IL-10–Producing Regulatory B10 Cells Ameliorate Collagen-Induced Arthritis via Suppressing Th17 Cell Generation

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IL-10–producing CD1d^{hi}CD5⁺ B cells, also known as B10 cells, have been shown to possess a regulatory function in the inhibition of immune responses, but whether and how B10 cells suppress the development of autoimmune arthritis remain largely unclear. In this study, we detected significantly decreased numbers of IL-10–producing B cells, but increased IL-17–producing CD4⁺ T (Th17) cells in both spleen and draining lymph nodes of mice during the acute stage of collagen-induced arthritis (CIA) when compared with adjuvant-treated control mice. On adoptive transfer of *in vitro* expanded B10 cells, collagen-immunized mice showed a marked delay of arthritis onset with reduced severity of both clinical symptoms and joint damage, accompanied by a substantial reduction in the number of Th17 cells. To determine whether B10 cells directly inhibit the generation of Th17 cells in culture, naive CD4⁺ T cells labeled with carboxyfluorescein succinimidyl ester (CFSE) were co-cultured with B10 cells. These B10 cells suppressed Th17 cell differentiation via the reduction of STAT3 phosphorylation and retinoid-related orphan receptor γ t (ROR γ t) expression. Moreover, Th17 cells showed significantly decreased proliferation when co-cultured

with B10 cells. Although adoptive transfer of Th17 cells triggered the development of collagen-induced arthritis in IL-17^{−/−}DBA/1J mice, co-transfer of B10 cells with Th17 cells profoundly delayed the onset of arthritis. Thus, our findings suggest a novel regulatory role of B10 cells in arthritic progression via the suppression of Th17 cell generation. (Am J Pathol 2012, 180:2375–2385; <http://dx.doi.org/10.1016/j.ajpath.2012.03.010>)

Human rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by extensive synovial hyperplasia, cartilage damage, bone erosion, and joint functional disability. Despite the unknown etiology of RA, IL-17–producing CD4⁺ T (Th17) cells have been shown to be associated with the severity of RA.^{1,2} The pathogenic roles of IL-17 in arthritis development have been delineated in mouse models with experimentally induced arthritis.^{3,4} Interestingly, newly reported findings that Th17 cells, but not IL-17⁺ γ δ T cells,⁵ play an essential role in driving arthritic bone destruction in mice and humans further highlight a key role of Th17 cells in the pathogenesis of autoimmune arthritis.

Recently, certain IL-10–producing B cells have been identified as regulatory B (Breg) cells that can negatively regulate immune responses.^{6–8} There is growing evidence on the existence of Breg cells in various autoimmune mouse models including experimental autoimmune encephalomyelitis,^{9–12} murine lupus,^{13–15} as well as experimental arthritis.^{7,16–18} Among which, CD1d^{hi}CD5⁺ B10 cells, one of IL-10–producing Breg cell subsets, have been shown to play a critical role in controlling the

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initial stage of experimental autoimmune encephalomyelitis by altering the interferon- γ and tumor necrosis factor- α secretion and suppressing T-cell proliferation through down-regulating the ability of dendritic cells.¹⁰ Transitional 2-marginal zone precursor B cells (T2-MZP), another IL-10-producing Breg cell subset, have been shown to suppress experimental arthritis in mice significantly.⁷ Moreover, transfer of T2-MZP Breg cells into arthritic IL-10^{-/-} mice increased regulatory T (Treg) cells and decreased Th1 and Th17 cells, which suggest that T2-MZP Breg cells may regulate the immune balance between Treg and Th1/Th17 cells.¹⁹ An elegant study by Yanaba et al,²⁰ using CD20 monoclonal antibodies for B-cell depletion in collagen-induced arthritis (CIA) mice, clearly revealed a crucial role of B cells at the initial stage of arthritis development, but it currently is unclear whether IL-10-producing B cells show any kinetic changes during arthritis progression. Several studies using adoptive transfer of T2-MZP B cells from remission arthritic mice or total B cells from apoptotic cell-treated mice have defined the suppressive function of these B cells during autoimmune pathogenesis in mice via influencing antigen-specific effector T cells.^{7,18} However, it has been unclear whether adoptive transfer of *in vitro* expanded Breg cells could be used as a therapeutic strategy for targeting autoimmune disease. Recently, we have reported a novel function of B-cell activating factor (BAFF) in the induction of IL-10-producing CD1d^{hi}CD5⁺ B10 cells that inhibit T-cell proliferation and possess a potent regulatory function *in vivo*,¹⁷ but it remains to be further validated whether and how adoptive transfer of BAFF-induced B10 cells can ameliorate the disease progression of autoimmune arthritis.

