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Mice Lacking Endogenous IL-10–Producing Regulatory B Cells Develop Exacerbated Disease and Present with an Increased Frequency of Th1/Th17 but a Decrease in Regulatory T Cells

Natalie A. Carter,* Rita Vasconcellos,*† Elizabeth C. Rosser,* Calogero Tulone,* Alba Muñoz-Suano,§ Masahito Kamanaka,*,‖ Michael R. Ehrenstein,* Richard A. Flavell,¶ and Claudia Mauri*

IL-10–producing B cells, also known as regulatory B cells (Bregs), play a key role in controlling autoimmunity. In this study, we report that chimeric mice specifically lacking IL-10–producing B cells (IL-10−/−B cells) developed an exacerbated arthritis compared with chimeric wild-type (WT) B cell mice. A significant decrease in the absolute numbers of Foxp3 regulatory T cells (Tregs), in their expression level of Foxp3, and a marked increase in inflammatory Th1 and Th17 cells were detected in IL-10−/−B cell mice compared with WT B cell mice. Reconstitution of arthritic B cell deficient (μMT) mice with different B cell subsets revealed that the ability to modulate Treg frequencies in vivo is exclusively restricted to transitional 2 marginal zone precursor Bregs. Moreover, transfer of WT transitional 2 marginal zone precursor Bregs to arthritic IL-10−/−mice increased Foxp3+ Tregs and reduced Th1 and Th17 cell frequencies to levels measured in arthritic WT mice and inhibited inflammation. In vitro, IL-10−/−B cells established longer contact times with arthritogenic CD4+CD25− T cells compared with IL-10−/−B cells in response to Ag stimulation, and using the same culture conditions, we observed upregulation of Foxp3 on CD4+ T cells. Thus, IL-10–producing B cells restrain inflammation by promoting differentiation of immunoregulatory over proinflammatory T cells. The Journal of Immunology, 2011, 186: 5569–5579.

Interleukin-10–producing B cells have been shown to be essential for the restoration of tolerance in several autoimmune disorders and in the promotion of leukemia (1–4). These cells can interfere with the development of several autoimmune diseases, including experimental autoimmune encephalomyelitis (5) and collagen-induced arthritis (6), by suppressing the development of autoreactive Th1 responses (7). Similar mechanisms are exploited by pathogens or in cancer to subvert host protective responses (8). Several regulatory B cell (Breg) subsets have now been identified, and most share the release of IL-10 as a common mechanism of action. In experimental arthritis, we have shown that the transfer of CD19+CD21hiCD23hiCD1dhi transitional 2 marginal zone precursor B cells (T2-MZP), which were identified as the major producers of IL-10, prevents or ameliorates established disease (6). Similarly, transfer of CD5+CD1dhi B cells (B10) controls the development of the contact hypersensitivity response (3). In each instance, Bregs isolated from IL-10–deficient mice (IL-10−/−) failed to suppress the development of autoimmune diseases (5, 7, 9, 10).

IL-10 mediates suppression of inflammation by a number of mechanisms, including downregulation of the production of proinflammatory cytokines, such as IFN-γ and IL-17, and the expression of MHC class II (11). In addition, it has been recently shown that the IL-10 produced by myeloid cells is crucial for the maintenance of Foxp3 expression and regulatory T cell (Treg) function and ultimately controls the severity of colitis (12). μMT mice display reduced numbers of Tregs (13) and develop exacerbated Ag-induced arthritis (AIA) compared with wild-type (WT) mice (A. Bosma, N.A. Carter, E.C. Rosser, and C. Mauri, unpublished observations). Altogether, this is suggestive that IL-10 produced by B cells may be important for maintaining the balance between Tregs and Th1/Th17 cells.

Adoptive transfer of Bregs has been preferentially used as a method to dissect the function of these cells in vivo. Although this approach has provided immunological insights into the mechanisms of action of Bregs, the extent to which endogenous Bregs play a role in suppressing autoreactive responses in vivo remains completely unknown. Given the possible existence of several Breg subsets, and to avoid the exclusion of any of these subsets, we have addressed this question using mixed bone marrow chimeric mice with an IL-10 deficiency restricted to B cells. Chimeric mice provide an invaluable tool for dissecting the effect of the lack of specific genes in B cell populations during in vivo responses (14, 15).

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Address correspondence and reprint requests to Dr. Claudia Mauri, University College London, 46 Cleveland Street, London W1T 4JF, United Kingdom. E-mail address: c.mauri@ucl.ac.uk

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Abbreviations used in this article: AIA, Ag-induced arthritis; Breg, regulatory B cell; FO, follicular; IL-10−/−, IL-10–deficient; LN, lymph node; mBSA, methylated BSA; MZ, marginal zone; RA, rheumatoid arthritis; T2-MZP, transitional 2 marginal zone precursor; Tr1, regulatory 1; Treg, regulatory T cell; WT, wild-type; WTB, wild-type B.

