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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

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

Yoshiyuki Mishima,[1] Bo Liu,[1,2] Jonathan J. Hansen,[1,3] and R. Balfour Sartor[1,3]

[1]*Center for Gastrointestinal Biology and Disease, Department of Medicine, Division of Gastroenterology and Hepatology, University of North Carolina, Chapel Hill, North Carolina;* [2]*Institute of Zoonesis, College of Animal Science and Veterinary Medicine, Jilin University, Changchun, People's Republic of China;* [3]*Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina*

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 unfractionated, 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 T_H1/T_H17-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 both 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)*

Keywords: Experimental Colitis; IBD; Immunoregulation; Regulatory B Cells.

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 ($Foxp3^{neg}$), 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 $CD45RB^{high}$ T cells,[5] and enteric bacterial antigen-specific Tr-1 cells ameliorate colitis induced by pathogenic T helper 1 (T_H1) 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 IBD[9] and potentiate murine experimental colitis,[10] suggesting that B cells are protective in IBD. However, the

Abbreviations used in this paper: APC, antigen-presenting cell; Blimp-1, B-lymphocyte-induced maturation protein-1; CBL, cecal bacterial lysate; DKO, double-knockout; ELISA, enzyme-linked immunosorbent assay; Foxp3, forkhead box P3; GF, germ-free; HBSS, Hanks' balanced salt solution; IFN, interferon; IL, interleukin; IL10R, interleukin-10 receptor; LP, lamina propria; LPL, lamina propria lymphocytes; MLN, mesenteric lymph nodes; PBS, phosphate-buffered saline; Rag2, recombination-activating gene 2; SPF, specific pathogen-free; T_H, T helper cell; Tr-1, T regulatory-1; Treg, T regulatory; UNC, University of North Carolina; WT, wild-type.

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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.[11] IL10-deficient (*Il10*$^{-/-}$) and IL10-receptor-deficient mice develop resident enteric bacteria-dependent T_H1/T_H17-mediated colitis.[12] 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.[9,10,13,14] 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 T_H1 cell development,[15] but it can also suppress the development of T_H17 cells by decreasing *Rorγt* gene expression and increasing the number of IL10-producing Tr-1 cells but not Foxp3$^+$ Treg.[16–20] IL27 promotes the expansion of Tr-1 cells by up-regulating c-Maf and aryl hydrocarbon receptor in naïve T cells.[20] There is conflicting evidence regarding the role of IL27 in colitis, with some studies showing that IL27 has proinflammatory effects[21–23] and others demonstrating that IL27 is anti-inflammatory.[24–26] Thus, IL27 likely plays a pivotal role in regulating the delicate balance between proinflammatory T_H1/T_H17 cells and anti-inflammatory IL10-producing T-cell populations in the intestine. However, it is unknown whether IL10-producing mucosal B cells affect the regulatory or proinflammatory functions of IL27.

This study addresses the mechanisms by which IL10-secreting B cells influence regulatory T-cell differentiation and ameliorate T-cell-mediated colitis. We show that IL10-producing B cells suppress wild-type (WT) but not *Il10*$^{-/-}$CD4$^+$ T-cell-mediated colitis and are associated with increased frequency of intestinal Tr-1 cells and decreased T_H1/T_H17 cytokine profiles. Moreover, we demonstrate that regulatory functions of IL10-secreting B cells are mediated by IL27-signaling in T cells. Together, these findings elucidate novel regulatory mechanisms of IL10-secreting B cells, help explain mechanisms of mucosal homeostasis, and could be exploited to treat IBD.

Materials and Methods

Mice

We purchased C57BL/6 (B6).WT, B6.*Il10*$^{-/-}$, B6.*Rag2*$^{-/-}$, and B6.*Il27ra (WSX-1)*$^{-/-}$ mice from Jackson Laboratories (Bar Harbor, ME). We purchased 129S6/SvEv (129).WT mice from Taconic Farms (Germantown, NY). The 129.*Il10*$^{-/-}$ mice were obtained from Dr Donna Rennick (DNAX Laboratories, Palo Alto, CA). The *Il10*$^{-/-}$*Rag2*$^{-/-}$ double-knockout (DKO) mice were generated by crossing B6.*Il10*$^{-/-}$ with B6.*Rag2*$^{-/-}$ mice or 129.*Il10*$^{-/-}$ with 129.*Rag2*$^{-/-}$ mice. The B6.*Il10*$^{+/EGFP}$ reporter (Vert-X) mice were obtained from Dr Christopher Karp.[27] 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, *Il10*$^{-/-}$, 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.

Cell Isolation

Mononuclear cells were isolated from the colon lamina propria (LP), mesenteric lymph nodes (MLN), and spleen, as described elsewhere.[13] The MLN were pressed through 70-μm filters into phosphate-buffered saline (PBS) with 2.5% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA). Spleens were mechanically dissociated, and the red blood cells were lysed with red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO). For isolation of colonic LP lymphocytes (LPL), the large intestines were washed with cold PBS, opened longitudinally, and cut into 10-mm pieces. Then the intestines were incubated in 1 mM dithiothreitol (Sigma-Aldrich) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS; Gibco/Invitrogen) for 15 minutes at room temperature. Next, the tissues were incubated in 1 mM EDTA (Sigma-Aldrich) in HBSS for 20 minutes at 37°C with shaking, which was repeated after a thorough washing. The cell suspensions were removed, and the remaining fragments were transferred to flasks containing HBSS with 1 mg/mL of collagenase type 3 (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco/Invitrogen), then stirred gently for 60 minutes at 37°C. The cell suspensions containing LPL were filtered through a nylon mesh and centrifuged, then the LPL were purified using a 44%–70% discontinuous Percoll gradient (GE Healthcare, Buckinghamshire, UK). After centrifugation at 800*g* for 20 minutes at 22°C, the mononuclear cells were collected from the interface.

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, unfractionated 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$^+$).

Table 1. Polymerase Chain Reaction Primers Used in this Study

Gene	Sense 5′-3′	Antisense 5′-3′	Reference
Il27p28	GGCCAGGTGACAGGAGACC	CAGCTTGTACCAGAAGCAAGGG	Collison et al[41]
Ebi3	AGCAGCAGCCTCCTAGCCT	ACGCCTTCCGGAGGGTC	Collison et al[41]
Il12a	TGGCTACTAGAGAGACTTCTTCCACAA	GCACAGGGTCATCATCAAAGAC	Collison et al[41]
Il23a	ATCCAGTGTGAAGATGGTTGTGA	GCAAGCAGAACTGGCTGTTG	Collison et al[41]
Il17a	CTCCAGAAGGCCCTCAGACTAC	AGCTTTCCCTCCGCATTGACACAG	Qu et al[42]
Il6	CCGGAGAGGAGACTTCACAG	TCCACGATTTCCCAGAGAAC	Bao et al[43]
Cmaf	AAATACGAGAAGCTGGTGAGCAA	CGGGAGAGGAAGGGTTGTC	Hiramatsu et al[44]
Ahr	ACATCACCTATGCCAGCCGC	TCTGTGTCGCTTAGAAGGAT	Wurster et al[45]
Il21	AAGATTCCTGAGGATCCGAGAAG	GCATTCGTGAGCGTCTATAGTGTC	Wurster et al[45]
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT	Wan and Flavell[46]
Rorgt	TGAGGCCATTCAGTATGTGG	CTTCCATTGCTCCTGCTTTC	Ano et al[47]
Tbet	TTCCCATTCCTGTCCTTCAC	CCACATCCACAAACATCCTG	Ano et al[47]
Actb	AGCCATGTACGTAGCCATCCAG	TGGCGTGAGGGAGAGCATAG	Liu et al[28]
Blimp1	TTCTTGTGTGGTATTGTCGGGACTT	TTGGGGACACTCTTTGGGTAGAGTT	Miyauchi et al[48]
Il12b	CGCAAGAAAGAAAAGATGAAGGAG	TTGCATTGGACTTCGGTAGATG	Made ourselves
Ifng	CTTCCTCATGGCTGTTTCTGG	CGCTTATGTTGTTGCTGATGG	Made ourselves
Il10	GTCATCGATTTCTCCCCTGTG	CCTTGTAGACACCTTGGTCTTGG	Made ourselves

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 ware 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 of protein. We used B6.CBL and 129.CBL in the culture medium with the cells purified from B6 and 129 mice, respectively.

