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T regulatory cells and B cells cooperate to form a regulatory loop that maintains gut homeostasis and suppresses dextran sulfate sodium-induced colitis

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Regulatory T cells (Tregs) and B cells present in gut-associated lymphoid tissues (GALT) are both implicated in the resolution of colitis. However, how the functions of these cells are coordinated remains elusive. We used the dextran sulfate sodium (DSS)-induced colitis model combined with gene-modified mice to monitor the progression of colitis, and simultaneously examine the number of Tregs and B cells, and the production of IgA antibodies. We found that DSS-treated mice exhibited more severe colitis in the absence of B cells, and that the adoptive transfer of B cells attenuated the disease. Moreover, the transfer of IL-10$^{-/-}$ B cells also attenuated colitis, suggesting that B cells inhibited colitis through an interleukin-10 (IL-10)-independent pathway. Furthermore, antibody depletion of Tregs resulted in exacerbated colitis. Intriguingly, the number of GALT Tregs in B cell-deficient mice was significantly decreased during colitis and the adoptive transfer of B cells into these mice restored the Treg numbers, indicating that B cells contribute to Treg homeostasis. We also found that B cells induced the proliferation of Tregs that in turn promoted B-cell differentiation into IgA-producing plasma cells. These results demonstrate that B cells and Tregs interact and cooperate to prevent excessive immune responses that can lead to colitis.

INTRODUCTION

Inflammatory bowel disease is a multifactorial inflammatory disorder characterized by intestinal inflammation and mucosal damage, followed by remissions, that leads to symptoms of wasting, diarrhea, and hemafecia, and presents as Crohn's disease or ulcerative colitis.[1] Although the pathogenesis of inflammatory bowel disease remains poorly understood, an overactive immune response to intestinal bacteria within the gut is one of the pathologic features.[2] Both the gut epithelium and the gut-associated lymphoid tissues (GALT) are important for the maintenance of intestinal homeostasis.[3,4] The GALT consists of Peyer's patches, lamina propria (LP), and mesenteric lymph nodes (MLNs). B cells are prominent within the GALT and the production of IgA is primarily initiated within the Peyer's patches and following upregulation of the gut-homing receptors $\alpha_4\beta_7$ and CXCR9 IgA plasmablasts migrate to the LP where they complete their differentiation and secrete IgA into the gut lumen.[4–6] Although a number of mechanisms are important for the generation of IgA within the GALT tissues, one essential cytokine is transforming growth factor-β (TGF-β).[7,8] A number of cell types within the GALT tissues produce TGF-β, including dendritic cells, B cells, T follicular cells, and Foxp3$^+$ T regulatory cells (Tregs).[4] Tregs play an essential role in immune tolerance and in their absence both humans and mice spontaneously develop autoimmune disorders at a young age.[9] Another essential cytokine in the maintenance of gut homeostasis is interleukin-10 (IL-10) and mice deficient in this cytokine spontaneously develop colitis, with Tregs thought to be the major contributor of the protective IL-10.[10–12] In this regard, Tregs have been shown to suppress the production of IL-17 during colitis in an IL-10-dependent manner.[13,14] There are two major populations of Tregs. Natural Tregs develop in the thymus and induced Tregs develop at sites of inflammation in the presence of IL-2 and TGF-β.[15–18] Both

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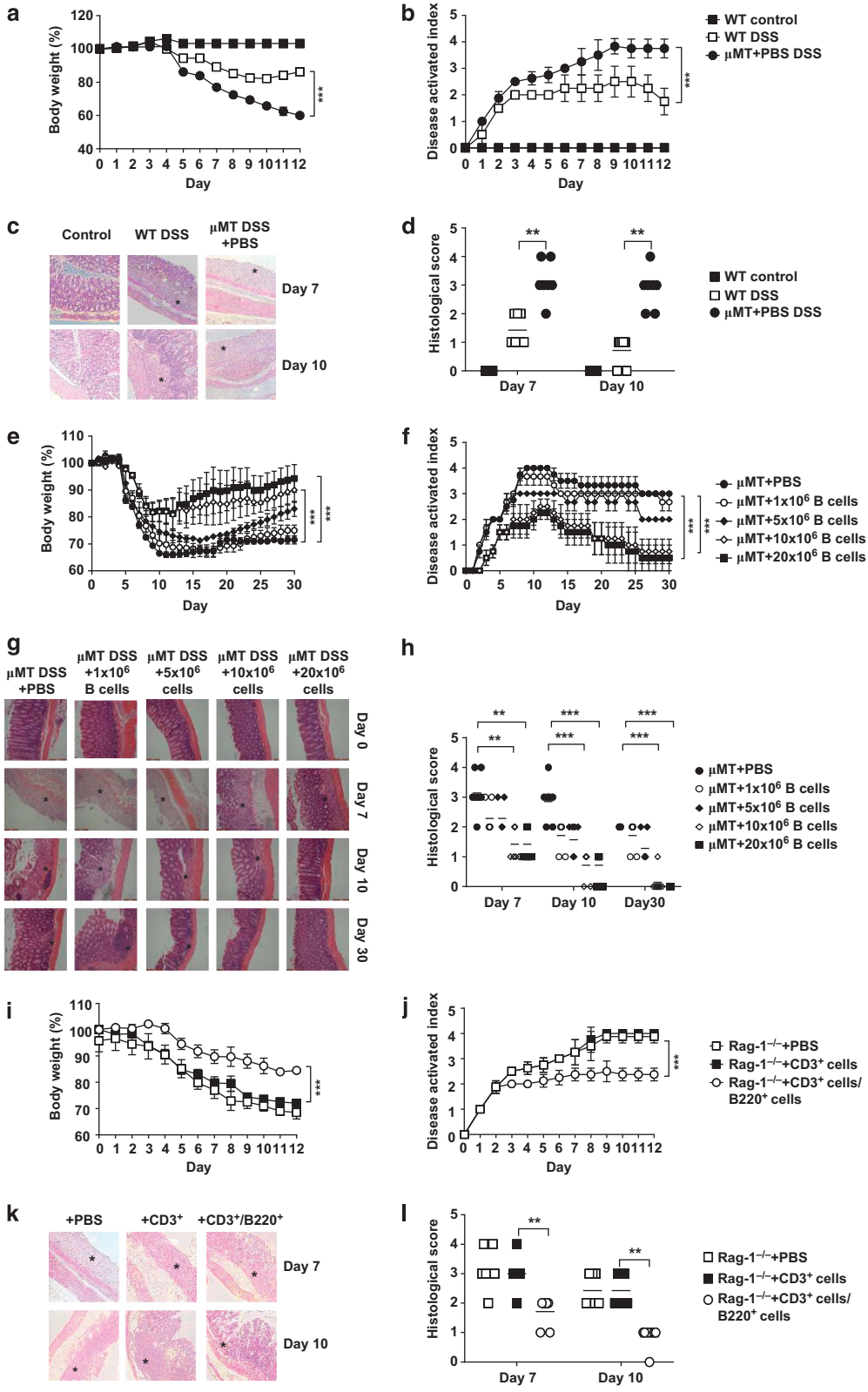
Treg subpopulations have been shown to play a role in colitis suppression.[19] In addition, Tregs were shown to be important for the maintenance of IgA$^+$ B cells and IgA within the gut.[20] Although the exact mechanisms whereby Tregs contribute to IgA homeostasis is not known, a recent *in vitro* study showed that they can produce TGF-β and promote IgA class switching,[21] suggesting that a similar mechanism may exist *in vivo* in the gut.



The administration of dextran sulfate sodium (DSS) into the drinking water of mice results in a disease similar to ulcerative colitis and leads to weight loss, diarrhea, and rectal bleeding, and is associated with histopathology that includes crypt abscesses and acute and chronic inflammation.[22,23] The onset of DSS colitis in severe combined immunodeficient (SCID) mice does not require the presence of T or B cells, making it an excellent model in which to study specific immune regulation.[24] In this regard, the expansion of Tregs with a superagonist CD28 antibody led to a reduction in the severity of DSS colitis.[25] A regulatory role for B cells in colitis was first shown in TCR$\alpha^{-/-}$ mice that spontaneously develop chronic colitis, exhibiting more severe disease in the absence of B cells.[26] Similarly, the severity of spontaneous colitis in SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{hi}$ cells was attenuated by the cotransfer of B cells.[27] Furthermore, altered B-cell development and function was shown to be the primary cause of spontaneous colitis in mice deficient in the *Gαi2* gene.[28] In addition, IL-10 production by splenic $CD19^+CD5^+CD1d^+$ regulatory B cells was shown to be important in attenuating the severity of DSS colitis in mice in which B cells were functionally impaired by a deficiency in CD19.[29] Recently, Sattle *et al.*[30] demonstrated that IL-10-producing regulatory B cells induced by IL-33 can rescue mucosal inflammation. Previously, we uncovered a link between B cells and Tregs; whereby, we showed that μMT mice deficient in peripheral B cells had a significant reduction in Tregs.[31] Partial reconstitution of the B-cell pool by the adoptive transfer of B cells resulted in the proliferation of Tregs in a GITR/GITRL-dependent manner.[31] Furthermore, we showed that the depletion of B cells with anti-CD20 accelerated the onset of spontaneous colitis in IL-10-deficient mice.[31] However, it is unclear how the regulatory functions of B cells and Tregs are interconnected and coordinated in the GALT during colitis.

In this study, we asked whether B cells and Tregs interact to attenuate the severity of acute DSS colitis. To address this question, we first showed that μMT mice exhibit a more severe colitis as compared with control mice, with a quicker and accelerated weight loss that included more severe intestinal pathology. The transfer of wild-type (WT) or IL-$10^{-/-}$ splenic B cells was sufficient to attenuate disease back to control levels. Thus, we investigated IL-10-independent B-cell mechanisms and found that although Treg numbers were not reduced in the GALT tissues in μMT mice, their numbers did not increase during colitis as observed in WT mice. B-cell reconstitution restored the expansion of Tregs in the GALT during colitis. Furthermore, we showed that the depletion of Tregs increased the severity of colitis and led to a decrease in the number of IgA^+ B cells and IgA in the gut and that TGF-β production by Tregs promoted IgA isotype class switching by B cells. Using the SCID model of colitis, we showed that both B cells and Tregs were required for the attenuation of disease and the production of IgA in the gut. Cumulatively, these data demonstrate that B cells promote the expansion of Tregs in the GALT tissues during acute colitis, essential for the generation of IgA-producing plasma cells in the gut. Thus, we have uncovered a regulatory loop in the gut whereby B cells and Tregs are critical for the homeostasis of the other cell type that together help to maintain immune tolerance.

