Resident Bacteria-Stimulated Interleukin-10-Secreting B Cells Ameliorate T-Cell-Mediated Colitis by Inducing T-Regulatory-1 Cells That Require Interleukin-27 Signaling

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SUMMARY
Regulatory mechanisms of interleukin-10 (IL10)-producing B cells in mucosal homeostasis are not fully understood. This study shows that IL10-secreting B cells activated by resident bacteria ameliorate T-cell-mediated colitis by inducing T regulatory-1 cells via an IL27-dependent mechanism.

BACKGROUND & AIMS: The regulatory roles of interleukin-10 (IL10)-producing B cells in colitis are not fully understood, so we explored the molecular mechanisms by which these cells modulate mucosal homeostasis.

METHODS: CD4+ T cells from wild-type (WT), Il10−/−, or Il27ra−/− mice were cotransferred with B cells from specific pathogen-free (SPF) or germ-free (GF) WT or Il10−/− mice into Rag2−/− Il10−/− (double-knockout) mice, and the severity of colitis and intestinal regulatory T-cell populations were characterized. In vitro, WT or Il10−/− B cells were cocultured with unfractonated, naïve or regulatory T cells plus Il10−/− antigen-presenting cells and stimulated with cecal bacterial lysate (CBL) with or without IL27 or anti-IL10R blockade. Gene expressions, cytokines in the supernatant and cell populations were assessed.

RESULTS: WT but not Il10−/− B cells attenuated T helper cell Tn1/Tn17-mediated colitis in double-knockout mice that also received WT but not Il10−/− T cells. In vitro, CBL-stimulated WT B cells secrete abundant IL10 and suppress interferon-γ (IFNγ) and IL17a-production by T cells without requiring cell contact. Although both WT and Il10−/− B cells induced Foxp3+ CD4+ T-regulatory cells, only WT B cells induced IL10-producing (Foxp3-negative) T regulatory-1 (Tr-1) cells in vivo and in vitro. However, IL10-producing B cells did not attenuate colitis or induce Tr-1 cells in the absence of T cell IL27 signaling in vivo. WT B cell-dependent Tr-1 induction and concomitant decreased IFNγ-secretion were also mediated by T-cell IL27-signaling in vitro.

CONCLUSIONS: IL10-secreting B cells activated by physiologically relevant bacteria ameliorate T-cell-mediated colitis and contribute to intestinal homeostasis by suppressing effector T cells and inducing Tr-1 cells via IL27-signaling on T cells. (Cell Mol Gastroenterol Hepatol 2015;1:295–310; http://dx.doi.org/10.1016/j.jcmgh.2015.01.002)

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Inflammatory bowel diseases (IBD) are chronic, T-cell-mediated intestinal disorders characterized by loss of tolerance to resident enteric bacteria and aggressive inflammatory responses. 1,2 Regulatory T cells (Treg) have a well-described role in attenuating experimental colitis and IBD. Treg help maintain intestinal homeostasis by preventing inappropriate innate and adaptive immune responses against resident bacteria. CD4+ T cells that express forkhead box P3 (Foxp3) and T regulatory-1 (Tr-1) cells that lack Foxp3 expression (Foxp3−/−), but produce interleukin-10 (IL10) comprise major regulatory T-cell populations in the intestine.3,4 CD25+ Foxp3+ CD4+ Treg prevent colitis in severe combined immunodeficiency mice cotransferred with CD45RBHIg T cells,5 and enteric bacterial antigen-specific Tr-1 cells ameliorate colitis induced by pathogenic T helper 1 (Th1) cells.6

In parallel with Treg cells, B cells contribute to intestinal homeostasis by secreting immunoglobulins that decrease mucosal translocation of luminal bacteria and producing regulatory cytokines that inhibit effector mucosal immune responses.7 Moreover, antigen presentation by B cells promotes the differentiation of tolerogenic CD4+ T cells.8 B-cell depletion may contribute to the development of human IBD9 and potentiate murine experimental colitis,10 suggesting that B cells are protective in IBD. However, the
mechanisms by which B cells attenuate intestinal inflammation are not entirely clear.

B cells secrete IL10 as do many other cell types including Treg cells, macrophages, mast cells, epithelial cells, and dendritic cells. IL10 reduces inflammation by inhibiting effector T-cell activation. IL10-deficient (Il10−/−) and IL10-receptor-deficient mice develop resident enteric bacteria-dependent Tπ1/Tπ17-mediated colitis. The role of IL10 derived from intestinal T-cells and myeloid cells in maintaining mucosal homeostasis is well studied, but relatively little is known about the importance of IL10-producing B cells in IBD and experimental colitis. Moreover, the mechanisms of how IL10-producing B cells potentially maintain mucosal homeostasis in the intestine are poorly understood.

IL27, a member of the IL12 family, consists of Epstein-Barr virus induced gene-3 (EBI3) and p28 subunits and has pleiotropic effects on the immune system. IL27 was originally reported to induce TH1 cell development, but it has pleiotropic effects on the immune system. IL27 was Barr virus induced gene-3 (EBI3) and p28 subunits and potentially maintain mucosal homeostasis in the intestine. Moreover, the mechanisms of how IL10-producing B cells attenuate intestinal inflammation are not entirely clear.

