GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Helminth Antigen—Conditioned Dendritic Cells Generate Anti-Inflammatory Cd4 T Cells Independent of Antigen Presentation via Major Histocompatibility Complex Class II

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A recently identified feature of the host response to infection with helminth parasites is suppression of concomitant disease. Dendritic cells (DCs) exposed to antigens from the tapeworm *Hymenolepis diminuta* significantly reduce the severity of dinitrobenzene sulfonic acid–induced colitis in mice. Here we elucidate mechanisms underlying this cellular immunotherapy. We show a requirement for Ccr7 expression on transferred *H. diminuta* antigen–treated (HD)–DCs, suggesting that homing to secondary lymphoid tissues is important for suppression of colitis. Furthermore, sodium metaperiodate–sensitive helminth-derived glycans are required to drive the anti-colitic response in recipient mice. Induction of Th2-type cytokines and Gata-3⁺Cd4⁺ cells in secondary lymphoid tissues is dependent on major histocompatibility complex class II (MHC II) protein expression on transferred DCs, although remarkably, transfer of MHC II^{−/−} HD-DCs still attenuated dinitrobenzene sulfonic acid–induced colitis in recipient mice. Moreover, transfer of Cd4⁺ splenic T cells retrieved from mice administered MHC II^{−/−} HD-DCs suppressed dinitrobenzene sulfonic acid–induced colitis in recipient mice. Our studies reveal that HD-DCs can suppress colitis via an alternative MHC II–independent pathway that involves, in part, mobilization of T-cell responses. These data support the utility of HD-DCs in blocking colitis, revealing a requirement for Ccr7 and providing for HD-DC autologous immunotherapy for disease in which MHC II expression and/or function is compromised. (*Am J Pathol* 2018, 188: 2589–2604; <https://doi.org/10.1016/j.ajpath.2018.07.008>)

Adoptive transfer of autologous dendritic cells (DCs) can enhance immunogenicity toward tumor antigens in patients with a wide variety of cancers.¹ Furthermore, DC vaccination has been evaluated in the context of infectious diseases; clinical trials have shown that autologous DCs loaded with heat-inactivated HIV can reduce viral load in patients with HIV.^{2,3} Preclinical studies support DC immunotherapy in inflammatory bowel disease via the development of a tolerogenic DC phenotype.⁴ Also, DCs pulsed with bacterial proteins, extracts of *Enterobacteria* spp., or the neuropeptide vasoactive intestinal peptide, or engineered to express FasL all have regulatory/tolerogenic properties that protect mice from colitis.^{5–7}

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Substantial data from animal models and a few small clinical trials indicate that infection with helminth parasites can ameliorate autoimmune diseases, including inflammatory bowel disease.⁸ Although promising, helminth therapy in a larger trial in Crohn disease⁹ and multiple sclerosis¹⁰ provided no major benefit to the patient. Even though it is well tolerated, helminth therapy is not without caveats: There are reports of unwanted side effects (eg, transient diarrhea, anemia, gastrointestinal pain), iatrogenic disease is a concern,^{11,12} and the putative psychological discomfort associated with harboring a live parasite should not be discounted. Such concerns are mitigated when helminth-derived molecules,^{13,14} or adoptive transfer of helminth antigen-treated cells, are used to treat disease.^{8,15–17} Murine bone marrow–derived DCs exposed to a crude extract of the tapeworm *Hymenolepis diminuta* (HD-DCs) reduce the severity of dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice via the mobilization of adaptive immunity—specifically, splenic Cd4⁺ T cells—and via Il-4 and Il-10.^{18,19} These findings raise a number of mechanistic issues that are important to address if helminth-based cellular immunotherapy is to become a therapeutic option: What is the molecular basis of the HD extract that drives a DC to evoke anti-colitic events, and how is this mediated?

Utilizing this model, in an effort to understand the mechanistic basis of HD-DC suppression of colitis as proof of concept to apply this approach to human disease, we show that a sodium metaperiodate–sensitive glycan(s) in the HD extract is required for the anti-colitic effect of HD-DCs. A requirement for Ccr-7 expression on the DC is observed and, by implication, homing to secondary lymphoid organs (SLOs) is important in the suppression of chemical-induced colitis. Moreover, and uniquely in the realm of helminth therapy, we have discovered a novel anti-inflammatory pathway, operating via Cd4⁺ T cells in a major histocompatibility complex class II (MHC II)-independent manner that was not accompanied by increased production of Il-10 or Il-4 by splenocytes from the HD-DC–recipient mice. The latter data not only indicate the putative utility of HD-DCs to block colitis, they also suggest that autologous immunotherapy for HD-DC may be of value in the treatment of disease in which MHC II expression/function is compromised, as is the case in some viral infections, including herpes and HIV.^{20,21}

Materials and Methods

Mice

Animal experiments followed protocols approved by the University of Calgary's Health Science Animal Care Committee (protocol AC13-0015), which conformed to the Canadian Council on Animal Care's guideline on animal research. Male BALB/C (Charles River Laboratories, St. Constant, Canada), MHC II knockout (KO) and wild-

type (WT) controls (C57BL/6 background; The Jackson Laboratory, Bar Harbor, ME), Ccr7 KO and their WT littermate controls [C57BL/6 background; gift from Dr. Paul Kubes (University of Calgary, Calgary, AB, Canada); as previously published²²], and C57BL6 recombination-activating gene 1 KO mice (colony at University of Calgary) were maintained at the Animal Research Center, University of Calgary, with free access to chow and water. Mice were used between 8 and 12 weeks of age, with only WT mice exposed to the colitis protocol.

HD Extract

Adult HD were flushed from the small intestine of rats, washed thoroughly, homogenized in phosphate-buffered saline (PBS), and centrifuged (3220 × g, 30 minutes, 4°C). The PBS-soluble component was recentrifuged, protein content was measured via Bradford assay, and aliquots were frozen at –80°C. Bioactivity of the extract was standardized by assessment of its capacity to suppress lipopolysaccharide-induced tumor necrosis factor α production (48 hours, 1 μ g/mL) by THP-1 macrophages by at least 40%.²³ Trace levels of endotoxin were present in the antigen preparation (65 pg/mg extract; ToxinSensor Chromogenic LAL kit; GenScript, Piscataway, NJ).¹⁸ For the assessment of the role of proteins, 100 μ g/mL worm extract was treated with 1 mg of proteinase K (InvitroGen Canada Inc., Burlington, ON, Canada) for 3 hours at 37°C, then boiled for 20 minutes. To assess the role of glycans, 100 μ g/mL worm extract was treated with 5 mg/mL sodium metaperiodate (Alfa Aesar, Tewksbury, MA) for 24 hours at room temperature, and subsequently dialyzed using 10K MWCO Slide-A-Lyzer G2 dialysis cassettes (Thermo Scientific, Rockford, IL) following the manufacturer's instructions; dialyzed-only extract was used as a sham-treated control.