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CD4+ T cells displayed decreased levels of Foxp3 expression compared with WT B (WTB) cell control mice. In addition, these CD4+ T cells displayed decreased levels of Foxp3 expression compared with the levels expressed in WTB cell control mice. The reduced number of Tregs in IL-10−/− B cell mice was mirrored by an accumulation of Th1 and Th17 cells in the lymph nodes (LN) draining the site of inflammation. We have also shown that only T2-MZP Bregs, and not marginal zone (MZ) or follicular (FO) B cell subsets, upregulated the frequency of Tregs after adoptive transfer into B cell-deficient mice. In addition, we showed that transfer of T2-MZP Bregs to IL-10−/− mice increased the Treg pool and reduced the severity of arthritis. In vitro analysis of the cross-talk between B and CD4+ T cells revealed that, upon Ag stimulation, IL-10+/+ B cells established contacts of longer duration with arthritogenic WT T cells than B cells isolated from IL-10−/− mice. Of note, in the same culture conditions, upregulation of Foxp3 on CD4+ T cells, concomitantly to a downregulation of proinflammatory cytokines. In this study, we demonstrate a novel function of Bregs in the primary induction of Tregs.

Materials and Methods

Abs
The following Abs were used: anti-CD3, anti-CD4, anti-CD19, anti-CD21, anti-CD23, anti-CD24, anti-Foxp3, anti-IL-10, anti-IFN-γ and anti-IL-17, purchased either from BD Biosciences or eBioscience.

Mice, induction, and assessment of arthritis
C57/B6, mtMT/B6, and IL-10−/− B6 mice were bred and maintained under specific pathogen-free conditions at the animal facility at University College London, London, U.K. Female C57/B6 mice (8–12 wk old) were immunized with 1 μg/ml methylated BSA (mBSA; Sigma-Aldrich) in CFA. This was followed, 7 d later, by intra-articular injection of 10 μl 20 μg/ml mBSA (disease onset). Knee swelling was measured using calipers and calculated as the percentage increase in knee size as compared with day 0 (preinjection). The walking score was graded as follows: 0, no walking; 1, walking on three legs; 2, limping with the leg in inflammation; 1, limping with the leg with inflamed knee only after pressure on the knee; and 0, normal walking (16). Affected joints were removed postmortem, fixed in 10% (w/v) buffered formalin, and decalcified in 5% EDTA. The joints were subsequently embedded in paraffin, sectioned, and stained with H&E. Normal = no damage; mild = minimal synovitis, cartilage loss, and bone erosion limited to discrete foci; moderate = synovitis and erosion present, but normal joint architecture intact; and severe = extensive erosion and joint architecture disrupted.

Generation of chimeric mice
Chimeric mice were generated as previously described (14). Recipient mtMT mice received 800 cGy gamma-irradiation via a caesium source. Five hours following irradiation, recipients received 2 × 106 donor bone marrow cells. Bone marrow preparations were depleted of T cells by negative selection with an MACS magnetic column (Miltenyi Biotec). To generate mice in which the absence of IL-10 was exclusively restricted to B cells, mtMT mice were reconstituted with mixture of bone marrow consisting of 80% from mtMT (no B cell differentiation) with 20% from IL-10−/− mice. Control mice received 80% from mtMT and 20% bone marrow from WT mice (to give a normal B cell compartment). Two additional control groups were included: 100% of bone marrow from mtMT into WT recipients (control for the absence of B cells) or 80% WT and 20% IL-10−/− bone marrow into mtMT recipients (this will assess the effect of 20% reduction in IL-10 production by non-B cell lymphocytes in the response observed). Chimeras were left to fully reconstitute over at least 8 wk before use in AIA experiments. The absence of B cells in the group reconstituted with 100% mtMT bone marrow confirmed the total ablation of the host bone marrow by irradiation. In contrast, the three other groups showed numbers of CD19+ B cells and CD4+ T cells equivalent to numbers found in non-chimeric WT B6 mice.

Dissection and digestion of the synovial membrane
Synovial cells were isolated from the synovial membrane on day 5 post-disease onset as described (17). Briefly, knee joints were removed, and the synovial membrane was excised under a dissecting microscope and digested with collagenase A (1 mg/ml, Sigma-Aldrich) and DNase type IV (150 μg/ml Roche) for 45 min at 37°C. Then cells were washed and passed through a cell strainer before being analyzed by flow cytometry.

Peritoneal macrophages enrichment
A total of 1.5 ml 4% thiglycolate fluid medium (Difco) was injected into the mice i.p. After 4 d, peritoneal lavage fluid was collected and cultured for 4 h. Cells adhering to the culture plate were used as the macrophage population (an aliquot was stained with CD11b and analyzed by flow cytometry). Macrophages were subsequently stimulated with purified Escherichia coli 0111:B4 LPS (1 μg/ml; Sigma-Aldrich) for 48 h. IL-10 in the supernatant was measured by ELISA.

Flow cytometric analysis of intracellular cytokine synthesis and ELISA

Intracellular cytokine analysis was performed as previously described (2). Splenocyte or LN cultures were suspended at 5 × 106 cells in complete medium where PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) and GolgiPlug (BD Biosciences) for 5 h, unless otherwise stated. Cells were then stained with surface markers followed by permeabilization and incubation with intracellular Abs. The cells were acquired with an FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Supernatants from cell cultures were harvested and analyzed for cytokines using standard sandwich ELISA kit (R&D Systems) and performed according to the manufacturer’s instructions.

Treg suppression assay
Spleens or LN were removed postmortem, and Treg and CD4+CD25+ T cells were isolated using the CD4+CD25+ Regulatory T Cell isolation kit for mouse (Miltenyi Biotec). Cells were cultured in round-bottom 96-well plates (Nunc) for 60 h with either complete medium alone or anti-CD3 (1 mg/ml; BD Biosciences). Cultures were pulsed overnight with 1 μCi [3H] thymidine, harvested, and counted in a scintillation counter (LKB Instruments). Supernatants were also harvested for cytokine detection by ELISA prior to addition of thymidine.