In this study, we first characterized the kinetic changes of Th17 cells and IL-10-producing B cells in peripheral lymphoid organs during CIA development. It was found that at the acute phase of CIA (ie, within 2 weeks after the second immunization), IL-10⁺ B cells were decreased whereas Th17 cells were increased significantly. On adoptive transfer of BAFF-induced B10 cells on the day of the second immunization, B10 cells effectively suppressed the development of arthritis with a remarkable reduction of Th17 cells. In addition, *in vitro* expanded B10 cells directly inhibited Th17 cell differentiation from naive CD4⁺ T cells via reducing STAT3 phosphorylation and ROR γ t expression. Furthermore, although transfer of purified Th17 cells promoted arthritis development in IL-17-deficient DBA mice, co-transfer of Th17 cells with B10 cells profoundly delayed the development of arthritis. Taken together, our findings have shown that BAFF-induced B10 cells can ameliorate arthritis development via suppressing Th17 cell generation, which may facilitate the development of a new cellular therapy for human RA.

Materials and Methods

Mice

DBA/1J and IL-10^{-/-} B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-17^{-/-} B6 mice

were kindly provided by Professor Yoichiro Iwakura (University of Tokyo). IL-10^{-/-} mice and IL-17^{-/-} mice in DBA/1J background were generated as previously described.¹⁷ All mice were maintained in a specific pathogen-free animal facility at the University of Hong Kong. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

CIA Induction and Assessment of Arthritis

CIA was induced in DBA/1J mice or IL-17^{-/-}DBA/1J mice (8 to 12 weeks old) as previously described.²¹ With the same protocol using PBS in place of bovine collagen type II (CII), adjuvant-treated DBA mice served as controls. Beginning on day 1 after the second immunization, mice were observed daily for signs of joint inflammation and scored for clinical signs. The scoring system for the severity of arthritic symptoms was the same as previously described.²¹

Flow Cytometric Analysis

Intracellular staining was performed as previously described.¹⁷ Briefly, cells from spleen or lymph nodes were suspended in complete medium with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma-Aldrich, St. Louis, MO), ionomycin (500 ng/mL; Sigma-Aldrich), and monensin (2 μ mol/L; Biolegend, San Diego, CA) for 5 hours. Cells then were stained with surface markers followed by permeabilization and incubation with intracellular antibodies. Phosphorylated STAT3 staining was performed according to the manufacturer's instructions by fixation of treated cells immediately with BD Phosflow Fix buffer and Perm Buffer III (BD Biosciences, San Jose, CA). ROR γ t staining was performed with the transcription factor staining buffer set (eBioscience, San Diego, CA). Stained cells were analyzed by a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (Treestar, Ashland, OR). All antibodies used for flow cytometry staining were purchased from Biolegend, eBioscience, or BD Biosciences.

Purification of CD4⁺ T Cells from Joint Tissues

After removing skin, muscle, and bone under a dissecting microscope, joint samples of both front and hind paws were minced and digested with collagenase (Calbiochem, Billerica, MA) for 1 hour at 37°C. Cell suspensions were filtered through a cell strainer. After lysis of red blood cells, the remaining cells were incubated with CD4-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes on ice and run through a column. The purity of collected CD4⁺ T cells was routinely >95%.

Enzyme-Linked Immunosorbent Spot Assay

The numbers of IL-17-producing CD4⁺ T cells from joint tissue were determined by enzyme-linked immunosorbent spot assay. The 96-well, multiscreen, nitrocellulose, flat-bottom plates (Millipore, Billerica, MA) were coated

with anti-mouse IL-17 (R&D Systems, Minneapolis, MN) in coating buffer containing anti-CD3 and anti-CD28 (1 μ g/mL) at 4°C overnight. Plates were washed and then blocked with RPMI 1640 with 10% fetal bovine serum (R10) at room temperature for 1 hour. Purified CD4⁺ T cells (1 $\times$ 10⁴) were seeded into wells and stimulated with PMA and ionomycin at 37°C for 4 hours. Plates were washed thoroughly before the addition of biotinylated anti-IL-17 in R10 overnight at 4°C. After washing, plates were incubated for 1 hour at room temperature with alkaline phosphatase-conjugated streptavidin (1:1000) in R10. Then, plates were washed and developed by adding NBT/BCIP solution (Sigma-Aldrich) and the reaction was stopped by washing with tap water.

Expansion of Murine B10 Cells and Adoptive Transfer of B-Cell Subsets

Splenic B220⁺ B cells from DBA and IL-10^{-/-}DBA mice were purified by B220-microbeads (Miltenyi Biotec) and cultured in the presence of recombinant murine BAFF (20 ng/mL; R&D systems) for 72 hours. Cultured B cells then were stained with CD19, CD1d, and CD5; CD1d^{hi}CD5⁺ B cells (B10) and CD1d^{lo}CD5⁻ B cells (B-ve) were sorted with an Epics Altra flow cytometer (Beckman Coulter, Fullerton, CA). The purity of sorted B-cell subsets was routinely >95%. These sorted B cells (1 $\times$ 10⁶) were transferred intravenously into DBA/1J mice or IL-17^{-/-} mice on the day of the second immunization. The sham control group was injected with the same volume of PBS only. In separate experiments, CFSE-labeled sorting-purified B10 cells (1 $\times$ 10⁶) were transferred into DBA/1J mice on the day of the second immunization for monitoring the migration route of transferred B10 cells.