B Cell, T Cell, and Antigen-Presenting Cell Cultures

The 2.5 × 10^5 unfractionated $CD4^+$ T cells isolated from 129.WT or *Il10*$^{-/-}$ mice were cocultured with 5 × 10^5 129.WT or *Il10*$^{-/-}$ B cells along with 2 × 10^5 129.*Il10*$^{-/-}$ APC at 200 μL/well (96-well plates) for 72 hours at 37°C with 5% CO_2. In some experiments, 5 × 10^5 naïve WT $CD25^-$ with or without 2.5 × 10^5 $CD25^+CD4^+$ T cells from 129.WT or *Il10*$^{-/-}$ mice were cocultured with or without 1 × 10^6 129.WT or *Il10*$^{-/-}$ B cells along with 2 × 10^5 129.*Il10*$^{-/-}$ 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

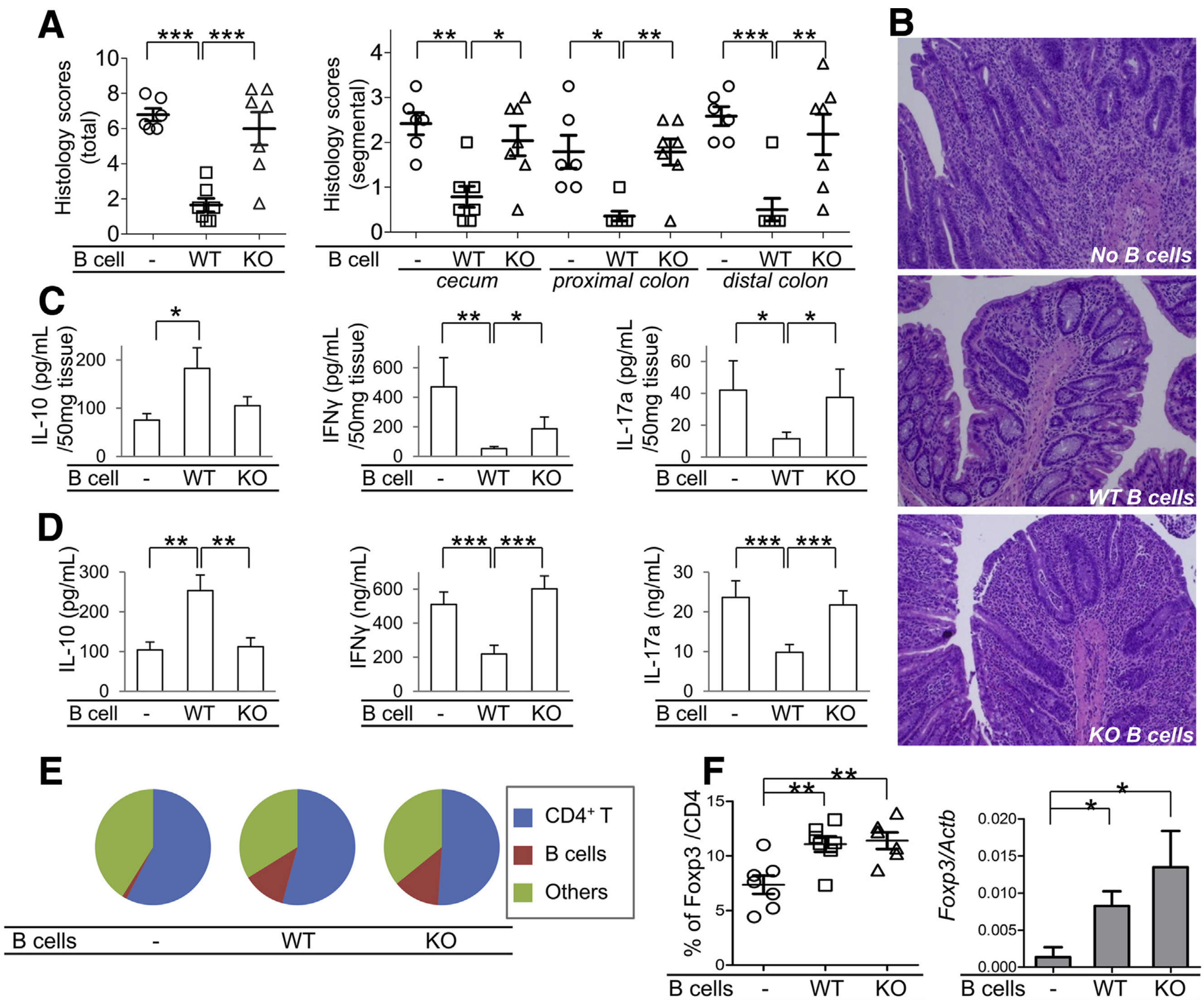

Figure 1. IL10-secreting B cells ameliorate intestinal inflammation. The 5×10^5 129.WT (wild type) unfractionated $CD4^+$ T cells were cotransferred with or without 1×10^6 B cells from 129.WT or *Il10*$^{-/-}$ knockout (KO) mice into specific pathogen-free (SPF) 129.*Rag2*$^{-/-}$*Il10*$^{-/-}$ mice. Six weeks later, colonic inflammation was quantified by (*A*) histology (total and segmental scores), photomicrographs (*B*), and cytokine-secretion from unstimulated colon explant cultures (*C*) and from cecal bacterial lysate (CBL)-stimulated mesenteric lymph nodes (MLN) cell cultures (*D*). MLN cells were evaluated for (*E*) the reconstitution of $CD4^+$ T cells ($CD3^+CD4^+$) and B cells ($CD19^+B220^+$) and (*F*) the frequency of $Foxp3^+CD4^+$ T-regulatory cells by flow cytometry. mRNA level of *Foxp3* in the distal colon was assessed by real-time polymerase chain reaction. N = 6–7/group, two replicates. Data are presented as mean ± standard error. **P* < .05; ***P* < .01; ****P* < .001.

B6.*Il10*$^{-/-}$ APC. In certain experiments, 2.5×10^5 APC from B6 (*Il10*$^{+/+}$) *Rag2*$^{-/-}$ or *Il10*$^{-/-}$*Rag2*$^{-/-}$ mice were cocultured without or with 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 in triplicate culture supernatants by comparison with 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, and Foxp3 (eBioscience), 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 with a fixation and permeabilization solution kit (BD Bioscience) according to the manufacturer's instructions. For intracellular cytokine 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 T_H1/T_H17-mediated colitis by 6 weeks as assessed by histologic scores (Figure 1*A* and 1*B*) and spontaneous IFNγ and IL17a-secretion by colonic tissue explants (Figure 1*C*); 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 1*C*). 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 1*D*).

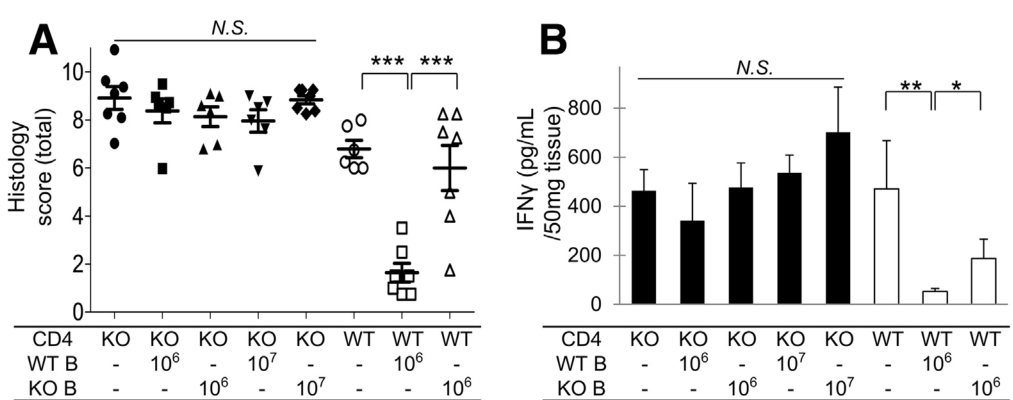

Figure 2. IL10-secreting B cells regulate intestinal inflammation in the presence of T-cell-derived IL10. 5×10^5 CD4$^+$ T cells from SPF 129.WT or *Il10*$^{-/-}$ knockout (KO) mice were cotransferred with or without different numbers of B cells from 129.WT or *Il10*$^{-/-}$ mice into SPF 129.*Rag2*$^{-/-}$*Il10*$^{-/-}$ 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 1*E*). 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 1*F*). 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 2*A* 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 3*A* 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 preexistence of inflammation in the *Il10*$^{-/-}$ donor mice (Figure 3*A* and *B*).