Cell Purification

Splenic B cells were purified magnetically by positive selection with anti-CD19 microbeads after negative selection by a mixture of anti-CD90.2, anti-CD11c, and anti-Ter119 microbeads (Miltenyi Biotec, Auburn, CA) (greater than 99.5% pure and 90% viable). The CD4+ T cells were isolated by a CD4+ T-cell isolation kit (Miltenyi Biotec) (more than 94.7% pure and 95% viable). In some experiments, unfractonated CD4+ T cells were further fractionated into CD25+ and CD25− T cells by PE-conjugated anti-CD25 antibody with anti-PE microbeads. Red blood cell lysed-unfractionated splenocytes from (Il10−/−)Rag2−/− and DKO mice were used for WT and Il10−/− antigen-presenting cells (APC), respectively (more than 88.4% CD11b+).

Materials and Methods

Mice

We purchased C57BL/6 (B6), WT, B6.II10−/−, B6.Rag2−/−, and B6.II7/ra (WSX-1)−/− mice from Jackson Laboratories (Bar Harbor, ME). We purchased 129S6/SvEv (129) WT mice from Taconic Farms (Germantown, NY). The 129.II10−/− mice were obtained from Dr Donna Rennick (DNA Laboratories, Palo Alto, CA). The II10−/−Rag2−/− double-knockout (DKO) mice were generated by crossing B6.II10−/− with B6.Rag2−/− mice or 129.II10−/− with 129.Rag2−/− mice. The B6.II10−/−Rag2−/− reporter (Vert-X) mice were obtained from Dr Christopher Karp. These mice were originally maintained in the specific pathogen-free (SPF) facility at the University of North Carolina (UNC), then all 129 strains, B6.WT, II10−/−, DKO, and Vert-X mice were derived into germ-free (GF) conditions by embryo transfers, and breeding colonies were established in the GF facility at the UNC. Afterward, the mice were transferred to a SPF room and colonized with SPF feces to maintain SPF colonies. The mice used in this study were born from parents that had also been born and raised in SPF conditions over six generations, and they were used at 8 to 16 weeks of age. These studies were approved by the UNC-Chapel Hill Institutional Animal Care and Use Committee (ACUC) Protocol no. 12–300.0.
Adoptive Cell Transfer
The 5 × 10^5 unfractionated splenic CD4^+ T cells from 129.WT or Il10^−/− mice were cotransferred intraperitoneally with or without 1 × 10^6 B cells from 129.WT or Il10^−/− mice into 129.DKO mice. The 5 × 10^5 CD25^+ CD4^+ T cells from B6.WT, Il27ra^+/−, or Vert-X mice were injected intraperitoneally into B6.DKO mice with or without 1 × 10^6 B cells from B6.WT or Il10^−/− donors. The severity of colitis and intestinal cell populations were assessed 3 to 6 weeks after the cell transfer.

Assessment of Colitis
We evaluated the severity of colitis by blinded histologic scoring, colonic tissue explant cultures, and MLN cell cultures, as described elsewhere.28

Blinded histologic scoring. Intestinal tissues were removed and fixed in 10% buffered formalin. Histologic inflammation was quantified in paraffin-embedded, H&E-stained sections of cecum, proximal colon, and distal colon in a blinded fashion, with each region being graded from 0 to 4 based on the degree of LP and submucosal mononuclear cellular infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion. The total histology score represents the summation of the scores for cecum, proximal colon, and distal colon (maximum score 12).

Colonic tissue explant cultures. Colonic tissues were thoroughly washed with cold PBS, shaken at room temperature in RPMI containing 50 μg/mL gentamicin for 30 minutes at 280 rpm, cut into 0.5-cm fragments, and weighed. Colonic tissue fragments were distributed (0.05 g per well) into 24-well plates and incubated in 1 mL of RPMI 1640 medium supplemented with 5% fetal bovine serum, 50 μg/mL gentamicin, and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B; Gibco, Grand Island, NY) for 20 hours at 37°C. Supernatants were collected and stored at −20°C before use for cytokine quantification.

Mesenteric lymph node cell cultures. The 5 × 10^5 unfractionated MLN cells of individual recipient animals were stimulated in 96-well flat-bottom plates with 10 μg/mL CBL at 37°C, 5% CO_2 in a humidified incubator. After 72 hours, the culture supernatants were collected for cytokine assays.

Cecal Bacterial Lysate
Cecal bacterial lysate (CBL) was prepared from the contents of ceca from SPF B6 or 129.WT mice as described elsewhere.28 The protein concentration of the lysate was determined, and the cells were stimulated with 10 μg/mL CBL at 37°C, 5% CO_2 in a humidified incubator. In some experiments, 5 × 10^5 naive WT CD25^− T cells isolated from Vert-X mice, Il27ra^+/− or (Il27ra^+/−/−) WT mice were cocultured with or without 1 × 10^6 129.WT or Il10^−/− B cells along with 2 × 10^5 129.I10^−/− APC at 200 μL/well (96-well plates) for 72 hours at 37°C with 5% CO_2. In some experiments, 5 × 10^5 naive WT CD25^− with or without 2.5 × 10^5 CD25^+ CD4^+ T cells from 129.WT or Il10^−/− mice were cocultured with 2 × 10^5 129.I10^−/− APC. In selected experiments, 2.5 × 10^5 CD25^+ CD4^+ T cells isolated from Vert-X mice, Il27ra^+/− or (Il27ra^+/−/−) WT mice were cocultured with 5 × 10^5 B6.WT or Il10^−/− B cells along with 2 × 10^5

