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Splenic B Cells from *Hymenolepis diminuta*-Infected Mice Ameliorate Colitis Independent of T Cells and via Cooperation with Macrophages

José L. Reyes,* Arthur Wang,* Maria R. Fernando,* Rabea Graepel,* Gabriella Leung,* Nico van Rooijen,† Mika Sigvardsson,‡ and Derek M. McKay*

Helminth parasites provoke multicellular immune responses in their hosts that can suppress concomitant disease. The gut lumendwelling tapeworm *Hymenolepis diminuta*, unlike other parasites assessed as helmint therapy, causes no host tissue damage while potently suppressing murine colitis. With the goal of harnessing the immunomodulatory capacity of infection with *H. diminuta*, we assessed the putative generation of anti-colitic regulatory B cells following *H. diminuta* infection. Splenic CD19+ B cells isolated from mice infected 7 [HdBc(7d)] and 14 d (but not 3 d) previously with *H. diminuta* and transferred to naive mice significantly reduced the severity of dinitrobenzene sulfonic acid (DNBS)-, oxazolone-, and dextran-sodium sulfate–induced colitis. Mechanistic studies with the DNBS model, revealed the anti-colitic HdBc(7d) was within the follicular B cell population and its phenotype was not dependent on IL-4 or IL-10. The HdBc(7d) were not characterized by increased expression of CD1d, CD5, CD23, or IL-10 production, but did spontaneously, and upon LPS plus anti-CD40 stimulation, produce more TGF-β than CD19+ B cells from controls. DNBS-induced colitis in RAG1−/− mice was inhibited by administration of HdBc(7d), indicating a lack of a requirement for T and B cells in the recipient; however, depletion of macrophages in recipient mice abrogated the anti-colitic effect of HdBc(7d). Thus, in response to *H. diminuta*, a putatively unique splenic CD19+ B cell with a functional immunoregulatory program is generated that promotes the suppression of colitis dominated by TH1, TH2, or TH1-plus-TH2 events, and may do so via the synthesis of TGF-β and the generation of, or cooperation with, a regulatory macrophage. The *Journal of Immunology*, 2015, 194: 000–000.

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61460) under accession number GSE61460.
colitis that accompanies infection with the intestinal nematode *Heligmosomoides polygyrus* (16, 17), and macrophages recruited to the lamina propria after infection with *Schistosoma mansoni* inhibited dextran sodium sulfate (DSS)-induced colitis (18).

*Hymenolepis diminuta* is a largely innocuous cestode that is expelled from mice with within 12–14 d of a primary infection (19). Infection with *H. diminuta* significantly reduces the severity of dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice, an effect that involved IL-10 likely from CD4+ T cells. Extrapolating from the observation of increased expression of markers of AAMs in the gut of parasitized mice, we showed that adoptive transfer of ex vivo–differentiated AAMs inhibited DNBS-induced colitis (20). Because the immune response to combat helminths is a coordinated multicellular one, the accompanying or subsequent immunoregulatory events are likely to be equally complex; therefore, it is highly likely that other immunoregulatory cells, in addition to CD4+ T cells and AAMs, are mobilized after infection with *H. diminuta*.

Subpopulations of B cells with immunosuppressive properties have been identified (21); for example, B cells that arise in response to infection with helminths can suppress airway inflammation and murine experimental autoimmune encephalitis, which is a model of multiple sclerosis (22–24). There is, at best, a rudimentary knowledge of regulatory B cell subtypes that arise upon infection with helminths, noting that the few studies available have used nematodes or the trematode *S. mansoni*. Thus, in ongoing analyses to dissect the multiple mechanisms by which infection with helminth parasites can suppress colitis, the current study assessed the nature and potential anti-inflammatory effect of splenic B cells harvested from mice after a primary infection with *H. diminuta*.

**Material and Methods**

**Mice, parasites, and infection**

Male BALB/c or C57BL/6 mice (7–8 wk old) were purchased from Charles River Laboratories (Quebec, Canada). IL-4 receptor–a knockout (KO) mice (BALB/c background) were a gift from Dr. F. Brombacher (University of Cape Town, South Africa), RAG1 KO-mice (C57BL/6 background; lack T and B cells) and mast cell deficient (Kit−/+ mice were from breeding colonies provided by Dr. P. Santamaria and Dr. M. Kelly (University of Calgary). IL-10 KO (BALB/c background) and additional RAG1−/− mice were purchased from Jackson Laboratories. Mice were housed under a light-dark cycle of 12:12 h with free access to food and water. Animal experiments were conducted with approval from the University of Calgary Health Science Animal Care Committee conforming to national guidelines.

The life cycle of *H. diminuta* was maintained in the laboratory by cecal passage through the immediate invertebrate host, the flour beetle, *Tribolium confusum*. Mice were infected with five cysticercoids in 100 µl of sterile 0.9% NaCl by oral gavage (25).

**B cell isolation and experimental design**

Splenocytes were isolated from mice infected with *H. diminuta* 3, 7 (He/BeCt(7)), or 14 (Hel/BeCt(14)) and CD19+ B cells were obtained using magnetic separation and a mouse B cell negative selection kit (StemCell Technologies, Vancouver, Canada) following the manufacturer’s instructions. Purified splenic B cells were washed in sterile PBS, and 5 × 10^6 B cells (90–92% CD19+) or 3 × 10^6 follicular B cells (see below) were given i.p. to recipient mice immediately after the intrarectal (i.r.) delivery of either DNBS or oxazolone (pilot studies with 3 × 10^6/ml splenic CD19+ cells produced inconsistent data) or when regular drinking water was replaced with 5% v/v DSS-drinking water. In addition, B cells from naive mice were treated in vitro for 48 h with either murine recombinant IL-4 plus IL-13 (both 10 ng/ml; Cedarlane Laboratories, Burlington, ON, Canada), referred to as BcIL-4/13 or 1 mg/ml of a PBS-soluble crude Ag extract of whole adult *H. diminuta* (referred to as HdAg) (26). The cells were rinsed extensively with PBS and used in transfer experiments.