Bone Marrow–Derived Dendritic Cells and Adoptive Transfer

DCs were generated from bone marrow stem cells as described.^{18,24} Briefly, bone marrow stem cells were harvested from the femurs of mice and seeded at 5×10^6 cells/10 mL of RPMI medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 2% Pen/Strep, 1% GlutaMAX, and 1% sodium pyruvate (all, Gibco, Grand Island, NY) and 20 ng/mL recombinant murine granulocyte macrophage colony-stimulating factor (Pepro-Tech, Montreal, Canada). On days 3 and 6, cells were given an additional 10 and 5 mL of supplemented medium containing 20 and 40 ng/mL granulocyte macrophage colony-stimulating factor, respectively. On day 8, floating cells were collected, washed, and resuspended in 10% dimethyl sulfoxide in fetal bovine serum (5 to 10×10^6 cells/mL) for storage in liquid nitrogen. For adoptive transfer experiments, DCs were thawed, resuspended in supplemented

RPMI medium, and rested overnight before treatment with worm extract (100 µg/mL) for 24 hours. On day 10, cells were >90% Cd11c⁺, as determined by flow cytometry. DCs were washed three times in PBS, and resuspended in 500 µL PBS (2 to 4 × 10⁶ cells/mL). For localization experiments, a single i.p. injection of 2 × 10⁶ cells was administered; for all other adoptive transfer experiments, a single dose of 1 × 10⁶ cells was transferred i.p.

Isolation of Cd4⁺ T Cells

WT and MHC II^{-/-} HD-DCs were transferred to mice, and 5 days later splenocytes were isolated and enriched for Cd4⁺ T cells, following protocols provided with the Easy-Sep Mouse Cd4⁺ T-cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada). For adoptive transfer experiments, 1 × 10⁶ Cd4⁺ T cells were resuspended in 500 µL of sterile PBS and administered by i.p. injection.

Induction and Assessment of Colitis

Colitis was induced in anesthetized (4% inhaled isoflurane) mice through intrarectal (i.r.) instillation of 3 mg (BALB/C mice) or 4 mg (C57BL/6 mice) of DNBS (MP Biomedicals, Santa Barbara, CA) dissolved in 100 µL of 50% ethanol using a polyethylene catheter inserted 3 cm from the anus²⁵; negative control mice received 100 µL of PBS. In mice that received adoptive cellular therapy, DNBS was administered 48 hours after cell transfer, with the exception of the recovery study, in which DCs were given directly after the DNBS.

Mice were examined daily for signs of disease and weight loss. At 72 hours after DNBS, colons were removed for macroscopic assessment, and a disease score was calculated based on previously published criteria¹⁸: visceral adhesions, where 0 indicates absent, 1 indicates moderate, and 2 indicates severe; colonic shortening, calculated as the percentage relative to negative controls multiplied by 0.1; colonic inflammation, calculated as percentage relative to total length of the colon, multiplied by 0.05; diarrhea, fecal blood, and erythema, where 0 indicates absent and 1 indicates present; and ulceration, where 1 point was calculated per 1 cm of ulceration. A 1-cm portion 20% from the distal end was removed, fixed in formalin, sectioned (5 µm), and stained with hematoxylin and eosin. Histopathology score was calculated in a blinded fashion (C.E.M., A.W., and D.M.M.) using a 12-point scale²⁵: cellular infiltration, where 0 indicates absent and 1 to 3 indicates increasing severity; loss of architecture, where 0 indicates absent and 1 to 3 indicates increasing severity; muscle thickening, where 0 indicates absent and 1 to 2 indicates increasing severity; and goblet cell depletion, crypt abscess, edema, and ulcers, where 0 indicates absent and 1 indicates present. In other studies, when DCs were administered directly after DNBS, necropsy was performed 5 or 7 days later, as a treatment/recovery protocol.

Splenocyte Cytokine Synthesis

Spleens were aseptically removed, and forced through a 100-µm nylon mesh strainer using the plunger from a 10-mL syringe. Erythrocytes were lysed with ammonium potassium chloride buffer for 90 seconds. Cytokine levels were assessed in the supernatants of splenocytes (5 × 10⁶) stimulated with concanavalin A (2 µg/mL) for 48 hours; enzyme-linked immunosorbent assays for interferon-γ, Il-4, Il-10, Il-12p40, Il-17, transforming growth factor (Tgf)-β, and tumor necrosis factor α were performed using DuoSet kits (R&D Systems, Minneapolis, MN), as per the manufacturer's instructions. Cytokine levels in colonic homogenates were also examined.

In Vivo Imaging

In vivo imaging protocols followed those previously described.²⁶ To reduce background fluorescence, mice were fed a modified AIM-93M diet (catalog number TD.00102; Harlan Laboratories, Frederick, MD) 7 days before experimentation. Mice were shaved 24 hours before imaging. Cells (8 to 10 × 10⁶ DCs or splenocytes) were stained with VivoTrack 680 NIR (PerkinElmer, Waltham, MA) following the manufacturer's instructions, before adoptive cell transfer (2 × 10⁶ i.p.). Noninvasive whole-body imaging was performed using the In-Vivo Xtreme 4MP imaging platform (Bruker, Billerica, MA). Cells were visualized using 670-nm excitation and 750-nm emission wavelengths. Animals were imaged every 24 hours after cell transfer; mice were anesthetized (2% isoflurane) and kept at 37°C during the imaging protocol, which included reflectance imaging (2-second exposure), fluorescence imaging at 670/750 nm (6-second exposure), and X-ray imaging (10-second exposure). At 5 days after cell transfer, mice underwent necropsy: the heart, lung, liver, spleen kidneys, small intestine, colon, and mesenteric lymph nodes (MLNs) were removed for additional reflectance and fluorescence imaging. All images were acquired and analyzed using MI SE molecular imaging software version 7.1.3.20550 (Bruker).

Flow Cytometry

For cell localization experiments, splenocytes were stained for Cd11c [clone N418 (APC); BioLegend, San Diego, CA] after blocking with Cd16/32 (TruStain; BioLegend), and analyzed for Cd11c and VivoTrack 680 expression on an Acoustic Attune Flow Cytometer, using Attune software version 1.2 (Life Technologies, Carlsbad, CA). For analysis of T-cell phenotypes, the following fluorophore-conjugated antibody clones were used: Cd4 BV786 (catalog number GK1.5; BD Biosciences, San Jose, CA), Cd8α AF700 (catalog number 53-6.7; BD Biosciences), trans-acting T-cell-specific transcription factor Gata-3 (Gata-3) PerCP/Cy5.5 (catalog number 16E10A23; BioLegend), T-box protein expressed in T cells (T-bet)

BV421 (catalog number 04-46; BD Biosciences), ROR γ t PE (catalog number Q31-378; BD Biosciences), forkhead box protein P3 (Foxp3) fluorescein isothiocyanate (catalog number FJK-16s; eBioscience, San Diego, CA), and Helios Alexa Fluor 647 (catalog number 22F6; BD Biosciences). Cells were stained with a viability dye (catalog number FVS 780; BD Biosciences) before Fc blocking with Cd16/32 (TruStain; BioLegend) for 15 minutes, and stained for surface antibodies for 30 minutes. Cells were fixed and permeabilized following the manufacturer's instructions using the Foxp3 Fix/Perm kit (eBioscience), blocked with 5% rat serum for 15 minutes, and stained with intracellular antibodies overnight at 4°C. Data were collected using the Canto II system (BD Biosciences) and analyzed by FlowJo software version 9 (Tree Star,

Ashland, OR). All lymphocytes were gated on viability, and doublets were excluded before assessment of T-cell phenotypes.