Adoptive transfer of B cell subsets
B cells isolated from the spleens of WT or IL-10−/− mice in the remission phase of AIA were stained with CD19, CD23, CD21, and IL-24. B cell subsets were FACs sorted using gates drawn according to previous reports (6). Seven days prior to transfer/intra-articular injection, all recipients were immunized with mBSA in CFA. T2-MZP Breg, FO, and MZ B cells (2 × 106) were transferred i.v. to syngeneic IL-10−/− C57/BL6 mice, mtMT C57/BL6 mice, or C57/B6 WT mice on the day of intra-articular injection. The control group (no transfer) received a PBS injection.

Flow cytometric analysis of B–T cell conjugate formation
B and CD4+CD25+ T cells were isolated from spleens of WT B6 and IL-10−/− mice immunized with mBSA/CFA by negative selection. CD4+ T cells were stained using Cell Tracker Green (0.5 μM; Invitrogen) and B cells with Cell Tracker Blue (0.5 μM; Invitrogen). Stained B and CD4+ T cells were combined at a ratio of 1:1 (total number of cells/well = 1 × 106 cells/ml) and cultured with or without 10 μg/ml mBSA (Sigma-Aldrich) and neutralizing anti-IL-10 (clone JES5-2A5 rat IgG1). Cells were incubated at 37°C for up to 40 min. Cells were then fixed with chilled paraformaldehyde. Conjugates were defined as Green CD4+ T cells containing with Blue-CD19+ B cells. The cells were acquired with an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Real-time imaging
B and CD4+ T cells were isolated from mBSA-immunized WT B6 and IL-10−/− mice using negative selection separation kits from Miltenyi Biotec. B cells were incubated at 37°C for 1 h with 10 μg/ml mBSA and CD4+
T cells with media alone. T cells were then stained using CFSE (5 μM; Sigma-Aldrich) and B and T cells resuspended in RPMI 1640 clear (Life Technologies) for imaging. Cells were then plated out at 10,000 T cells and 10,000 B cells per well with 10 μg/ml mBSA and imaged in real time over 20 min at 37°C. Imaging was done using Nikon Eclipse TE 300 microscope, Plain Fluor ELWD 20×0.45 (Nikon) lens, and LaserSharp2000 acquisition software. The camera used was Laser System Radiance2100 (Bio-Rad). Analysis of cell interactions was done using Velocity (Perkin-Elmer) software, and for each condition, all cells in the frame of view were numbered, and then 40 cells, picked using random number tables, were blindly analyzed for cell–cell contacts.

Statistical analysis
For the statistical analysis of the data, the Mann–Whitney *U* test and Fisher exact test were applied to analyze clinical results. Unpaired *t*-tests were applied in all other experiments. A *p* value < 0.05 was considered significantly different.

Results
IL-10–competent B cells limit the severity and damage caused by the inflammatory response
First, we assessed the effect that the lack of endogenous IL-10–producing B cells has on the severity of inflammatory arthritis. Bone marrow chimera mice, in which B cells lack the capacity to release IL-10, were generated as previously described (14) (summarized in Supplemental Fig. 1A). Briefly, μMT mice were reconstituted with either a mixture of bone marrow consisting of 80% from μMT with 20% from IL-10−/− mice (IL-10−/− B cell) or with 80% from μMT and 20% bone marrow from WT (WTB cell) mice. One group received 100% of bone marrow from μMT (control for the absence of B cells). The results in Supplemental Fig. 1B confirm that B cells from IL-10−/− B cell mice do not produce IL-10 in response to CpG stimulation. In addition, no significant differences were identified between the absolute numbers and the frequencies of repopulated CD4+ B cells, CD19+ B cells and different B cell subsets among the WT B cells and IL-10−/− B cell chimeras compared with nonchimera WT mice (Supplemental Fig. 1C). T cell proliferative responses to anti-CD3 stimulation were equivalent in IL-10−/− B cell and WT B cell chimeric mice (Supplemental Fig. 1D). The results in Supplemental Fig. 1E show that peritoneal macrophages from IL-10−/− B cell mice secrete similar amount of IL-10 to macrophages isolated from WT cell chimeric mice and WT nonchimeric mice after stimulation with LPS in vitro. These results confirm that the IL-10 production by the non-B cell compartment is quantitatively equivalent in all groups.

In the current study, we took advantage of the AIA model, as this model recapitulates both the DTH response and the development of an autoimmune-like disease. AIA was induced in chimeric mice by immunization with mBSA/CFA, followed 1 wk later by intraarticular injection with mBSA. Unlike the experimental autoimmune encephalomyelitis model, in which the absence of IL-10–producing B cells exclusively affected the recovery phase of disease (14), in AIA, the severity of arthritis was significantly exacerbated in mice lacking IL-10–producing B cells (Fig. 1A; for clarity of representation, only days 3 and 7 are shown hereafter). Histological analysis revealed severe damage in 75% of the joints of IL-10−/− B cell mice as a result of the extensive expansion of the synovial pannus, fibrin exudate, and massive infiltration of mononuclear cells (Fig. 1B, 1E; an enlarged representative area of infiltration is shown in Supplemental Fig. 2A, 2B). In contrast, WTB cell mice developed mild to moderate arthritis, with only 20% of the joints showing a severe loss of bone architecture (Fig. 1B, 1E). IL-10−/− B cell chimeric mice have populations of hematopoietic cells that are IL-10–deficient besides B lymphocytes. Although these account for only 20% of the total population for each cell type, to further confirm that the effect that we observed is entirely due to the lack of IL-10–producing B cells, we have included an additional group of control mice. We reconstituted irradiated μMT recipients with 80% WT and 20% IL-10−/− bone marrow. This group of mice developed arthritis with the same severity as WTB cell chimeric mice and WT nonchimeric mice, which was significantly less severe than IL-10−/− B cell chimeric mice (data not shown).