Experiments were performed to assess any relevant participation of the 8–10% contaminant cells after magnetic purification. Cell sorting (FACSaria II machine and FACS Diva version 6.1.3. software, BD Biosciences; at University of Calgary flow cytometry facilities) was performed, yielding a 99.1% CD19+ cell population. Next, splenic follicular B cells were isolated by cell sorting based on CD23 IgM surface expression (27).

To track He/BeCt(7) after transfer, cells were stained with CellTrace CFSE labeling kit (Molecular Probes). Splenocytes were adjusted to 5 × 10^6/ml PBS/BSA 0.01% and stained with CellTrace CFSE for 10 min at 37°C. The reaction was quenched with FBS-medium, cells were spun down, and B cells were purified as above.

In other experiments, mice treated with DNSB ± HdBc(7) also received anti–TGF-β neutralizing Abs (200 µg i.v.; R&D Systems) immediately after i.r. delivery of DNBS and colitis assessed 72 h after DNBS administration.

**Induction and assessment of experimental colitis and monoarthritis**

Colitis was induced in anesthetized mice by i.r. DNSB (3 mg) or oxazolone (5 mg; both from Sigma, St. Louis, MO) in 100 µl of 50% ethanol via a 3-cm catheter or by a 5-d treatment with 5% DSS (MP Biochemicals, Solon, OH) (25, 28). Mice were sacrificed at 72 h after DNBS–oxazolone administration, and disease severity was assessed with a five-point disease activity score, based on animal weight and general condition, colon length, diarrhea, and macroscopic evidence of ulceration. At necropsy, the colon was removed, measured, and divided based on percentage length, such that: 1) the terminal 20% was snap-frozen for the determination of myeloperoxidase (MPO) activity using a colorimetric assay in which 1 U of MPO enzyme activity is required to degrade 1 µM H2O2 per minute; 2) the next 10% was fixed in 10% neutral buffered formalin, and histopathology was assessed after processing using a 12-point scale on H&E-stained sections in a blinded fashion; and 3) the next 10% was snap frozen in liquid nitrogen for either extraction of mRNA for quantitative polymerase chain reaction (qPCR) analysis or homogenized in PBS containing a protease inhibitor mixture (Roche, Mississauga, ON, Canada) and cytokine levels measured by ELISA (28).

Monoarthritis was induced via the intra-articular injection of 10 µl CFA (Sigma) into the ipsilateral joint and 10 µl sterile PBS into the contralateral joint as control. Knee joint swelling was monitored over a 6-d period by an observer blinded to the experimental treatment using mechanical Vernier calipers as described previously (6).

**Flow cytometry**

Total cells were gated in a forward light scatter–versus-CD19 plot to identify B cells, and specific surface markers were analyzed. The cell surface Ags CD5 and CD1d are prominent markers of regulatory B cells, and Tim-1 (29) and CD23 (24) have also been associated with specific regulatory B cell subtypes. One million B cells were treated with anti-CD16/32 (10 min, 4°C) to block nonspecific binding, and then standard flow cytometry staining protocols were conducted with the following fluorochrome-conjugated Abs: APC-CD19, FITC-CD5, PE-CD1d, PE-Tim-1, PE-CD23, PE-CD138, PE-CD80, PerCP-MHC II, FITC-B220, FITC-IgM, PE-programmed death ligand (PD1)-1, and PE-PDL-2 (all from BioLegend, San Diego, CA). APC-F4/80 and PE-GM1 were used as markers of macrophages and neutrophils, respectively. Data were analyzed using the Attune flow cytometer and Attune cytometric software version 1.25 (R&D systems).

**qPCR**

Colonie tissue was homogenized in TRIZol reagent (Life Technologies, Carlsbad, CA). Total RNA was quantified using a NanoDrop (Thermo Fischer Scientific), and 1 µg isolated RNA was used to generate cDNA with an iScript RT Kit (Bio-Rad, Mississauga, ON, Canada) in a MyCycler thermocycler (Bio-Rad). cDNA was added to 500 nM gene-specific primers for murine chitinase 3–like protein (Ym1), resistin-like molecule α (Relmα), choline acetyltransferase (ChAT), and Foxp3, IL-1β, IFNγ, IL-17, and IL-10 to a 1× SYBR green reaction mix (Bio-Rad). Data were analyzed using the 2−ΔΔct method using 18S as a housekeeping gene.

**Analysis of cytokine, Ig, and NO production**

Isolated splenocytes (1 × 10^6/ml) were stimulated with Con A (2 µg/ml) for 48 h, and supernatants were collected. A panel of cytokines were screened in the Lumines 32-plex (Eve Technologies, University of California). Supernatants from CD19+ B cells (1 × 10^5) stimulated in vitro with LPS (1 µg/ml) ± anti-CD40 (1 µg/ml) for 48 h were assessed for IFN-γ, IL-4, IL-10, and TGF-β by sandwich ELISA (R&D Systems) and NO by the Griess reaction (measures nitrite as a surrogate for NO). Macrophage (MO) production of TNF-α was assessed with ELISA.
Bone marrow–derived MØ–HdBc(7d) cocultures

Bone marrow MØ were grown as described (30). Femur and tibia from naive BALB/c mice were flushed with sterile RPMI medium containing HEPES (20 mM), penicillin-streptomycin (2%), and 1× Glutamax (all from Life Technologies). Total bone marrow cells were treated with M-CSF (20 ng/ml; Cedarlane Laboratories, Burlington, ON) on days 0, +2 and +4; cells were harvested at day 7, and 2.5 × 10^5 cells were seeded into 12-well plates. HdBc(7d) were retrieved and cultured with MØ or LPS-activated MØ (classically activated macrophages [CAM]) for 48h, the B cells were retrieved and assessed by flow cytometry, and spontaneous IL-10 and TGF-β production was measured with ELISA.