Histopathologic Analysis

Joint samples were fixed in 10% formalin for 3 days, followed by decalcification in 15% formic acid overnight before being embedded in paraffin. Tissue sections were prepared for H&E or Safranin O staining.

Microcomputed Tomography

Microcomputed tomography of mouse paws was performed using a SkyScan 1076 scanner (SkyScan, Kontich, Belgium). Briefly, mice were anesthetized with Hypnorm (0.5 mL/kg; Elmet, Leeds, UK) and Dormicum (0.5 mL/kg). One of the legs was fixed in a polystyrene foam tube and then the mouse was placed horizontally in a 1076 scanner sample chamber for microcomputed tomography imaging (Roche, Basel, Switzerland). The image reconstruction was performed by using a modified Feldkamp1 algorithm using the SkyScan Nrecon2 software.

Enzyme-Linked Immunosorbent Assay

Serum levels of bovine collagen type II-specific total immunoglobulin (Ig)G, IgM, and IgG isotype were mea-

sured by enzyme-linked immunosorbent assay (ELISA), as previously described.²² Levels of IL-17A in culture supernatant and synovial fluid were measured by using an IL-17A ELISA kit (Biolegend), following the manufacturer's protocol.

T-Cell Subset Induction and Purification

For Th17 cell induction, CD4⁺CD25⁻CD62L⁺ naive T cells isolated by the CD4⁺CD62L⁺ T-Cell Isolation Kit II (Miltenyi Biotec) were cultured in a 6-well plate precoated with anti-CD3 (1 μ g/mL) and anti-CD28 (1 μ g/mL) in the presence of recombinant human-transforming growth factor- β (3 ng/mL), recombinant murine IL-6 (20 ng/mL), and rIL-23 (20 ng/mL) for 3 days. For Treg cell induction, CD4⁺CD25⁻CD62L⁺ naive T cells were cultured in the presence of rh-transforming growth factor- β (5 ng/mL) with plate-bound anti-CD3 (1 μ g/mL) and anti-CD28 (1 μ g/mL) for 3 days. Purification of Th17 cells were performed by using the mouse IL-17 secretion and enrichment assay kit (Miltenyi Biotec), according to the manufacturer's instructions.

B10 Suppresses Th17 Cell Generation

Sorting-purified B10 cells from DBA and IL-10^{-/-}DBA mice and B-ve cells from DBA mice were co-cultured with CFSE-labeled purified naive CD4⁺ T cells in Th17 cell polarization medium at a ratio of 1:1 for 72 hours before flow cytometric analysis for Th17 cell generation.

Statistical Analysis

Data are expressed as the mean $\pm$ standard error of the mean. Unless otherwise indicated, statistical analysis was performed using the unpaired Student's *t*-test. A *P* value < 0.05 was considered significantly different.

Results

Kinetic Changes of Th17 Cells and IL-10⁺ B Cells During CIA Development

Although the pathogenic role of Th17 cells in autoimmune arthritis has been well recognized, only recently has the immunoregulatory function of Breg cells been appreciated in autoimmune diseases. To characterize the kinetic changes of Th17 cells and IL-10⁺ B cells during the development of experimental arthritis, both frequencies and absolute numbers of Th17 cells and IL-10⁺ B cells in the spleen and draining lymph nodes of CIA mice were determined by flow cytometric analysis. At the acute phase of CIA (ie, within 2 weeks after the second immunization), both frequencies and total numbers of Th17 cells from the spleen and draining lymph nodes showed an approximate twofold increase when compared with those from adjuvant-treated control DBA mice (Figure 1, A and C). In contrast, the frequencies of IL-10⁺ B cells in both spleen and draining lymph nodes were decreased significantly (Figure 1B), with almost 50% of reduction in