It has been previously shown that IL-10−/− mice develop exacerbated autoimmune diseases compared with WT mice (18, 19). Similarly, IL-10−/− mice develop a more severe arthritis as compared with WT mice (Fig. 1C–E; an enlarged representative area of inflammation is shown in Supplemental Fig. 2C, 2D). It is interesting to note that IL-10−/− B cell and IL-10−/− mice develop arthritis with equivalent severity, suggesting that B cells producing IL-10, in addition to other IL-10–producing cells (i.e., T cells) (11, 20), have an integral role in restraining the severity of the inflammatory responses (Fig. 1C–E; an enlarged region of interest is shown in Supplemental Fig. 2).

IL-10 produced by B cells maintains Treg number and Foxp3 expression during inflammation
Next we investigated the effect that IL-10–producing B cells have on the maintenance of Treg number and on the expression of Foxp3 in CD4+ T cells during the acute phase of arthritis. A decrease in the percentage and absolute number of CD4+Foxp3+ Tregs was observed in the draining LNs of IL-10−/− B cell mice compared with the numbers measured in the LNs isolated from control WTB cell mice (Fig. 2A). In addition, the levels of Foxp3 expression were significantly reduced in Tregs from IL-10−/− B cell mice compared with Tregs from WTB cell mice (Fig. 2B). Identical results were obtained if we measured the levels of Foxp3 on CD4+CD25+ T cells (data not shown). Despite the reduced expression of Foxp3, CD4+CD25+ T cells isolated from IL-10−/− B cell mice efficiently suppressed the proliferation of CD4+CD25+ T cells isolated from WT mice (Fig. 2C). The numerical defect in Foxp3+ Tregs and reduced levels of Foxp3 expression were confined to the Treg population isolated from the draining inguinal LN and were not found in Tregs isolated from non-draining LN (data not shown), which is in agreement with the current concept of surveillance by Tregs in the draining LN (21, 22).

The reduced number of Tregs observed in the LNs of IL-10−/− B cell mice could reflect their increased capacity to migrate and accumulate in the target tissues. In general, IL-10−/− B cell mice show a significantly increased accumulation of cells in the synovium compared with control mice (Fig. 2D). The frequency of Foxp3+CD4+ T cells among synovial cells was significantly reduced in the IL-10−/− B mice compared with control WTB cell mice (Fig. 2E). The ratio between CD4+Foxp3+ Tregs and synovial cells is reduced by more than half in IL-10−/− B cell mice compared with WTB cell mice (Fig. 2F). However, equivalent absolute numbers of Tregs were measured in the two groups of chimeric mice (Fig. 2G). We could argue that the differences in percentage observed in the joints is still of physiological relevance as a dilution in the numbers of Tregs may reflect a reduced chance
**FIGURE 1.** Endogenous B cell-derived IL-10 constrains the severity of arthritis. AIA was induced in chimeric mice by immunization with mBSA/CFA, followed 1 wk later by intra-articular injection with mBSA. A, Bar charts represent walking score and knee swelling, calculated as a percentage increase relative to joint size prior to disease onset, of IL-10−/− B cell or WTB cell chimeric mice with AIA. Days 3 and 7 were chosen as representative days. One independent experiment, representative of three, is shown (n = 5). Data were compared by statistical analysis using the Fisher test. B, Representative histology of arthritic joints isolated from IL-10−/− B or WTB cell chimeric mice 7 d postdisease onset. Joints were processed as described in the Materials and Methods section (original magnification ×10). The arrows indicate the areas where there has been an infiltration of cells into the joint. C, Bar charts represent walking score and knee swelling, calculated as a percentage increase relative to joint size prior to disease onset, for WT and IL-10−/− mice with AIA on day 3 postonset. One independent experiment, representative of three, is shown (n = 10). Data were compared by statistical analysis using the Fisher test. D, Representative histological analysis comparing arthritic knees of WT and IL-10−/− mice 7 d postdisease onset. The arrows indicate the areas where there has been an infiltration of cells into the joint. Original magnification ×10. E, Bar chart shows the severity of damaged joints in WTB cell and IL-10−/− B cell chimeric mice as well as IL-10−/− and WT mice with AIA. Histological analysis was done 7 d postdisease onset, and five mice per group were examined. One experiment representative of three is shown.

**IL-10−producing B cells suppress IL-17 and IFN-γ production at the site of inflammation**

The involvement of the Th1 and Th17 subsets in the pathogenesis of arthritis is supported by accumulation of these cells and upregulation of expression of their signature cytokines, IL-17 and IFN-γ, in the synovial fluid of individuals with rheumatoid arthritis (RA) (23, 24). To address the effect that the lack of B cell-derived IL-10 has on the proinflammatory arm of the immune response, we next measured the frequencies of Th1 and Th17 in draining LN of IL-10−/− B cell versus control WTB cell mice. We found that both Th1 and Th17 responses were significantly increased in the draining LN of IL-10−/− B cell compared with WTB cell mice (Fig. 3A, 3B). This increase was mirrored by a dramatic decrease in the frequency and in the number of CD4+ IL-10−/− T cells cells (Fig. 3C, 3D). An overall increase in IFN-γ and IL-17 production and a decrease of IL-10 released into the supernatant was measured from the same LN culture upon stimulation with mBSA (Fig. 3E).