With respect to the MØ: 1) mRNA was extracted for qPCR, 2) cells stimulated with LPS (1 μg/ml) for cytokine production, and 3) in additional studies MØ were stimulated with LPS for 24 h, HdBc(7d) at 1:2, 1:4, and 1:8 ratios (MØ:B cells), supernatants were collected, and TNFα, IL-10, and NO were measured.

Microarray assay

Splenic CD19+ B cells from 7-d H. diminuta–infected and uninfected mice (as controls) were isolated by cell sorting (purity = 99.1%). Cells were maintained in RLT buffer (Qiagen, Valencia, CA) and RNA extraction and microarray analysis (Affymetrix GeneChip Mouse Genome Array 430.2) were conducted at Linköping University. Data were analyzed with Partek Genomics Suite 6.6 software (Partek, Chesterfield, MO) at the University of Calgary.

Data analysis

Data are mean ± the SEM. Multiple group comparisons were performed using one-way ANOVA followed by post hoc Tukey test, whereas comparison uninfected and H. diminuta-infected used Student t test and GraphPad Prism 5 software (La Jolla, CA). In both analyses, p < 0.05 was accepted as a statistically significant difference.

Results

H. diminuta–elicited B cells ameliorate colitis

Infection with H. diminuta results in a TH2-dominated immune response (19), and enumeration revealed an ∼25% increase in CD19+/CD45R(B220)+ splenic B cells 7 d postinfection: 48.8 ± 2.8 versus 38.5 ± 1.6 × 10^6 (n = 5; p < 0.01). Splenic B cell purified with magnetic beads yielded a population composed of 90–92% CD19+ cells, with the remainder being predominantly

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**FIGURE 1.** (A–D) B cells isolated by magnetic beads or cell sorting suppress DNBS-induced colitis. Splenic B cells (5 × 10^6) isolated 7 d after H. diminuta infection (HdBc) by magnetic bead (mag. isol) selection (90–92% CD19+) or cell sorting (99.1% CD19+; 3 × 10^6) have similar capacities to suppress DNBS (3 mg, i.r., 72 h)-induced colitis in BALB/c mice (mean ± SEM; n = 4–5). (E) Original magnification ×200. *p < 0.05, †p < 0.05 compared with control (con) and DNBS only groups, respectively. L, lumen; M, muscle.
CD3+ cells. This population was used in the majority of the experiments; however, it was possible that the contaminating 8–10% of CD3+ cells mediated the effect and not the B cells. Thus, cell sorting was used to isolate an ∼99.1% CD19+ splenic B cells for adoptive transfer experiments in direct comparison with the B cell population isolated by magnetic bead separation (Fig. 1).

Mice treated with DNBS (i.r.) developed colitis as assessed by wasting, shortening of the colon, colonic MPO activity, disease activity score, and histopathology. Concomitant administration of splenic CD19+ B cell isolated from H. diminuta–infected mice by cell sorting or magnetic beads significantly inhibited DNBS-induced colitis (Fig. 1). The similar anti-colitic outcomes of transferring cell-sorted and magnetic bead–isolated CD19+ B cells indicated that the contaminating CD19− cells (∼4 × 10^5 cells) in the latter were unlikely to be driving the anti-colitic effect; therefore, subsequent experiments used CD19+ cells isolated with the magnetic bead method.

**FIGURE 2.** Splenic B cells from H. diminuta–infected mice reduce DNBS-induced colitis. Splenic CD19+ B cells (5 × 10^6, i.p.) isolated from H. diminuta–infected mice 7 [Hd/Bc(7)] but not 3 d previously inhibit DNBS-induced (3 mg, i.r., 72 h) colitis as gauged by (A) weight loss, (B) colon length, (C) disease activity score, (D) colonic MPO activity, and (E and F) histopathology (mean ± SEM, n = 11–15 mice from four experiments). Original magnification ×200. *p < 0.05, #p < 0.05 compared with control (con) and DNBS only, respectively. Bc, B cells from naive mice; L, lumen, M, external muscle layers.
In contrast to \(HdBc(7^d)\), B cells from naive mice or mice infected 3 d previously \([HdBc(3^d)]\) did not affect the severity of DNBS-induced colitis (Fig. 2). Splenic CD19+ B cells from mice infected 14 d previously with \(H. diminuta\) \((n = 5)\) protected recipients from DNBS-induced colitis (data not shown). Adoptive transfer of \(HdBc(7^d)\) significantly inhibited oxazolone- (see below) and DSS-induced colitis \((n = 7–9;\) Supplemental Fig. 1). In accordance with the reduced MPO activity in the colon of \(HdBc(7^d)\) DNBS-treated mice (Fig. 2D), these animals also had fewer Gr1+ peritoneal granulocytes (data not shown). In accordance with these data, qPCR of colonic tissue from DNBS-treated mice revealed increased IL-1\(\beta\), IFN-\(\gamma\), and IL-17 mRNA that was reduced in mice receiving DNBS plus \(HdBc(7^d)\); there was no statistical increase in IL-10 mRNA in the latter group (Fig. 3). Con A stimulation revealed reduced IFN-\(\gamma\) and increased IL-4 and IL-10 production by spleen cells isolated from \(HdBc(7^d)\)-treated mice compared with those from DNBS naïve B cell–treated mice (Fig. 4A–C). In contrast, colonic levels of IL-10 were reduced in DNBS-treated mice compared with controls (Fig. 4D), independent of B cell treatment.