Table 1. Polymerase Chain Reaction Primers Used in this Study

| Gene | Sense 5’-3’ | Antisense 5’-3’ | Reference |
|------|-------------|-----------------|-----------|
| Il2p28 | GCCAGGTGACAGGAGACC | CAGCGTTGACAGGAGACC | Collison et al.41 |
| Ebi3 | AGCGACGTTCCTCTAAGCTT | AGCGACGTTCCTCTAAGCTT | Collison et al.41 |
| Il12a | TGGCTACTAGAGAGATCTTCCAA | GCCACGGGTACATCATCAAAGAC | Collison et al.41 |
| Il12a | ATCCAGTGGAGAATGTTGTTGA | GCAAGCAGAACTGCTGCTTGG | Collison et al.41 |
| Il17a | CTCGAGAAGGCCTCTAGTAC | AGGTTTCCCTCGGATGACAG | Qu et al.45 |
| Il6 | CGCGAGGAGGACAGTTCAC | TCCACGATTTCCGAGAAC | Bao et al.43 |
| Cmaf | AAATACGAGAAGCTGTTGACAA | CGGAGAGGAGAAGTTGTC | Hiroset et al.44 |
| Ahn | ACATACCATGTGATCGGC | TCTGTGCTGGTAGAGGAT | Wurster et al.45 |
| Il21 | AAGATTAGTGGAGTCGATCAGAG | GATTCGAGCGTATAGGTGTC | Wurster et al.45 |
| Foxp3 | GCCGCTCTCCAGCAGCAAC | GCTGATCATGGCTGGTTTG | Wan and Flavell46 |
| Rorgt | TGAGCGCATTGATATGGTG | CCTCCATGTCGTCGGTTC | Ano et al.47 |
| Tbet | TTCCCATCGGTCTCCACAC | CCCATCACCAAAACATCCTG | Ano et al.47 |
| Actb | AGCGATATCTAGCAGATCCAG | TGCGCTGAGGAGAGCATAG | Liu et al.48 |
| Blnp1 | TCTGTTGTTGTATTGTGGGACCTT | TGAGGAGACTCTTCTGAGTT | Miyazaki et al.49 |
| Il12b | CGCAAGAAGAAGATGGAAGGA | TTGAGTGAGGACCTGATGAG | Made ourselves |
| Il7g | CTTTCCATGCTGGTTTCCGT | CGGTTATGTTGCTGATG | Made ourselves |
| Il10 | GTCTAGATTCCTCCCCTTG | CCTGATGACACTTCTGCGTTG | Made ourselves |
In certain experiments, 2.5 × 10^5 APC from B6 (Il10^-/-) Rag2^-/- or Il10^-/- Rag2^-/- mice were cocultured with or without 5 × 10^6 B cells from B6 WT or Il10^-/- mice.

In another experiment, 5 × 10^5 naïve T cells isolated from Vert-X mice and 5 × 10^5 B6 Il10^-/- APC were cocultured without or with 1 × 10^6 B6.WT or Il10^-/- B cells at 400 µL/well using 48-well transwell plates (0.4-µm pore size) (Costar, Corning, NY). The culture medium was RPMI 1640 (Gibco/Invitrogen) containing 10% fetal bovine serum, 1% penicillin/streptomycin/amphotericin B (Gibco/Invitrogen), and 5 × 10^5 mol/L 2-mercapt ethanol (Sigma-Aldrich), with 10 µg/mL CBL from either WT 129 or B6 mice or without bacterial lysates.

In selected experiments, 10 µg/mL anti-IL10R neutralizing antibody (BD Biosciences, San Jose, CA), anti-IL27 neutralizing antibody (eBioscience, San Diego, CA), isotype control [purified rat IgG1 (BD Biosciences) or mouse IgG2a (eBioscience)], or recombinant murine IL10 or IL27 at 10, 1, or 0.1 ng/mL (PeproTech, Rocky Hill, NJ) was added. After 72 hours, the supernatants were collected for measurement of cytokines by enzyme-linked immunosorbent assay (ELISA), and cells were analyzed by flow cytometry. For bacteria-activated T-cell RNA analysis, CBL-stimulated naïve T cells were reisolated using a
CD4+ T-cell isolation kit with LD columns (Miltenyi Biotec) 24 hours after coculture with B cells and APC.

Cytokine Measurements
To evaluate the production of cytokines, ELISAs were performed by the Immunoassay Core of the CGIBD at UNC according to the manufacturer’s instructions with the following products: mouse anti-IL10, IL12/23p40, interferon-γ (IFNγ), and IL17 (BD Biosciences), and IL27 (eBioscience). Concentrations of cytokines were established following products: mouse anti-IL10, IL12/23p40, interferon-γ (IFNγ), and IL17 (BD Biosciences), and IL27 (eBioscience). Concentrations of cytokines were established using standard curves generated using the appropriate recombinant cytokine.

Real-Time Polymerase Chain Reaction
Total RNA was isolated from reisolated CD4+ cells using RNeasy Micro Kit (Qiagen, Valencia, CA) and real-time polymerase chain reaction (RT-PCR) was performed as described elsewhere.28 The primer sequences used in this study are shown in Table 1.

Flow Cytometry
The antibodies we used in this study were anti-mouse CD4, CD19, CD45, B220, CD3, F4/80, CD11c, CD11b (BD Bioscience), IL10, IL17, IFNγ, IL27p28, Ebi3 (R&D Systems, Minneapolis, MN), and live/dead cell viability kits (Invitrogen/Life Technologies, Carlsbad, CA). MLN, or colonic LP cells were collected and incubated for 15 minutes at 4°C with anti-CD16/CD32 (BD Bioscience) and then for 20 minutes at 4°C with antibodies for cell surface and live/dead cell viability kits to evaluate the cell phenotype and CD4+ T-cell/B-cell reconstitution. Enumeration of cells expressing IL10, IL17, IFNγ, IL27p28, Ebi3, and Foxp3 was performed by intracellular staining, 100 ng/mL phorbol myristate acetate, 1 μg/mL ionomycin (Sigma-Aldrich), and GolgiStop (BD Biosciences) were added into the medium during the last 4 hours of the culture period. The cells were washed and then analyzed on a CyAn flow cytometer (Beckman Coulter, Brea, CA). Proper isotype antibodies were used as a control, and gated live CD45+ cells were analyzed with Summit 5.2 software (Beckman Coulter).