**Transfer of T2-MZP Bregs into IL-10−/− hosts resets the balance between Tregs and Th1/Th17**

Our data so far suggest that endogenous Bregs actively participate in the maintenance or differentiation of Foxp3+ Tregs and in constraining Th1/Th17 response and the severity of arthritis. Other IL-10−producing cells, including dendritic cells and myeloid cells, have been shown to contribute to the maintenance of healthy numbers of Tregs via the release of IL-10 (12). Next we set out to understand and weight the sole contribution of IL-10−producing B cells in the maintenance/differentiation of Tregs in arthritis and in the inhibition of Th1/Th17 response. T2-MZP Bregs cells, which we have previously shown to be the major producers of IL-10 in arthritis (6) (Supplemental Fig. 3A), were isolated from WT mice in the remission phase of the disease and transferred i.v. to either syngeneic IL-10−/− mice or to WT mice on the day of disease onset. The purity of the T2-MZP Bregs, after FACS sorting, is shown in Supplemental Fig. 3B. A group of IL-10−/− and WT mice were also left untreated and used as a control. Similar to our results in collagen-induced arthritis, transfer of T2-MZP Bregs protects WT mice from developing severe arthritis (6) (Fig. 4A). Interestingly, despite IL-10−/− mice developing an exacerbated disease compared with WT mice (Figs. 1C–E, 4A), transfer of T2-MZP Bregs conferred a modest, yet significant, protection from AIA as shown by the reduction in knee swelling compared with IL-10−/− mice treated with PBS (no cells transferred) (Fig. 4A). Analysis of T cell differentiation revealed that the protective effect was accompanied by increased percentages of Foxp3+CD4+ T cells and a suppression of Th1/Th17 cell dif-
Differentiation in LN draining the arthritic joint compared with the levels detected in untreated IL-10−/− mice (Fig. 4B,4C). Interestingly, this increase in Foxp3+CD4+ T cells following T2-MZP Breg transfer can only be seen in the IL-10−/− mice and not WT recipients (Fig. 4B). This suggests that IL-10 produced by the T2-MZP Breg population is able to normalize Foxp3+CD4+ T cells levels in the IL-10−/− recipients (to WT levels); however, it cannot further upregulate Foxp3+Tregs once they have reached this threshold.

Transfer of IL-10−/− deficient T2-MZP Bregs failed to suppress arthritis development in both WT and IL-10−/− mice (we have only included the results for WT for clarity of presentation in Fig.

**FIGURE 2.** Lack of B cell-derived IL-10 results in a decrease of Foxp3+ Tregs. AIA was induced in chimeric mice by immunization with mBSA/CFA, followed 1 wk later by intra-articular injection with mBSA. A, Representative dot plots showing the percentage of Foxp3+CD4+ Tregs in inguinal draining LN 5 d postdisease onset in WTB cell and IL-10−/− B cell chimeric mice. Bar chart shows mean ± SEM (n = 5) of percentage and absolute numbers of Foxp3+CD4+ Tregs and is representative of three separate experiments. The p values were determined by unpaired t test. B, Fluorescence intensity, normalized to 100%, of Foxp3 on CD4+T cells. One of five representative histograms from one experiment is shown. Mean fluorescence intensity of Foxp3 gated on CD4+Foxp3+ (n = 5) is shown in the bar graph, representative of three independent experiments. The p values were determined by unpaired t test. C, CD4+CD25− Tregs were isolated, respectively, from draining LN of arthritic IL-10−/− B or WTB cell chimeric mice and cocultured (1:1) with CD4+CD25− T cells isolated from draining LN of arthritic WT mice for 72 h with anti-CD3 (1 μg/ml). [3H]thymidine was added 12 h before harvesting. Data shown are mean ± SEM of triplicate wells and are representative of two independent experiments. D, Bar chart shows the live cells present in the synovia of IL-10−/− B cell and WTB cell chimeric, removed 5 d after disease onset, counted by trypan blue. One out of three independent experiments is shown. The p values were determined by unpaired t test. E, Representative dot plot showing the percentage of Foxp3+CD4+ T cells in the synovia. The synovial cells from five mice were pooled, and one independent experiment, representative of four, is shown. F, Bar chart showing the ratio of live synovial cells: synovial Tregs present in the IL-10−/− B cell and WTB cell chimeric 5 d after disease onset. Data shown are mean ratio ± SEM from four independent experiments. For each experiment, the synovial cells from five mice were pooled for each group. The p values were determined by unpaired t test. G, Bar chart showing the absolute numbers of Foxp3+CD4+ T cells in the synovia. The synovial cells from five mice were pooled, and one independent experiment, representative of four, is shown. H, Representative histogram showing the fluorescence intensity, normalized to 100%, of Foxp3 in CD4+ T cells in the synovia of IL-10−/− B and WTB cell mice. The synovial cells from five mice were pooled, and one independent experiment, representative of four, is shown.
4D), IL-10−/− T2-MZP Bregs also fail in upregulating Foxp3 Treg numbers and suppressing Th1/Th17 differentiation in both WT and IL-10−/− recipient mice, respectively (Fig. 4E and data not shown), whereas T2-MZP Bregs from WT animals can suppress Th1 and Th17 cells in these recipients.