In vitro stimulation of the CD19+ B cells with LPS revealed few differences in cytokine output between the groups (Supplemental Fig. 2). Anti-CD40 plus LPS stimulation did not reveal enhanced IL-10 production by \(HdBc(7^d)\) (Fig. 4E), but there was an ~3-fold statistically significant spontaneous release of TGF-\(\beta\) by these cells; this was not further enhanced by anti-CD40 plus LPS (Fig. 4F). Cotreatment with anti-TGF-\(\beta\) Abs significantly reduced the anti-colitic effect of \(HdBc(7^d)\); however, mice treated with only DNBS had enhanced disease when given TGF-\(\beta\)–neutralizing Abs (Fig. 5).

NO has been reported to be produced by IgA+ B cells (31); however, no differences in spontaneous or stimulated NO production between \(HdBc(7^d)\) and naïve B cells were observed (data not shown). qPCR \((n = 3–5)\) revealed no significant expression of ChAT mRNA in CD19+ B cells from infected or control mice. Similarly, measurement of IgG\(_1\) production after LPS plus anti-CD40 stimulation revealed no differences between...
the groups: naive B cell = 3.4 ± 1.5 ng/ml versus HdBc(7d) = 2.3 ± 1.3 ng/ml.

Despite the consistent and robust ability of HdBc(7d) to inhibit DNBS-induced colitis, flow cytometric analysis did not reveal any significant upregulation of CD1d, CD5, CD21, CD23, PDL1, PDL2, or Tim1 expression (markers associated with regulatory B cells [Bregs]) (21, 29) on freshly isolated cells compared with CD19+ splenic B cells from naive mice (Supplemental Fig. 3A). However, based on the total increase in splenic CD19+ B cells in infected mice this translated into a significant increase in CD19+/CD5+ cells: 6.0 ± 0.6 versus 10.2 ± 0.7 × 10^6 (n = 5; p = 0.002). Furthermore, adoptive transfer of 3 × 10^6 CD19+ follicular B cells from mice infected 7 d previously with H. diminuta resulted in significant inhibition of DNBS-induced colitis (Fig. 6).

In addition, microarray-based global gene expression analysis revealed upregulation of 700 and 71 genes in HdBc compared with naive B cells when 1.5 fold-changes and 1.8 fold-changes were set as the threshold for analysis, respectively (Supplemental Fig. 3B; data deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61460). For example, calcyclin, complement component 1 q subcomponent b-polypeptide, mitochondrial ribosomal protein L52, C-type lectin domain family 2, signal transducer and activator of transcription 4, and peroxisome proliferative activated receptor γ coactivator 1a genes were significantly upregulated. Although such analysis gives no indication of protein expression or function, it attests to the activation of the HdBc, and the appropriate cluster analysis could reveal a set of mRNA changes as signature molecules for the H. diminuta–induced Bregs.
A large proportion of HdBc(7d) delivered i.p. remains in the peritoneal cavity

Four, 24, 48, and 72 h after i.p. injection of CFSE-labeled HdBc(7d), peritoneal lavages were obtained, the omentum was observed microscopically, and CD19+ B cells were isolated from the spleen, mesenteric lymph node (MLN), and colon. At all time-points, considerable numbers of HdBc(7d) were found in the peritoneal cavity. For example, at 72 h after treatment, 13–22% of CFSE-labeled HdBc(7d) (~750,000 cells) remained in the peritoneal cavity (n = 3). Accumulation in the omentum was inconsistent, and appreciable numbers of labeled HdBc(7d) were below the level of detection in the spleen, MLN, and colon at this time point.

Anti-colitic B cells generated in response to infection with H. diminuta are functionally different from IL-4−/−IL-13 or worm Ag–treated B cells

Postulating that the H. diminuta–evoked Bregs could be elicited by a direct effect of worm Ag exposure to TH2 cytokines, CD19+ B cells from naive mice were treated in vitro with worm Ag or IL-4 plus IL-13 for 48 h and their anti-colitic potential compared with HdBc(7d). Worm Ag–treated B cells did not inhibit DNBS-induced colitis, whereas BcIL-4/13 significantly protected against this colitis, they were, on average, less effective than HdBc(7d) (Fig. 7A, 7B). When oxazolone was used to evoke colitis, there was a statistically significant difference in the protection afforded by HdBc(7d) compared with BcIL4/13 (Fig. 7C, 7D).

Mast cells are increased by helminth and are an important arm of the TH2 response (33); it was recently shown that they can regulate immune functions performed by myeloid derived suppressor cells (34). We tested the ability of HdBc(7d) obtained from Kit−/− mast cell–deficient mice to regulate colitis. Splenic B cells from infected Kit−/− mice suppressed DNBS-induced colitis to a degree comparable to cells from wild-type (WT) mice (Fig. 7E). The reciprocal direction of communication was also tested, and it was found that HdBc(7d) from WT mice when transferred into mast cell–deficient mice protected the recipients from colitis, indicating that HdBc(7d)–mast cell communication is not required for the suppression of DNBS-induced colitis (Fig. 8).