Statistical Analysis

Data are presented as arithmetic means $\pm$ SEM, and an α level of 0.05 was accepted as statistically significant. For parametric data, statistical outliers were removed using the Grubb test. For comparisons between two groups, a two-tailed unpaired *t*-test was performed. For multiple groups, a one-way analysis of variance was used; for post hoc analysis among all groups, the Tukey test was used; to compare two specific groups, the Sidak test was performed. Significant differences in nonparametric data were assessed using the

Figure 1 Localization of transferred cells. Noninvasive whole-body imaging was performed on BALB/C mice that received splenocytes, dendritic cells (DCs), and *Hymenolepis diminuta* antigen-treated (HD)-DCs (2×10^6 i.p.) 48 hours after cell transfer. **A**: Representative composites of fluorescence overlaid onto reflectance and X-ray images of ventrally placed mice, 5 days after cell transfer. **B**: Representative images of fluorescence overlaid onto reflectance images of organs removed 5 days after cell transfer (72 hours after DNBS challenge). **C**: Representative images of fluorescence overlaid onto reflectance images of the mesenteric lymph nodes (MLNs) and gastrointestinal tract. **D–F**: Quantification of fluorescence signal of the spleen, MLNs, and colon, respectively. Data are expressed as means $\pm$ SEM (**D–F**). $n = 3$ (naïve DCs); $n = 4$ (HD-DCs, DCs + DNBS, HD-DCs + DNBS); $n = 5$ (control, DNBS, splenocytes + DNBS); $n = 7$ (splenocytes), all from one to two experiments. * $P \leq 0.05$ versus negative control (one-way analysis of variance with Dunnett posttest); $\dagger P \leq 0.05$ versus positive control (one-way analysis of variance with Dunnett posttest); $\ddagger P \leq 0.05$ DC versus DC + DNBS, or HD-DC versus HD-DC + DNBS (one-way analysis of variance with Sidak posttest). AU, arbitrary fluorescence units.

U-test or the Kruskal-Wallis test with Dunn posttest. For macroscopy and histology damage scores, analyses were conducted in all DNBS-treated groups; all other analyses included the negative control. Analyses were conducted with Prism software version 6.0 (GraphPad Software, La Jolla, CA).

Results

Adoptively Transferred HD-DCs Localize to the Spleen, MLN, and Colon, with Enhanced Recruitment in DNBS Colitis

To determine where HD-DCs exert their effect, a fluorescent lipophilic membrane dye was used to mark and then visualize the localization of i.p.-injected cells. Whole-body imaging of control or DNBS-treated mice displayed no detectable fluorescence, with the exception of a small signal in the cecum likely due to residual food (Figure 1, A–C). In contrast, a consistent fluorescence signal was obvious in the peritoneal cavity of mice that received labeled cells 1 to 4 days (not shown) and 5 days (Figure 1A) after cell transfer. A faint signal was detected in the lungs and occasionally the kidneys, with a strong signal in the liver.

Notable fluorescence occurred in the spleen, MLNs, and gastrointestinal tract of mice that received labeled DCs or HD-DCs (Figure 1, B and C), with the pattern and intensity of fluorescence being similar in both cell types under noninflamed (ie, no DNBS) and colitic (DNBS-treated) conditions (Figure 1, D–F). Statistical comparisons revealed that recruitment of DCs and HD-DCs to the spleen was significantly enhanced in mice with colitis (Figure 1D); HD-DC recruitment to the MLNs was similarly enhanced in DNBS-treated mice relative to recruitment under non-inflamed conditions (Figure 1E). Treatment with DNBS increased the recruitment of only DCs, not HD-DCs, to the colon relative to healthy recipient mice (Figure 1F).

HD-DC Suppression of Colitis Is Ccr7 Dependent

In a prophylactic regimen, HD-DCs significantly reduced the severity of DNBS-induced disease¹⁸; here those observations were extended by showing enhanced recovery from colitis when the HD-DCs were given immediately after the DNBS (Supplemental Figure S1). Given that i.p.-administered HD-DCs migrate to the spleen and MLNs in recipients, with greater localization to these organs in DNBS-treated mice, the importance of lymphatic recruitment in HD-DC suppression of colitis was assessed. Ccr7 plays a key role in DC migration to SLOs, via chemokine (C-C motif) ligand (Ccl) 19 and Ccl21, which are constitutively expressed by endothelial cells of lymphatic vessels, high endothelial venules, and stromal cells in the T-cell zone. Proinflammatory cytokines increase Ccl21 expression to enhance the migration efficiency of DCs to SLOs.²⁷ Fluorescence quantification of whole spleens revealed a slightly reduced signal among

Ccr7^{−/−} HD-DC recipients, relative to WT controls ($P = 0.11$) (Figure 2, A and B). A greater proportion and total number of VivoTrack 680⁺ cells were detected in splenocytes isolated from mice receiving WT HD-DCs relative to Ccr7^{−/−} HD-DCs (Figure 2C). Whole-organ imaging of MLNs and colons revealed no significant difference in fluorescence intensity between WT and Ccr7^{−/−} HD-DC recipients (data not shown). The absence of Ccr7 on the DCs abrogated the protection against DNBS-induced colitis afforded by HD-DCs (Figure 2, D–F). Non-worm-antigen-treated Ccr7^{−/−} or MHC II^{−/−} DCs did not affect the severity of colitis and so these controls were not included in subsequent analyses [mean $\pm$ SEM disease activity scores: DNBS, 6.4 ± 1.1 ($n = 4$); DNBS + Ccr7^{−/−} DCs, 5.9 ± 0.5 ($n = 3$); and DNBS + MHC II^{−/−} DCs, 7.8 ± 0.4 ($n = 4$)]. Concanavalin-A stimulation of splenocytes isolated from recipients of WT HD-DCs, but not Ccr7^{−/−} HD-DCs, resulted in increased splenic IL-4 (Figure 2G) and elevated IL-10 (Figure 2H), consistent with previous findings.¹⁸

HD-DCs Drive a Th2 Immune Response in the Spleen and MLNs of Recipient Mice

Treatment with HD-DCs evoked a significant increase in the proportion of Cd4⁺Gata-3⁺ T cells in the spleen and MLNs of recipients. Changes in the proportion of Foxp3⁺ cells were not observed: Gata-3 was induced in the Foxp3[−] population after WT HD-DC treatment, indicating the induction of a classic Th2 response, whereas levels of Foxp3⁺ T-regulatory cells remained similar among groups (Figure 3, A–C). *In vitro* stimulated splenocytes from HD-DC recipients synthesized significantly higher levels of IL-4, with IL-10 also being increased but failing to reach statistical significance in this set of mice (Figure 3D). Mitogen-stimulated splenocytes from HD-DC recipients are not compromised in their capacity to produce interferon- γ or tumor necrosis factor α ¹⁸; similar results were observed and additionally it was found that splenocyte output of IL-17 was not different between mice treated with DCs or HD-DCs ($n = 3$ to 4; data not shown). [Analysis of cytokines (both protein and mRNA) in colonic homogenates yielded inconsistent results ($n = 4$ to 7; data not shown), as has been our experience with this model in the past (personal observations)]. None of the other Cd4⁺ T-cell compartments in the spleen or MLNs (eg, ROR γ t, T-bet) were significantly different between the experimental groups, although the proportion of Cd4⁺ T cells was slightly increased in the spleen after HD-DC treatment (Table 1).