Figure 1. Kinetic changes of Th17 cells and IL-10⁺ B cells during CIA development. **A** and **B**: Single-cell suspensions from spleen and draining lymph nodes (DLN) of adjuvant-treated DBA mice, CIA mice at the acute phase, and CIA mice at the chronic phase were prepared for immunostaining and analyzed by flow cytometry for CD4 and IL-17 expression (**A**) and CD19 and IL-10 expression (**B**). The representative flow results indicate the frequencies of Th17 cells and IL-10⁺ B cells in spleen and DLN. **C** and **D**: The total number of Th17 cells (**C**) and IL-10⁺ B cells (**D**) (mean $\pm$ standard error of the mean) from 5 independent experiments with 5 mice in each group were statistically analyzed by the Student's *t*-test. **P* < 0.05, ***P* < 0.01. **E**: Joint samples from adjuvant-treated DBA mice and CIA mice at the acute and chronic phases were digested for CD4⁺ T-cell isolation. CD4⁺ T cells (1×10^4) were seeded in each well. IL-17-spot-forming cells (SFC) were counted and compared. Data are derived from 5 independent experiments. **P* < 0.05, ****P* < 0.001.

total number of splenic IL-10⁺ B cells (Figure 1D). However, on the progression of arthritis into a chronic phase (ie, within 8 to 10 weeks after the second immunization), both frequencies and numbers of Th17 cells were reduced substantially whereas IL-10⁺ B cells gradually were increased when compared with CIA mice at an acute phase.

To further examine the dynamic changes of Th17 cells in inflamed joints, CD4⁺ T cells from joint tissues of control DBA mice and CIA mice at both acute and chronic phases were purified and the number of IL-17-spot-forming cells

was determined by enzyme-linked immunosorbent spot analysis. Consistent with the pattern of their kinetic changes in either spleen or lymph nodes, Th17 cells also showed a profound increase in inflamed joints of CIA mice at the acute phase, followed by a significant decrease at the chronic phase (Figure 1E). These results indicate that some correlated changes exist between Th17 cells and IL-10⁺ B cells during the progression of arthritis.

Notably, we examined DBA mice with a similar age as chronic CIA mice to exclude the possibility that aging may affect the kinetic change of Th17 cells and/or IL-10⁺ B cells.

Figure 2. BAFF treatment induces the expansion of IL-10-producing B10 cells in culture. **A:** Flow cytometric analysis of IL-10-producing B cells. Purified splenic B cells from wild-type DBA and IL-10^{-/-}DBA mice were cultured with BAFF for 72 hours, treated with PMA, ionomycin, and monensin for 5 hours and then immunostained with anti-CD19 and anti-IL-10. In the flow profiles, frequencies of IL-10-producing B cells (mean ± standard error of the mean) were indicated, representative of 10 separate experiments. **B:** CD1d^{hi}CD5⁺ B cells are the major subset for IL-10 production. The gates of BAFF-induced B10 and B-ve cells were shown for electronic cell sorting. Percentages of IL-10⁺ B cells (mean ± standard error of the mean) were indicated for CD1d^{hi}CD5⁺ and CD1d^{lo}CD5⁻ B-cell subsets, representative of 10 separate experiments.

Similar frequencies and absolute numbers of Th17 cells and IL-10⁺ B cells were found between older DBA mice (24 to 28 weeks old) and control DBA mice (12 to 16 weeks old) used in this study (data not shown). Thus, the dynamic change of Th17 cells and IL-10⁺ B cells during the development of arthritis appeared to be associated closely with disease progression in CIA mice.

Adoptive Transfer of B10 Cells Ameliorates Arthritis Development in CIA Mice

We previously found that BAFF-induced IL-10-producing B cells displayed a typical phenotype of CD1d^{hi}CD5⁺B10 cells with a regulatory function.¹⁷ To further validate the therapeutic application of B10 cells *in vivo*, we optimized the protocol for the efficient expansion of BAFF-induced B10 cells in culture. Splenic B cells were purified and cultured in the presence of BAFF for 72 hours, during which the percentage of IL-10-producing B cells was increased significantly to 8.15% ± 2.18%, although approximately 20% of these BAFF-treated B cells were CD1d^{hi}CD5⁺ B cells as detected by flow cytometric analysis (Figure 2, A and B). Notably, IL-10-producing B cells substantially were expanded with a 10-fold increase in absolute numbers after 72 hours of culture. Moreover, approximately 30% of these CD1d^{hi}CD5⁺ B cells were found to be IL-10⁺ B cells, representing a major IL-10-producing B-cell subset with a potent regulatory function in suppressing T-cell proliferation. In sharp contrast, CD1d^{lo}CD5⁻ B cells showed little IL-10 production with no immunosuppressive function (data not shown).