Moreover, CD19+ B cells from H. diminuta–infected IL-4−/− mice also inhibited DNBS-induced colitis (Fig. 7). Spleen cells from IL-4−/− mice produced IL-10 (~1 ng/ml), and although less than that from infected WT mice, this is still a substantial amount of cytokine. Thus, although IL-4 likely plays a role in educating B cells in H. diminuta–infected immunocompetent mice, signaling through the IL-4R is not an absolute requirement for the generation of H. diminuta–induced Bregs with the capacity to inhibit colitis. These data are consistent with the finding that IL-4Rα−/− mice infected with H. diminuta (these mice fail to expel the worm; data not shown) are somewhat protected from DNBS-induced colitis (Supplemental Fig. 4).

In contrast to these analyses of colitis, HdBc(7d) did not protect mice from the monoarthritis induced by direct intra-articular injection of CFA (data not shown).

Inhibition of DNBS colitis by HdBc(7d) does not require recipient T or B cells

A variety of Tregs can block colitis in murine model systems (35–37), and Bregs can induce Tregs (38, 39); however, at necropsy, Foxp3 mRNA expression was not increased in colonic tissue, spleen, or MLNs of mice treated with DNBS plus HdBc(7d) (Fig. 9A). To test a role for other Tregs or involvement of the recipient’s T or B cells in the suppression of disease by HdBc(7d), colitis was elicited in RAG1−/− mice. Reconstitution of RAG1−/− mice with naive B cells did not affect the outcome of DNBS-induced colitis, whereas mice given HdBc(7d) developed significantly less macroscopic and histologic disease (Fig. 9B, 9C). Flow cytometry analysis of spleen cells from the RAG1−/− recipients failed to detect any transferred CFSE-labeled CD19+ HdBc(7d) (n = 3; data not shown).

Despite the fact that the HdBc(7d) were not producing enhanced amounts of IL-10, this cytokine is implicated in helminth modulation of disease and the suppression of colitis (25, 40, 41) in immunocompetent mice. Therefore, it was important to test a role for IL-10 in the current paradigm. IL-10−/− mice expel H. diminuta (D. McKay, personal communication) and Con A stimulation of B cell–depleted splenocytes from infected IL-10−/− mice resulted in ~2-fold and ~9-
fold increases in IL-4 and IL-5 production compared with cells from uninfected mice, respectively. Adoptive transfer of \( \text{HdBC}(7^d) \) from IL-10 \(^{-/-} \) mice into BALB/c mice suppressed DNBS-induced colitis to a degree not significantly different from \( \text{HdBC}(7^d) \) from WT donors, indicating that IL-10 is dispensable in the \( \text{HdBC}(7^d) \) that drives the suppression of colitis (Fig. 9D, 9E).

HdBC(7\(^d\)) cooperate with MØ to suppress DNBS-induced colitis

Macrophages are a diverse population of cells that can be skewed in favor of a proinflammatory or anti-inflammatory phenotype (12, 20). Testing the hypothesis that \( \text{HdBC}(7^d) \) suppression of colitis required or promoted the presence of an anti-inflammatory or
proresolution MØ, experiments were conducted in mice depleted of MØ using clodronate-liposomes (MØ depletion confirmed by reduction in F4/80+ cells to ~2% of the cell content of the spleen and peritoneal cavity from ~25% and ~22% in controls, respectively; Gr1+ cell numbers were negligibly affected by the procedure; in vitro observation indicated a lack of cytotoxic effects of

**FIGURE 7.** Naive B cells treated in vitro with TH2 cytokines but not *H. diminuta* Ag suppress colitis. Colitis was elicited by i.r. (A and B) DNBS (3 mg) or (C and D) oxazolone (oxazo; 4 mg), and mice were treated simultaneously with B cells from mice infected with *H. diminuta* 7 d previously (*Hd*Bc(7d)) or B cells from naive mice treated for 48 h in vitro with either a crude PBS-soluble extract of *H. diminuta* (Bc-Ag; 1 mg/ml) or IL-4 plus IL-13 (both 10 ng/ml; Bc [IL4/13]). (E and F) *Hd*Bc(7d) from mast cell deficient mice (Kitw-sh) or lacking the IL-4R α-chain are as efficient as cells from WT mice at suppressing DNBS-induced colitis (mean ± SEM; n = 8–10 mice from two experiments). *p < 0.05, *p < 0.05, *p < 0.05 compared with control, DNBS or oxazo., and oxazo. plus Bc IL4/13, respectively.)
cotreated with loss, (mice are protected from DNBS-induced colitis. Analysis of communication).

**FIGURE 8.** Mast cell–deficient mice (Kitw−/−) given HdBc(7d) from WT mice are protected from DNBS-induced colitis. Analysis of (A) weight loss, (B) disease activity scores, and (C) colonic MPO activity reveal significantly less severe DNBS-induced (3 mg, i., 72 h) colitis in mice cotreated with HdBc(7d) (5 × 10⁶, i.p.; mean ± SEM; n = 3–15 mice. *p < 0.05, #p < 0.05 compared with control and DNBS only, respectively.

clostronate liposomes on CD19+ B cells) (J.L. Reyes, personal communication).

Colitis was not appreciably different among RAG1−/− mice treated with DNBS, DNBS plus clodronate, and DNBS plus HdBc(7d) plus clodronate (Fig. 10A), whereas mice administered HdBc(7d) and the control, PBS-containing liposomes had less severe disease (Fig. 10A–C). Quantitative PCR of colon extracts for arginase-1 (not shown), Ym1, and Relmα (Fig. 10D) revealed no consistent changes in the mRNA levels of these markers for AAMs (12) between the groups, suggesting that the MØ population required for the HdBc(7d) suppression of colitis is not the IL-4/IL-13 driven AAM.