Protein-Degraded HD Antigens Drive an Anti-Colitic DC

The induction of Gata-3⁺ T cells by HD-DCs, coupled with the increased expression of MHC II and Cd80 on HD-DCs,¹⁸ led to the hypothesis that the interaction between HD-DCs and Cd4⁺ T cells operates via cognate MHC II–T-

Figure 2 Suppression of colitis by *Hymenolepis diminuta* antigen-treated dendritic cells (HD-DCs) is C-C chemokine receptor type 7 (Ccr7) dependent. Wild-type (WT) or Ccr7 knockout (KO) HD-DCs (C57BL/6 background) stained with VivoTrack 680 dye (PerkinElmer, Waltham, MA) were transferred (2×10^6 i.p.) to C57BL/6 mice 48 hours before challenge with dinitrobenzene sulfonic acid (DNBS; 4 mg i.r.). **A** and **B**: Spleens were removed 72 hours after DNBS challenge, fluorescence images were acquired and overlaid onto reflectance photographs, and the fluorescence signal was quantified. Capped horizontal line indicates *t*-test analysis. **C**: Flow-cytometric analysis of the percentage and total number of Cd11c⁺ VivoTrack 680⁺ splenocytes in recipients of WT HD-DCs and Ccr7 KO HD-DCs. **D** and **E**: Disease severity was assessed 72 hours after DNBS challenge via macroscopy and histology damage scores, and analyzed by the Kruskal-Wallis test with Dunn posttest; capped horizontal line indicates *U* comparison. **F**: Representative micrographs of hematoxylin and eosin-stained cross sections of colonic tissue. **G** and **H**: Supernatants from stimulated splenocytes (5×10^6 , 2 μ g/mL concanavalin A, 48 hours) were measured for IL-4 and IL-10 by enzyme-linked immunosorbent assay, and analyzed by a one-way analysis of variance with Tukey posttest. Data are expressed as means $\pm$ SEM. *n* = 3 (**G** and **H**, control); *n* = 4 (**C**, Ccr7 KO HD-DCs + DNBS; **G** and **H**, DNBS, Ccr7 KO HD-DCs + DNBS); *n* = 5 (**C**, **G**, and **H**, WT HD-DCs + DNBS), all from one experiment. **P* $\leq$ 0.05 (**C**, two-tailed *t*-test); **P* $\leq$ 0.05 versus positive control (**G** and **H**). Scale bar = 300 μ m. AU, arbitrary fluorescence units; L, lumen; M, muscle.

cell receptor interaction, and therefore would be dependent on intact protein components of the HD extract. Mice injected with DCs pulsed with proteinase K and heat-treated HD antigen were protected from DNBS-induced colitis, as

assessed by reduced macroscopic damage (Figure 4A), but did not display increased concanavalin A-induced IL-4 and IL-10 by splenocytes (Figure 4, B and C). The absence of IL-4 and IL-10 production suggested the possibility of an

Figure 3 *Hymenolepis diminuta* antigen-treated dendritic cells (HD-DCs) drive a Th2 immune response in the spleen and mesenteric lymph nodes (MLNs) in recipient mice. **A:** Representative dot plots of Gata-3⁺ and forkhead box protein P3 (Foxp3)⁺ expression in Cd4⁺ T cells in the spleen and MLNs of control BALB/C mice and mice receiving naïve DCs or HD-DCs (1×10^6 i.p.) are shown. **B:** Percentage of Gata-3⁺ and Foxp3⁺ Cd4⁺ T cells in the spleen. Data were analyzed by one-way analysis of variance with Sidak posttest between cell-treated groups. Absolute numbers $\pm$ SEM of Gata-3⁺Cd4⁺ splenocytes: control, $6.76 \pm 0.727 \times 10^5$; naïve DCs, $1.30 \pm 0.337 \times 10^6$; and HD-DCs, $2.80 \pm 0.384 \times 10^6$. Absolute numbers $\pm$ SEM of Foxp3⁺Cd4⁺ splenocytes: control, $3.64 \pm 0.0727 \times 10^6$; naïve DCs, $4.93 \pm 0.313 \times 10^6$; and HD-DCs, $4.25 \pm 0.378 \times 10^6$. **C:** Production of IL-4 and IL-10 in the supernatant of stimulated splenocytes (5×10^6 , $2 \mu\text{g/mL}$ concanavalin A, 48 hours) as measured by enzyme-linked immunosorbent assay, and analyzed by one-way analysis of variance with Tukey posttest. **D:** Percentages of Gata-3⁺ and Foxp3⁺ Cd4⁺ T cells in MLNs. Data analyzed by one-way analysis of variance with Sidak posttest between cell-treated groups. Data are expressed as means $\pm$ SEM unless otherwise specified. $n = 4$, from one experiment. * $P \leq 0.05$.

anti-colitic pathway alternative to the one described previously.^{18,19}

MHC II KO HD-DCs Suppress Colitis and Drive Splenic Tgf- β Production but Not a Th2 Phenotype

To further test the requirement for cognate MHC II–T-cell receptor interaction in the HD-DC-initiated suppression of colitis, MHC II^{-/-} DCs were used. Delivery (i.p.) of WT and MHC II^{-/-} HD-DCs resulted in

virtually identical suppression of DNBS-induced colitis (Figure 5, A–C), yet MHC II^{-/-} HD-DCs did not elicit increases in splenic IL-4 and IL-10, indicating a reduced Th2 response (Figure 5, D and E). Also, Cd4⁺Gata-3⁺ T-cell concentration was not increased in the spleen and MLNs of naïve mice given MHC II^{-/-} HD-DCs (Figure 6 and Table 2). However, a small but significant increase in Foxp3⁺Helios⁻ cells was observed in the MLNs of MHC II^{-/-} HD-DC recipients.

Table 1 T-Cell Phenotype in Mice Receiving WT HD-DCs

Study group	Cd8, %	Cd4, %	Cd4 ⁺ cells		Cd4 ⁺ Foxp3 ⁺ cells		Cd4 ⁺ cells	
			Gata-3, %	Foxp3, %	Helios [−] , %	Helios ⁺ , %	T-bet, %	RORγt, %
Spleen								
Control	12.56 ± 0.48	21.03 ± 1.07	1.25 ± 0.16	6.26 ± 0.30	1.79 ± 0.11	4.94 ± 0.15	2.19 ± 0.44	0.57 ± 0.057
WT DCs	10.64 ± 0.50	18.48 ± 0.78	1.95 ± 0.49	7.43 ± 0.27	1.96 ± 0.05	5.92 ± 0.25	1.82 ± 0.28	0.55 ± 0.075
WT HD-DCs	12.05 ± 0.54	22.78 ± 0.81*	4.15 ± 0.32*	6.41 ± 0.57	1.69 ± 0.05	5.11 ± 0.52	1.70 ± 0.29	0.49 ± 0.10
MLNs								
Control	27.1 ± 0.82	48.4 ± 1.10	0.17 ± 0.02	1.91 ± 0.30	1.41 ± 0.15	1.81 ± 0.27	0.30 ± 0.09	0.38 ± 0.04
WT DCs	26.4 ± 1.03	45.08 ± 0.81	0.33 ± 0.04	2.97 ± 0.16	1.96 ± 0.10	2.93 ± 0.17	0.69 ± 0.30	0.40 ± 0.06
WT HD-DCs	28.1 ± 0.85	44.83 ± 0.80	0.73 ± 0.05*	3.14 ± 0.03	2.02 ± 0.06	3.20 ± 0.04	0.45 ± 0.10	0.40 ± 0.06

C57BL/6 mice received 1×10^6 naïve WT DCs or HD-DCs in phosphate-buffered saline; controls received an equal volume of vehicle. All cells are gated on the viable lymphocyte population, before doublet exclusion. Gata-3 and Foxp3 populations are expressed as the percentage of Cd4⁺ cells, whereas T-bet and RORγt are expressed as the percentage of Cd4⁺Gata-3⁺Foxp3⁺ cells. Data are expressed as means ± SEM.

