL-17+IL-21+ TFH cells (Fig. 7B), it was difficult to conclude that IL-12 had an effect on such cells. Because IL-12 and IL-23 share the p40 subunit, and no additive effect was found between the two neutralized mAbs, the effects caused by neutralization of IL-12p40 can be through blockade of IL-23 signaling. Additionally, rIL-12 did not significantly induce IL-17+IL-21+ TFH cells. Thus, DCs could promote the B cell help function of CD4+ T cells by increasing IL-6 and IL-23 secretion.

**Discussion**

IL-10 is a pleiotropic cytokine produced by multiple cell types that has an array of actions on T, B, NK, and myeloid cells (2). Among its actions, IL-10 has immunostimulatory effects that augment the number of B cells activated via the BCR and costimulatory signals; however, it also has immunosuppressive properties related to its direct effects on Th cells and its ability to inhibit the production of multiple cytokines and chemokines (2, 28). In polarized Th1 (25) and Th17 cells (29), an IL-10 signal during activation of these cells is thought to be an important regulatory mechanism to avoid excessive immune responses. However, in TFH cells, the role of IL-10 was controversial. Early studies showed that TFH cells in tonsils with high expression of ICOS and CD45RO secreted moderate amounts of IL-10 (30), where it is crucial for costimulation in T and B cell collaboration. However, a recent study found that regulatory T cell-derived IL-10 reduced the production of autoimmune Abs in mouse lupus models by inhibiting IL-17-producing CD4+CXCR5+ICOS+ cells (31). Additionally, Chacón-Salinas et al. (32) demonstrated that mast cell-derived IL-10 affected TFH cell function and suppressed GC formation, a process associated with UV radiation-mediated immune suppression in skin cancer. Those studies outlined the complex role of IL-10 in TFH cell-mediated effects.

In this study, in vitro and in vivo experiments under conditions of both IL-10 sufficiency and deficiency clearly showed that IL-10 inhibited Ab responses by regulating TFH cells. The regulatory roles of IL-10 on TFH cells were mediated by at least two aspects:

**Increased production of IL-17+IL-21+ TFH cells contributes to IBD in Il10rb−/− mice**

Because IL-10 signaling-deficient mice spontaneously develop IBD, and IgG increased in the intestinal tract in this model (12), we wondered whether increased production of TFH cells contributed to IBD. Because the Il10rb−/− mice spontaneously develop IBD after 12 wk of age, we compared the mice at age 6 and 16 wk. Compared with allogeneic healthy control mice (age 6 wk), IBD mice (age 6 wk) showed significant inflammatory cell infiltration into the intestinal mucosa and disorganization of intestinal wall construction (Supplemental Fig. 2A). Many more IL-17–producing cells were found in the mesenteric lymph nodes of Il10rb−/− mice at age 16 wk than in the same mice at age 6 wk (Supplemental Fig. 2B). Intracellular staining (Supplemental Fig. 2C) also showed greater amounts of IL-17– and IL-21–producing CD4+ CXCR5+ cells in mice of older age.

**FIGURE 7.** Il10rb−/− DCs promote generation of TFH cells through IL-6 and IL-23 secretion. (A) The mRNA level of possible cytokines secreted by CD11c+ DCs from Il10rb−/− (KO) and WT mice. DCs were stimulated with LPS (1 μg/ml) for 48 h and collected for RNA extraction. Results are expressed as mean ± SD of triplicates. *p < 0.05 versus active WT. (B) The protein level of IL-12, IL-6, IL-10, and IL-23 in supernatant of culturing DCs, as described above, was evaluated by ELISA. Results are expressed as mean ± SD of triplicates. Similar results were obtained in three additional experiments. *p < 0.05 versus active WT. (C) Representative dot plots show flow cytometric analysis of IL-21 and IL-17 expression in TFH cells, which were gated on CD4+CXCR5+ cells. The numbers in the dot plot quadrants represent percentages. Data are representative of three experiments.
IL-4 secreted from T FH cells were demonstrated to shape the Ab B cell differentiation and/or shaping the Ab repertoire. IFN-γ could regulate the quantity of T FH cells by prohibiting their differentiation. Furthermore, Il10rb2−/− T FH cells, either induced in vitro or purified from immunized mice, showed superior B cell help compared with WT controls, suggesting that IL-10 also regulated the quality of T FH cells. T FH cells express higher levels of many costimulatory molecules, such as ICOS (18–20), CD154 (21), OX40 (22), and CD84 (23), than do other differentiated Th cell subsets. This reflects both the sustained multisignal integration required for T FH cell generation and their unique association with B cells (33). The association of T and B cells was demonstrated to facilitate both eutopic and ectopic GC responses and then promote Ab production (33). Furthermore, IL-10, as an inhibitor of some immune reactions, was described to regulate Th cells expressing costimulatory molecules (34). Thus, we investigated whether the effects of IL-10 on T FH cell B cell help were mediated by regulating those costimulatory molecules. However, the superior B cell help function of Il10rb2−/− T FH cells did not seem to be related to those molecules; no significant difference in either RNA or protein level of those molecules was found between Il10rb2−/− and WT T FH cells.