In addition, in vitro coculture of HdBc(7d) with MØ for 24 or 48 h affected neither the MØ-constitutive or LPS-stimulated expression of arginase-1 and iNOS mRNA nor production of NO and TNF-α (n = 4–6; data not shown); however, IL-10 output was increased (Fig. 10E). Although IL-10 is not a requirement in the HdBc(7d), it might be an active anti-inflammatory component in the MØ effector cell.

HdBc(7d) cocultured with MØ show increased expression of activation markers

Having identified the need for MØ in the suppression of colitis initiated by HdBc(7d), we queried the ability of MØ to affect the B cells. Coculture of HdBc(7d) with MØ resulted in significant upregulation of the B cell activation markers CD5, CD1d (that would allow interaction with NKT cells), and PDL1 (that would promote cell killing/anergy; Fig. 11), whereas levels of CD80 (Fig. 11) and CD23 (not shown) were unchanged. Coculture with CAM to mimic the likelihood of encountering LPS-exposed MØ in vivo resulted in similar changes (of lesser magnitude) in these markers on the B cells. Coculture with MØ or CAM did not affect the spontaneous production of IL-10 or TGF-β by the HdBc(7d) (n = 4; data not shown).

**Discussion**

Although B cells participate in autoimmunity, their absence can render mice susceptible to some inflammatory diseases (42). Infection with helminth parasites is a potent immunoregulatory force that can suppress inflammatory disease via many mechanisms, including mobilization of heterogeneous Breg populations that can be equally diverse in their mechanism of immune suppression. For example, suppression of inflammation by infection with S. mansoni (trematode) or H. polygyrus (nematode) is IL-10 dependent and IL-10 independent, respectively (15, 24).

Expulsion of *H. diminuta* from mice has the bystander benefit of limiting the severity of DNBS-induced colitis (25). Given that worm expulsion is a multicellular event, many immune cell phenotypes could contribute to the suppression of colitis, and we focused on Bregs because of the paucity of data on the helminth-evoked Breg and the complete lack of data on Bregs after infection with cestodes. Splenic CD19+ B cells were increased in infected animals and adoptive transfer of HdBc(7d) and HdBc(14d), but not HdBc(3d), reduced the severity of DNBS-, oxazolone-, and DSS-induced colitis. Consistent with this, HdBc(7d) transfer resulted in reduced IL-1β and IFN-γ mRNA in the colon, and reduced IFN-γ and enhanced IL-4 and IL-10 output by splenocytes from the recipients. To our knowledge, this is the first demonstration of Bregs in response to cestodes, and we are unaware of any other study showing helminth-induced Bregs suppressing diseases associated with TH1, TH2, and TH1/2 immunity.

The induction of a Breg would be via contact with worm Ag or conditioning by the immune milieu, in which IL-4 and IL-13 are likely candidates given the stereotypic TH2 response to infection with helminths. B cells from naive mice exposed to *H. diminuta* extracts displayed no ability to suppress colitis. IL-4 plus IL-13 in vitro–educated B cells had a different regulatory program from Bregs in response to cestodes, and we are unaware of any other study showing helminth-induced Bregs suppressing diseases associated with TH1, TH2, and TH1/2 immunity.

**FIGURE 8.** Mast cell–deficient mice (Kitw−/−) given HdBc(7d) from WT mice are protected from DNBS-induced colitis. Analysis of (A) weight loss, (B) disease activity scores, and (C) colonic MPO activity reveal significantly less severe DNBS-induced (3 mg, i., 72 h) colitis in mice cotreated with HdBc(7d) (5 × 10⁶, i.p.; mean ± SEM; n = 3–15 mice. *p < 0.05, #p < 0.05 compared with control and DNBS only, respectively.

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addition, stimulated splenocytes from IL-4Rα−/− mice produced less IL-10 than those from WT mice did, suggesting that even in the presence of low amounts of IL-10, *Hd*Bc(7d) drive anti-colitic activity. Thus, despite a role for IL-10 in specific helminth-driven Bregs (22), *H. diminuta* suppression of DNBS colitis (25), and CFA monoarthritis (6), IL-10 is neither the key differentiation factor nor...
effector molecule in the $HdBc(7d)$. Stimulated $HdBc(7d)$ did not preferentially produce IL-10, and more conclusively, $HdBc(7d)$ from IL-10^{-/-} mice suppressed DNBS-induced colitis to a degree indistinguishable from cells from WT mice.

A small but significant increase in TGF-β was noted in $HdBc(7d)$. TGF-β has been implicated in helminth-driven immunosuppression (43), and human B cells can produce TGF-β (44); therefore, it is tempting to speculate that the anti-colitic effect of $HdBc(7d)$ may be due, in part, to TGF-β. The use of anti-TGFβ neutralizing Abs supports this supposition: disease in mice treated with DNBS plus $HdBc(7d)$ plus anti–TGF-β was not statistically different from mice treated with only DNBS; however, mice treated with DNBS plus

FIGURE 10. Depletion of macrophages in recipient mice prevents the suppression of DNBS-induced colitis by B cells from $H. diminuta$–infected mice. RAG1^{-/-} mice were treated with clodronate-containing liposomes (clodL) to deplete macrophages or PBS-liposomes (PBSL) as a control prior to the induction of colitis with DNBS (3 mg, i.r., 72 h) ± CD19+ B cells ($5 \times 10^6$, ip.) from $H. diminuta$–infected mice [7 d postinfection; $HdBc(7d)$]. (A and B) Disease activity and histopathology scores were not different between DNBS, DNBS + clodL, and DNBS + $HdBc(7d)$+clodL treatment groups ($n = 4$–9 mice from two experiments. (C) Representative histologic images. Original magnification $\times 200$. (D) Graph showing disease activity scores.$^*$p < 0.05, #p < 0.05 compared with control (con) and DNBS group, respectively. (E) qPCR of tissue from WT mice revealed no differences in colonic mRNA expression of Ym1 or Relmα, markers indicative of alternatively activated macrophages, between the groups ($n = 4$). (F) Coculture of bone marrow-derived MØ with $HdBc(7d)$ from WT mice enhances IL-10 production by LPS-stimulated MØ (100 ng/ml, 24 h; mean ± SEM; $n = 6$–10). *p < 0.05, **p < 0.05 compared with control (con) and DNBS group, respectively. L, gut lumen; M, external muscle layers. 