RESULTS

DSS-induced colitis is more severe in B cell-deficient mice

Because colitis in TCR-$\alpha^{-/-}$ and IL-$10^{-/-}$ mice in the absence of B cells was reported to be either more severe or accelerated,[26,31] we investigated whether B cells play a similar role in DSS-induced colitis. Compared with WT mice, B cell-deficient mice (μMT) administered DSS for 7 days exhibited significantly more weight loss (**Figure 1a**) and more severe disease as measured by the disease activity index score (**Figure 1b**). WT mice developed diarrhea and lost >10% of their body weight starting on day 8 (peak phase) that was accelerated in μMT mice to day 5 (**Figure 1a**). WT mice began to recover starting on day 10, whereas μMT mice continued to progress with no diminution of disease symptoms or severity (**Figure 1a,b**). Histologically, acute colitis in WT mice is characterized by epithelial damage, focal crypt injury, goblet cell depletion, and inflammatory cell invasion that resolved in the recovery phase of WT mice (**Figure 1c,d**). However, these tissue changes were more severe in μMT mice with inconspicuous recovery (**Figure 1c,d**). To confirm a role for B cells in the attenuation of colitis severity, we adoptively transferred fluorescence-activated cell sorting (FACS)-purified WT splenic B cells ($B220^+$) into μMT mice 2 days before the administration of DSS. By performing a dose–response curve, we found that administration of $10–20 \times 10^6$ B cells was optimal with 1 and 5×10^6 B cells mediating little protection (**Figure 1e–h**). The purity of the B cells was >97%, with few to no $CD4^+Foxp3^+$ Tregs or $CD11b^+Gr1^+$ myeloid suppressor cells present to account for the protection afforded by the B-cell transfer (**Supplementary Figure S1** online). Our finding that the transfer of up to 10×10^6 B cells was required to observe

Figure 1 B-cell deficiency enhanced the severity of dextran sulfate sodium (DSS)-induced colitis. Colitis was induced in (**a–h**) wild-type (WT), (**a–d**) μMT, or (**i–l**) Rag1$^{-/-}$ mice by administration of 2% DSS in the drinking water for 7 days followed by regular drinking water for an additional 5 days. Controls only received regular drinking water. PBS, phosphate-buffered saline. (**e–h**) On day −2 of DSS-induced colitis, a group of μMT mice were intravenously (i.v.) administered 1, 2, 10, or 20×10^6 splenic fluorescence-activated cell sorting (FACS)-purified WT B cells ($B220^+$) and (**i–l**) Rag1$^{-/-}$ mice were similarly administered splenic $CD3^+$ T cells or $CD3^+$ T cells and $B220^+$ B cells. Mice were evaluated daily for (**a, e, i**) weight loss and (**b, f, j**) disease activity index (DAI) scores were calculated. Representative colon histological sections stained with hematoxylin and eosin (H&E) are shown at (**c, k**) days 0, 7, and 10 or (**g**) 0, 7, 10, and 30 after colitis induction. Images are shown at original magnification ×200. An asterisk indicates the position of the leukocyte infiltration in the histological images. (**d, h, l**) Histological sections were blindly scored on a scale of 0 to 4 to generate a histological score and individual mouse scores are shown with each data point representing a single mouse. The data shown are the mean ± s.e.m. of one experiment with (**a, b, e, f**) five or (**i, j**) eight mice and was repeated three times with similar results. $^{**}P<0.01$; $^{***}P<0.001$.

protection suggests that only a small subset of the total B-cell population has the capacity to regulate DSS colitis.

To further confirm that B cells play a crucial role in the suppression of colitis, purified splenic $CD3^+$ cells with or without $B220^+$ cells from naive WT mice were adoptively transferred into lymphocyte-deficient Rag-$1^{-/-}$ recipient mice 2 days before DSS administration. Rag-$1^{-/-}$ mice that received phosphate-buffered saline or $CD3^+$ cells alone exhibited severe disease and delayed resolution of colitis that was attenuated by the adoptive transfer of B cells (**Figure 1i–l**). These results demonstrate that B cells play an important role in suppressing colitis development and reducing disease severity. Using flow cytometry (**Supplementary Figure S2A**) the protective role for B cells in colitis was correlated with a significant increase in the percentage of total B cells ($B220^+$) in the MLN, intra-epithelial lymphocyte (iEL), and lamina propria lymphocyte (LPL) populations, but not in the Peyer's patches, which exhibited a decrease in B cells, at various time points during DSS-induced colitis (**Supplementary Figure S2B**). The increase in B cells was offset by a decrease in the percentage of CD4 and/or CD8 T cells in the MLNs and iELs and a decrease in $CD11b^+$ myeloid cells in the LPLs (**Supplementary Figure S2B**). These data suggest that B cells accumulate within the GALT during colitis.

IL-10 production by B cells is not required for their attenuation of DSS-induced colitis

Because IL-10 production by $CD1d^{hi}CD5^+$ B cells has been shown to attenuate DSS-induced colitis in $CD19^{-/-}$ mice,[29] we next determined whether total B cells similarly attenuated disease in μMT mice by the same mechanism. The adoptive transfer of either WT or IL-$10^{-/-}$ B cells into μMT mice attenuated DSS-induced colitis in a similar manner, reducing weight loss (**Figure 2a**), the disease activity index (**Figure 2b**), and the histological score (**Figure 2c,d**) as compared with the WT control mice. This result was confirmed by performing the identical experiment in Rag-$1^{-/-}$ mice that were cotransferred with $CD3^+$ T cells with either WT or IL-$10^{-/-}$ B cells (**Figure 2e–h**). To determine which cells produced IL-10 in the gut following the administration of DSS, we used IL-10-IRES-EGFP reporter mice.[32,33] Although we were able to detect low numbers of IL-10-producing B cells ($B220^+$ IL-10^{eGFP}) in the spleen, MLNs, Peyer's patches, iELs, and LPLs by flow cytometry (**Figure 3a**), their absolute numbers did not increase over the course of the experimental period (**Figure 3b**). However, we did detect IL-10 production in $CD4^+$ T cells on day 10 of colitis, indicating that the reporter was functional (**Figure 3c**). Although IL-10 production by $CD4^+$ T cells was significantly increased in the MLNs, iELs, and LPLs on day 10 following colitis induction (**Figure 3d**), we did not detect an increase in the expression of IL-10 message in the same tissue populations over the disease course (**Figure 3e**). These cumulative data suggest that IL-10 production by B cells is dispensable for attenuation of DSS-induced colitis and that $CD4^+$ T cells are a major contributor to the IL-10 pool in the gut.

B cells regulate colitis by maintenance of Tregs

As we have previously shown that B cells can resolve experimental autoimmune encephalomyelitis (EAE) by the maintenance of Tregs,[31] we next determined whether a similar mechanism existed in DSS-induced colitis. First we used flow cytometry (**Figure 4a**) to confirm that μMT mice have a significant reduction in the absolute number of Tregs in the spleen as compared with WT control mice (**Figure 3b**).[31] Interestingly, a similar reduction was not observed in the iEL, MLN, or LPL populations (**Figure 4b**). Although the adoptive transfer of B cells into the μMT mice significantly increased splenic Treg numbers, there was no change in Treg numbers in the GALT tissues (**Figure 4b**).[31] On day 10 after colitis induction, there was a significant increase in the absolute number of Tregs in the iELs, MLNs, and LPLs of WT, but not μMT mice, as compared with the controls (**Figure 4b**). The adoptive transfer of B cells before colitis induction resulted in a significant increase in Treg numbers in the MLNs and LPLs, but not the iELs (**Figure 4b**). However, splenic Treg numbers were not significantly altered by the induction of DSS colitis in any of the conditions (**Figure 4b**). These data are consistent with B cell-dependent GALT-specific expansion of Tregs during colitis. To validate whether the increase in Tregs in mice receiving B cells was relevant to the suppression of colitis, we treated mice with anti-CD25 (PC61) that resulted in the depletion of Tregs from the blood, Peyer's patches, spleen, iELs, MLNs, and LPLs as compared with the isotype control (**Supplementary Figure S3A, B**). However, the absolute number of conventional $CD4^+$ T cells was not altered (**Supplementary Figure S3B**). As expected, the depletion of Tregs ablated the protection conferred by the transfer of B cells into μMT mice (**Figure 4c–f**).