Statistical Analysis
Statistical analysis was performed using Prism 5 software (GraphPad, San Diego, CA) to compare the mean between two groups with two-tailed, unpaired Student’s t tests; comparisons of the mean from multiple groups were analyzed with one-way analysis of variance (ANOVA) and the Bonferroni posttest. P < .05 was considered statistically significant.

Results
Interleukin-10-Producing B Cells Attenuate T-Cell-Mediated Colitis via Interleukin-10 Secreting T Cells
To evaluate the role of IL10-producing B cells in regulating colitis in vivo, we cotransferred SPF 129.WT or Il10−/−CD4+ T cells with or without B cells from 129.WT or Il10−/− mice into 129.Rag2−/−Il10−/− (DKO) recipients in SPF conditions. The DKO mice that received unfractionated WT CD4+ T cells developed moderate Th1/Th17-mediated colitis by 6 weeks as assessed by histologic scores (Figure 1A and 1B) and spontaneous IFNγ and IL17a-secretion by colonic tissue explants (Figure 1C); the cotransferred WT, but not Il10−/−, B cells suppressed all measured parameters of inflammation. Spontaneous secretion of IL10 in colonic explant cultures was increased in the mice that received WT B cells (Figure 1C). Likewise, CBL-stimulated MLN cells from mice that received WT B cells secreted more IL10 and less IFNγ and IL17a compared with mice that received Il10−/− or no B cells (Figure 1D).

![Figure 2](image-url)

**Figure 2.** IL10-secreting B cells regulate intestinal inflammation in the presence of T-cell-derived IL10. 5 × 10^6 CD4+ T cells from SPF 129.WT or Il10−/− knockout (KO) mice were cotransferred with or without different numbers of B cells from 129.WT or Il10−/− mice into SPF 129.Rag2−/−Il10−/− mice. Six-weeks later, histologic total colon inflammation scores (A) and IFNγ-secretion in colon explant cultures (B) were measured. N = 6–7/group, two replicates. Data are presented as mean ± SE, *P < .05, **P < .01, ***P < .001 (n = 6–7/group). N.S: not statistically significant.
We found that the percentages of T and B cells in MLN from mice that received WT B cells were not statistically significantly different compared with the percentages in those that received Il10/-/- B cells (Figure 1E). The percentages of MLN Foxp3+CD4+ Treg and Foxp3 mRNA levels in the distal colon were statistically significantly increased in the mice that received either WT or Il10/-/- B cells compared with the mice that did not receive B cells (Figure 1F). On the other hand, cotransfer of WT B cells had no effect on histologic inflammation or IFNγ-secretion by colonic tissue explants in mice that received Il10/-/- CD4+ T cells even when 10 times more WT B cells were transferred (Figure 2A and B), suggesting that IL10-producing B cells inhibit experimental colitis caused by T-cell-transfer only when the T cells are capable of secreting IL10. Moreover, the B cells from the GF and SPF B6.WT mice exhibited similar anti-inflammatory properties (Figure 3A and B). Our transferred CD19+ B220+ population that contained mainly naïve B cells, even in SPF mice (more than 95%), received physiologic bacteria stimulation in SPF recipients to stimulate IL10-secretion and regulatory function. On the other hand, B cells from noninflamed GF and inflamed SPF B6.Il10/-/- mice similarly lacked anti-inflammatory properties in vivo and in vitro, suggesting that the difference in colitis between mice that received WT versus Il10/-/- B cells is not due to the pre-existence of inflammation in the Il10/-/- donor mice (Figure 3A and B).

Figure 3. Germ-free (GF) wild-type (WT) B cells have anti-inflammatory properties similar to specific pathogen-free (SPF) WT B cells, but GF Il10/-/- B cells are unable to regulate intestinal inflammation. (A) In vivo, 5 x 10^6 splenic CD25-CD4+ T cells from GF B6.WT mice were cotransferred with or without 1 x 10^6 B cells from GF or SPF B6.WT or Il10/-/- knockout (KO) mice into SPF B6.Rag2/-/- Il10/-/- mice. Six weeks after the cell-transfer, the severity of colitis was assessed by total histologic scores and cytokine measurement by colonic tissue explant cultures. N = 6-7/group. Mean ± standard error (SE). *P < .05. (B) In vitro, 2.5 x 10^5 splenic CD25-CD4+ T cells from GF B6.WT mice were cocultured with or without 5 x 10^5 B cells from GF or SPF conditions along with 2 x 10^5 GF Il10/-/- Antigen-presenting cells 48 hours later, cytokines in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA). N = 6/group, two replicates. Mean ± SE, *P < .05; **P < .01; ***P < .001.
Physiologically Activated Interleukin-10-Producing B Cells Inhibit Proinflammatory Cytokine-Secretion by T Cells In Vitro