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anti–TGF-β displayed exaggerated disease. Whereas TGF-β may be a component of the HdBc(7d) suppression of colitis the possibility that the anti–TGF-β worked by blocking recipient rather than donor cell TGF-β cannot be dismissed.

The interaction of *H. diminuta*, a gut lumen-dwelling parasite, with its host is different from that of abrasive nematodes and helminth species that cause tissue damage via migration through the host; this tissue damage has been posited as a driving force for anti-inflammatory events (45). Given this substantial difference in host–parasite interaction, the generation of a unique splenic CD19+ Breg in response to *H. diminuta* is plausible. Furthermore, descriptions of lymphocyte (including Bregs), innate adaptive lymphocytes, and macrophage subtypes (46, 47) attest to this possibility, supporting the contention that unique B cells could be mobilized in response to infection with cestode parasites.

A spectrum of Bregs have been identified based on expression of CD1d, CD5, CD23, FasL, Tim-1, and others (21, 44); for example, CD23 hi CD5+ and CD1d hi CD21 hi CD23 hi Bregs have been described in mice following infection with *H. polygyrus* and *S. mansoni*, respectively (22, 24). Whereas the total number of splenic CD19+CD5+ cells was increased in infected mice, flow cytometry analyses did not reveal significant upregulation of canonical Breg markers on the HdBc(7d) Breg in response to *H. diminuta* is plausible. Furthermore, descriptions of lymphocyte (including Bregs), innate adaptive lymphocytes, and macrophage subtypes (46, 47) attest to this possibility, supporting the contention that unique B cells could be mobilized in response to infection with cestode parasites.

Mechanistically, Bregs can have intrinsic anti-inflammatory activity or could mobilize other immunoregulatory cells such as Foxp3+ Tregs (22). Foxp3 mRNA was not increased in mice receiving *HdBc(7d)*, but this does not reflect function nor negate the possibility of recruitment of other regulatory T or B cells into the anti-colitic response. Although there may be differences in the mechanism of DNBS-induced colitis in WT and T cell–depleted mice, RAG1−/− mice display a DNBS phenotype and histopathology that are highly similar to WT mice. Suppression of DNBS-induced colitis in RAG1−/− mice that received *HdBc(7d)* revealed that recipient T and B cells were not required for the inhibition of colitis.

FIGURE 11. *HdBc(7d)* are activated by coculture with MØ. Freshly isolated *HdBc(7d)* display no obvious up-regulation of activation or Breg markers (see Supplemental Fig. 3), but after 48 h of coculture with bone-marrow derived MØ (250,000) they have increased expression of CD5 (A), CD1d, and programmed death ligand-1 (PDL1), whereas CD80 levels remained unchanged (B). A similar pattern was noted after coculture with LPS-treated MØ (1 µg/ml; mean ± SEM; n = 4. *p < 0.05 compared with medium controls.)
The data from RAG1−/− mice indicate either an intrinsic anti-colitic capacity in the HbBc(7d) or interaction with innate immune cells. We found that DNBS-induced colitis in RAG1−/− mice treated with clodronate and receiving HbBc(7d) was not different from mice treated with DNBS ± clodronate. Administration of clodronate-liposomes depleted phagocytes, predominately MØ (20, 53) (confirmed by F4/80 staining), indicating B cell–MØ communication in the amelioration of colitis. There are few data on B cell–MØ cross-talk, but it is not unprecedented; peritoneal MØ from B cell–deficient mice produced more TNF-α and IL-1β in response to LPS (54), and monocyte recruitment in murine myocardial infarction was triggered by B cells (55). The current study demonstrates B cell–MØ cooperation in the suppression of colitis. Alternatively activated macrophages can block colitis (20), but the induction of AAMs is unlikely to underlie the HbBc(7d) anti-colitic effect because qPCR revealed no increase in arginase, Ym1, and Relmα mRNA expression in MØ co cultured with HbBc(7d) or colonic extracts of mice given HbBc(7d). Indeed, an anti-inflammatory MØ that was Ym1−/Relmα− was implicated in the suppression of DSS-induced colitis following infection with S. mansoni (18). Coculture of HbBc(7d) with bone marrow-derived MØ resulted in increased LPS-induced IL-10 production by the MØ. So whereas HbBc(7d) appear to be neither a target nor a source of IL-10, their induction of MØ-derived IL-10 would contribute to the suppression of colitis, which is compatible with the role for IL-10 in the H. diminuta suppression of DNBS-induced colitis (25).