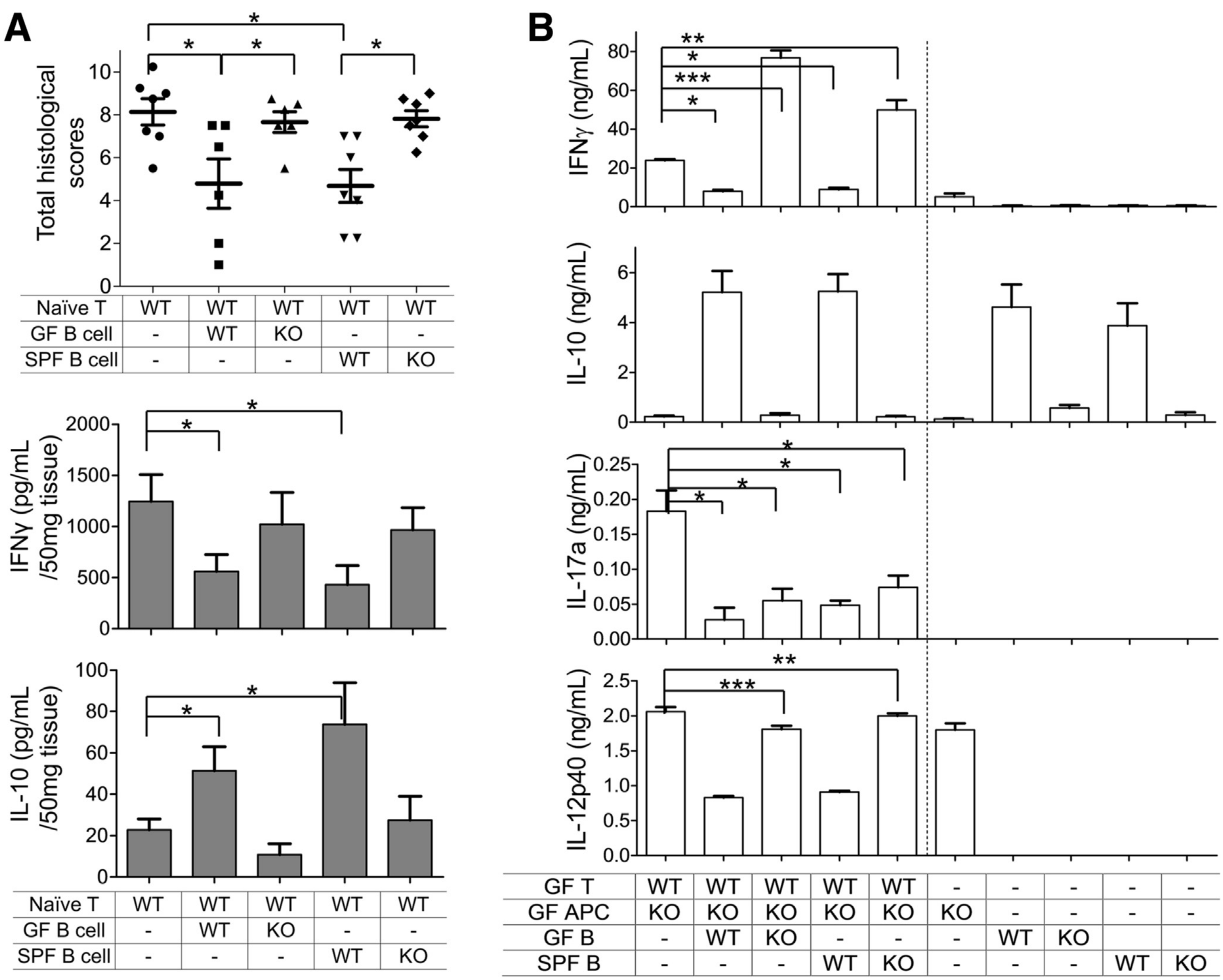

Figure 3. Germ-free (GF) wild-type (WT) B cells have anti-inflammatory properties similar to specific pathogen-free (SPF) WT B cells, but GF *Il10*$^{-/-}$ B cells are unable to regulate intestinal inflammation. (*A*) In vivo, 5×10^5 splenic CD25$^-$CD4$^+$ T cells from GF B6.WT mice were cotransferred with or without 1×10^6 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, 2.5×10^5 splenic CD25$^-$CD4$^+$ T cells from GF B6.WT mice were cocultured with or without 5×10^5 B cells from B6.WT or *Il10*$^{-/-}$ mice housed in GF or SPF conditions along with 2×10^5 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 4*A*). 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 *Il10*$^{-/-}$ or no B cells (Figure 4*A* and 4*B*). 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 *Il10*$^{-/-}$ T cells (Figure 4*A* 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 *Il10*$^{-/-}$ APC, whereas exogenous recombinant-IL10 decreased IFNγ and IL17a-secretion in the cultures containing *Il10*$^{-/-}$CD4$^+$ T cells, *Il10*$^{-/-}$ B cells with *Il10*$^{-/-}$ 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

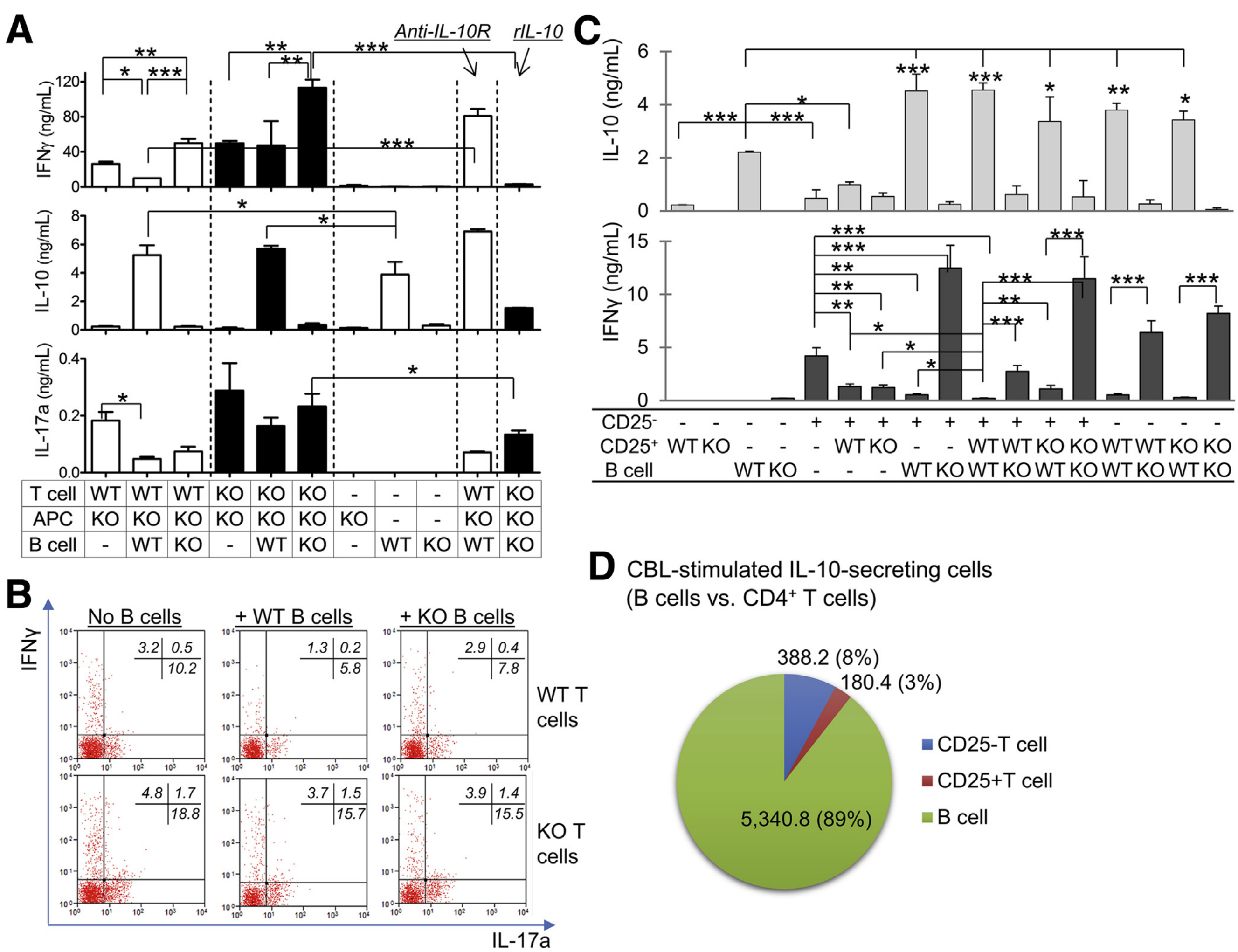

Figure 4. Cecal bacteria–stimulated interleukin-10 (IL10)-secreting B cells suppress proinflammatory cytokines to a similar degree as CD25$^+$CD4$^+$ T-regulatory cells in vitro. This protection is eliminated by interleukin-10 (IL10)-signaling blockade. Cytokine secretion was measured in cecal bacterial lysate (CBL)-stimulated cultures containing 2.5 $\times$ 10^5 unfractionated 129.WT or *Il10*$^{-/-}$ KO (knockout) CD4$^+$ T cells, 5 $\times$ 10^5 129.WT (wild type) or *Il10*$^{-/-}$ B cells along with 2.5 $\times$ 10^5 129.*Il10*$^{-/-}$ 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 *Il10*$^{-/-}$ B cells, and WT or *Il10*$^{-/-}$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 $\pm$ standard error. *$P < .05$; **$P < .01$; ***$P < .001$.