We next sought to determine mechanisms by which IL10-producing B cells inhibit proinflammatory cytokine-secretion by T cells. We measured cytokines in physiological CBL-stimulated cocultures of unfractionated CD4⁺ T and B cells from 129.WT or Il10⁻/⁻ mice and Il10⁻/⁻ APC. We detected increased IL10 in cultures containing WT B cells and the amounts were even higher when cocultured with WT or Il10⁻/⁻ T cells (Figure 4A). In cultures containing WT CD4⁺ cells, the presence of IL10-producing B cells was associated with decreased levels of IFNγ and IL17a compared with cultures that had II10⁻/⁻ or no B cells (Figure 4A and 4B). In contrast, consistent with the inability of WT B cells to attenuate experimental colitis in the absence of IL10-producing T cells, WT B cells were unable to suppress IFNγ and IL17a when cocultured with II10⁻/⁻ T cells (Figure 4A and B). Blockade of IL10-signaling by anti-IL10R antibody increased IFNγ-secretion in the cultures containing WT CD4⁺ T cells, WT B cells, and II10⁻/⁻ APC, whereas exogenous recombinant-IL10 decreased IFNγ and IL17a-secretion in the cultures containing II10⁻/⁻ CD4⁺ T cells, II10⁻/⁻ B cells with II10⁻/⁻ APC. These data indicate that the anti-inflammatory function of IL10-producing B cells may be due in part to their ability to suppress IFNγ and

Figure 4. Cecal bacteria–stimulated interleukin-10 (IL10)-secreting B cells suppress proinflammatory cytokines to a similar degree as CD25⁺ CD4⁺ T-regulatory cells in vitro. This protection is eliminated by interleukin-10 (IL10)-signaling blockade. Cytokine secretion was measured in cecal bacterial lysate (CBL)-stimulated cultures containing 2.5 x 10⁵ unfractionated 129.WT or II10⁻/⁻ KO (knockout) CD4⁺ T cells, 5 x 10⁵ 129.WT (wild type) or II10⁻/⁻ APC along with 2.5 x 10⁵ 129.II10⁻/⁻ antigen-presenting cell (APC) by enzyme-linked immunosorbent assay (ELISA) (A) and flow cytometry (B). We selectively added 10 μg/mL IL10 receptor (IL10R) antibody or 5 ng/mL recombinant IL10. (N = 6/group, three replicates.) In parallel, IL10 and interferon-γ (IFNγ) were assessed in CBL-stimulated cultures containing WT CD25⁺ CD4⁺ T cells, WT or II10⁻/⁻ B cells, and WT or II10⁻/⁻ CD25⁺ CD4⁺ T-regulatory cells (Treg) by ELISA (C) (N = 9/group, three replicates). The average numbers of live (live/dead cell staining kit) CBL-stimulated IL10-secreting T and B cells in the cultures containing WT CD25⁺ CD4⁺ T cells, WT B cells, and WT CD25⁺ CD4⁺ Treg were analyzed by flow cytometry (D). Mean ± standard error. *P < .05; **P < .01; ***P < .001.
IL10a-secretion and that suppression of IFNγ and IL17 requires the presence of IL10-producing T cells.

Next, we investigated the regulatory function of B cells on T cell subsets. Because CD25CD4+ T cells have previously been shown to attenuate chronic T-cell-mediated colitis,29 we explored whether the regulatory role of IL10-producing B cells was mediated by IL10-secreting CD25CD4+ Treg. We measured cytokines in CBL-stimulated cocultures of naïve CD25CD4+ T cells from WT mice, CD25+CD4+ Treg from WT or Il10−/− mice, and B cells from WT or Il10−/− mice with Il10−/− APC. We found that WT B cells produced more IL10 than WT CD25 CD4+ or CD25 CD4+ T cells alone and that the total amount of IL10 was even higher when cocultured with WT CD25 CD4+ and/or CD25 CD4+ T cells (Figure 4C). The majority of IL10-positive cells in the cultures containing WT CD25 CD4+, WT CD25 CD4+, and WT B cells were B cells (Figure 4D). The WT but not Il10−/− B cells suppressed IFNγ-production by CD25 CD4+ T cells to a similar degree as WT or Il10−/−CD25 CD4+ Treg cells. CD25 CD4+ Treg and WT B cells each suppressed IFNγ-secretion, and when added together, the degree of suppression was greater (Figure 4C). On the other hand, the presence of Il10−/− B cells enhanced IFNγ-production by both CD25 CD4+ and CD25 CD4+ T cells compared with no B cells (Figure 4C). These data suggest that IL10-producing B cells inhibit Tn1 function to a similar degree as CD25 CD4+ Treg cells.

Because IL10-producing myeloid cells also regulate effector T cells,30,31 we compared the functional capacities of IL10-producing B cells with APC. Interestingly, while WT B cells suppress both IFNγ and IL17, WT APC decreased IFNγ but paradoxically enhanced IL17-secretion despite their IL10-secreting capacity (Figure 5), suggesting that myeloid WT APC are unable to suppress Tn17-mediated immune reactions that are regulated by B cells.