B cells contribute to Treg homeostasis in the GALT by inducing their proliferation

The increase in Tregs in the GALT could be due to increased local proliferation of Tregs. This was determined by quantitating the number of proliferating Tregs that had incorporated bromodeoxyuridine during a 4-day pulse by flow cytometry (**Figure 5a**). In WT mice, ~22% of Tregs were undergoing proliferation in the MLNs, and this was significantly decreased to ~12% in μMT mice (**Figure 5a**). WT levels of Treg proliferation were restored in μMT mice following the adoptive transfer of B cells (**Figure 5a**). A similar trend was observed in the LPL population (**Figure 5a**). To further confirm that B cells promoted Treg expansion, we utilized an *in vivo* approach using Rag-$1^{-/-}$ mice. Carboxyfluorescein succinimidyl ester-labeled $CD4^+CD25^+$ T cells were transferred into Rag-$1^{-/-}$ mice alone or with B cells 2 days before DSS administration. We observed that both splenic and MLN Tregs had undergone significantly more proliferation on day 10 in the presence of B cells (**Figure 5b**). To determine whether B cell-induced Treg proliferation required cell–cell contact, we performed an *in vitro* Treg proliferation assay co-culturing B cells and carboxyfluorescein succinimidyl ester-labeled Tregs stimulated with anti-CD3 in the same well or separated by a transwell. As

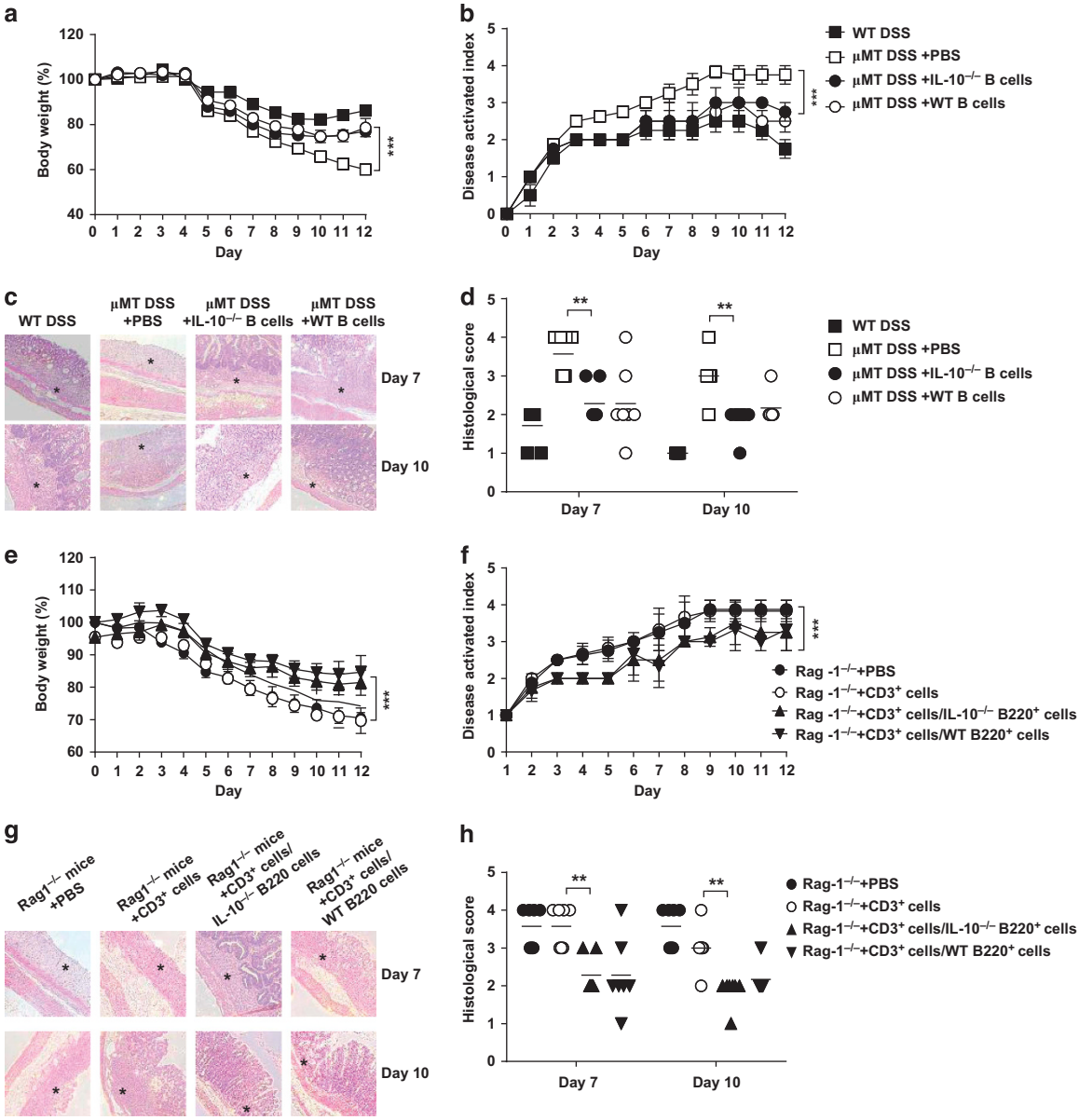


Figure 2 Attenuation of colitis by B cells does not require their production of interleukin-10 (IL-10). Colitis was induced in (**a–d**) wild-type (WT), (**a–d**) µMT, or (**e–h**) Rag1$^{-/-}$ as for **Figure 1**. PBS, phosphate-buffered saline. (**a–d**) On day −2 of dextran sulfate sodium (DSS)-induced colitis, µMT mice were intravenously (i.v.) administered 2×10^7 splenic WT or IL-10$^{-/-}$ B cells (B220$^+$) and (**e–h**) Rag1$^{-/-}$ mice were similarly administered splenic CD3$^+$ T cells or CD3$^+$ T cells and either WT or IL-10$^{-/-}$ B cells. Mice were evaluated daily for (**a, e**) weight loss and (**b, f**) disease activity index (DAI) scores were calculated. Representative colon histological sections stained with hematoxylin and eosin (H&E) are shown for the (**c**) WT and µMT and (**g**) Rag1$^{-/-}$ groups of mice at days 7 and 10 after colitis induction. Images are shown at original magnification ×200. An asterisk indicates the position of the leukocyte infiltration in the histological images. (**d, h**) Histological sections were scored as for **Figure 1** with each data point representing a single mouse. (**a, b, e, f**) The data shown are the mean ± s.e.m. from one experiment with seven mice and was repeated three times with similar results. $^{**}P<0.01$; $^{***}P<0.001$).

shown in **Figure 5c**, Tregs underwent a significant increase in cell division only when they were in direct contact with B cells. These cumulative data suggest that the increased numbers of Tregs in the GALT during colitis are due to interactions with B cells driving their expansion.

Tregs promote the production of colitis-protective IgA

Our data suggest that Tregs are required for attenuation of colitis in the presence of B cells. Interestingly, the adoptive transfer of Tregs alone into Rag-1$^{-/-}$ mice did not alter the course of colitis (**Supplementary Figure S4A–D**), suggesting that Tregs are essential but not sufficient for the suppression of colitis. We next investigated whether the known colitis regulatory factors IL-10, TGF-β, and IgA were deficient in Rag-1$^{-/-}$ mice receiving Tregs alone.[7,8,10,34] By both enzyme-linked immunosorbent assay (ELISA; **Figure 6a**) and intracellular staining using flow cytometry (**Figure 6b**), we found that both IL-10 and TGF-β production in the gut mucus

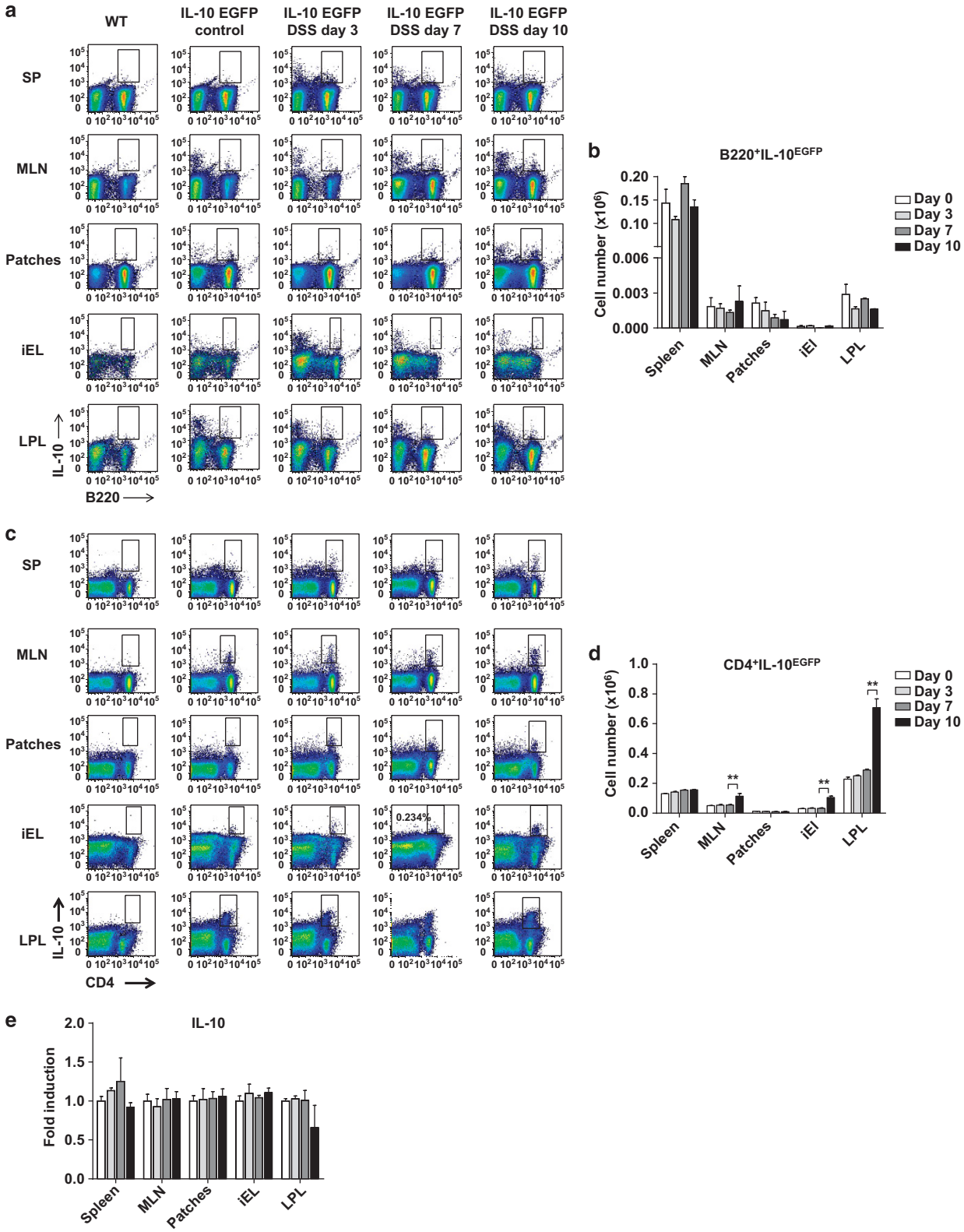


Figure 3 CD4 + T cells, but not B cells, contribute to interleukin-10 (IL-10) production in the gut-associated lymphoid tissues (GALT) of mice with dextran sulfate sodium (DSS)-induced colitis. Cells were isolated from the spleen (SP), mesenteric lymph nodes (MLNs), Peyer's patches (Patches), intraepithelial lymphocytes (iELs), and lamina propria (LP) of DSS-treated wild-type (WT) and IL-10EGFP mice (IL-10-IRES-EGFP) on days 0, 3, 7, and 10 and flow cytometry was used to determine the absolute number of (**a, b**) IL-10 (EGFP$^+$)- producing B cells (B220$^+$) and (**c, d**) CD4$^+$ T cells. Representative dot plots show IL-10 production by (**a**) B and (**c**) CD4$^+$ T cells. (**b**, **d**) Data shown are the mean ± s.e.m. of one experiment with three mice in each group. (**e**) IL-10 mRNA expression by fluorescence-activated cell sorting (FACS)-purified WT B cells (B220$^+$) was examined by quantitative reverse transcriptase (RT)-PCR analysis. IL-10 expression was normalized to β-actin mRNA. Data are the mean ± s.e.m. from one experiment with five mice and was repeated at least three times with similar results. $^{**}P < 0.01$.

were dose-dependently increased during colitis. Representative dot plots showing the intensity of the IL-10, TGF-β, and IgA intracellular staining are shown in **Supplementary Figure S5**.