IL17a-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 $CD25^+CD4^+$ 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 $CD25^+CD4^+$ Treg. We measured cytokines in CBL-stimulated cocultures of naïve $CD25^-CD4^+$ 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 $CD25^+CD4^+$ 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 4*C*). The majority of IL10-positive cells in the cultures containing WT $CD25^-CD4^+$, WT $CD25^+CD4^+$, and WT B cells were B cells (Figure 4*D*). 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 4*C*). 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 4*C*). These data suggest that IL10-producing B cells inhibit T_H1 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 T_H17-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 $Foxp3^+$ or IL10-producing ($Foxp3^{neg}$) 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 $IFN\gamma^+$ and $IL17^+$ cells (Figure 6*A*). Both WT and *Il10*$^{-/-}$ B cells significantly increased the percentage of $Foxp3^+$ T cells (Figure 6*A*) and *Foxp3* mRNA in reisolated T cells (Figure 6*B*) 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 1*E* 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 6*B*). Of considerable interest, WT

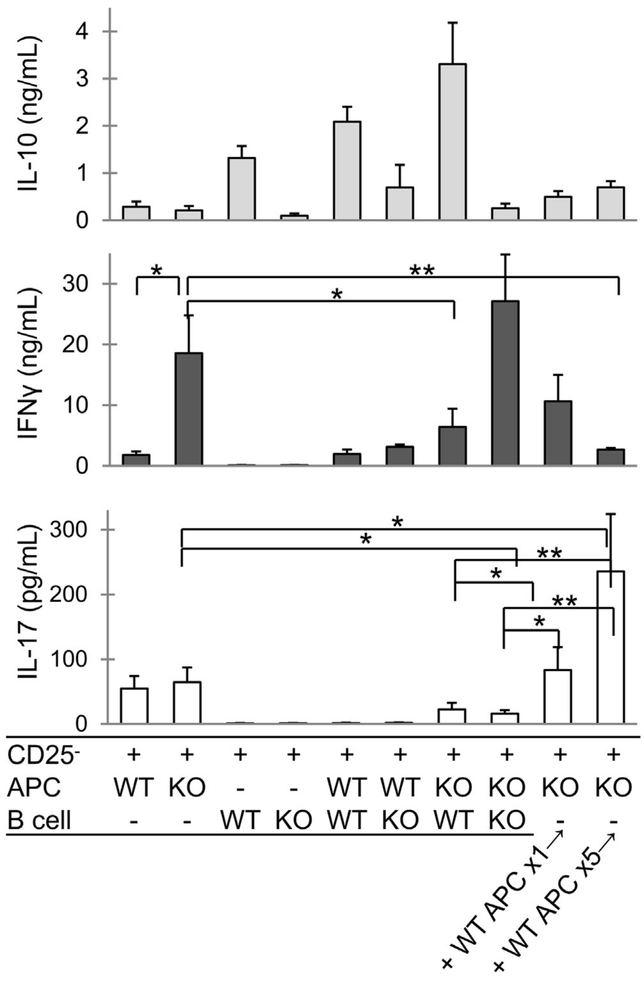

Figure 5. Interleukin-10 (IL10)-producing B cells have greater ability to suppress IL17a than IL10-producing antigen-presenting cells (APC). We cocultured 5 × 10^5 splenic B6.WT (wild-type) $CD25^-CD4^+$ T cells with and without 1 × 10^6 B cells from B6.WT or *Il10*$^{-/-}$ knockout (KO) mice along with 2 × 10^5 B6.WT or *Il10*$^{-/-}$ APC. In parallel, 2 × 10^5 (×1) or 1 × 10^6 (×5) WT APC were added to the cultures of WT $CD25^-CD4^+$ T cells and *Il10*$^{-/-}$ APC. Cells were stimulated with cecal bacterial lysate for 72 hours and supernatant levels of IL10, interferon-γ (IFNγ) and IL17a were measured by enzyme-linked immunosorbent assay (ELISA). N = 9/group, three replicates; mean ± standard error. **P* < .05; ***P* < .01.

but not *Il10*$^{-/-}$ B cells enhanced differentiation of naïve T cells into IL10-producing Tr-1 cells (Figure 6*A*) and up-regulated *Il10* mRNA expression in reisolated $CD4^+$ T cells (Figure 6*B*). 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 6*C*). 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,