The aforementioned experiments suggest that T2-MZP Bregs are responsible for the modulation of Treg numbers in vivo. However, MZ B cells have also been previously shown to produce IL-10 in arthritis (25). To address whether additional B cell subsets contribute to the induction of Treg in vivo, next we transferred FO, MZ, and T2-MZP Bregs isolated from AIA mice into arthritic μMT mice. We used μMT mice as recipients in this set of experiments to eliminate the potential participation of endogenous B cells interacting with transferred IL-10+ B cells in the induction of Tregs. The results in Fig. 4F report a significant decrease in CD4+Foxp3+ Treg frequencies and absolute numbers in
FIGURE 4. Transfer of T2-MZP Bregs resets the balance between Tregs and Th1/Th17. T2-MZP cells (2 × 10^6), isolated from WT mice in AIA remission, were transferred i.v. to syngeneic IL-10−/− mice or to WT mice on the day of disease onset. Control group received a PBS injection. A, The bar chart shows the percentage increase in knee size on day 3 relative to the size of the same knee prior to disease onset. One independent experiment, representative of three, is shown (n = 5 for each group). All data were compared by statistical analysis using the Fisher test. B, Representative dot plots showing CD4+Foxp3+ T cells in draining LNs (isolated from different groups) and corresponding bar chart showing mean ± SEM. One independent experiment, representative of three, is shown (n = 5 for each group). The p values were determined by unpaired t test. C, Bar chart shows mean ± SEM of Th1 and Th17 frequencies in the draining LNs of IL-10−/− mice either with or without transfer of WT T2-MZP Bregs (2 × 10^6). One independent experiment, representative of three, is shown (n = 5 for each group). The p values were determined by unpaired t test. D, T2-MZP B cells (2 × 10^6), isolated from WT and IL-10−/− mice in the remission phase of arthritis, were transferred i.v. to syngeneic WT mice on the day of disease onset. Control group (no cell transfer) received a PBS injection. Chart shows the percentage increase in knee size on day 3 relative to the size of the same knee prior to disease onset.
B cell-deficient mice compared with WT mice in both draining LN and the affected joints. Adoptive transfer of T2-MZP Bregs alone, and not any of the other B cell subsets transferred, induced an increase in the percentages and absolute numbers of CD4+ Foxp3+Tregs as compared with the levels found in μMT mice in both draining LN and the affected joints (Fig. 4G, Supplemental Fig. 4A). The increase in Treg frequencies was also IL-10 dependent, as T2-MZP Bregs isolated from IL-10−/− mice fail to upregulate CD4+Foxp3+T cells in μMT mice (Fig. 4H, Supplemental Fig. 4B for relative absolute numbers). Together, these results strongly suggest that the IL-10 produced by T2-MZP Bregs induces Tregs and is also responsible for the suppression of cytokines that drive inflammation.

Several reports suggest that different modes of cell interaction are pivotal in determining a tolerogenic versus activatory outcome (26, 27). IL-10−/− B cells and IL-10−/−B cells isolated from arthritic mice were cultured with arthritogenic WT CD4+CD25+ T cells (1:1) and stimulated with mBSA for up to 30 min. Upon Ag stimulation, IL-10−/− B cells form a significantly higher number of conjugates with WT CD4+ T cells than IL-10−/− B cells (Fig. 5A). In addition, to further confirm the role of IL-10 in this interaction we neutralized IL-10 production in WT CD4+CD25− T cells and IL-10−/− B cells coculture and found that the numbers of conjugates were significantly reduced (Fig. 5A). Next, we addressed the effect of IL-10 produced by B cells on the duration of Treg contact time established with CD4+CD25+ T cells. A. One independent experiment, representative of three, is shown (n = 4 for each group). All data were compared by statistical analysis using the Fisher test. E. Bar chart shows mean percentage of Foxp3+CD4+ Tregs ± SEM (n = 6) from inguinal draining LN 5 d postdisease onset and is representative of three separate experiments. In the same inguinal draining LN, Th1 and Th17 frequencies were assessed following 5 h culture with PMA/ionomycin and brefeldin A. One independent experiment, representative of three, is shown (n = 4 for each group). The p values were determined by unpaired t test. F. Bar chart shows mean percentage of Foxp3+CD4+ Tregs ± SEM (n = 4) from inguinal draining LN 5 d postdisease onset and is representative of three separate experiments. G. T2-MZP Breg, FO, and MZ B cells (2 × 10⁵) were isolated from WT mice in the remission phase of AIA and were transferred i.v. to syngenic μMT mice at the day of disease onset. A control group of arthritic μMT mice received a PBS injection. The percentage of Foxp3+CD4+ Tregs in the inguinal draining LN 5 d postdisease onset was calculated. The results display the mean ± SEM percentage of increase/decrease of Foxp3+CD4+ Tregs in each group (recipient of a different B cell subset) compared with the percentage of Treg in control μMT group. H. T2-MZP B cells (2 × 10⁶), isolated from WT or IL-10−/− mice in AIA remission were transferred i.v. to syngenic μMT mice on the day of disease onset. Bar chart shows mean percentage of Foxp3+CD4+ Tregs ± SEM (n = 4) from inguinal draining LN 5 d postdisease onset and is representative of two separate experiments.
whether the lack of IL-10 production by B cells altered the duration of interactions with arthritogenic WT CD4⁺ T cells in response to mBSA stimulation. B cells were isolated from the spleens of arthritic IL-10⁻/⁻ or WT mice and cocultured with arthritogenic WT CD4⁺CD25⁻ T cells, also Foxp3⁺ (purity of Foxp3⁺ T cells are shown in Supplemental Fig. 4C). Contacts between B and T cells were monitored for the first 20 min of culture. Contacts were defined as two cells in close proximity with their membranes flattened against each other (Supplemental Fig. 4D, 4E) as previously described (28, 29). The analysis of the overall time that the cells spent in contact revealed a quantitative difference between the two groups (Fig. 5B). We observed a higher percentage of long contacts (contacts exceeding 800 s) between IL-10⁺/⁺ B cells and WT CD4⁺ T cells compared with IL-10⁻/⁻ B cells and WT CD4⁺ T cells, whereas an increased percentage of brief contacts was observed between IL-10⁻/⁻ B cells and WT CD4⁺ T cells (<100 s) (Fig. 5C). Very minimal interaction was observed between B and T cells cocultured without any stimulation (data not shown).