To evaluate the therapeutic potential of *in vitro* expanded B10 cells in suppressing autoimmune arthritis, we intravenously transferred various B-cell subsets into DBA mice on the day of second immunization. As shown in Figure 3A, CIA mice were treated with sorting-purified BAFF-induced CD1d^{hi}CD5⁺ B10 cells, CD1d^{lo}CD5⁻ B-ve cells, or CD1d^{hi}CD5⁺ B10 cells

from IL-10^{-/-}DBA mice [B10(IL-10^{-/-})], as well as PBS only as sham control. Among four experimental groups with different treatments, B10 cell-treated mice showed an approximate 6-day delay in arthritis onset, with only 75% of mice developing arthritis on day 20. However, the incidence of arthritis development in the other three control groups reached 100% on day 14 after the second immunization. As shown in Figure 3B, the clinical scores for arthritis severity were reduced markedly in B10 cell-treated CIA mice. On day 20 after the second immunization, joint samples of CIA mice from each group were prepared for H&E staining and Safranin O staining. Consistently, histopathologic analysis also showed remarkably reduced synovial hyperplasia, lymphocyte infiltration, cartilage damage, and bone erosion in the group of B10 cell-treated CIA mice when compared with other control groups (Figure 3, C and D). As shown in Figure 3D, the intact cartilage layers were observed only in joint tissue sections from CIA mice treated with B10 cells. To verify whether adoptive transfer of B10 cells can reduce bone destruction during CIA development, CIA mice from each group on day 40 after the second immunization were examined by microcomputed tomography analysis. As shown in Figure 3E, B10 cell-treated mice showed greatly reduced bone erosion with well-preserved joint structure as compared with control groups. Serum levels of total bovine collagen type II-specific IgG production were significantly lower in B10 cell-treated CIA mice than those in control groups. Further analysis on IgG isotypes revealed that serum levels of IgG1 and IgG2a were decreased substantially in the group of B10 cell-treated mice, whereas no significant difference in serum levels of bovine collagen type II-specific IgG2b and IgG3 was detected between the B10 cell-treated group and the other control groups (Figure 3F). Moreover, transferred B10 cells were found to be accumulated in inflamed joints (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Together, these results clearly showed the therapeutic effects of B10 cells on suppressing the development of autoimmune arthritis in mice.

Figure 3. Adoptive transfer of B10 cells ameliorates CIA development in mice. **A:** Sorting-purified B10 cells, B-ve cells, and B10 cells from IL-10^{-/-} DBA mice [B10 (IL-10^{-/-})] were injected intravenously into CIA mice on the day of the second immunization. Control CIA mice were injected with PBS only. The incidences of arthritis development in bovine collagen type II (CII)-immunized mice were measured. Statistical analysis of the data was performed by using the Fisher exact test ($n = 8$ for each group). * $P < 0.05$. **B:** The clinical score for arthritis severity in various groups as described earlier was assessed daily for 20 consecutive days after the second immunization. Values (mean $\pm$ standard error of the mean) are from the control group and treatment groups. The P value was determined by the Mann-Whitney test ($n = 8$ for each group). * $P < 0.05$, ** $P < 0.01$. **C** and **D:** Representative images of H&E staining (**C**) and Safranin O staining (**D**) are as shown (original magnification, $\times 200$). Cartilage is stained with an orange to red color. **E:** Representative microcomputed tomography scan photographs of hind paws of CIA mice on day 40 after the second immunization are shown. **Red arrows** indicate the sites of bone erosion. **F:** Serum levels of CII-specific total IgM, total IgG, IgG1, IgG2a, IgG2b, and IgG3 were measured by ELISA in CIA mice (white box), adoptive transfer of B10 (black box), B-ve (gray box), or B10 (IL-10^{-/-}) (hatched box). Values of mean $\pm$ standard error of the mean are derived from 8 mice per group. * $P < 0.05$, ** $P < 0.01$.

Figure 4. Adoptive transfer of B10 cells suppresses Th17 cell generation in CIA mice. **A:** Single-cell suspensions from spleen and draining lymph nodes (DLN) were collected on day 14 after the second immunization from CIA mice adoptive transfer of B10, B-ve, B10 (IL-10^{-/-}) cells, and PBS only. Cells were stained with anti-CD4 and followed by intracellular staining with anti-IL-17. The representative profiles show the frequencies of Th17 cells in the spleen and DLN of CIA mice with or without treatment. **B:** The total numbers of Th17 cells in spleen and DLN were enumerated. Values are the mean $\pm$ standard error of the mean derived from 4 mice in each group. * $P < 0.05$, ** $P < 0.01$. **C:** Joint tissues from control CIA mice and CIA mice treated with different B-cell populations were digested for CD4⁺ T-cell isolation. CD4⁺ T cells (1×10^4) were seeded in each well, and IL-17-spot-forming cells (SFC) were counted and compared from 4 independent experiments. * $P < 0.05$.