* $P < 0.05$ versus WT DC treatment (one-way analysis of variance with Sidak posttest); $n = 4$, from one experiment.

DCs, dendritic cells; Foxp3, forkhead box protein P3; HD, *Hymenolepis diminuta* antigen treated; MLNs, mesenteric lymph nodes; T-bet, T-box protein expressed in T cells; WT, wild type.

Although only the adoptive transfer of WT HD-DCs drove the production of Il-4 and Il-10 in concanavalin A-stimulated splenocytes, the transfer of MHC II^{-/-} HD-DCs significantly increased the synthesis of Tgf-β by concanavalin A-stimulated splenocytes relative to recipients of naïve WT, naïve MHC II^{-/-}, or HD-DC WT DCs (Figure 7). In contrast, *in vitro* co-culture of splenic Cd4⁺ T cells with HD-DCs did not consistently result in an increase in Tgf-β production, suggesting the absence of a key factor *in vitro* that was available to the cells *in vivo* (data not shown).

To test whether protection by MHC II^{-/-} HD-DCs was mediated by Cd4⁺ T cells, as was the case with WT HD-DCs,¹⁸ Cd4⁺ T cells isolated from the spleen of MHC II^{-/-} HD-DC-treated mice 5 days after cell transfer were administered i.p. to naïve mice that were

immediately challenged with DNBS (Figure 8A). The adoptive transfer of MHC II^{-/-} HD-DC-educated Cd4⁺ T cells significantly attenuated the severity of colitis, as shown by reduced damage on macroscopy (Figure 8B) and histology (Figure 8, C and D). The T-cell transfer did not influence the production of Il-4, Il-10, or Tgf-β by concanavalin A-stimulated splenocytes from the recipient mice (Figure 8, E–G). Supportive of these data, the transfer of MHC II^{-/-} HD-DC to recombination-activating gene 1^{-/-} mice did not result in an amelioration of DNBS-induced colitis (5 mg i.r., 72 hours) [mean ± SD disease activity scores: control, 0 ± 0 ($n = 3$); DNBS, 6.7 ± 2.7 ($n = 3$); DNBS + MHC II^{-/-} HD-DC, two of four mice required early euthanasia because of disease severity and the other two had disease activity scores of 8.2 and 4.5].

Figure 4 Proteinase K (proK)- and heat-degraded *Hymenolepis diminuta* (HD) antigens drive an anti-colitic dendritic cell (DC). BALB/C mice were administered 1×10^6 DCs i.p. previously treated with intact HD extract, or boiled and proK-digested HD extract (100 μg/mL, 24 hours), and challenged with dinitrobenzene sulfonic acid (DNBS) 48 hours later. **A:** Macroscopic damage scores were assessed 72 hours later, and analyzed by Kruskal-Wallis test with Dunn posttest. **B and C:** Splenocytes were stimulated (5×10^6 , 2 μg/mL concanavalin A, 48 hours) and supernatants assessed for levels of Il-4 and Il-10 by enzyme-linked immunosorbent assay. Data are expressed means ± SEM. $n = 3$ (**B and C**, control, DNBS, from one experiment); $n = 5$ (**B and C**, HD-DCs + DNBS, proK HD-DCs + DNBS, from one experiment); $n = 6$ (**A**, control, from two experiments); $n = 7$ (**A**, DNBS, from two experiments); $n = 9$ (**A**, HD-DCs + DNBS, proK HD-DCs + DNBS, from two experiments). * $P \leq 0.05$ versus positive control. b.d., below detection threshold.

Figure 5 Major histocompatibility complex class II knockout (MHC II KO) *Hymenolepis diminuta* antigen-treated dendritic cells (HD-DCs) suppress colitis. MHC II KO and wild-type (WT) HD-DCs (1×10^6 i.p.) were adoptively transferred to C57BL/6 mice 48 hours before dinitrobenzene sulfonic acid (DNBS; 4 mg i.r.) challenge. **A** and **B**: Macroscopy and histology damage scores were analyzed by Kruskal-Wallis test with Dunn posttest to positive control. **C**: Representative micrographs of hematoxylin and eosin-stained cross sections of colonic tissue. **D** and **E**: Supernatants from stimulated recipient splenocytes (5×10^6 , 2 μ g/mL concanavalin A, 48 hours) were analyzed for levels of IL-4 and IL-10 by enzyme-linked immunosorbent assay, and analyzed by one-way analysis of variance with Tukey posttest. Data are expressed as means $\pm$ SEM. $n = 5$ (**D** and **E**, control); $n = 6$ (**A** and **B**, control, DNBS; **D** and **E**, DNBS); $n = 7$ (**A** and **B**, DNBS; **D** and **E**, HD-DCs + DNBS); $n = 8$ (**A** and **B**, HD-DCs + DNBS); $n = 9$ (**A** and **B**, MHC II KO HD-DCs + DNBS; **D** and **E**, MHC II KO HD-DCs + DNBS), all from two experiments. * $P \leq 0.05$ versus DNBS; $^\dagger P \leq 0.05$ versus MHC II KO HD-DCs + DNBS; $^\ddagger P \leq 0.05$ versus all groups. Scale bar = 300 μ m. L, lumen; M, muscle.

Intact HD Glycans Are Required to Induce Anti-Colitic WT and MHC II KO DCs

Helminth-derived glycans modify the immunomodulatory properties of DCs,^{28–30} as determined by the ablation of the bioactivity in helminth-derived extracts by treatment with sodium metaperiodate.^{31–33} When WT DCs (Figure 9, A–C) or MHC II^{−/−} DCs (Figure 9, F–H) were exposed to sodium metaperiodate-treated HD extract, both cell types failed to inhibit DNBS-induced colitis (adoptive transfer of DCs pulsed with sham-treated worm extract reduced the severity of DNBS-induced tissue damage). In accordance with these indices of colonic disease, WT sodium metaperiodate-treated HD-DCs did not elicit an increase in IL-4 or IL-10 from recipient splenocytes (Figure 9, D and E). As before (Figure 5, D and E), MHC II^{−/−} HD-DCs did not elicit a splenic IL-4 response in recipient mice (Figure 9I) independent of sodium metaperiodate treatment, although in this instance there was a moderate increase in splenic IL-10 above that of DNBS-only treated mice (Figure 9J).¹⁸

Discussion

Helminth and cellular immunotherapy are being assessed in preclinical and clinical trials in inflammatory disease, including inflammatory bowel disease.⁸ Combining these approaches, we previously showed that HD-DCs protected mice from DNBS-induced colitis.¹⁸ Expanding on this finding, the current data support three main conclusions. First, the expression of Ccr7 and hence homing to SLOs is important for the maximum anti-colitic effect of HD-DCs. Second, the suppression of colitis initiated by the WT HD-DCs promotes splenic Cd4⁺Gata3⁺ Th2 cells; yet, in the absence of MHC II, an alternative anti-colitic pathway emerges that is independent of Th2 immunity but is transferred by Cd4⁺ splenic T cells. Finally, a key molecule(s) in the worm extract that facilitates the DC immunoregulatory/anti-colitic phenotype is a heat- and proteinase K-insensitive glycan(s).