**Physiologically activated Interleukin-10-Producing B Cells Promote the Differentiation of Naïve CD4+ T Cells into Tr-1 cells by Interleukin-10-Dependent Mechanisms**

We next determined the influence of IL10-secreting B cells on the development of regulatory T cells in vitro. To investigate this, we quantified induction of Foxp33 or IL10-producing (Foxp33/) Tr-1 cells and T-cell transcripts in CBL-treated B6.Il10−/− APC cocultures with CD25 CD4+ T cells from Vert-X mice (Il10−/−EGFP reporter, IL10-sufficient) and B cells from B6.WT or Il10−/− mice. The WT but not Il10−/− B cells significantly suppressed differentiation of CD25 CD4+ T cells into IFNγ+ and IL17+ cells (Figure 6A). Both WT and Il10−/− B cells significantly increased the percentage of Foxp3+ T cells (Figure 6A) and Foxp3 mRNA in reisolated T cells (Figure 6B) consistent with the up-regulation of intestinal Foxp3+ T cells in DKO mice that received WT or Il10−/− B cells compared with no B cells (Figure 1E and F). Il17a expression was lower in the presence of either WT or Il10−/− B cells whereas Ifng expression was decreased only in the presence of WT but not Il10−/− B cells (Figure 6B). Of considerable interest, WT but not Il10−/− B cells enhanced differentiation of naïve T cells into IL10-producing Tr-1 cells (Figure 6A) and up-regulated Il10 mRNA expression in reisolated CD4+ T cells (Figure 6B). The frequency of Tr-1 cells and IL10-secretion increased and IFNγ and IL17a-secretion decreased in the presence of increasing numbers of WT but not Il10−/− B cells (Figure 6C). Together, these data suggest that the regulatory capability of IL10-producing B cells is due in part to their ability to inhibit differentiation of certain effector T-cell subtypes and promote differentiation of discrete regulatory T cell subtypes, including Tr-1 cells.

Whether the observed regulatory features of IL10-producing B cells, including the expansion of Tr-1 cells,
was directly due to secreted IL10 or indirectly due to other factors remains unknown. Therefore, we quantified the development of Tr-1 cells in CBL-stimulated cocultures of CD25 CD4+ T cells from Vert-X mice, IL10−/− B cells and IL10−/− APC in the presence or absence of exogenous recombinant IL10. Adding increasing amounts of recombinant IL10 significantly decreased IFNγ and IL17a-secretion and increased Tr-1 cells (Figure 6D). These data indicate that B cell secretion of IL10 is capable of down-regulating IFNγ and IL17 and inducing Tr-1 cells.

Next, we wanted to determine whether IL10-producing B cells require physical cell contact with T cells to induce regulatory T cells and suppress inflammation. We cocultured naive CD4+ T cells from Vert-X mice and IL10−/− APC with or without B cells from WT or IL10−/− mice using transwell plates. Interestingly, WT B cells did not require cell contact with T cells for Tr-1 induction nor suppression of proinflammatory cytokines, indicating that IL10 secretion by B cells confers protection (Figure 7A).

**Figure 6.** Resident bacteria-activated interleukin-10 (IL10)-producing B cells promote T regulatory-1 (Tr-1) cell-induction and suppress T helper cell TH1/TH17 differentiation of naïve CD4+ T cells, in vitro. (A) IL10EGFP reporter Vert-X CD4+ T cells were cocultured with B6.WT (wild type) or IL10−/− (knockout) B cells plus B6.II10−/− antigen-presenting cell (APC) with cecal bacterial lysate (CBL) for 72 hours and percentages of IL17+, interferon-γ (IFNγ+), Foxp3+ CD4+ T cells, and Tr-1 (IL10+, Foxp3−) cells were analyzed by flow cytometry phorbol myristate acetate, ionomycin, and GolgiStop were added in the last 4 hours of cultures. (N = 9/group, three replicates.) (B) Transcript levels were evaluated in CD4+ T cells that were reisolated after 24 hours of coculture as described in (A). (N = 6/group, two replicates.) (C) Numbers of Tr-1 cells and cytokine concentrations in supernatants of CBL-stimulated cocultures containing WT CD4+ T cells, II10−/− APC and no B cells or increasing numbers of WT or II10−/− B cells are shown. (N = 6/group, two replicates.) (D) Frequency of Tr-1 cells and cytokine concentrations in supernatants of CBL-stimulated cocultures containing WT CD4+ T cells, II10−/− B cells and II10−/− APC with or without increasing concentrations of recombinant IL10. N = 6/group, two replicates; data presented as mean ± standard error. *P < .05; **P < .01; ***P < .001.
Because IL10-secreting B cells expand Tr-1 cells in vitro, we hypothesized that WT B cells are also associated with increased Tr-1 cells in vivo. To test this, we cotransferred CD25^+ CD4^+ T cells from Vert-X mice with or without B cells from WT or Il10^−/− mice into DKO recipients and quantified colonic and MLN Tr-1 cells and Foxp3^+ Treg. While WT, but not Il10^−/−, B cells significantly induced Tr-1 cells in the colonic LP and MLN, Foxp3^+ T cells were induced by B cells in an IL10-independent fashion (Figure 7B and C). These results suggest that physiologically activated IL10-producing B cells may attenuate colitis by selectively promoting the expansion of immunoregulatory IL10-producing Tr-1 cells in the intestine.