As expected, IgA was completely absent because of the deficiency in B cells (**Figure 6a,b**). To investigate whether Tregs provide help for IgA secretion, WT mice were depleted of

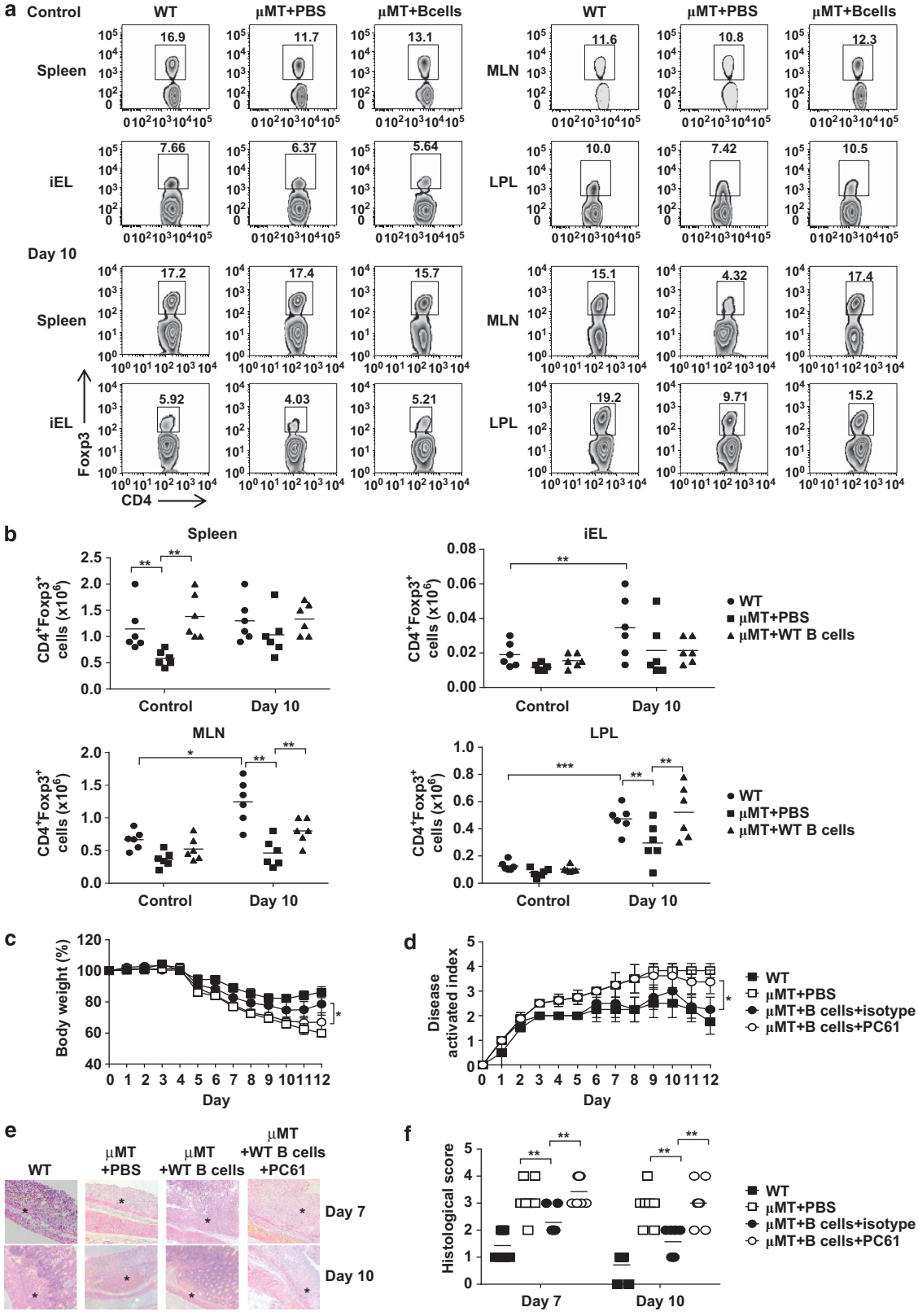


Figure 4 For caption see page 1304.

Tregs with anti-CD25 and then 10 days later the presence of LPL $B220^-IgA^+$ plasma cells was quantitated during colitis (**Figure 6c**). As previously reported,[20] we also observed a significant decrease in IgA plasma cells when Tregs were depleted (**Figure 6c**). We then confirmed that the transfer of B cells into μMT mice would lead to the generation of IgA plasma cells (**Figure 6c**) and the production of IgA (**Figure 6d**) in the LPLs during colitis. This increase in IgA plasma cell number (**Figure 6c**) and IgA production (**Figure 6d**) was greatly inhibited when Treg depletion accompanied the B-cell transfer. The inclusion of anti-CD25 did not affect T-cell activation as assessed by CD69 expression (data not shown). Similarly, B-cell activation was also not altered as determined by CD69, CD80, or CD86 expression (data not shown). We then performed *in vitro* experiments to confirm that Tregs promote B-cell secretion of IgA. To address this question, we purified B cells and $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from the Peyer's patch and co-cultured the cells in various combinations. Using flow cytometry to identify IgA-expressing B cells (**Figure 6e**), we found that B cells cultured alone or with $CD4^+CD25^-$ T cells did not lead to the generation of IgA-expressing B cells or the secretion of IgA (**Figure 6e**). However, when B cells were cultured with $CD4^+CD25^+$ Tregs, there was a significant increase in both IgA^+ B cells and IgA secretion (**Figure 6d**). The inclusion of both $CD4^+CD25^-$ and $CD4^+CD25^+$ T subpopulations in the B-cell culture elicited the highest level of IgA production (**Figure 6d**). As a control, we confirmed that $CD4^+CD25^-$ T cells induced IgM production (**Figure 6d**). In comparison, $CD4^+CD25^+$ Tregs were poor inducers of IgM production by B cells and they were able to significantly inhibit its induction by $CD4^+CD25^-$ T cells (**Figure 6d**). The inclusion of a TGF-β blocking antibody to cultures containing B cells and both $CD4^+CD25^-$ and $CD4^+CD25^+$ T subpopulations significantly inhibited the generation of IgA, but not IgM, suggesting that Tregs can provide the TGF-β necessary to drive IgA isotype class switching. To further show that IgA is protective in DSS-induced colitis, we depleted B cells with anti-CD20 (**Figure 7a**) and found that this abrogated the production of IgA in the gut (**Figure 7b**), resulting in more severe colitis (**Figure 7c–f**).

B-cell and Treg coordination is required for colitis attenuation

To further confirm a role for B-cell and Treg coordination in attenuation of colitis, we utilized the $CD4^+CD45RB^{hi}$ T-cell SCID model of colitis.[35,36] The severe disease driven by the adoptive transfer of $CD4^+CD45RB^{hi}$ T cells was attenuated by the cotransfer of B cells that was abrogated when Tregs were depleted with anti-CD25 as determined by body weight (**Figure 8a**), survival (**Figure 8b**), and histological score (**Figure 8c**). The levels of secretory IgA were increased in mice that received $CD4^+CD45RB^{hi}$ T cells and B cells and decreased by Treg depletion (**Figure 8d**).

DISCUSSION

In this study, we investigated the regulatory function of B cells in DSS colitis and the mechanism underlying their inhibitory function. By using μMT and $Rag1^{-/-}$ mice combined with adoptive transfer, we found that B-cell deficiency exacerbated the severity of colitis and that B cells inhibited colitis in an IL-10-independent manner. Furthermore, we demonstrated *in vitro* and *in vivo* that B cells promoted Treg expansion and that Tregs in turn enhanced B-cell IgA production. Thus, Tregs and B cells cooperate to form a regulatory loop to maintain gut homeostasis and suppress colitis.