In addition to costimulatory molecules, cytokines provide important signals for B cell help. T FH cells were found to produce a heterogeneous pattern of cytokines, such as IL-4, IL-10, IL-21, IFN-γ, and IL-17. Among these cytokines, IL-21 was expressed most highly in T FH cells, which suggested a specialized role for IL-21 in T FH cells (33). IL-21 not only had a crucial role in T FH cell differentiation, it also had a well-established role in B cell proliferation and differentiation (33). In accordance with these findings, we showed that greater IL-21 secretion from IL-10 signal-defective T FH cells could lead to increased Ig production. This suggested that T FH cell-mediated immune effects were regulated by inhibitory features of IL-10 on its distinctive cytokine, IL-21, similar to the way in which IL-10 regulates Th1- and Th17-mediated immune responses by inhibiting IFN-γ and IL-17.

In addition to IL-21, other cytokines played important roles in B cell differentiation and/or shaping the Ab repertoire. IFN-γ and IL-4 secreted from T FH cells were demonstrated to shape the Ab repertoire (35), and IL-17 was also reported to be capable of inducing B cell activation (26, 27). In the current study, intracellular staining of splenocytes did not show any definitive IFN-γ or IL-4–producing CD4+CXCR5+ cells, but moderate IL-17–producing CD4+CXCR5+ cells were found in immunized Il10rb2−/− mice. Moreover, these IL-17–producing CD4+CXCR5+ cells also produced IL-21. As anticipated, IL-17 secreted by these cells was not only involved in B cell help, but also added the effects of IL-21 on B cell help, which suggests that the superior ability of Il10rb2−/− T FH cells to provide B cell help was not completely dependent on IL-21 signaling. The observation that IL-17–producing T FH cells were controlled by IL-10 was also consistent with the finding in Icos−/− mice, in which the presence of IL-17–producing CD4+ CXCR5+ cells in the spleen and draining lymph nodes was related to a decrease in IL-10–producing regulatory T cells (20).

The cellular interactions underlying T FH differentiation are not fully elucidated, but they appear to involve sequential priming on DCs. IFN-α/β (36) and IL-6 (37) were demonstrated to stimulate mouse T FH cell differentiation, and so did IL-12 (38) to human T FH, and those signals in DCs were important for the effect. In this study, we first demonstrated that DC signals contributed to the generation of IL-17–producing T FH cells when there was an IL-10 deficiency and then observed an increase in the secretion of IL-6 and IL-23 by DCs associated with this process. Because previous reports described crucial roles for IL-6 and IL-23 in the induction and expansion of Th17 cells (29, 39, 40), our findings might suggest that IL-17–producing T FH cell differentiation overlapped with Th17 cell differentiation. Through comparison with IL-17+CD4+CXCR5+ cells, we precluded that the origin of IL-17+IL-21+ T FH cells was classic Th17 cells, because these cells expressed IL-23R and RORγt but inhibited the expression of CCR6 and IL-22. Under the condition of IL-10 deficiency, Th17 cells may accumulate in secondary lymphoid organs, enter into B cell follicles, and differentiate to T FH cells. This is in line with a report that CXCR5+CD4+ T cells contain specific subsets, including Th1, Th2, and Th17, which differentially supports Ab secretion (38). However, a more detailed study is needed to determine their developmental relationship, because IL-6 and IL-23 did not appear to increase CXCR5 expression.

In many inductive and spontaneous autoimmune models (20, 41), IL-17–producing T FH cells could be found in the spleen or draining lymph nodes. In this study, we found that IL-17–producing T FH cells accumulated in the spleen of immunized Il10rb2−/− mice, as well as in the mesenteric lymph nodes of Il10rb2−/− mice during spontaneous IBD. It is possible that such IL-17–producing T FH cells played roles in the pathogenesis of IBD in IL-10 signaling-deficient mice. Thus, our study may provide an explanation for the relationship between IL-10 and IBD.

In summary, our study indicated that IL-10, which was traditionally regarded as a costimulator of Ab production, could also inhibit Ab responses indirectly through regulating a subset of T FH cells that produces IL-17 and IL-21. The IL-17–producing T FH cells played roles in the pathogenesis of IBD in IL-10 signaling-deficient mice. Thus, our study may provide an explanation for the relationship between IL-10 and IBD.

Disclosures
The authors have no financial conflicts of interest.

References