Adoptive Transfer of B10 Cells Suppresses Th17 Cell Generation in CIA Mice

To further determine the immune mechanisms underlying the regulatory function of B10 cells in suppressing experimental arthritis, we sought to examine whether adoptive transfer of B10 cells affected Th17 cell generation *in vivo*. To monitor the frequency and cell numbers of Th17 cells in the spleen and draining lymph nodes, cell suspensions from these lymphoid organs of CIA mice transferred with different B-cell populations were prepared for the detection of Th17 cells by flow cytometric analysis. As shown in Figure 4, A and B, both frequencies and total cell numbers of Th17 cells were reduced significantly in B10 cell-treated CIA mice when compared with CIA mice treated with B-ve cells, B10 cells from IL-10^{-/-} DBA mice or PBS only. To further evaluate the infiltration of Th17 cells in joint tissues, CD4⁺ T cells from joint samples were isolated for the detection of IL-17-secreting T cells by enzyme-linked immunosorbent spot assay. We also detected profoundly reduced Th17 cells in joints of B10 cell-treated CIA mice as compared with other control groups (Figure 4C). Thus, our findings suggest that adoptive transfer of B10 cells can affect the population size of Th17 cells *in vivo*.

B10 Cells Suppress Th17 Cell Differentiation from Naive CD4⁺ T Cells in Culture

To investigate whether B10 cells exert any direct effects on suppressing Th17 cell proliferation and/or differentiation *in vitro*, naive CD4⁺ T cells were cultured in the Th17 cell polarization medium. When co-cultured with B10 cells, an approximate 60% reduction of Th17 cells in both frequencies and total cell numbers was observed, as compared with naive T-cell induction alone or co-culturing with B-ve and B10 cells from IL-10^{-/-} mice (Figure 5, A and B). Moreover, enzyme-linked immunosorbent analysis confirmed the significantly reduced levels of IL-17 in the supernatant of T cells co-cultured with B10 cells (Figure 5C). Interestingly, when B10 cells were co-cultured with naive CD4⁺ T cells in Treg cell polarization medium, an approximate threefold increase of Treg cells in both frequencies and absolute numbers was observed when compared with naive T-cell induction alone or co-culturing with B-ve cells (see Supplemental Figure S2, A and B at <http://ajp.amjpathol.org>).

To further examine whether B10 cells can suppress Th17 cell proliferation, we used CFSE-labeled naive CD4⁺ T cells for

Figure 5. B10 cells suppress Th17 cell differentiation from naive CD4⁺ T cells in culture. **A:** Purified naive CD4⁺ T cells were cultured alone or with B10 (CD1d^{hi}CD5⁺ B), B-ve (CD1d^{lo}CD5⁺), B10 (IL-10^{-/-}) cells CD1d^{hi}CD5⁺ B cells from IL-10^{-/-} DBA mice in Th17 polarization medium for 72 hours, then cells were restimulated with PIM for 5 hours, stained intracellularly with anti-IL-17A, and analyzed by flow cytometry. Numbers in flow charts indicate the frequencies of Th17 cells. **B:** The total numbers of generated Th17 cells in each treatment were enumerated. Values (mean $\pm$ standard error of the mean) are derived from five independent experiments. * P < 0.05, ** P < 0.01. **C:** ELISA determinants of IL-17A secretion in the supernatant from earlier-described co-culture conditions were shown as mean $\pm$ standard error of the mean (n = 5). * P < 0.05. **D:** CFSE-labeled purified naive splenic CD4⁺ T cells were cultured alone or with B10, B-ve, B10 (IL-10^{-/-}) cells in Th17 polarization medium for 72 hours, then cells were restimulated with PIM for 5 hours, stained with anti-IL-17A, and analyzed by flow cytometry. Gating on Th17 cells, each peak of Th17 cells indicates the division of Th17 cells in different culture conditions. Numbers in the graph represent the percentage of cells in each division. Results are representative of three independent experiments. **E:** CFSE-labeled CD4⁺ T cells were treated as described earlier. Each numeric figure in the graph represents the percentage of divisions in the gated area. Results are representative of three independent experiments. **F:** Flow cytometric analysis of phosphorylated STAT3 (pSTAT3) (Y705) expression level is shown after naive T cells at 0 minutes (red line), naive T cells without treatment (blue line) or co-culturing with B10 cells (orange line), or co-culturing with B10 (IL-10^{-/-}) cells (green line) in the Th17 cell polarization medium for 15 minutes. **G:** Flow cytometric analysis of ROR γ t expression level is shown after naive T cells were treated as described earlier.

co-culture. As revealed by flow cytometric analysis on Th17 cell divisions, co-culturing with B10 cells resulted in a marked reduction in Th17 cell proliferation (Figure 5D). Moreover, the frequency of Th17 cells generated in each division profoundly was decreased in B10 cell-treated culture, indicating that B10 cells suppress the generation of Th17 cells from naive T cells. Importantly, either B-ve cells or B10 cells from IL-10-deficient mice showed no or little effect on suppressing Th17 cell proliferation, which suggests that IL-10 is an important mediator for B10 cells exerting their suppressive function on Th17 cells (Figure 5E).