In a seminal paper, a protective role for B cells and serum Ig was described in the TCR-$\alpha^{-/-}$ spontaneous model of colitis.[26] In a follow-up study, it was found that the MLNs harbored a $CD1d^+$ B cell that produced IL-10 that suppressed colonic inflammation.[37] In our study, we used the DSS model of colitis to further investigate mechanisms whereby B cells attenuate the severity of colitis. To address this question, we used μMT mice and $Rag^{-/-}$ mice reconstituted with $CD3^+$ T cells to study disease severity in the absence of B cells. We found that the colitis disease activity index score was significantly higher as compared with control mice in both B cell-deficient mice. These data are consistent with the more severe disease previously described in TCR-$\alpha^{-/-}$ × μMT mice.[26] To confirm a protective role for B cells, we adoptively transferred splenic B cells before the induction of DSS colitis and showed that this was sufficient to alleviate severe disease back to that observed in WT control mice. We obtained the same result in two different laboratories using C57BL/6 μMT mice in China and B10.PL μMT mice in the United States[38] (data not shown), indicating that our findings are not likely influenced by differences in the microbiome. This distinction is important because in DSS colitis Myd88 signaling in B cells has been shown to be important for their protective function whereby they seem to control the dissemination of gut bacteria via production of complement activating IgM.[39] Because both IL-10 treatment

Figure 4 Expression and function of $CD4^+CD25^+$ regulatory T cells (Tregs) during colitis. Colitis was induced in wild-type (WT) and μMT mice as for **Figure 1**. Flow cytometry was used to determine the (**a**) percentage and (**b**) absolute number of Tregs ($CD4^+Foxp3^+$) within the CD4-gated population in the spleen, intraepithelial lymphocytes (iELs), mesenteric lymph nodes (MLNs), and lamina propria lymphocytes (LPLs) of WT and μMT mice and μMT mice that were intravenously (i.v.) administered 2×10^7 $B220^+$ WT splenic B cells (day −2) in controls and 10 days after colitis induction (**a, b**). (**a**) Representative contour plots are shown. (**b**) Data are pooled from two independent experiments with six mice in each group and expressed as mean ± s.e.m. (**c–f**) Colitis was induced in unmanipulated WT mice and μMT mice that were administered phosphate-buffered saline (PBS) or 2×10^7 $B220^+$ WT splenic B cells on day −2 with or without the injection of the anti-CD25 antibody (500 μg) on days −2 and 0. Mice were evaluated daily for (**c**) weight loss and (**d**) disease activity index (DAI) scores were calculated. (**e**) Representative colon histological sections stained with hematoxylin and eosin (H&E) are shown for the WT and μMT groups. Images are shown at original magnification ×200. An asterisk indicates the position of the leukocyte infiltration in the histological images. (**f**) A histological score was generated as for Figure 1. The data shown are the mean ± s.e.m. from one experiment with seven mice and was repeated three times with similar results. (**b, f**) Each data point represents a single mouse. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

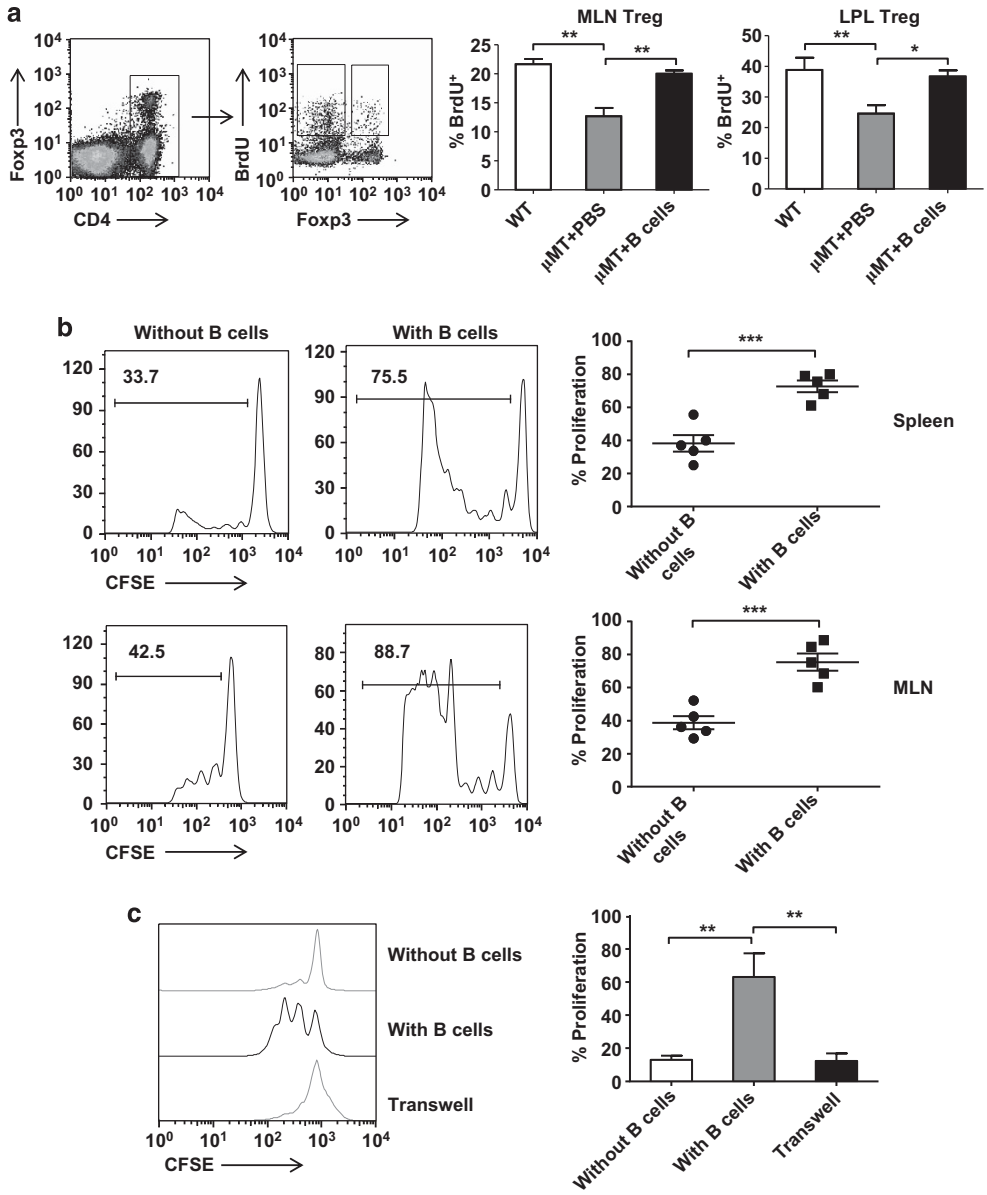


Figure 5 B cells promote regulatory T cell (Treg) proliferation. Colitis was induced as for **Figure 1** in (**a, b**) wild-type (WT) mice and (**a**) μMT and (**b**) $Rag^{-/-}$ mice that had received either phosphate-buffered saline (PBS) or 2×10^7 WT splenic B cells on day −2. (**a**) Bromodeoxyuridine (BrdU) was added to the drinking water on day 3 and continued for 7 days. The percentage of $BrdU^+$ $CD4^+$ $Foxp3^+$ Tregs was determined by flow cytometry with the gating strategy shown in the left two panels. The bar graphs show the percentage of $BrdU^+$ Tregs in the mesenteric lymph nodes (MLNs) and lamina propria lymphocytes (LPL) as the mean ± s.e.m. of six mice from one experiment, and was performed three times with similar results. (**b**) Splenic $CD4^+CD25^+$ Tregs were fluorescence-activated cell sorting (FACS) purified and labeled with carboxyfluorescein succinimidyl ester (CFSE) before intravenous (i.v.) transfer into Rag-$1^{-/-}$ mice with or without purified splenic $B220^+$ B cells. On day 10, Treg proliferation was determined by dye dilution using flow cytometry (left histograms). The percentage of Tregs that had undergone proliferation in the spleen and MLNs is shown in the right scatter graphs, with each data point representing a single mouse. The data shown are the mean ± s.e.m. from one experiment with five mice and was repeated twice with similar results. (**c**) Splenic Tregs ($CD4^+CD25^+$) were purified by FACS and labeled with CFSE and cultured alone (2×10^5 cells) or with splenic $B220^+$ B cells (2×10^5) that were placed either in the culture well or in a transwell in the presence of anti-CD3 (1 μg ml^{-1}) and anti-CD28 (5 μg ml^{-1}) in the presence or absence of B cells for 72 h after which Treg proliferation was determined by CFSE dye dilution by flow cytometry. A representative histogram is shown on the left and the bar graph is the mean ± s.e.m. of three independent experiments each with three mice. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

and B-cell production of IL-10 have been shown to attenuate DSS colitis,[29,40–42] we tested whether IL-10 production by B cells was required for their regulatory activity. By transferring splenic B cells from IL-$10^{-/-}$ mice, we were able to demonstrate that IL-10 production by B cells is dispensable for their regulatory activity in DSS colitis (**Figure 2**). Although the approach of using B cells from IL-$10^{-/-}$ mice does not preclude that the genetic ablation altered their function and thus the experimental outcome our study, the approach we used is standard to the regulatory B-cell field where IL-10 dependency has been established. Similar results were obtained in both C57BL/6 and B10.PL μMT mice, further