Adoptive transfer of T cells, DCs, macrophages, and mesenchymal stem cells suppresses inflammation in a cell- and disease-specific manner via activity in the target organ, SLOs, or the peritoneum.^{15,26,34–36} Immunolocalization revealed HD-DCs preferentially trafficked to the spleen, MLN, and colon of recipient mice; recruitment of HD-DCs to these SLOs, but not the colon, was significantly enhanced in colitic mice. The functional significance of the high level of fluorescence observed in the liver was not evaluated but likely reflects drainage from the peritoneum.^{26,37} Considering its role as an immunologic organ,³⁸ it cannot be ruled out that DC activity in the liver could have anti-colitic consequences; however, previous studies revealed that HD-DCs drive production of Th2 cytokines by splenocytes, and that Cd4⁺ splenocytes from recipient mice block

Figure 6 Major histocompatibility complex class II knockout (MHC II KO) *Hymenolepis diminuta* antigen–treated dendritic cells (HD-DCs) do not drive Th2 response in recipients. **A–H:** Flow-cytometric quantification of the percentages of Gata-3⁺, forkhead box protein P3 (Foxp3)⁺, Foxp3⁺Helios⁻, and Foxp3⁺Helios⁺ Cd4⁺ cells in the spleen (**A–D**) and mesenteric lymph nodes (MLNs) (**E–H**) of C57BL/6 mice receiving MHC II KO HD-DCs (1×10^6 i.p.) 5 days previously. Mean $\pm$ SEM absolute numbers of Gata-3⁺Cd4⁺ splenocytes: control, $1.04 \pm 0.357 \times 10^5$; naïve MHC II^{-/-} DCs, $3.53 \pm 0.503 \times 10^5$; and MHC II^{-/-} HD-DCs, $2.39 \pm 0.341 \times 10^5$. Mean $\pm$ SEM absolute numbers of Foxp3⁺Cd4⁺ splenocytes: control, $7.43 \pm 1.77 \times 10^6$; naïve MHC II^{-/-} DCs, $9.48 \pm 0.922 \times 10^6$; and MHC II^{-/-} HD-DCs, $8.09 \pm 1.67 \times 10^6$. Data are expressed as means $\pm$ SEM. $n = 4$ (control); $n = 5$ (HD-DC, MHC II KO HD-DCs), from one experiment. * $P < 0.05$ (one-way analysis of variance with Sidak posttest between cell-treated groups).

colitis,¹⁸ suggesting that nonhepatic mechanisms are responsible for disease suppression.

The absence of increased HD-DC recruitment to the colon in DNBS-induced inflammation suggests that homing to the gut is not as important as recruitment to SLOs in this model, although this remains to be tested. Since HD-DCs require adaptive immune responses in recipients to suppress

colitis,^{18,26} recruitment of HD-DCs to SLOs to suppress colitis is not surprising. Ccr7 is important for DC migration to SLOs,²⁷ directing chemotaxis via a G protein and mitogen-activated protein kinase–dependent process, while mobilizing a Rho/protein–tyrosine kinase 2/cofilin-dependent pathway to control the speed of cell migration.³⁹ Adoptive transfer of Ccr7^{-/-} HD-DCs failed to attenuate DNBS-induced colitis, yet the reduced homing of Ccr7^{-/-} HD-DCs to the spleen was not statistically significant, and recruitment of WT and Ccr7^{-/-} HD-DCs to the MLN was equivalent. However, deletion of Ccr7 does not fully impair DC migration to SLOs.^{40,41} In the absence of Ccr7, DCs have been reported to increase chemotaxis to the C-X-C chemokine receptor type 4 ligand Cxcl12; however, as the Ccr7 ligand Ccl19 is approximately 10-fold more potent than Cxcl12, it seems unlikely that HD-DCs lacking Ccr7 would localize in SLOs with the equivalent speed and efficiency as WT HD-DCs.⁴² Thus, the inability of Ccr7^{-/-} HD-DCs to ablate the severity of DNBS-induced colitis in our study is in accordance with recruitment to SLOs (specifically the spleen) being of central importance in the anti-colitic effect of HD-DCs. These findings, however, do not discount the potential importance of local cytokine responses; the kinetics of such responses and the source of these cytokines within the lamina propria should be considered in future studies.

HD-DCs do not attenuate colitis in T cell–deficient mice, and Cd4⁺ T cells from the spleens of HD-DC–treated mice suppress disease.¹⁸ Characterizing the T-cell phenotypes induced by HD-DC transfer revealed a significant increase in the proportion of Gata3⁺ (Th2 master transcription factor)/Cd4⁺ splenic and MLN T cells in response to HD-DC treatment. In contrast, the expression of neither T-bet, ROR γ t (Th1 and Th17 transcription factors, respectively), nor Foxp3⁺ in Cd4⁺ cells was increased in the SLOs after delivery of HD-DCs. These results are in accordance with studies showing helminth antigen–loaded DCs drive Th2 responses.^{43–47} Others report that helminth-educated DCs can increase the numbers of T-regulatory cells,^{15,48} and *Foxp3* mRNA was subtly increased in the colon of mice infected with HD.⁴⁹

Helminths produce immunomodulatory proteins, such as the cathelicidin-like peptides from the trematode *Fasciola hepatica*, which impede antigen processing and presentation by macrophages.⁵⁰ Sensitivity to boiling and enzymatic digestion (typically trypsin or proteinase K) are often used to characterize proteinaceous helminth-derived immunomodulatory molecules. This approach revealed protein- and heat-sensitive molecules from *Onchocera volvulus* and HD, respectively, which suppressed T-cell proliferation^{41,51,52}; schistosome egg antigen proteins, which induced DC secretion of Il-1 β ⁵³; and *Nippostrongylus brasiliensis* antigens, which, when applied to DCs, drove Th2 responses in recipient mice.^{46,54} Although DCs treated with heat- and proteinase K–degraded HD extract retained their ability to suppress DNBS-induced colitis, they did not enhance