IL-10-producing B cells convert effector CD4⁺ T cell to Foxp3⁺CD4⁺ T cells

Our data so far suggest that longer contact time between IL-10⁺/⁺ B cells and CD4⁺CD25⁻ T cells might result in regulatory cell differentiation, whereas shorter interaction time, such as that established between IL-10⁻/⁻ B cells and CD4⁺CD25⁻ T cells, results in the induction of inflammatory cell types. IL-10⁻/⁻ B cells and IL-10⁻/⁻ B cells isolated from arthritic mice were cultured with arthritogenic WT CD4⁺CD25⁻ T cells and stimulated with mBSA. WT CD4⁺CD25⁻ T cells were cultured alone with mBSA as a control. Minute amounts of IL-17, but high secretion of IL-10, was measured when WT CD4⁺CD25⁻ T cells were differentiated in the presence of WT IL-10⁺/⁺ B cells; in contrast, a dramatic interaction was observed between B and T cells cocultured without any stimulation (data not shown).

**FIGURE 6.** IL-10 produced by B cells influences Treg differentiation. A, As described in Fig. 5, spleens from WT and IL-10⁻/⁻ mice with AIA were excised 5 d postdisease onset, and CD4⁺CD25⁻ T cells and B cells were negatively purified. Total of 1.5 × 10⁵ IL-10⁻/⁻ and IL-10⁺/⁺ B cells were then cultured with WT CD4⁺CD25⁻ T cells for 48 h with mBSA (10 μg/ml) (1:1). Total of 3 × 10⁵ WT CD4⁺CD25⁻ T cells, 3 × 10⁵ WT B cells, and 3 × 10⁵ IL-10⁻/⁻ B cells were also cultured alone with mBSA (10 μg/ml) as controls. Supernatants were collected, and the production of IL-17 and IL-10 was measured by ELISA. Bar charts show mean ± SEM, and one experiment, representative of three, is shown (n = 5 for each group). The p values were determined by unpaired t test. B, Foxp3 expression on CD4⁺ T cells was assessed by FACS, and representative plots are also shown. Bar chart shows mean ± SEM, and one experiment, representative of three, is shown (n = 5 for each group). The p values were determined by unpaired t test. C, CD4⁺CD25⁻ T cells were isolated by negative selection from IL-10 GFP reporter animals (TIGER mice) 5 d postdisease onset. Spleens from WT and IL-10⁻/⁻ mice were excised 5 d postdisease onset, and B cells were negatively purified. Total of 1.5 × 10⁵ IL-10⁻/⁻ and IL-10⁺/⁺ B cells were then cultured with WT CD4⁺CD25⁻ T cells for 48 h with mBSA (10 μg/ml) (1:1). Expression of IL-10 (GFP⁺ cells) and Foxp3 on CD4⁺ gated T cells was assessed by FACS. The plots are representative of three independent experiments and for each group n = 5. ND, not detectable.
increase in IL-17 production was observed when WT CD4+ T cells were cultured with IL-10-/- T cells (Fig. 6A). It is possible that B cells isolated from IL-10-/- mice induce IL-17 production because they might be more proinflammatory than IL-10-producing B cells. However, B cells isolated from IL-10-/- mice and WT produce similar amounts of IFN-γ and IL-1β upon Ag stimulation (data not shown), suggesting that it is the IL-10 that directly or indirectly modulates the production of IL-17.

This reduction in proinflammatory T cells was mirrored by an increase in the Foxp3+ T cell population (Fig. 6B). Interestingly, only IL-10–competent B cells were capable of converting CD4+ CD25- (Foxp3-) T cells to Foxp3+CD4+ T cells in response to mBSA stimulation (Fig. 6B, Supplemental Fig. 4C for CD4+ CD25 Foxp3 purity). Moreover, by taking advantage of GFP- IL-10 reporter mice (30), which permit a higher resolution of IL-10–producing B cells, we demonstrated that the IL-10+ CD4+ T cell population differentiating in the IL-10+/− B cells cultured with WT CD4+ T cells is Foxp3- (Fig. 6C), confirming our and others previous findings showing that Bregs are also important in the induction of T regulatory 1 (Tr1) populations in vitro (25, 31).