**Induction of T Regulatory-1 Cells by Interleukin-10-Secreting B Cells Requires T Cell Interleukin-27 Signaling**

Others have shown that IL27 inhibits differentiation of naïve T cells into Th17 cells and promotes the expansion of Tr-1 cells through up-regulating c-Maf and aryl hydrocarbon receptor in naïve T cells. Iwasaki et al. demonstrated that IL27-driven Tr-1 induction requires early growth response protein 2 (EGR-2)-mediated B-lymphocyte-induced maturation protein-1 (Blimp-1) induction. Because the presence of IL10-producing B cells in T-cell cocultures is associated with increased gene expression of Il10, Cmaf, Ahr, Blimp1, and Il21 in T cells (Figure 6B), we hypothesized that IL10-secreting B cells increase Tr-1 cells in an IL27-dependent manner. To test this, we cotransferred naïve T cells from WT or Il27ra^−/− mice with or without B cells from WT or Il10^−/− mice into B6 DKO mice. Interestingly, the ability of WT B cells to inhibit colitis was abrogated in the absence of IL27-signaling on CD4^+ T cells (Figure 8A). Spontaneous IFNγ and IL17a-secretion by colonic tissue explants was higher and IL10-secretion was lower in mice that received WT B cells along with Il27ra^−/−CD4^+ T cells versus WT T cells (Figure 8B). Moreover, WT B cells were unable to induce intestinal Tr-1 cells in the absence of IL27-signaling (Figure 8C and D). In vitro, neutralization of IL27 diminished the ability of WT B cells to suppress IFNγ-secretion by T cells and to induce Tr-1 cells (Figure 9A), corresponding with decreased Ahr and Il10-expression in reisolated T cells (Figure 9B). Moreover, CD25^+ CD4^+ T cells from Il27ra^−/− mice were unable to differentiate to Tr-1 cells and produced more IFNγ in the presence of IL10-secreting B cells (Figure 9C). These data suggest that the full ability of IL10-secreting B cells to stimulate Tr-1-development requires IL27-signaling in T cells.
Figure 8. Interleukin-10 (IL10)-producing B cells are unable to attenuate intestinal inflammation in the absence of T cell IL27-signaling, in vivo. 5 × 10⁵ B6.WT (wild-type) or Il27ra⁻/⁻ CD25 CD4⁺ T cells were cotransferred with or without 1 × 10⁶ B6. B cells from WT or Il10⁻/⁻ knockout (KO) mice into specific pathogen-free (SPF) B6.Rag2⁻/⁻ Il10⁻/⁻ mice. Six weeks later, colonic inflammation was quantified by total histologic score (A) and cytokine-secretion from colon explant cultures (B). Frequency of T regulatory-1 (Tr-1) and Foxp3⁺ CD4⁺ T-regulatory cells in mesenteric lymph nodes (MLN) and colon LP CD4⁺ T cells was assessed by flow cytometry (C, D). N = 8–9/group, two replicates; mean ± standard error. *P < .05; **P < .01.

Figure 9. Blockade of interleukin-27 (IL27) decreases IL10-secreting B-cell-mediated T regulatory-1 (Tr-1) induction in vitro. (A) Frequency of Tr-1 cells and cytokine concentrations in cecal bacterial lysate (CBL)-stimulated cocultures containing 2.5 × 10⁵ B6.WT (wild-type) CD25 CD4⁺ T cells, 5 × 10⁵ B6.WT or Il10⁻/⁻ B cells and 2.5 × 10⁵ B6.Il10⁻/⁻ antigen-presenting cells (APC) with the indicated blocking-antibodies (anti-IL27 or isotype control). N = 9/group, three replicates. (B) Transcript levels in CD4⁺ T cells that were reisolated after 24 hours from CBL-stimulated cocultures that contained WT CD25 CD4⁺ T cells, WT B cells, Il10⁻/⁻ APC, and the indicated blocking antibodies. (N = 6/group, two replicates.) (C) Frequency of Tr-1 cells and cytokine levels in CBL-stimulated cocultures containing 2.5 × 10⁵ B6.WT or Il27ra⁻/⁻ CD25⁻ CD4⁺ T cells, 5 × 10⁵ B6.WT or Il10⁻/⁻ B cells, and 2.5 × 10⁵ B6.Il10⁻/⁻ APC. N = 6/group, two replicates; mean ± standard error. *P < .05; ***P < .001.
To investigate whether various levels of IL27 were associated with differential Tr-1-induction and anti-inflammatory response by IL10-secreting B cells, naïve B6.WT T cells and Il10−/− APC were cocultured with or without B6.WT or Il10−/− B cells in the presence or absence of different doses of recombinant IL27. Increasing concentration of recombinant IL27 promoted IFNγ-secretion and decreased IL17a-secretion (Figure 10), whereas additional recombinant IL27 was not associated with further Tr-1-induction by WT B cells. These data indicate that low to moderate concentrations of IL27 are sufficient for IL10-secreting B cells to induce Tr-1 cells and confer protection whereas excessive IL27 enhances IFNγ-secretion that may exacerbate TH1-mediated intestinal inflammation.

Because IL27 is implicated in the development of Tr-1 cells, we sought to identify the cell-type that secretes IL27 in our in vitro coculture system. Although others have previously identified myeloid cells as the primary source of IL27,15 we detected IL27-secretion by CBL-stimulated B cells, with no difference between Il10−/− and WT cells (Figure 11A). We also found markedly higher levels of IL27 with cocultured Il10−/− APC and Il10−/− B cells, which was abrogated by recombinant IL10. Moreover, the addition of anti-IL10R-blocking antibody to cocultured WT B cells and WT APC enhanced IL27-secretion (Figure 11A). The IL27+ B cells, but not the IL27+ myeloid cells, were expanded in CBL-stimulated versus nonstimulated cocultures, suggesting that increased IL27 in the cultures containing Il10−/− B cells and Il10−/− APC was mainly produced by CBL-stimulated Il10−/− B cells (Figure 11B and C). Together, these data indicate that CBL induces B cells to secrete IL27, especially in the presence of APC and absence of IL10, and that IL10, regardless of the source, controls IL27 secretion.

**Discussion**

We demonstrate a key role for physiologically stimulated IL10-secreting B cells in mucosal immune homeostasis and provide a mechanism by which resident intestinal microbial components induce IL10-dependent regulatory immune responses that prevent intestinal inflammation. Our in vivo data show that IL10-producing B cells ameliorate T-cell-mediated experimental colitis depending on IL10-production by both cotransferring B cells and CD4+ T cells in the absence of IL10-producing APC.