Table 2 T-Cell Phenotype in Mice Receiving MHC II KO HD-DCs

Study group	Cd8, %	Cd4, %	Cd4 ⁺ cells		Cd4 ⁺ Foxp3 ⁺ cells		Cd4 ⁺ cells	
			Gata-3, %	Foxp3, %	Helios [−] , %	Helios ⁺ , %	T-bet, %	RORγt, %
Spleen								
Control	13.90 ± 0.17	21.65 ± 0.21	0.14 ± 0.02	10.27 ± 0.26	3.32 ± 0.03	6.56 ± 0.29	2.97 ± 0.11	1.34 ± 0.27
KO DCs	13.05 ± 0.28	20.80 ± 0.58	0.45 ± 0.09	11.60 ± 0.33	3.37 ± 0.13	7.81 ± 0.18	4.61 ± 0.61	2.10 ± 0.49
KO HD-DCs	13.92 ± 0.54	21.10 ± 0.79	0.37 ± 0.06	11.66 ± 0.36	3.35 ± 0.07	7.71 ± 0.44	4.3 ± 0.38	0.98 ± 0.30
MLNs								
Control	26.65 ± 1.48	39.93 ± 2.28	0.05 ± 0.01	5.82 ± 0.14	2.41 ± 0.05	3.48 ± 0.49	0.25 ± 0.02	0.96 ± 0.24
WT DCs	27.75 ± 1.05	42.53 ± 1.42	0.10 ± 0.03	6.57 ± 0.38	2.80 ± 0.19	3.42 ± 0.03	0.30 ± 0.07	0.56 ± 0.05
WT HD-DCs	27.64 ± 0.80	40.06 ± 1.17	0.21 ± 0.06	7.62 ± 0.27*	3.26 ± 0.09*	3.85 ± 0.18	0.68 ± 0.24	0.72 ± 0.14

C57BL/6 mice received 1×10^6 naïve MHC II KO DCs or HD-DCs in phosphate-buffered saline; controls received an equal volume of vehicle. All cells are gated on the viable lymphocyte population, before doublet exclusion. Gata-3 and Foxp3 populations are expressed as the percentage of Cd4⁺ cells, whereas T-bet and RORγt are expressed as the percentage of Cd4⁺Gata-3⁺Foxp3⁺ cells. Data are expressed as means ± SEM.

* $P < 0.05$ versus KO DC treatment (one-way analysis of variance with Sidak posttest). $n = 4$ (control); $n = 5$ (HD-DCs and MHC II KO HD-DCs), all from one experiment.

DCs, dendritic cells; Foxp3, forkhead box protein P3; HD, *Hymenolepis diminuta* antigen treated; KO, knockout; MHC II, major histocompatibility complex class II; MLNs, mesenteric lymph nodes; T-bet, T-box protein expressed in T cells; WT, wild type.

splenocyte production of Il-4 and Il-10, important cytokines in this model for disease suppression.^{18,19} Adoptive transfer of heat and proteinase K–treated HD-DCs may still be compatible with these cells' activation of Th2 immunity via MHC II interaction with T cells, since antigenic peptides remained in the helminth extracts; however, the lack of Il-4 and Il-10 responses from splenocytes in mice given boiled + proteinase K–treated HD-DCs suggested an alternative pathway of the suppression of colitis.

Adoptive transfer of MHC II^{−/−} HD-DCs unexpectedly resulted in the suppression of DNBS-induced colitis; in separate experiments, the transfer of these cells was accompanied by an increased capacity of isolated splenocytes to produce Tgf-β, a cytokine that can ameliorate colitis.^{55,56} Helminth induction of Tgf-β is not unprecedented.^{15,57,58} However, *in vitro* co-culture of naïve splenic Cd4⁺ T cells and HD-DCs did not result in increased Tgf-β (personal observations), suggesting that an environmental

factor available to the cells *in vivo* was absent *in vitro*. Furthermore, when DNBS was introduced into the model, levels of splenic Tgf-β in MHC II KO mice were inconsistent and variable, possibly due to altered Tgf-β availability under inflammatory conditions. Consequently, conclusions on the role of Tgf-β, if any, in the suppression of DNBS-induced colitis by MHC II^{−/−} HD-DCs would be premature, and an extensive and rigorous series of experiments are required to determine whether this preliminary observation on increased Tgf-β is relevant as an alternative pathway of the suppression of colitis in mice given MHC II^{−/−} HD-DCs. Il-4 and Il-10 signaling is required for WT HD-DCs to inhibit DNBS-induced colitis,^{18,19} so in the absence of either (or both), it is unclear why this alternative pathway of immune suppression is not mobilized. It is likely that the absence of MHC II that is crucial in the activation of the alternative pathway may not be recapitulated by deficiencies in Il-4, Il-10, or their respective receptors.

Figure 7 Major histocompatibility complex class II knockout (MHC II KO) *Hymenolepis diminuta* antigen–treated dendritic cells (HD-DCs) drive splenic transforming growth factor (Tgf)-β. Wild-type (WT) or MHC II KO untreated DCs, or HD-DCs (100 μg/mL) were adoptively transferred (1×10^6 i.p.) to C57BL/6 mice. **A–C:** Splenocytes were isolated 5 days later, stimulated (5×10^6 , 2 μg/mL concanavalin A, 48 hours), supernatants were collected, and levels of Il-4, Il-10, and Tgf-β were measured by enzyme-linked immunosorbent assay and analyzed by one-way analysis of variance with Sidak posttest between the WT HD-DC and MHC II KO HD-DC groups. Data are expressed as means ± SEM. $n = 8$ (MHC II KO naïve DCs); $n = 11$ (naïve DCs); $n = 16$ (MHC II KO HD-DCs); $n = 18$ (HD-DCs), all from three or four experiments. * $P \leq 0.05$.

Figure 8 Major histocompatibility complex class II knockout (MHC II KO) *Hymenolepis diminuta* antigen-treated dendritic cells (HD-DCs) educate an anti-colitic Cd4⁺ splenocyte. **A:** C57BL/6 mice received Cd4⁺ splenocytes (1 × 10⁶) from the mice that were given MHC II KO HD-DCs (1 × 10⁶ i.p.) 5 days previously, and were immediately challenged with dinitrobenzene sulfonic acid (DNBS) (4 mg i.r.). **B** and **C:** Severity of colitis was assessed by macroscopy (**B**) and histology (**C**) damage scores, and analyzed by Kruskal-Wallis test with Dunn posttest. **D:** Representative hematoxylin and eosin-stained cross sections of colonic tissue. **E–G:** Supernatants of recipient stimulated splenocytes (5 × 10⁶, 2 μg/mL concanavalin A, 48 hours) were analyzed for levels of IL-4, IL-10, and latent transforming growth factor (Tgf)-β. Data are expressed as means ± SEM. *n* = 6 (control, MHC II KO HD-DC-educated Cd4⁺ + DNBS); *n* = 8 (DNBS), from two experiments. **P* < 0.05 versus positive control. Scale bar = 300 μm. L, lumen; M, muscle.

Characterization of the effect of MHC II^{-/-} HD-DCs revealed that, unlike WT HD-DCs, they failed to induce Gata-3 expression in the spleen or MLNs of recipient mice. MHC II^{-/-} HD-DCs evoked a small but significant increase in the percentage of Cd4⁺Foxp3⁺Helios⁻ cells in the MLN, characteristic of peripheral induction of T-regulatory cells, rather than the recruitment of natural T-regulatory cells.⁵⁹ Interestingly, although infection with *Heligmosomoides polygyrus* similarly resulted in a subtle (approximately 0.13-fold) increase in colonic Cd4⁺Foxp3⁺

cells, only transfer of colonic Cd4⁺ cells from *H. polygyrus*-infected, but not control, mice, attenuated colitis.⁶⁰ This finding suggests that helminth parasites (and, by extension, their antigens) have the potential to alter the immunosuppressive capacity of Foxp3⁺ cells in the absence of significant proliferation.