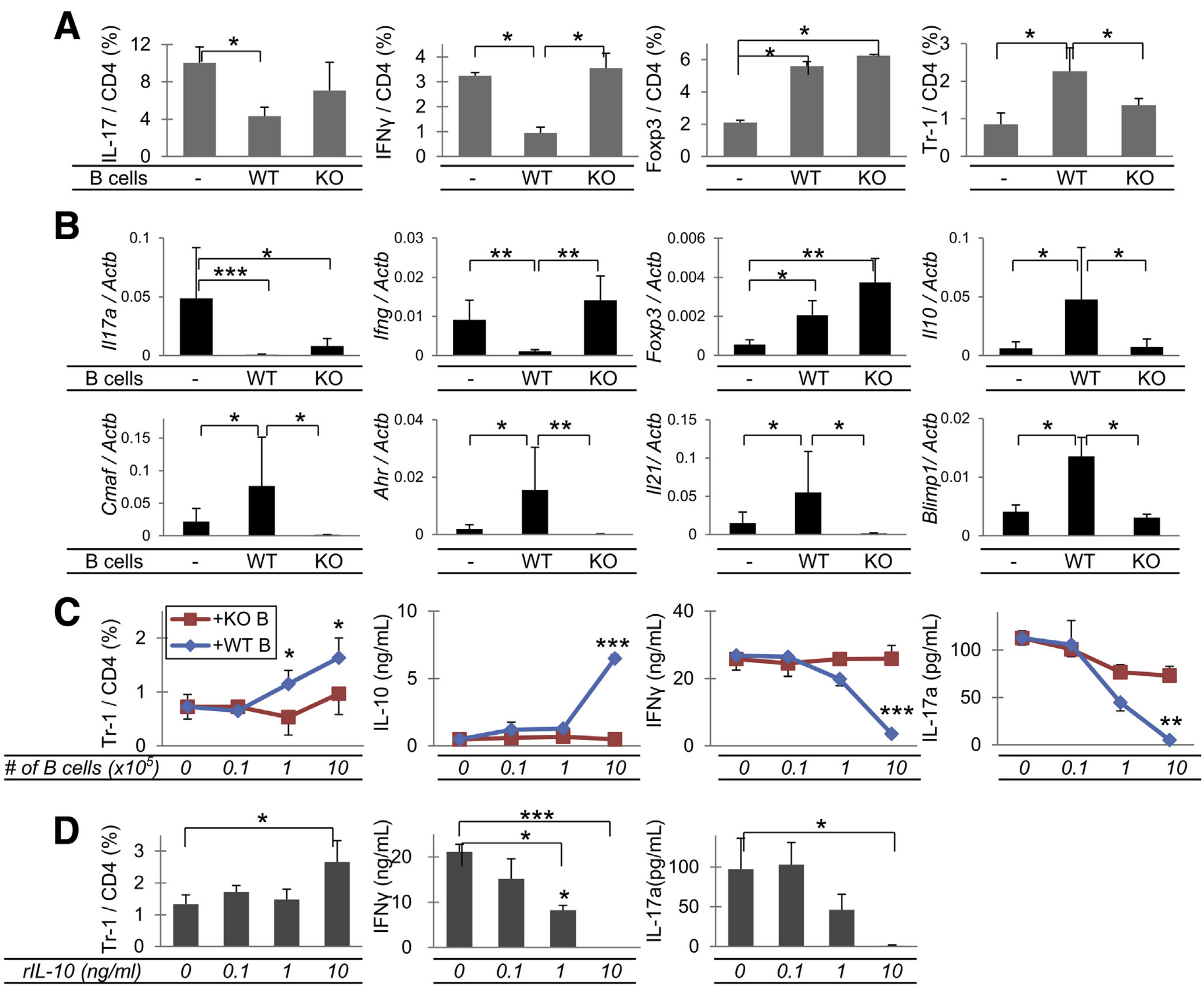

Figure 6. Resident bacteria-activated interleukin-10 (IL10)-producing B cells promote T regulatory-1 (Tr-1) cell-induction and suppress T helper cell T_H1/T_H17 differentiation of naïve $CD4^+$ T cells, in vitro. (*A*) *Il10*^{EGFP} reporter Vert-X $CD4^+$ T cells were cocultured with B6.WT (wild type) or *Il10*$^{-/-}$ (knockout) B cells plus B6.*Il10*$^{-/-}$ antigen-presenting cell (APC) with cecal bacterial lysate (CBL) for 72 hours and percentages of $IL17^+$, interferon-γ^+ ($IFN\gamma^+$), $Foxp3^+CD4^+$ T cells, and Tr-1 ($IL10^+$, $Foxp3^{neg}$) 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, *Il10*$^{-/-}$ APC and no B cells or increasing numbers of WT or *Il10*$^{-/-}$ 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, *Il10*$^{-/-}$ B cells and *Il10*$^{-/-}$ 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.

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 6*D*). 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 naïve $CD4^+$ 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 7*A*).

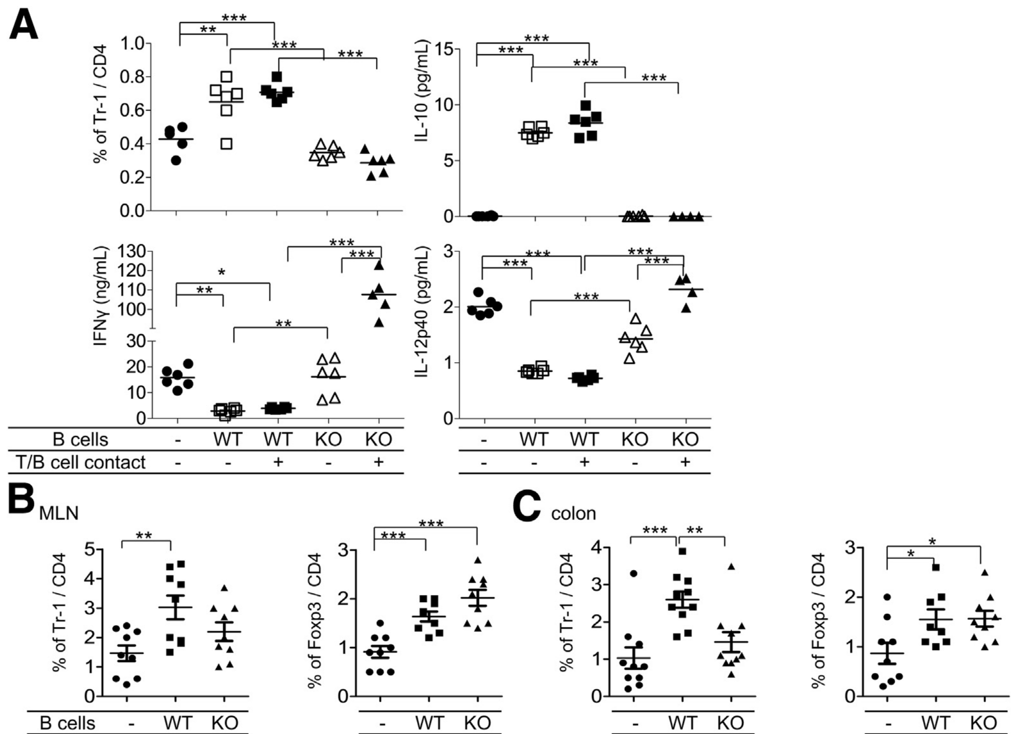

Figure 7. Interleukin-10 (IL10)-producing B cells do not require physical cell contact with T cells to induce T regulatory-1 (Tr-1) cells and regulate inflammation. (*A*) 5 × 10^5 naïve Vert-X $CD4^+$ T cells and 5 × 10^5 B6.*Il10*$^{-/-}$ antigen-presenting cell (APC) (in the lower chamber) were cocultured without or with B6.WT (wild-type) or *Il10*$^{-/-}$ KO (knockout) B cells (either in the upper or lower chamber) with cecal bacterial lysate (CBL) for 72 hours. (N = 5–6/group, two replicates; mean ± SD. **P* < .05, ***P* < .01.) (*B*, *C*) IL10-producing B cells promote Tr-1 cell-induction in vivo. We cotransferred 5 × 10^5 WT naive Vert-X $CD4^+$ T cells with or without 1 × 10^6 B6. B cells from WT or *Il10*$^{-/-}$(KO) mice into SPF B6.*Rag2*$^{-/-}$*Il10*$^{-/-}$ mice. Three weeks later, the frequency of Tr-1 and $Foxp3^+CD4^+$ T-regulatory cells in mesenteric lymph nodes (MLN) (*B*) and colon lamina propria (LP) (*C*) from *Rag2*$^{-/-}$*Il10*$^{-/-}$ mice were assessed by flow cytometry ($CD4^+$ cells gated). N = 8–9/group, two replicates; mean ± standard error. ***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 7*B* 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 T_H17 cells and promotes the expansion of Tr-1 cells through up-regulating c-Maf and aryl hydrocarbon receptor in naïve T cells.[20] Iwasaki et al[32] 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 6*B*), 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 8*A*). 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 8*B*). Moreover, WT B cells were unable to induce intestinal Tr-1 cells in the absence of IL27-signaling (Figure 8*C* 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 9*A*), corresponding with decreased *Ahr* and *Il10*-expression in reisolated T cells (Figure 9*B*). 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 9*C*). These data suggest that the full ability of IL10-secreting B cells to stimulate Tr-1-development requires IL27-signaling in T cells.

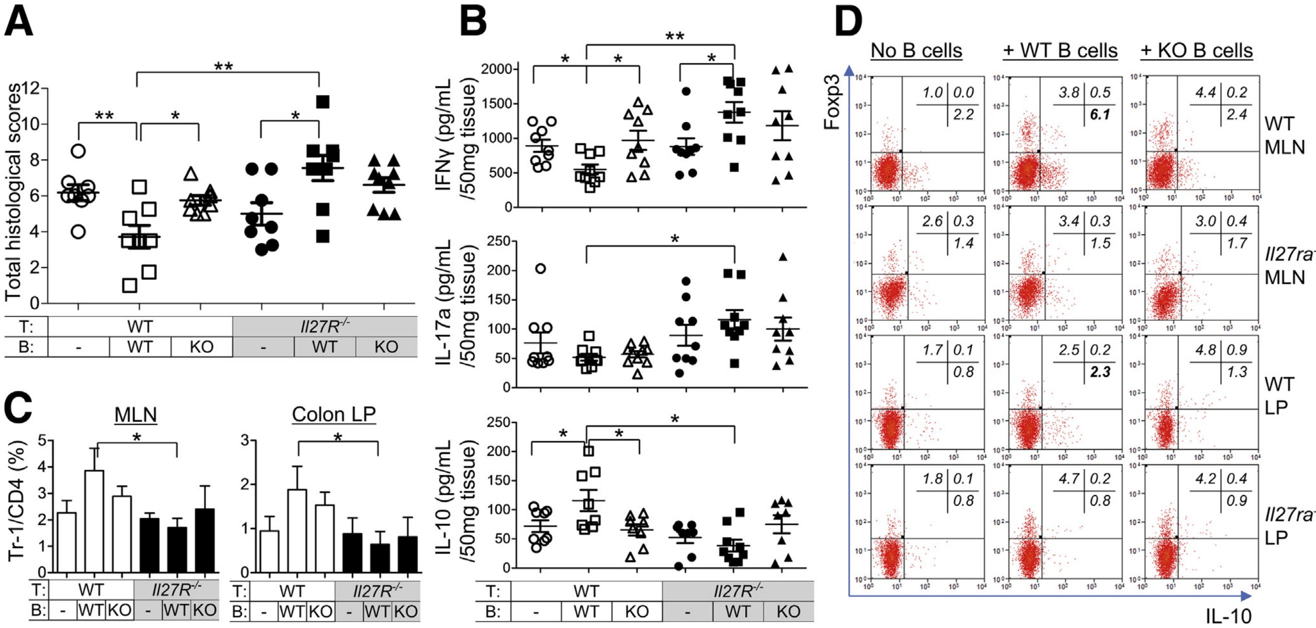

Figure 8. Interleukin-10 (IL10)-producing B cells are unable to attenuate intestinal inflammation in the absence of T cell IL27-signaling, in vivo. 5×10^5 B6.WT (wild-type) or *Il27ra*$^{-/-}$CD25$^-$CD4$^+$ T cells were cotransferred with or without 1×10^6 B6. B cells from WT or *Il10*$^{-/-}$ knockout (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. *$P < .05$; **$P < .01$.