Discussion
Results from multiple studies have demonstrated that Breg-derived IL-10 is important in the prevention of autoimmune disorders including arthritis or in the promotion of cancer (1, 2, 14, 31, 32). Adoptive transfer of in vitro–manipulated B cells has been shown to recruit Tregs to the site of inflammation (33). Hence, the relevance of immunoregulation mediated by endogenous B cell–derived IL-10 in vivo remains completely uninvestigated. Our results revealed the existence of a previously unappreciated regulatory pathway that maintains the number of Tregs and downregulates the differentiation of Th1 and Th17 pathogenic cells in vivo. We have previously shown that transferrer Bregs can suppress, even if Tregs were depleted prior to transfer, which suggested that Bregs work independently from thymically derived Tregs (6). In this study, we show that IL-10-/- B cells fail in converting CD4+ T cells into Foxp3+ Tregs in vitro and lack of B cell–derived IL-10 in vivo profoundly reduces Treg numbers. By taking advantage of the IL-10 GFP reporter mice, we have shown that, at least in vitro, the Breg–induced IL-10–secreting Tr1 cells are Foxp3+.

In addition, our data show that in vivo, the ability of IL–10–secreting B cells to induce Foxp3+ Tregs is confined to the T2-MZP Breg subset. Unfortunately, the currently available tools did not allow us to unequivocally discern in vivo whether the IL-10–producing T cells are Foxp3 negative. However, it is plausible to propose that Bregs, via the release of IL-10, are important in the maintenance of a peripherally induced suppressive CD4+ T cell pool comprising both Foxp3+ and CD4+IL-10+ Tr1 cells and the downregulation of the Th1/Th17 proinflammatory response. Furthermore, it will be integral for the understanding of Breg biology to ascertain whether these cells mediate this suppression by processing and presenting Ag in addition to the secretion of IL–10.

Our results could also have important implications in the cancer field, in which it has been suggested that Bregs play a pathogenic role by suppressing T cell activation (4, 32). Because a high number of Tregs are observed in several types of cancer, including lymphoma (34), it is tempting to intimate that there is a positive correlation between the upregulation of Bregs and Tregs.

A direct correlation between the numbers of DC and Tregs and an inverse correlation with Th1 and Th17 proinflammatory cytokines has been previously reported (35). In addition, DCs producing IL-10 induce expansion of CD4+ and CD8+ Tregs (36–38). Altogether, our results support the concept that the immune system has implemented a series of sophisticated check points consisting of several cell types that independently or synergistically contribute to the maintenance of Treg homeostasis in vivo. The results in Fig. 1 clearly show an exacerbated arthritis in mice lacking IL-10–producing B cells, demonstrating that these check points are still in place despite inflammation.

Our data in Fig. 2 showed that in the absence of endogenous IL-10 released by B cells, the few remaining Tregs display a reduced expression of Foxp3 but retained their suppressive function in vitro. Recent evidence shows that IL-10 produced by myeloid cells is crucial in the maintenance of the expression of Foxp3+ Tregs in mice with colitis. However, unlike in our model, Tregs in the colitis model have lost suppressive capacity (12). One possible explanation for these discordant results is that whereas in colitis model, the expression of Foxp3 is nearly abrogated, in IL-10-/− B cell mice, the expression of Foxp3 is reduced. Of note, Tregs isolated from peripheral blood of RA patients also display reduced numbers of Tregs compared with healthy controls, but retained the capacity to suppress proliferation (39, 40).

Our data are also in broad agreement with data from patients with active RA or juvenile arthritis showing that the frequencies of Tregs in the synovia exceed those present in peripheral blood (39, 41). It also remains to be established whether Tregs isolated from the joints of IL–10-/- B cell arthritic mice are less suppressive than those isolated from WT chimeric mice. It has been reported that addition of proinflammatory cytokines, which are usually increased in the joints of patients with active RA, to synovial Treg/Teffector cocultures impairs the suppressive capacity of Tregs (41). This may suggest that despite being numerically unchanged, the Tregs differentiating in IL–10-/- B cell mice may have lost their suppressive capacity due to the increased exposure to proinflammatory cytokines (Figs. 3E, 6A). Alternatively, as has been previously shown, effector T cells at the site of inflammation may be less susceptible to suppression (42). Unfortunately, due to the very low number of cells obtained from the inflamed joints, it is technically not possible to perform coculture assays to assess the suppressive capacity of Tregs from the synovia of these mice.

We have recently translated our mouse work into humans and demonstrated that CD19+CD24hiCD38hi B cells, previously described as B cells at the immature-transitional stage of development, comprised the highest percentage of IL–10–producing B cells in response to CD40 stimulation compared with other healthy peripheral blood B cells (43, 44). In addition, CD19+ CD24hiCD38hi B cells inhibited the differentiation of IFN-γ and TNF-α–producing CD4+ T cells (44). The existence of B cells with regulatory function in humans has also been recently confirmed in an independent study (45). The results presented in this study, together with our recent findings in man, are particularly interesting in view of the recent work showing that long-term renal transplant tolerance in humans is associated with an accumulation of CD19+CD24hiCD38hi B cells (also known as transitional B cells) and a greater number of transitional B cells expressing IL-10. In addition, the same set of patients also showed an increase in FOXP3+ Tregs (46). Therefore, it is tempting to speculate that the increase in FOXP3 observed in this set of patients might be directly related to the increase in transitional IL–10–producing B cells.

In conclusion, our results show the importance of Bregs as arbiters of the inflammatory response that can evoke other regulatory cells (including Tr1 and Foxp3+ Tregs) to act in unison for the reduction of proinflammatory cells types and prevention of tissue damage.
joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. Arthritis Rheum. 58: 875–887.