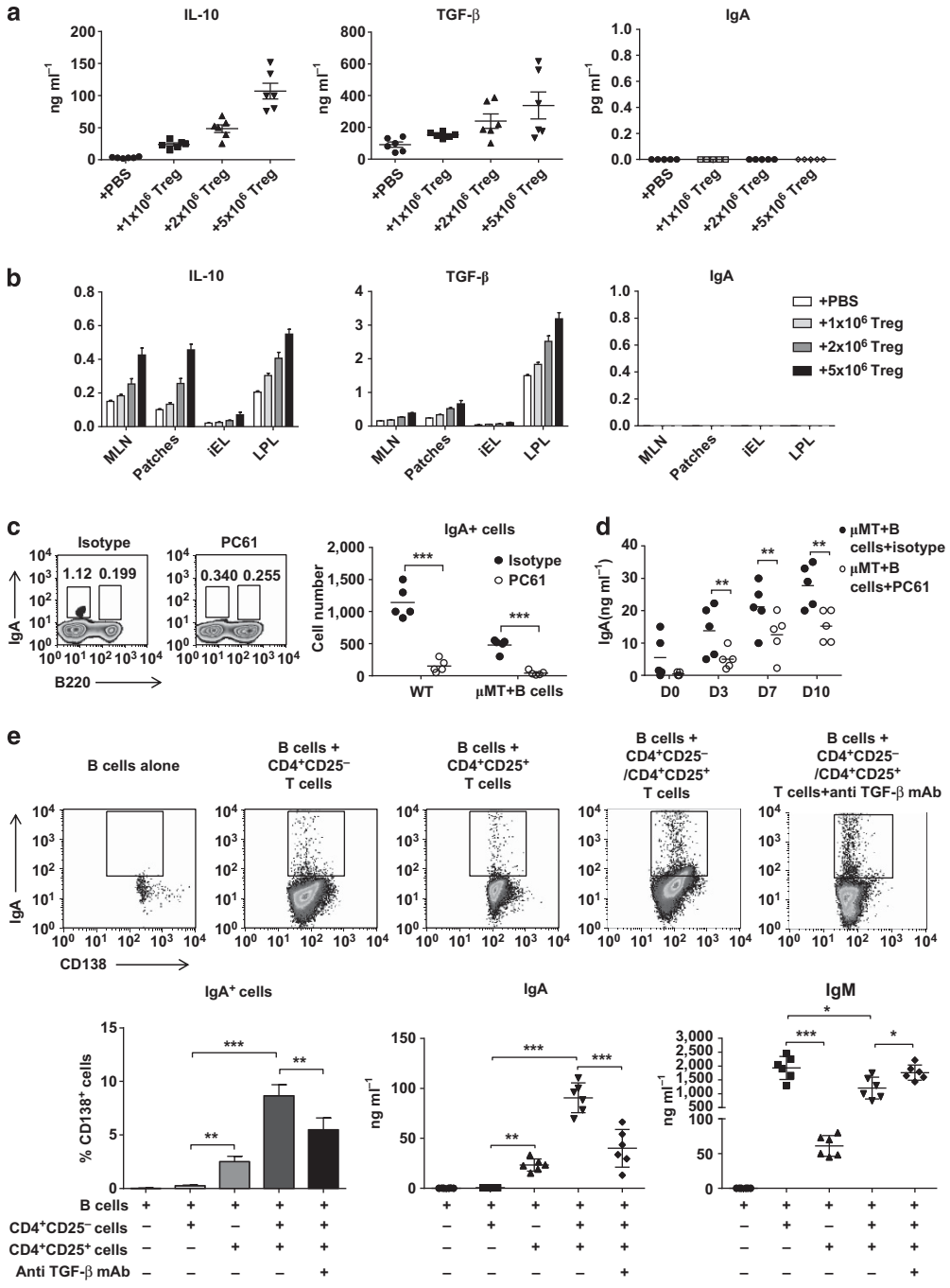


Figure 6 Regulatory T cells (Tregs) are required for IgA production by B cells in the gut-associated lymphoid tissues (GALT). (**a, b**) Rag1$^{-/-}$ mice were intravenously (i.v.) administered splenic CD8$^+$ (2×10^7) and CD4$^+$CD25$^-$ (2×10^7) T cells along with various doses of CD4$^+$CD25$^+$ Tregs (1×10^6, 2×10^6, and 5×10^6) and on the same day administered 2% dextran sulfate sodium (DSS) in the drinking water for 7 days followed by regular drinking water for an additional 5 days. (**a**) On day 10, the intestines were flushed with 20 ml endotoxin and fetal calf serum (FCS)-free phosphate-buffered saline (PBS). Mucus containing fluid was harvested and the level of interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and IgA in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA). (**b**) On day 10, GALT populations were harvested and the expression of IL-10, TGF-β, and IgA was determined by intracellular staining by flow cytometry. The bar graphs show the absolute number of IL-10$^+$ cells, TGF-β$^+$ cells, and IgA$^+$ cells. (**c, d**) Wild-type (WT) and μMT mice were depleted of Tregs by injection of the anti-CD25 antibody PC61 (500 μg) on days −2 and 0. (**c**) After 10 days, the number of LPL IgA$^+$ plasma cells was determined by flow cytometry with the gating strategy shown in the left panels. The percentage of B220$^-$IgA$^+$ plasma cells in the LPLs from WT and μMT mice i.v. administered 2×10^7 B220$^+$ WT splenic B cells on day −2 are shown. (**d**) Total IgA production within the gut was determined by ELISA from isotype or PC61-treated μMT mice that had received B cells on days (D) 0, 3, 7, and 10. Each data point represents a single mouse with the mean shown. The experiments were performed (**b**) two or (**a, c, d**) three times with similar results. (**e**) Fluorescence-activated cell sorting (FACS)-purified splenic B cells (B220$^+$) (2×10^5) were cultured alone or with FACS-purified Peyers' patch CD4$^+$CD25$^-$ conventional CD4$^+$ T cells (2×10^5) and/or CD4$^+$CD25$^+$ Tregs (2×10^5) and after 3 days the percentage of CD138$^+$IgA$^+$ plasma cells in the culture was determined by flow cytometry. Data shown are the mean ± s.e.m. of six mice. Representative dot plots are shown in the upper panels. Total IgA and IgM production in the supernatant was measured by ELISA with each data point representing a single mouse. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. iEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; mAb, monoclonal antibody; MLN, mesenteric lymph node.

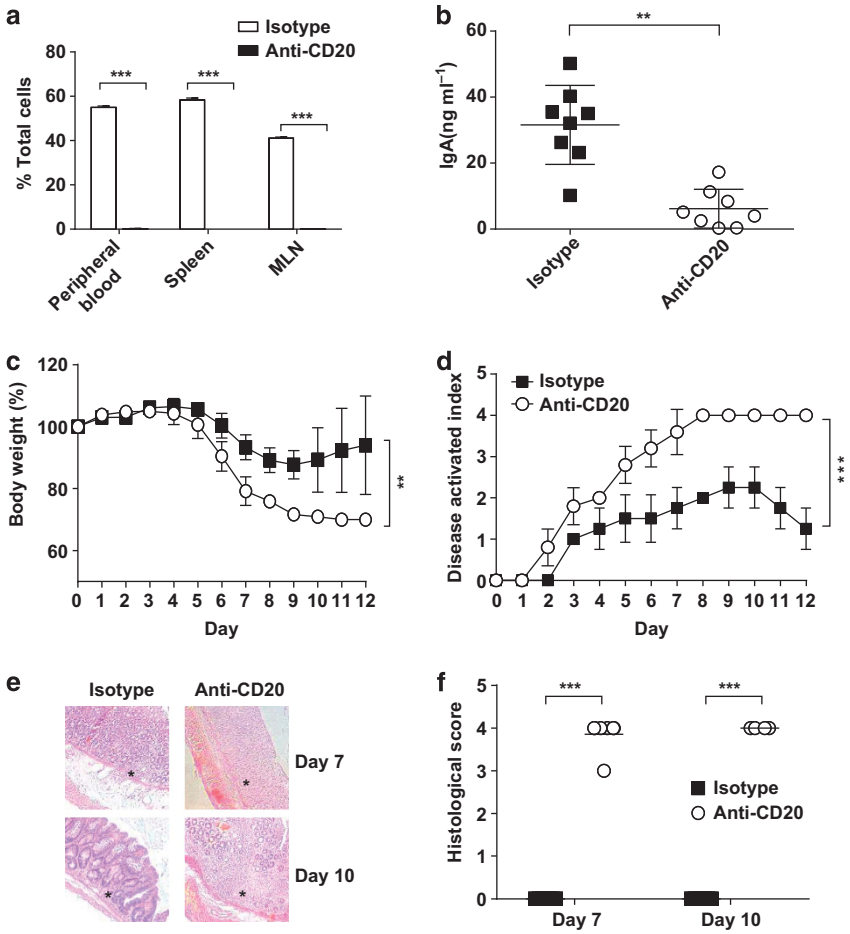


Figure 7 B-cell depletion led to reduced IgA production in the gut and increased colitis severity. Wild-type (WT) mice were administered either anti-CD20 (5D2) or its isotype control (IgG_1) (250 mg) three times on days −49, −21, and −7. Colitis was induced as for **Figure 1** on day 0. (**a**) B-cell depletion was assessed on day 0 in the peripheral blood, spleen, and mesenteric lymph nodes (MLNs) by flow cytometry with the percentage of $B220^+$ B cells of total mononuclear cells shown. (**b**) IgA levels within the gut on day 10 were measured by enzyme-linked immunosorbent assay (ELISA). (**c–f**) Mice were evaluated daily for (**c**) weight loss and (**d**) disease activity index (DAI) scores were calculated. (**e**) Representative colon histological sections stained with hematoxylin and eosin (H&E) on days 7 and 10 after colitis induction are shown. Images are shown at original magnification ×200. An asterisk indicates the position of the leukocyte infiltration in the histological images. (**f**) Histological sections were scored as for **Figure 1** with each data point representing a single mouse. The data shown are the mean ± s.e.m. of eight mice from a single experiment that was repeated with similar results. $^{**}P<0.01$; $^{***}P<0.001$.

supporting our conclusion of B-cell regulatory function independent of IL-10 in DSS colitis (data not shown).