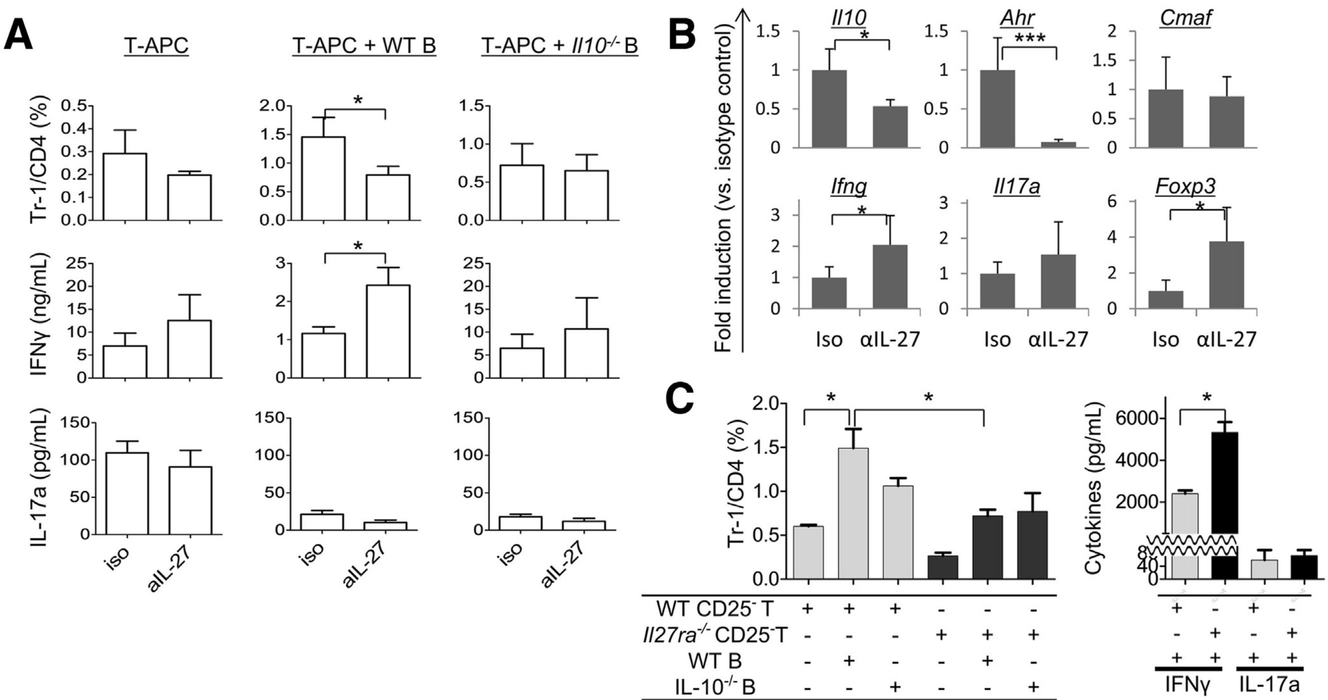

Figure 9. Blockade of interleukin-27 (IL27) decreases IL10-secreting B-cell-mediated T regulatory-1 (Tr-1) induction in vitro. (*A*) Frequency of Tr-1 cells and cytokine concentrations in cecal bacterial lysate (CBL)-stimulated cocultures containing 2.5×10^5 B6.WT (wild-type) CD25$^-$CD4$^+$ T cells, 5×10^5 B6.WT or *Il10*$^{-/-}$ B cells and 2.5×10^5 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 2.5×10^5 B6.WT or *Il27ra*$^{-/-}$CD25$^-$CD4$^+$ T cells, 5×10^5 B6.WT or *Il10*$^{-/-}$ B cells, and 2.5×10^5 B6.*Il10*$^{-/-}$ APC. N = 6/group, two replicates; mean ± standard error. *$P < .05$; ***$P < .001$.

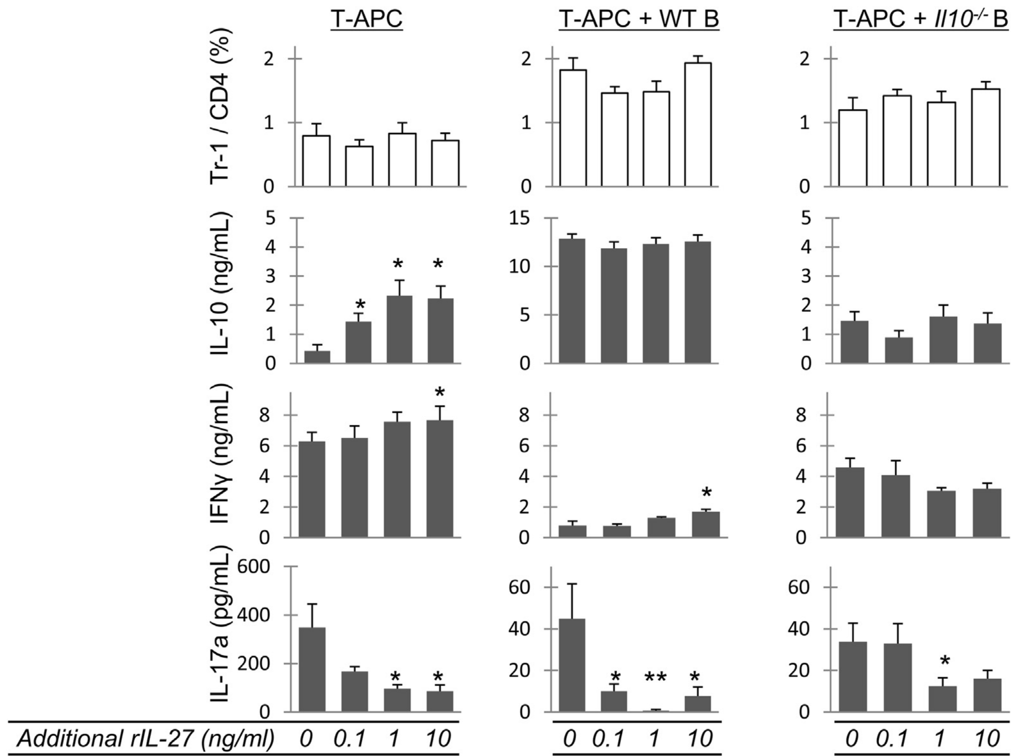

Figure 10. Added interleukin-27 (IL27) promotes T helper cell 1 (T_H1)-mediated immune responses without affecting wild-type (WT) B cell-induced T regulatory-1 (Tr-1) cells. 0, 0.1, 1, and 10 ng/mL of recombinant murine IL27 were added to the cecal bacterial lysate (CBL)-stimulated cell cultures containing 2.5×10^5 $CD25^-CD4^+$ T cells from Vert-X mice, 5×10^5 B cells from B6.WT or *Il10*$^{-/-}$ mice plus 5×10^5 B6.*Il10*$^{-/-}$ APC. Tr-1 induction was assessed by flow cytometry and IL10, IFNγ, and IL17a supernatant levels were measured by enzyme-linked immunosorbent assay (ELISA). N = 9/group, three replicates; mean ± standard error. $^*P < .05$ (vs no recombinant IL27).