Previous studies of regulatory B cells in experimental colitis have characterized subpopulations of B cells that secrete higher IL10 concentrations. For instance, intestinal CD11bhigh B cells produce IL10 and attenuate colitis.10 IL10-secreting splenic B10 cells ameliorate acute DSS-induced colitis,33 a model that does not require the presence of T cells and microbiota to develop colitis; and
peritoneal-derived IL10-producing B cells attenuate colitis in IL10\(^{-/-}\) mice and when cotransferred with CD45RB\(^{high}\) T cells into Rag2\(^{-/-}\) recipients. However, our unfractionated IL10-producing B cells were sufficient to suppress chronic colitis caused by bacteria-activated WT T cells in an IL10-deficient susceptible host. We found a relatively low percentage of B10 (CD1d\(^{high}\)CD5\(^{+}\)) phenotype among the MLN and colonic LP IL10-secreting B cells of recipient mice (data not shown). The anti-inflammatory B cells identified in our study likely have a broader phenotype than the small B10-subset and are most likely physiologically stimulated by resident microbiota in the recipient’s intestine to differentiate into resident IL10-secreting B cells or plasma cells that regulate inflammation.

Because several studies have suggested that regulatory B cells modulate inflammation by inducing Foxp3\(^{+}\) Treg through glucocorticoid-induced TNFR-related protein (GITR) or transforming growth factor-\(\beta\) (TGF-\(\beta\)), we expected that WT but not IL10\(^{-/-}\) B cells would attenuate inflammation by inducing intestinal Foxp3\(^{+}\) T cells. However, our in vivo and in vitro data show that B cells induce Foxp3\(^{+}\) T cells in an IL10-independent fashion as previously indicated, but IL10\(^{-/-}\) B cells were not fully protective in the absence of IL10-secreting APC. Thus, mechanisms by which WT but not IL10\(^{-/-}\) B cells suppressed mucosal inflammation required further explanation.

Although cotransferred CD25\(^{+}\)CD4\(^{+}\) Treg can ameliorate Th1/Th17-mediated colitis induced by naïve CD45RB\(^{high}\) T

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**Figure 11.** Bacterial lysates induce B cells to produce interleukin-27 (IL27) in the presence of antigen-presenting cell (APC). (**A**) IL27 concentrations in cecal bacterial lysate (CBL)-stimulated cocultures containing wild-type (WT) or Il10\(^{-/-}\) knockout (KO) APC and WT or Il10\(^{-/-}\) B cells, with 10 \(\mu\)g/mL of anti-IL10-receptor or isotype antibodies, or 10 ng/mL of recombinant IL10. (N = 9/group, three replicates; mean ± standard error. ***P < .001.) Representative figures (**B**) and cell numbers (**C**) for IL27\(^{+}\) (p28\(^{+}\)Ebi3\(^{+}\)) B cells and CD11b\(^{+}\) myeloid cells in CBL-stimulated or non-stimulated cocultures that contains WT B cells with WT APC or Il10\(^{-/-}\) APC with Il10\(^{-/-}\) B cells. N = 4/group, two replicates; mean ± standard error.
cells transferred into Rag−/− mice,29 our transferred unfractionated WT T cells (containing both naïve and Foxp3+CD25+CD4+ Treg) could not suppress colitis in Il10−/−Rag2−/− mice in the absence of IL10-producing B cells or IL10-secreting APC. Previous studies have implicated a requirement for protective IL10-producing myeloid cells that induce and maintain Foxp3+ Treg in T cell transfer colitis models.28,37 In our study, WT B cells ameliorated T cell-mediated colitis when cotransferred with WT but not Il10−/−CD4+ T cells, suggesting that IL10-producing B cells can replace IL10-secreting APC in activating protective IL10-producing regulatory CD4+ T cells to confer protection. Our in vitro transwell study demonstrated that IL10 secretion by B cells without cell-to-cell contact is sufficient to activate Tr-1 cells and suppress inflammatory cytokine production.

Several studies have demonstrated the capacity of B cells to activate Tr-1 cells. IL10-producing B cells induced Tr-1 cells in a rheumatoid arthritis model,38 and TLR2-B cells to activate Tr-1 cells. IL10-producing B cells induced Tr-1 cells and suppress inflammation in an IL27-signaling-dependent manner, but high concentrations of IL27, particularly in the presence of IL12, ameliorates colitis by multiple mechanisms, including IL10 and IL12p40 production.35 In conclusion, physiologically activated IL10-secreting B cells regulate mucosal inflammation by multiple mechanisms: suppressing effector APC:T cell activity, decreasing proinflammatory cytokine production, and expanding Tr-1 cells in association with immunosuppressive amounts of IL27 (Figure 12). B cells induce Foxp3+ Treg in an IL10-independent manner, but this is not sufficient to maximally protect against colitis. Overall, IL10-secreting B cells

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B cells is due to higher IL12 secretion by Il10−/− B cells. On the other hand, depleting Il10−/− B cells by anti-CD20-antibodies worsened mucosal inflammation in Il10−/− mice,45 and bacterial antigen-stimulated Il10−/− B cells have some protection against T-cell-mediated colitis,10 suggesting IL10-independent regulatory mechanisms of B cells. We likewise showed slight protection against colitis by Il10−/− B cells and IL10-independent induction of Foxp3+ Treg by B cells.

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have an important role in regulating T-cell function and ameliorating T-cell-mediated colitis. These findings provide new insights into the mechanisms of intestinal immune homeostasis and may provide new targets for IBD therapies.

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