Finally, the reciprocal MØ–B cell communication was considered. Coculture of HbBc(7d) with MØ or CAMs resulted in up-regulation of CD1d and PDL1 on the B cell. The former would allow engagement of NK T cells, and the latter could give the B cell an additional anti-colitic strategy as described for PDL1 and the anergy of T cells following infection with S. mansoni (56). As the mechanism of HbBc(7d) action is unraveled, it will be important to dissect MØ–B cell communication and the consequences of activation of each cell as elements of the suppression of colitis. A complex web of immunity arises following infection with helminth parasites, driven by the opposing forces of the host’s efforts to eliminate the parasite and the parasite’s attempts to circumvent the host’s anti-worm response. In this study, we identify a putatively unique splenic follicular zone CD19+ B cell mobilized in response to H. diminuta that requires neither IL-4 nor IL-10 for its differentiation and does not overexpress typical markers of Bregs (i.e., CD1d, CD23) or IL-10, and whose anti-colitic activity can be independent of T and B cells. Rather, it appears that this H. diminuta CD19+ Breg is a source of TGF-β and exerts an anti-colitic effect by cooperating with MØ whose immunoregulatory properties may be affected by TGF-β and other factors that await identification or cognate interaction with the HbBc(7d). Precise characterization of the phenotype and mechanism of action of this CD19+ HbBc(7d) coupled with the ability to reprogram human B cells into such a phenotype could be a valuable addition to the treatments available for mucosal inflammatory disease.

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Disclosures
The authors have no financial conflicts of interest.

References


Figure S1 – B cells from mice infected with *H. diminuta* 7 days previously (*HdBc*(7^d*)) suppress dextran-sodium sulphate (DSS)-induced colitis as assessed by (A) body weight, (B) colon length, (C) cumulative disease activity score and (D) representative H&E images (mean ± SEM; n=7-9 mice from 2 experiments; *, #, p<0.05 compared to control (con) and DSS only treated mice, respectively; L, lumen, M, muscle; original image magnification x200).
### Table: Cytokine Production

<table>
<thead>
<tr>
<th>Cytokine (pg/mL)</th>
<th>B cells from naïve mice</th>
<th>B cells from 7 d-p-i <em>H. diminuta</em></th>
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<tr>
<td></td>
<td>Non-stimulated</td>
<td>LPS (1 µg/mL)</td>
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<td>Eotaxin</td>
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**Figure S2** – Comparison of CD19+ B cells from naïve mice or those infected 7 day previously with *H. diminuta* using the luminex 32-cytokine multiplex revealed no differences between the groups in either the spontaneous or lipopolysaccharide (LPS)-stimulated production of the listed cytokines/chemokines/growth factors (n=3).
Reyes et al. 2014, supplementary Figure S3

A

Uninfected

Hd infected (7 dpi)

10.4±2.2 %

8.7±1 %

14.1±4.7

20.7±4.7

2.1±0.05

2.6±0.2

CD1d

CD5

CD138

CD19

Uninfected

Hd infected (7 dpi)

15.0±1.5

17.5±4.2

3.8±0.4

4.3±0.5

3.5±0.6

3.4±0.3

PDL1

PDL2

Tim1

B

Hierarchical Clustering

-1.80

0.00

1.80

group

Bc

Hd8c7d
Figure S3 – (A) Analysis of CD19+ splenic B cells (Bc) from naïve mice and those from *H. diminuta* (*Hd*) 7 days post-infection (7 pdi) by flow cytometry for surface expression of markers indicative of regulatory B cell phenotypes revealed no statistically significant differences between the groups (n=3-6; PDL, programmed death ligand; Tim1, T cell immunoglobulin mucin-1). (B) Heat map obtained from microarray assay (using 1.8 Fold change) analysis comparing B cell from uninfected and 7 dpi after *Hd* infection. Initial analysis with a 1.5 fold change difference between groups showed 700 genes up or down-regulated in *HdBc*(7d) compared to Bc. In order to narrow into the most significantly up-regulated genes, we increased the fold change to 1.8 and found 71 genes consistently up-regulated in *HdBc*(7d). Heat map shows 3 samples from uninfected animals (red group) and 3 samples from *H. diminuta* infected mice (blue group). Data deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61460
Reyes et al 2014, supplementary Figure S4

**IL-4 receptor-α knock-out mice**

A) Disease activity score

- **con**, **DNBS**, **H. diminuta**, **+ DNBS**

B) % weight change

- **con**, **DNBS**, **H. diminuta**, **+ DNBS**

C) Colon length (cm)

- **con**, **DNBS**, **H. diminuta**, **+ DNBS**

D) Histopathology score

- **con**, **DNBS**, **H. diminuta**, **+ DNBS**

E) Histology images

F) Splenocyte IL-10 (ng/ml)

**Control** vs **DNBS**

**DNBS+BcHd^{ IL-4Ra^{-/-}}** vs **WT**

**WT Hd^{ IL-4Ra^{-/-}}** vs **IL-4Ra^{-/-} Hd^{ IL-4Ra^{-/-}}**

**p=0.07**

**p=0.06**

**p=0.09**

**P<0.01**
Figure S4. Infection with *H. diminuta* protects IL-4Rα−/− mice from DNBS-induced colitis. Female (8-12 week old) IL-4Rα−/− mice were infected by oral gavage with 5 viable *H. diminuta* cysticercoids and 7 days later, colitis was induced by intra-rectal instillation of dinitrobenzene sulphonate acid (DNBS; 3 mg). Mice were necropsied 72 h post-DNBS and disease severity was significantly reduced in the *H. diminuta* treated mice as assessed by (A) the composite disease activity score, (B) body weight, (C) colon length and (D) a histopathology score that assesses degree of inflammatory cell infiltrate, edema, architectural destruction, loss of goblet cells and ulceration (data are mean ± SEM; n=6-7 mice from 2 experiments; * and #, p<0.05 compared to control (i.e. PBS treated mice) and DNBS-only, respectively). (E) Representative H&E stains sections showing colonic histology (original mag. = x200) and (F) measurement IL-10 levels in response to con A stimulation (48 hr) by spleen cells from both wild-type (WT) and IL-4Rα−/− mice harvested 7 days after *H. diminuta* infection.