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 T_H1-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 11*A*). 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 11*A*). 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 11*B* 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 cotransferred 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 $CD1d^{high}$ 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

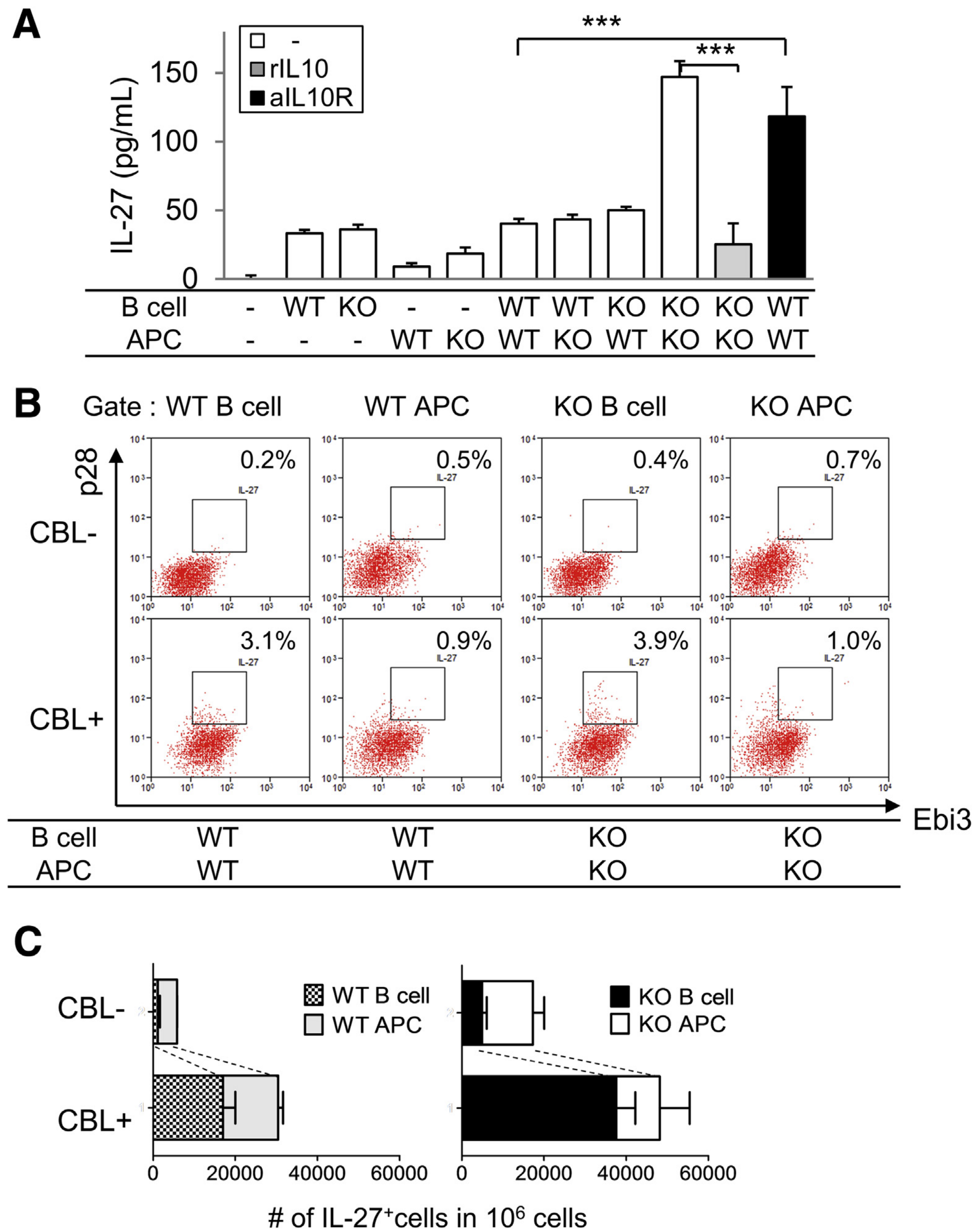

Figure 11. Bacterial lysates induce B cells to produce interleukin-27 (IL27) in the presence of antigen-presenting cell (APC). (*A*) IL27 concentrations in cecal bacterial lysate (CBL)-stimulated cocultures containing wild-type (WT) or $Il10^{-/-}$ knockout (KO) APC and WT or $Il10^{-/-}$ B cells, with 10 μg/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.

peritoneal-derived IL10-producing B cells attenuate colitis in $Il10^{-/-}$ mice and when cotransferred with $CD45RB^{high}$ T cells into $Rag2^{-/-}$ recipients.[34] 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-β (TGF-β),[35,36] 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,[35] 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 T_H1/T_H17-mediated colitis induced by naïve $CD45RB^{high}$ T 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-activated B cells regulated *Helicobacter*-induced gastritis by inducing $CD25^+CD4^+$ Tr-1-like cells.[39] These studies addressed the importance of IL10-producing B cells to control effector and regulatory T cells. Therefore, we focused on the key mechanisms by which IL10-producing but not $Il10^{-/-}$ B cells induced Tr-1 cells in IBD.

Key findings of our study were that physiologically stimulated IL10-secreting B cells induce mucosal Tr-1 cells in an IL27-signaling-dependent manner and decrease T_H1/T_H17 immune responses. The biologic role of IL27 in IBD remains uncertain because IL27 has been implicated as both a proinflammatory and anti-inflammatory cytokine.[22–27] However our data suggest that these pleiotropic protective and inductive effects may be due to different timing or concentrations of IL27 and variable presence of IL10 in the various colitis models. B cells appear to optimally control the regulatory functions of T cells by secreting IL10 with small amounts of IL27, but high concentrations of IL27, particularly in the absence of IL10, enhance IFNγ production.

Because IL10-producing myeloid cells can also induce Tr-1 cells and regulate effector T cells,[30,31] we compared the functional capacities of IL10-producing B cells with APC and found that WT B cells have a greater ability to induce Tr-1 cells than comparable myeloid APC (data not shown). Of note, although WT B cells suppress both IFNγ and IL17, WT APC decreased IFNγ but paradoxically enhanced IL17-secretion, despite their IL10-secreting capacity. Thus, IL10-secreting B cells appear to be more efficient immunoregulatory cells than myeloid WT APC in that they suppress both T_H1 and T_H17-mediated immune reactions. These data indicate that IL10 from several APC sources (myeloid and B cells) activate Tr-1 cells and that IL10-secreting B cells and myeloid cells differentially regulate effector T-cell subsets.

Our in vitro data show that IL10-deficient B cells have pleiotropic effects on CBL-stimulated $CD4^+$ T cells, decreasing IL17 while promoting IFNγ production. This effect is consistent with increased IL12p40 production by CBL-stimulated $Il10^{-/-}$ B cells versus WT B cells, suggesting that the observed increased IFNγ in the presence of $Il10^{-/-}$ 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,[35] and bacterial antigen-stimulated $Il10^{-/-}$ B cells have some protection against T-cell-mediated colitis,[40] 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.

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

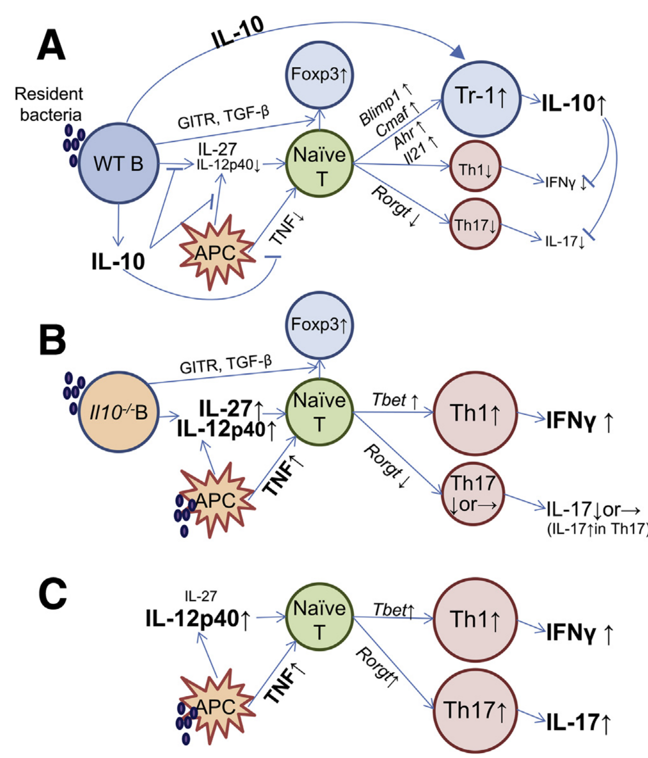

Figure 12. Proposed regulatory mechanisms of interleukin-10 (IL10)-producing B cells. Proposed regulatory mechanisms of wild-type (WT) B cells (*A*) compared with $Il10^{-/-}$ B cells (*B*) or antigen-presenting cell (APC) and T-cell subsets in the absence of B cells (*C*). B cells regulate mucosal inflammation by multiple mechanisms, including IL10 secretion-dependent 1) direct suppression of APC proinflammatory cytokines, 2) decreasing the proinflammatory cytokines from effector T cells, and 3) stimulating Tr-1 cells in collaboration with low to moderate concentrations of IL27 as well as IL10-independent induction of $Foxp3^+$ T-regulatory cells mediated by glucocorticoid-induced TNFR-related protein (GITR) or transforming growth fractor-β (TGF-β). In the absence of IL10 production, B cells produce higher concentrations of IL27 and IL12 p40 and activate T helper cells (T_H1) to secrete interferon-γ (IFNγ), even though they can include $Foxp3^+$ T-regulatory cells (*B*).