Similar to our finding, it was previously reported that $CD19^{-/-}$ mice also exhibited more severe DSS colitis as compared with WT.[29] Although these mice are not B cell deficient, the B cells have impaired B-cell receptor signaling.[43] Later studies reported that $CD19^{-/-}$ mice are largely deficient in $CD1d^{hi}CD5^+$ B cells that are thought to regulate inflammation via production of IL-10.[29] WT $CD1d^{hi}CD5^+$ B cells, but not those deficient in IL-10, were able to reduce the severity of DSS colitis in $CD19^{-/-}$ mice.[29] Interestingly, non-$CD1d^{hi}CD5^+$ B cells were unable to attenuated DSS colitis in $CD19^{-/-}$ mice. Although we did not specifically examine the role of $CD1d^{hi}CD5^+$ B cells in our study, we found that total splenic B cells that contain this population were able to attenuate colitis in μMT mice (**Figure 1**). As B-cell protection against severe colitis did not require IL-10 production, it is unlikely that $CD1d^{hi}CD5^+$ B cells are the relevant population of B cells in our study (**Figure 1**). In support of this finding, we were unable to detect IL-10-producing B cells in GALT tissues during colitis using an IL-10-EGFP reporter mouse (**Figure 3**). Interestingly, although the $CD1d^{hi}CD5^+$ B cells have been shown to produce IL-10 *in vitro* following stimulation with both lipopolysaccharide and phorbol 12-myristate 13-acetate/ionomycin,[29] to the best of our knowledge, substantial IL-10 production by these B cells has never been demonstrated in real time *in vivo* under any conditions including inflammation and autoimmunity. Thus, it is not clear where B cells produce IL-10 during DSS colitis. In our studies, although B cells accumulated in GALT tissues during colitis (**Supplementary Figure S2**) as previously reported,[44] they did not produce IL-10 (**Figure 3**). Our finding that $CD4^+$ T cells are the major producers of IL-10 in the GALT tissues during colitis in WT mice (**Figure 3**) is consistent with previous findings that

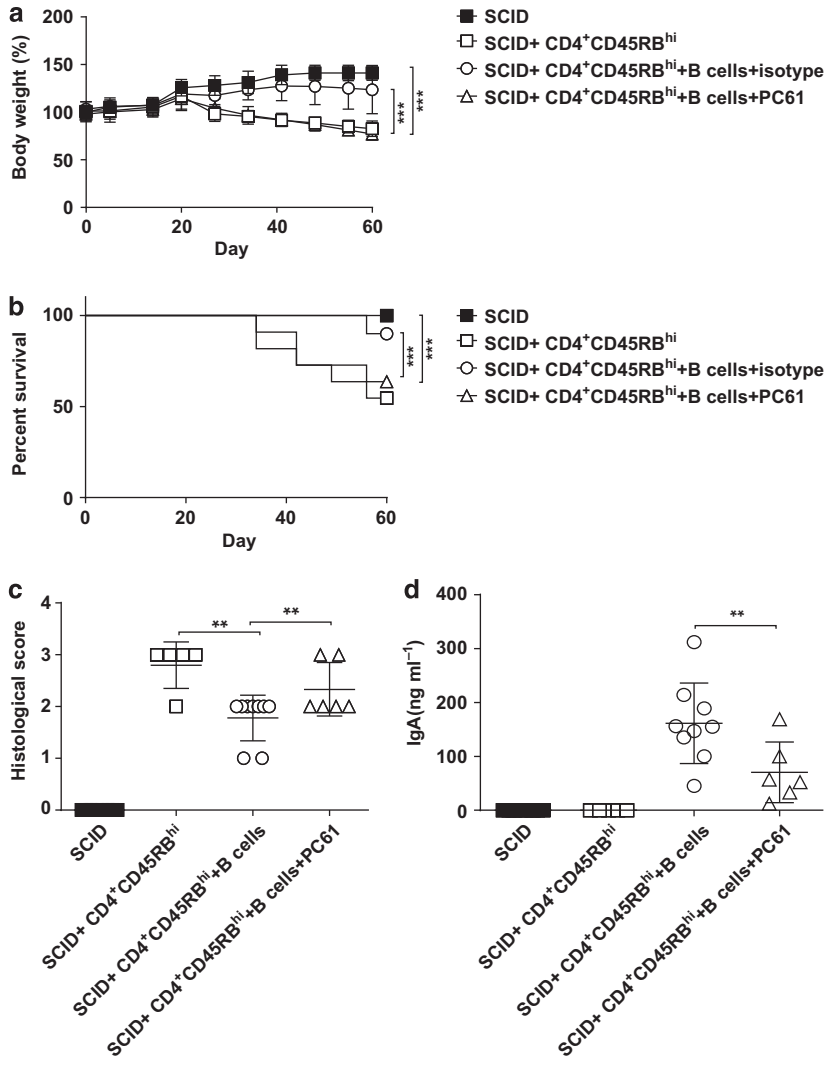


Figure 8 B cells and regulatory T cells (Tregs) are required for the attenuation of chronic colitis. Severe combined immunodeficient (SCID) mice were intravenously (i.v.) administered fluorescence-activated cell sorting (FACS)-purified splenic $CD4^+CD45RB^{hi}$ T cells (1×10^5) with or without $B220^+$ B cells (1×10^5) and colitis was induced as for **Figure 1**. On days −2, 0, 15, 30, and 45, some mice were intraperitoneally (i.p.) administered anti-CD25 (PC61) or its isotype control (500 mg). The severity of intestinal injury was evaluated by measuring the (**a**) body weight and (**b**) survival. (**c**) Histological sections were scored as for **Figure 1** with each data point representing a single mouse. (**d**) The secretion of IgA within gut was measured by enzyme-linked immunosorbent assay (ELISA) on day 60. The data shown are the mean ± s.e.m. of 10 mice from a single experiment that was repeated two times with similar results. $^{**}P<0.01$; $^{***}P<0.001$.

Tregs and not macrophages are the relevant source of protective IL-10.[11,12]

It is unclear why in our studies IL-10 production by B cells was not required, but a number of differences existed in our work and that of others. We used μMT mice that are deficient in all populations of peripheral B-cell subsets and introduced a diverse population of B cells from the spleen that likely contains a novel population of regulatory B cells, consistent with our previous studies in EAE.[31] Studies by the Bhan laboratory transferred MLN cells into TCR-$\alpha^{-/-}$ mice that likely do not contain the splenic regulatory B-cell population, allowing the IL-10-dependent mechanism to be revealed.[37] Another major difference is that the TCR-$\alpha^{-/-}$ model is spontaneous, whereas we used an inducible model. IL-10 is known to be a potent suppressor of inflammation and thus it is likely to be more important at keeping inflammation at bay in a model that takes weeks to develop as compared with DSS colitis that takes days.[45] In the Yanaba study, they transferred FACS-purified splenic B cells that were either $CD1d^{hi}CD5^+$ or depleted of this population into $CD19^{-/-}$ mice.[29] $CD19^{-/-}$ mice are not deficient in B cells, and thus they also likely harbor the splenic regulatory B cells that control Treg homeostasis, also allowing the IL-10-dependent mechanism to be revealed. Taken together, the cumulative data indicate that B cells can utilize multiple regulatory pathways to attenuate colitis severity.

In EAE, we previously showed that μMT mice deficient in B cells were unable to recover from disease.[38,46] Although other laboratories showed an importance for B-cell production of IL-10 in disease recovery, our EAE model was IL-10

independent.[31,47,48] In investigating this novel mechanism, we found that μMT mice had a significant reduction in the number of splenic Tregs and that reconstitution with either WT or IL-10$^{-/-}$ B cells restored both their numbers and the ability to recover from EAE.[31] In addition, we found that B cells promoted the homeostatic expansion of Tregs via a GITR/GITRL mechanism.[31] Here, we extended the analysis of Treg populations in the GALT tissues of μMT mice. In agreement with our previous study, splenic Tregs were significantly reduced.[31] Interestingly, we found no reduction in the absolute number of Tregs in the iELs, MLNs, or LPLs in μMT mice and the transfer of B cells did not increase their numbers as in the spleen (**Figure 3**).[31] Following colitis induction, both B-cell and Treg numbers significantly increased in the GALT of WT mice (**Figure 5** and **Supplementary Figure S2**). Treg expansion in μMT mice did not occur, suggesting that B cells, as in EAE, also promote Treg expansion in the gut (**Figure 5**).[31] This is supported by our findings that the transfer of B cells into μMT mice before colitis induction resulted in a significant increase in Tregs in the MLNs and LPLs because of their proliferation (**Figures 4 and 5**). Interestingly, a similar increase did not occur in the iEL population, indicating that Treg regulation of colitis severity has an anatomical locality. Tregs are considered one of the most important immune cell regulators of intestinal homeostasis with IL-10 as well as OX-40 and TGF-β important for their regulatory function.[11,49–52] In addition, to natural Tregs the gut is an important site for the generation of induced Tregs, both of which have been shown to be important in the resolution of disease in the CD45^{hi}CD4^{+} T-cell transfer model of colitis.[19,53] In addition to promoting the expansion of Tregs, B cells also have the potential to promote the generation of induced Tregs.[54–58] Although the transfer of Tregs and *in vitro* expansion has been shown to attenuate a variety of autoimmune diseases including EAE and colitis,[25,59–62] in our study the transfer of Tregs alone into RAG-1$^{-/-}$ mice was not sufficient to attenuate disease (**Supplementary Figure S4**). These data suggest that B-cell induction of Treg expansion alone is not the sole mechanism of their protection in DSS colitis. As Rag-1$^{-/-}$ mice deficient in B cells cannot generate protective IgA in the gut, we explored whether Tregs were essential for the IgA isotype class switching of B cells. Because IgA class switching is dependent upon TGF-β,[7,8] we first determined that the transfer of Tregs into Rag-1$^{-/-}$ mice led to the increased production of TGF-β in the intestinal lumen during colitis (**Figure 6**). In both WT and μMT mice reconstituted with B cells, the absolute number of IgA^{+} plasma cells and IgA levels in the intestine were significantly reduced during colitis in mice that were Treg depleted (**Figure 6**). Using an *in vitro* assay, we provide evidence that Treg production of TGF-β can drive IgA production (**Figure 6**). To demonstrate that IgA production occurs during colitis, we depleted B cells with anti-CD20 before colitis induction and found that IgA levels in the intestine were significantly reduced that correlated into more severe colitis (**Figure 7**). To further demonstrate that both Tregs and B cells are required to attenuate chronic colitis, we used the SCID model of colitis[36] and showed that only when B cells and functional Tregs were present chronic colitis was attenuated (**Figure 8**).

The depletion of B cells with the anti-CD20 therapeutic rituximab has shown efficacy in a number of autoimmune diseases including rheumatoid arthritis, systemic vasculitis, and multiple sclerosis.[63–65] However, in ulcerative colitis and Crohn's disease, rituximab had no beneficial effect.[66,67] Clinically, the depletion of B cells with anti-CD20 for a variety of disorders has been reported to either exacerbate or lead to the spontaneous onset of colitis.[68–74] To model this clinical finding, we previously demonstrated that the depletion of B cells in IL-10$^{-/-}$ mice accelerated the onset of spontaneous colitis.[31] These cumulative studies support our findings that B cells and Tregs are both indispensible in maintaining gut homeostasis. These data demonstrate a regulatory loop whereby B cells promote the expansion and perhaps induction of Tregs that in turn produce immunosuppressive IL-10 and TGF-β, promoting the generation of protective IgA.