B10 Cells Suppress STAT3 Phosphorylation and ROR γ t Expression in Naive CD4⁺ T Cells During Th17 Induction

Because the differentiation of Th17 cells is initiated by activation of STAT3, which further induces the expression of ROR γ t,²³ we sought to determine whether B10 cells can reduce the STAT3 phosphorylation in naive T cells at the initial stage of Th17 cell differentiation. As shown in

Figure 5F, the amount of phosphorylated STAT3 (Y705) was decreased in B10 cell-treated naive T cells as compared with naive T cell alone or with the addition of B10 (IL-10^{-/-}) cells in the Th17 cell polarization medium for a time interval of 15 minutes. Accordingly, the expression of ROR γ t in T cells also decreased after treatment with B10 cells for 15 minutes (Figure 5G). Thus, these data suggest that B10 cells also can affect Th17 cell differentiation via inhibiting the STAT3-ROR γ t pathway.

B10 Cells Suppress Th17 Cell-Mediated CIA Development in IL-17-Deficient Mice

Although it has been reported that IL-17^{-/-} DBA mice are resistant to CIA induction,³ we sought to examine whether adoptive transfer of Th17 cells from wild-type DBA mice can trigger the development of arthritis in collagen-immunized IL-17^{-/-} mice. By using a cytokine-capture assay, we first purified *in vitro* differentiated viable Th17 cells (Figure 6, A and B). Then, we transferred purified Th17 cells alone or with B10 cells from DBA mice

Figure 6. B10 cells suppress Th17 cell-mediated collagen-induced arthritis in IL-17^{-/-} mice. **A:** Naive CD4⁺ T cells were cultured in the Th17 cell polarization medium for 4 days, and then cells were stained with anti-IL-17 and anti-interferon (IFN)- γ , followed by flow cytometric analysis. Gated on CD4⁺ T cells, the percentage of induced IL-17⁺ CD4⁺ T cells and interferon- γ ⁺ CD4⁺ T cells are as shown. **B:** *In vitro* induced Th17 cells were purified further by IL-17 cytokine capture assay and examined by flow cytometry. The purity routinely is higher than 90%. **C:** IL-17^{-/-} DBA mice were used for CIA induction. Purified Th17 cells alone or with sorting-purified B10 cells, or B10 (IL-10^{-/-}) cells were co-transferred into IL-17^{-/-} mice on the day of the second immunization. Collagen II-immunized IL-17^{-/-} mice were injected with PBS as sham control. The incidence of arthritis development in collagen II-immunized IL-17^{-/-} mice were as shown ($n = 6$ for each group). **D:** ELISA determinants of IL-17A secretion in synovial fluid from the earlier described mice were shown as mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$, ** $P < 0.01$.

or IL-10^{-/-} DBA mice into IL-17^{-/-} mice on the day of the second immunization. As shown in Figure 6C, adoptive transfer of Th17 cells triggered the onset of arthritis in IL-17^{-/-} mice starting on day 7 after the second immunization, whereas no arthritic symptoms were observed in IL-17^{-/-} mice without transfer of Th17 cells during a period of 30 days for observation. Compared with B10

(IL-10^{-/-}) cell-treated mice, co-transfer of B10 cells with Th17 cells delayed the development of arthritis by 7 days and only 50% of B10 cell-treated mice developed clinical symptoms of arthritis. Moreover, levels of IL-17A in synovial fluid also were decreased in B10 cell-treated mice (Figure 6D). Thus, these findings provide strong evidence that B10 cells ameliorate the development of arthritis via the inhibition of Th17 cells *in vivo*.

Discussion

In this study, we detected significantly reduced IL-10⁺ B cells at the acute phase of CIA induction. Adoptive transfer of *in vitro* expanded B10 cells has effectively suppressed the development of arthritis with a marked reduction of Th17 cells in mice. By using IL-17-deficient DBA mice, we clearly show that IL-17-producing CD4⁺ T cells are critically involved in the development of arthritis. Moreover, we have shown that co-transfer of B10 cells can remarkably delay the onset of Th17 cell-driven CIA development in IL-17-deficient mice. Together, these results have shown that IL-10-producing B10 cells ameliorate the development of arthritis by suppressing Th17 cell generation.