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.

References

1. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature 2011; 474:307–317.
2. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577–594.
3. Gregori S, Goudy KS, Roncarolo MG. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. Front Immunol 2012;3:30.
4. Shale M, Schiering C, Powrie F. $CD4^+$ T-cell subsets in intestinal inflammation. Immunol Rev 2013;252:164–182.
5. Izcue A, Coombes J, Powrie F. Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol 2009; 27:313–338.
6. Cong Y, Weaver CT, Lazenby A, et al. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. J Immunol 2002;169:6112–6119.
7. Lund FE, Randall TD. Effector and regulatory B cells: modulators of $CD4^+$ T cell immunity. Nat Rev Immunol 2010;10:236–247.
8. Eynon EE, Parker DC. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. J Exp Med 1992;175:131–138.
9. Goetz M, Atreya R, Ghalibafian M, et al. Exacerbation of ulcerative colitis after rituximab salvage therapy. Inflamm Bowel Dis 2007;13:1365–1368.
10. Mizoguchi A, Mizoguchi E, Takedatsu H, et al. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity 2002;16:219–230.
11. Fuss IJ, Boirivant M, Lacy B, et al. The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis. J Immunol 2002;168:900–908.
12. Sellon R, Tonkonogy S. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun 1998;66:5224–5231.
13. Mishima Y, Ishihara S, Aziz MMM, et al. Decreased production of interleukin-10 and transforming growth factor-β in Toll-like receptor-activated intestinal B cells in SAMP1/Yit mice. Immunology 2010;131:473–487.
14. Fassi DEl, Nielsen CH, Kjeldsen J, et al. Ulcerative colitis following B lymphocyte depletion with rituximab in a patient with Graves' disease. Gut 2008;57:714–715.
15. Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive $CD4^+$ T cells. Immunity 2002;16:779–790.
16. Carrier Y, Whitters MJ, Miyashiro JS, et al. Enhanced GITR/GITRL interactions augment IL-27 expression and induce IL-10-producing Tr-1 like cells. Eur J Immunol 2012;42:1393–1404.
17. Jeon SG, Kayama H, Ueda Y, et al. Probiotic Bifidobacterium breve induces IL-10-producing Tr1 cells in the colon. PLoS Pathog 2012;8:e1002714.
18. Ansari NA, Kumar R, Gautam S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. J Immunol 2011;186:3977–3985.
19. Spolski R, Kim H-P, Zhu W, et al. IL-21 mediates suppressive effects via its induction of IL-10. J Immunol 2009;182:2859–2867.
20. Pot C, Jin H, Awasthi A, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol 2009;183:797–801.
21. Cox JH, Kljavin NM, Ramamoorthi N, et al. IL-27 promotes T cell-dependent colitis through multiple mechanisms. J Exp Med 2011;208:115–123.
22. Honda K, Nakamura K, Matsui N, et al. T helper 1-inducing property of IL-27/WSX-1 signaling is required for the induction of experimental colitis. Inflamm Bowel Dis 2005;11:1044–1052.
23. Kim G, Shinnakasu R, Saris CJM, et al. A novel role for IL-27 in mediating the survival of activated mouse CD4 T lymphocytes. J Immunol 2013;190:1510–1518.
24. Sasaoka T, Ito M, Yamashita J, et al. Treatment with IL-27 attenuates experimental colitis through the suppression of the development of IL-17-producing T helper cells. Am J Physiol Gastrointest Liver Physiol 2011; 300:G568–G576.
25. Troy AE, Zaph C, Du Y, et al. IL-27 regulates homeostasis of the intestinal $CD4^+$ effector T cell pool and limits intestinal inflammation in a murine model of colitis. J Immunol 2009;183:2037–2044.
26. Hanson ML, Hixon JA, Li W, et al. Oral delivery of IL-27 recombinant bacteria attenuates immune colitis in mice. Gastroenterology 2014;146:210–221.e13.
27. Madan R, Demircik F, Surianarayanan S, et al. Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. J Immunol 2009;183:2312–2320.
28. Liu B, Tonkonogy SLL, Sartor RBB. Antigen-presenting cell production of IL-10 inhibits T-helper 1 and 17 cell responses and suppresses colitis in mice. Gastroenterology 2011;141:653–662.e1–4.
29. Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. J Exp Med 2000;192:295–302.
30. Levings MK, Gregori S, Tresoldi E, et al. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not $CD25^+CD4^+$ Tr cells. Blood 2005;105:1162–1169.
31. Wu HY, Quintana FJ, da Cunha AP, et al. In vivo induction of Tr1 cells via mucosal dendritic cells and AHR signaling. PLoS ONE 2011;6:e23618.
32. Iwasaki Y, Fujio K, Okamura T, et al. Egr-2 transcription factor is required for Blimp-1-mediated IL-10 production in IL-27-stimulated $CD4^+$ T cells. Eur J Immunol 2013; 43:1063–1073.
33. Yanaba K, Yoshizaki A, Asano Y, et al. IL-10-producing regulatory B10 cells inhibit intestinal injury in a mouse model. Am J Pathol 2011;178:735–743.

34. Maseda D, Candando KM, Smith SH, et al. Peritoneal cavity regulatory B cells (B10 cells) modulate IFN-γ^+CD4$^+$ T cell numbers during colitis development in mice. J Immunol 2013;191:2780–2795.
35. Ray A, Basu S, Williams CB, et al. A novel IL-10-independent regulatory role for B cells in suppressing autoimmunity by maintenance of regulatory T cells via GITR ligand. J Immunol 2012;188:3188–3198.
36. Satoguina JS, Adjobimey T, Arndts K, et al. Tr1 and naturally occurring regulatory T cells induce IgG4 in B cells through GITR/GITR-L interaction, IL-10 and TGF-beta. Eur J Immunol 2008;38:3101–3113.
37. Murai M, Turovskaya O, Kim G, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 2009;10:1178–1184.
38. Carter NA, Rosser EC, Mauri C. Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. Arthritis Res Ther 2012;14:R32.
39. Sayi A, Kohler E, Toller IM, et al. TLR-2-activated B cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. J Immunol 2011;186:878–890.
40. Schmidt EGW, Larsen HL, Kristensen NN, et al. B cells exposed to enterobacterial components suppress development of experimental colitis. Inflamm Bowel Dis 2012;18:284–293.
41. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007;450:566–569.
42. Qu Z, Fu J, Ma H, et al. PDLIM2 restricts Th1 and Th17 differentiation and prevents autoimmune disease. Cell Biosci 2012;2:23.
43. Bao L, Tessier C, Prigent-Tessier A, et al. Decidual prolactin silences the expression of genes detrimental to pregnancy. Endocrinology 2007;148:2326–2334.
44. Hiramatsu Y, Suto A, Kashiwakuma D, et al. c-Maf activates the promoter and enhancer of the IL-21 gene, and TGF-beta inhibits c-Maf-induced IL-21 production in CD4$^+$ T cells. J Leukoc Biol 2010;87:703–712.
45. Wurster AL, Rodgers VL, Satoskar AR, et al. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. J Exp Med 2002;196:969–977.
46. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci USA 2005;102:5126–5131.
47. Ano S, Morishima Y, Ishii Y, et al. Transcription factors GATA-3 and RORγt are important for determining the phenotype of allergic airway inflammation in a murine model of asthma. J Immunol 2013;190:1056–1065.
48. Miyauchi Y, Ninomiya K, Miyamoto H, et al. The Blimp1-Bcl6 axis is critical to regulate osteoclast differentiation and bone homeostasis. J Exp Med 2010;207:751–762.

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Correspondence
Address correspondence to: R. Balfour Sartor, MD, Room 7309A MBRB, CB# 7032, Center for Gastrointestinal Biology and Disease, Department of Medicine, Division of Gastroenterology and Hepatology, University of North Carolina, Chapel Hill, North Carolina 27599-7032. e-mail: rbs@med.unc.edu; fax: (919) 843-6899.

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The authors disclose no conflicts.

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