METHODS

Mice. WT C57BL/6 and SCID mice were obtained from the Chinese Academy of Science (Shanghai, China). B6.129P2-Il10^{tm1cgn}/J (IL-10$^{-/-}$) and B6.129S7-Rag1^{tm1Mom}/J (Rag1$^{-/-}$) mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129S2-Ighm^{tm1cng}/J (μMT) mice on the C57BL/6 background were transferred from RIKEN Yokohama Institute, Yokohama City, Kanagawa, Japan. All mice used were at 8–12 weeks of age and were housed in the animal facility of Fudan University (Shanghai China) or in the Translational Biomedical Research Center of the Medical College of Wisconsin (Milwaukee, WI) under specific pathogen-free barrier conditions. IL-10-IRES-EGFP mice were obtained from Dr Christopher Karp and bred onto the B10.PL background.[32,33] All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. Animal studies conducted at BloodCenter of Wisconsin were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

DSS-induced colitis. Mice were given 2% DSS (molecular weight 36–50 kDa; MP Biomedicals, Santa Ana, CA) in their drinking water for 7 days followed by regular drinking water. Daily clinical evaluations, including the assessment of body weight, stool consistency, and detection of rectal bleeding, were conducted to generate a disease activity index score. Each parameter was given a grade from 0 to 4 and then averaged as follows: body weight loss (scored as: 0, none; 1, 1–5%; 2, 6–10%; 3, 11–15%; 4, >15%), stool consistency (scored as: 0, well-formed pellets; 2, loose stools; 4, diarrhea), and fecal blood (scored as: 0, negative hemoccult test; 1, positive hemoccult test; 2, blood visibly present in the stool; 3, blood visibly and blood clotting on the anus; 4, gross bleeding) as previously described.[75]

Induction of CD4^{+}CD45RB^{hi} transfer colitis. Colitis was induced in SCID mice as previously described.[35] Briefly, splenic CD4^{+}CD45RB^{hi} and B220^{+} subsets were purified by FACS with a MoFlo flow cytometer (Beckman, Coulter, Carlsbad, CA) using anti-CD4, anti-CD45RB, and anti-B220. SCID mice were intraperitoneally injected with 1×10^5 CD4^{+}CD45RB^{hi} cells with or without 1×10^5 B cells as previously described.[35]

Histology score. At various time points, mice were killed and the colon was removed and fixed in 10% buffered formalin. After paraffin embedding, 5 μm thick sections were cut transversely and stained with hematoxylin and eosin. Assessment of histological colon injury was performed at ×200 as previously described.[76] A histology score

reflecting infiltration of inflammatory cells and epithelial damage was generated on a scale from 0 to 4 as follows: 0, normal tissue; 1, inflammation with scattered infiltrating inflammatory cells and no signs of epithelial degeneration; 2, moderate inflammation with multiple foci and/or mild epithelial ulcerations; 3, severe inflammation with marked wall thickening and/or ulcerations in $>30\%$ of the tissue; and 4, inflammation with transmural inflammatory cell infiltration and/or $>75\%$ of the tissue section affected. An average of 10 fields of view per colon was evaluated blindly for each mouse.

Gut cell isolation. MLNs, Peyer's patches, iELs, and LPLs were isolated by the method of Lefrancois and Lycke.[77] Briefly, MLNs were isolated and then the intestine from the duodenum to the rectum was flushed with $Ca^{2+}Mg^{2+}$-free 100 mM CMF-HBSS (Ca2+, Mg2+ free Hank's Balanced Salt Solution) (Sigma-Aldrich, St Louis, MO). The colons were cleaned by removal of fat, fecal matter, and debris. RPMI was injected into the intestine to highlight the Peyer's patches that were then collected with a fine forceps and scissors. The intestines were opened longitudinally and cut into 0.5 cm sections, shook twice with CMF solution containing 10 mM HEPES, 25 mM $NaHCO_3$, 1 mM DTT, and 1 mM EDTA (all from Sigma-Aldrich), and 2% fetal bovine serum (Gibco, Portland, OR) at 37 °C. IELs were then purified using discontinuous Percoll gradients (GE Healthcare, Buckinghamshire, UK) by collecting mononuclear cells at the interface between the 40 and 70% layers. For the isolation of LPLs, intestinal pieces were digested for 70 min with complete RPMI-1640 containing 1 mg ml^{-1} collagenase (Roche Applied Science, Upper Bavaria, Germany), 40 µl ml^{-1} dispase (Sigma-Aldrich), and 4 µl ml^{-1} Dnase I (Sigma-Aldrich) at 37 °C. LPLs were then purified using 40%/70% discontinuous Percoll gradients.

Flow cytometry and antibodies. The anti-mouse antibodies B220-PE-Texas Red, CD4-APC-CyC7, CD8-PerCP-Cy7, Foxp3-APC, CD11b-Pacific Blue, CD45RB –FITC, CD138-APC, IgA-FITC, anti-α4β7-FITC, and CCR9-PerCP-Cy7 and their respective fluorochrome-conjugated isotypes were purchased from eBioscience (San Diego, CA). Intracellular Foxp3 staining was performed using the Foxp3-Transcription Factor Staining Buffer Set (eBioscience) as per the manufacturer's recommended protocol. Cells were acquired on an LSRII flow cytometer (BD Biosciences, San Diego, CA) or a CyAn ADP Analyzer (Beckman, Coulter) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Cell isolation and adoptive transfer. $CD3^+$ T cells or $B220^+$ B cells were purified from the spleen of WT or IL-$10^{-/-}$ mice using the mouse CD3ε MicroBead and the B cell isolation kits from Miltenyi Biotec (Bergisch-Gladbach, Germany), respectively, according to the manufacturer's instructions. The purity of both cell populations was $>95\%$. Purified single-cell suspensions (2×10^7 cells) were intravenous adoptively transferred 48 h after DSS administration. Controls received an equal volume of phosphate-buffered saline.

Cell depletion *in vivo*. B-cell depletion was performed using 250 µg per mouse anti-CD20 antibody (clone 5D2, gifted by Genentech, San Francisco, CA). The depletion kinetics was as previously reported.[78,79] For Treg depletion, the hybridoma cell line PC61.5.3 specific for mouse CD25 was purchased from the American Type Culture Collection (Manassas, VA) and monoclonal antibody was generated locally.[80] Tregs were depleted via injection of 500 µg per mouse of purified PC61.5.3.

ELISA. The intestines were isolated and flushed with endotoxin-free phosphate-buffered saline. Mucus containing fluid was harvested and stored at −80 °C until assayed. The concentration of TGF-β, IL-10, and IgA was evaluated using the Mouse Ready-SET-Go! ELISA kits from eBioscience according to the manufacturer's instructions.

Real-time reverse transcriptase-PCR analysis. Total RNA was isolated from the spleen and GALT B cells with spin columns (Applied Biosystems, Foster City, CA) and reverse transcribed into complementary DNA (Applied Biosystems). The expression of IL-10 was analyzed by real-time PCR. Real-time PCR amplifications were performed on the ABI 7500 Thermocycler (Applied Biosystems) in 20 µl reaction volumes containing complementary DNA, primers, and SYBR Green I Supermix. Thermal cycling parameters were: 10 min at 95 °C followed by 40 cycles each of 10 s at 9 °C and 60 s at 6 °C, and a final extension cycle for 5 min at 72 °C. Specificity of the reverse transcriptase-PCR reaction was controlled by the generation of melting curves. The ΔΔCt calculation method was used to compute relative gene expression as compared with β-actin. The sense IL-10 primer was 5′-GGTTGCCAAGCCTTATCGGA-3′ and the antisense primer was 5′-ACCTGCTCCACTGCCTTGCT-3′. The sense β-actin primer was 5′-CCAGCCTTCCTTCTTGGGTATG-3′ and the antisense primer was 5′-TGTGTTGGCATAGAGGTCTTTACG-3′.

***In vitro* Treg proliferation assay**. $B220^+$ splenic B cells were stimulated with lipopolysaccharide (10 µg ml^{-1}) (Sigma-Aldrich) for 48 h. $CD4^+CD25^+$ splenic Tregs (2×10^5) were stained with 3 µM carboxyfluorescein succinimidyl ester (Life Technologies, Carlsbad, CA) before stimulation with anti-CD3 (1 µg ml^{-1}) (eBioscience) and anti-CD28 (5 µg ml^{-1}) (eBioscience) in the presence of rIL-2 (60 U ml^{-1}) (R&D Systems, Minneapolis, MN) with or without stimulated B cells (2×10^5). Cells were cultured in 24-well plates for 3 days in complete RPMI-1640 (Gibco) containing 10% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, and 100 U ml^{-1} each of penicillin and streptomycin. Proliferation of Tregs was determined by carboxyfluorescein succinimidyl ester dye dilution by flow cytometry.

Peyer's patch T-cell and B-cell co-culture assay. $CD4^+CD25^-$ and $CD4^+CD25^+$ Tregs (2×10^5) from Peyer's patches were stimulated *in vitro* as per the Treg proliferation assay and cultured with or without $B220^+$ Peyer's patch B cells (2×10^5). After 24 h, cells were collected and expression of B220, CD138, and IgA were evaluated by flow cytometry. The concentration of IgA was determined in the supernatant using a Mouse Ready-SET-Go! ELISA kit from eBioscience.

Statistical analysis. Multiple group comparisons were performed using one-way analysis of variance or Kruskal–Wallis test, followed by Bonferroni correction or Mann–Whitney to compare two individual groups. Statistical analysis was performed using STATA 8.0 (Brazos, TX) or Prism 5.0 (La Jolla, CA). The *P*-values of <0.05 were considered statistically significant.

Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTION

Y.C., A.R., and B.N.D. designed the experiments; L.W., Y.C., and B.N.D. wrote the paper; L.W., A.R., X.J., S.B., X.L., and T.Q. performed the experiments; L.W. and A.R analyzed the data and R.H. and J.W. made important intellectual contributions.

DISCLOSURE

The authors declared no conflict of interest.

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