WT HD-DCs educate splenic Cd4⁺ T cells that suppress disease.¹⁸ Remarkably, we found that Cd4⁺ splenic T cells from recipients of MHC II^{-/-} HD-DCs significantly attenuated the severity of DNBS-induced colitis in the

Figure 9 *Hymenolepis diminuta* (HD)-derived glycans are indispensable for suppression of dinitrobenzene sulfonic acid (DNBS) colitis. Wild-type (WT) dendritic cells (DCs) (1×10^6) treated with sham-HD, or sodium metaperiodate (SMP)-HD (100 $\mu\text{g/mL}$, 24 hours) were transferred (i.p.) to C57BL/6 mice 48 hours before DNBS challenge (4 mg i.r.). Macroscopy (**A**) and histology (**B**) damage scores were analyzed by Kruskal-Wallis test with Dunn posttest to positive control. **C**: Representative micrographs of hematoxylin and eosin-stained cross sections of colonic tissue. **D** and **E**: Levels of IL-4 and IL-10 were measured from supernatants of stimulated splenocytes (5×10^6 , 2 $\mu\text{g/mL}$ concanavalin A, 48 hours) and analyzed by one-way analysis of variance with Tukey posttest. **G–J**: Major histocompatibility complex class II knockout (MHC II KO) DCs treated with sham HD, or SMP-HD were transferred to C57BL/6 mice 48 hours before DNBS challenge. Macroscopy (**F**) and histology (**G**) damage scores were analyzed by Kruskal-Wallis test with Dunn posttest to positive control. **H**: Representative micrographs of hematoxylin and eosin-stained cross sections of colonic tissue. **I** and **J**: Levels of IL-4 and IL-10 were measured from supernatants of stimulated splenocytes, and analyzed by one-way analysis of variance with Tukey posttest. Data are expressed as means $\pm$ SEM. $n = 3$ (**A**, **B**, **D–G**, **I**, and **J**, controls); $n = 5$ (**A**, **B**, **D**, and **E**, HD-DCs + DNBS, SMP HD-DCs + DNBS; **F**, **G**, **I**, and **J**, DNBS, MHC II KO SMP HD-DCs + DNBS); $n = 6$ (**A**, **B**, **D**, and **E**, DNBS; **F**, **G**, **I**, and **J**, MHC II KO HD-DCs + DNBS), all from one experiment. * $P \leq 0.05$ (**D** and **E**, versus all groups; **F** and **G**); † $P \leq 0.05$ versus DNBS group (**I** and **J**). Scale bars = 300 μm . b.d., below detection threshold; L, lumen; M, muscle.

Figure 10 Major histocompatibility complex class II (MHC II)-dependent and MHC II-independent suppression of colitis in *Hymenolepis diminuta* antigen-treated dendritic cells (HD-DCs). **A:** Adoptive transfer of wild-type (WT) HD-DCs induces GATA-3 expression in CD4⁺ cells, and drives splenic IL-4 and IL-10 in recipients. As previously reported, WT HD-DCs educate an IL-4-producing CD4⁺ cell that can suppress colitis in recipients via IL-10 production.^{18,19} **B:** In an alternative pathway, HD-DCs that lack MHC II do not up-regulate Gata-3⁺CD4⁺ cells, or drive splenic IL-4 or IL-10 in recipient mice, but inhibit colitis, in part through an anti-colitic CD4⁺ splenocyte. The bioactive molecule(s) in the HD extract involved in educating an immune-regulatory/anti-colitic DC are sodium metaperiodate (SMP) sensitive.

absence of Cd4⁺Gata-3⁺ cell induction and limited increases in Cd4⁺Foxp3⁺Helios⁺ cells in mice, a finding compatible with the observation that MHC II^{-/-} HD-DCs did not affect the outcome of disease in DNBS-treated recombination-activating gene 1^{-/-} mice. Thus, in the absence of cognate interaction via MHC II, HD-DCs instruct the development of an anti-colitic T cell and/or enhance the potency of low-frequency T-regulatory cells, as suggested by Hang et al.⁶⁰ Although surprising, this finding is not unprecedented: Walsh et al⁶¹ reported the induction of neuroprotective Cd4⁺ T cells in the central nervous system via an MHC II-independent mechanism. Although MHC II was absent from transferred HD-DCs, MHC II-competent antigen-presenting cells in the recipient mouse could be conditioned within the micro-environment, which may contribute to the observed Cd4⁺ T-cell-mediated effect in protection from colitis. Regardless, MHC II^{-/-} HD-DCs evoke, either directly or indirectly, a pathway of Cd4⁺ T cell-mediated protection from colitis.

Intriguingly, treatment of the HD extract with sodium metaperiodate, an oxidizing agent that alters glycan conformation by opening saccharide rings, rendered it incapable of evoking the anti-colitic phenotype in WT or MHC II^{-/-} DCs. Similarly, a sodium

metaperiodate-sensitive glycan was implicated in the suppression of macrophage activation by HD-derived antigens.²³ Although poorly characterized, a variety of bioactive immunoregulatory glycans from helminths can modify DC and other immune-cell activity.^{30,32,45,62} These data indicate that within the mixed molecule milieu of the HD extract, glycans are crucial in the anti-colitic effect that operates via both MHC II-dependent and MHC II-independent pathways.

Helminth extracts have been shown to activate Toll-like receptor 2,⁶³ raising the potential of HD extract education of WT or MHC II^{-/-} DCs occurring via these or other germ line-encoded sensors of pathogens and how microbial factors may influence the system. Moreover, the gut microbiota are a determinant of digestive health,⁶⁴ and infection with helminths results in shifts of the composition of the microbiota. It is unknown whether cellular immunotherapy would affect the composition or function of the gut microbiota, and any consequence of an altered microbiota on the inhibition of colitis also is unknown. The interaction of the gut microbiota and enteric helminths is an emerging and intriguing area that will undoubtedly affect digestive health and will have to be considered in the development of helminth therapy, the use of helminth-derived molecules in the treatment of disease, and helminth antigen-based cellular immunotherapy.⁸

In summary, in exploring the mechanism of HD-DC suppression of DNBS-colitis in mice, we found that protection from colitis was dependent on a heat- and proteinase K-insensitive, sodium metaperiodate-sensitive glycan(s) in the worm extract and on the expression of Ccr7 on DCs. With MHC II present, HD-DCs mobilized Cd4⁺Gata-3⁺ T cells and ameliorated colitis via IL-4 and IL-10 signaling from recipient and donor cells (Figure 10).^{18,19} However, in the absence of MHC II, the mechanism of inhibition of DNBS-induced colitis by HD-DCs is dramatically different: Neither splenic Cd4⁺Gata-3⁺ cells nor IL-10 or IL-4 is evoked. Although the signaling mechanism evoked by the transfer of MHC II⁻ HD-DCs is unclear, we suggest that redundancy exists within the immune system such that the absence of MHC II can, to some degree, be compensated. Considering the use of HD-DCs in human colitis, we speculate that the promotion of Ccr7 expression could enhance the efficacy of this potential novel therapy, which would be effective in patients who, for whatever reason (eg, concomitant viral infection) have a compromised ability to express MHC II.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.07.008>.

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