It has been well recognized that IL-23/Th17/IL-17 axis is critically involved in driving chronic inflammatory autoimmune diseases.^{24,25} The role of IL-17 in the pathogenesis of autoimmune arthritis in both human and mouse has been well demonstrated.^{1,26} IL-17 can increase the migration, chemokine expression, and invasiveness of synoviocytes, enhance metalloprotease secretion to cause cartilage damage,¹ and act as an angiogenic mediator in RA.²⁷ Previously, we have revealed a protective role of NK cells in the development of experimental arthritis, possibly via the suppression of Th17 cell generation.²² In the present study, we characterized the dynamic changes of Th17 cells during the progression of arthritis (Figure 1). Interestingly, the changes in both frequency and total cell numbers of Th17 cells in lymphoid organs and inflamed joints were correlated conversely with the kinetics of IL-10-producing B cells, especially at the acute phase of CIA development, which indicate a possible functional interaction between Th17 cells and B10 cells *in vivo*. Importantly, we also provide the evidence that adoptive transfer of purified *in vitro* induced Th17 cells can trigger the development of arthritis in IL-17-deficient DBA mice, which indicate an essential role of Th17 cells in CIA induction (Figure 6, C and D). To test whether B10 cells can directly affect Th17 cell generation, we have co-cultured naive CD4⁺ T cells with B10 cells in Th17 cell polarization medium. Notably, B10 cells directly can inhibit Th17 cell differentiation and proliferation in culture. Recent evidence indicates that the development and function of Th17 cells are critically dependent on STAT3. Selective depletion of STAT3 in T cells abrogates Th17 cell differentiation partially because the expression of ROR γ t and ROR α also is abrogated.²⁸ Here, we show that B10 cells can reduce STAT3 phosphorylation and further decrease ROR γ t expression at the initial stage of inducing Th17 cells from naive T cells

(Figure 5, F and G). Because it has become clear that activation of the STAT3-ROR γ t pathway is indispensable for Th17 cell differentiation, our current findings thus highlight a novel function of B10 cells in regulating Th17 differentiation. Future studies are warranted to elucidate the underlying mechanisms by which B10 cells regulate the activation of the STAT3-ROR γ t pathway.

T-cell plasticity has been well recognized. Many factors are known to influence the T-cell plasticity including cellular conditions, transcriptional circuitry, and chromatin modifications, and so forth.²⁹ Thus, our current data cannot exclude the possibility that B10 also may induce Th17 cell transdifferentiation into other T-cell subsets or preferentially promote Treg cell differentiation by up-regulating Foxp3 expression. Of note, we also have found that B10 cells increase Treg cell differentiation in culture (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Nevertheless, it is plausible to reason that B10 cells may play an important role in T-cell plasticity.

Recent studies have shown that Th17 cells can induce B-cell proliferation and isotype class switching both *in vitro* and *in vivo*.³⁰ Our findings that serum levels of IgG2a are decreased significantly in B10 cell-treated mice are consistent with the observed reduction in Th17 cells in these mice (Figures 3 and 4). However, it remains unclear whether B10 cells also directly can inhibit autoreactive B-cell proliferation and affect isotype class switch. Further studies may provide further insight in understanding the role of B10 cells in modulating autoimmune responses during arthritis development in both mouse and human.

The pathogenic roles of B cells in arthritis are characterized extensively,³¹ which have been illustrated further by the efficacy of B-cell depletion using rituximab (anti-CD20) in treating RA patients.³² Although the underlying mechanisms still largely are unclear, van de Veerdonk et al² showed that treatment with rituximab reduces the local Th17 response in RA patients, a potent effect associated with a better clinical outcome. However, this effect is abrogated in the absence of B cells, which suggests that rituximab exerts its effect by directly depleting B cells and reduces Th17 cells indirectly. Interestingly, this depletion therapy also has enhanced the generation of both Treg and Breg cell populations that may contribute to the long-term remission and maintenance of tolerance.^{33,34} Although B cell-targeted therapies in RA and other autoimmune diseases are very promising,^{35,36} long-term B-cell depletion possibly may lead to the development of immunopathology. Given that CD20 is expressed on B lymphocytes from the pre-B to mature B cells, but not on plasma cells,²¹ current approaches for B-cell targeting cannot distinguish pathogenic B cells from Breg cells. Moreover, both numeric change and functional impairment of human Breg cells have been implicated in the disease pathogenesis of RA.^{6,8} Thus, further studies are needed to address whether the depletion of Breg cells may affect the long-term efficacy of B cell-targeted therapy. Nevertheless, our current study has shown that direct transfer of B10 cells into collagen-immunized mice effectively can delay the development of arthritis and markedly reduce joint damage by suppressing Th17

cell generation (Figures 3, 4, and 6). Moreover, transferred B10 cells can be detected *in vivo* for >3 weeks (unpublished data), which further suggest that B10 cell therapy possibly may exert a longer suppressive effect on arthritis development than the direct administration of IL-10, because IL-10 is known to have a short half-life *in vivo*.³⁷

In summary, we have validated the efficacy of *in vitro* expanded B10 cells in ameliorating arthritis development in CIA mice. We further show that the therapeutic effects of BAFF-induced B10 cells on suppressing experimental arthritis possibly are mediated by inhibiting Th17 cell differentiation and proliferation *in vivo*. Therefore, further elucidation of functional interactions between T- and B-cell subsets may contribute to the development of new therapeutic strategies in treating human RA.

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