Chronic *Toxoplasma gondii* infection enhances susceptibility to colitis

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Oral infection with *Toxoplasma gondii* results in dysbiosis and enteritis, both of which revert to normal during chronic infection. However, whether infection leaves a lasting impact on mucosal responses remains uncertain. Here we examined the effect of the chemical irritant dextran sodium sulfate (DSS) on intestinal damage and wound healing in chronically infected mice. Our findings indicate that prior infection with *T. gondii* exacerbates damage to the colon caused by DSS and impairs wound healing by suppressing stem cell regeneration of the epithelium. Enhanced tissue damage was attributable to inflammatory monocytes that emerge preactivated from bone marrow, migrate to the intestine, and release inflammatory mediators, including nitric oxide. Tissue damage was reversed by neutralization of inflammatory monocytes or nitric oxide, revealing a causal mechanism for tissue damage. Our findings suggest that chronic infection with *T. gondii* enhances monocyte activation to increase inflammation associated with a secondary environmental insult.

monocytes | disbiosis | toxoplasmosis | nitric oxide | inflammation

Toxoplasma gondii is a common intracellular protozoan parasite that chronically infects approximately one-third of the human population (1, 2). Toxoplasma infection is typically acquired through exposure to soil, food, or water that is contaminated with oocysts from infected cat feces or ingestion of undercooked meat harboring tissue cysts. In an immunocompetent host, chronic infections are generally asymptomatic (3, 4). However, several studies indicate that latent toxoplasmosis plays a role in the pathogenesis of other diseases characterized by excessive inflammation. For example, it has been reported that antibodies directed toward *T. gondii* are more prevalent in patients with inflammatory bowel disease (IBD) (5). Another study reported that patients suffering from toxoplasmosis are at greater risk of developing sepsis (6).

Following oral infection, dendritic cells are among the first immune cells to encounter invading parasites in the intestine, with $CD11b^{+}CD103^{-}$ dendritic cells serving as the primary targets of infection (7). In the early phase of infection, inflammatory monocytes and neutrophils represent the dominant newly recruited cell populations in the gut (8). Dendritic cells, macrophages, and neutrophils produce interleukin-12 (IL-12) that activates interferon (IFN)-γ production initially by natural killer (NK) and innate lymphoid (ILC) cells and later by T cells (9, 10). Together, this IL-12–IFN-γ axis is critical for control of infection. Although parasite growth is controlled efficiently, severe immunopathology can also ensue in the small intestine due to overproduction of proinflammatory cytokines such as IFN-γ and TNF-α as well as free radical nitric oxide (NO) (11–13).

Oral infection in susceptible C57BL/6 mice can lead to enteritis driven by loss of Paneth cells (14), breach of the epithelial barrier, and leakage of luminal contents to the submucosa (15). Oral infection with *T. gondii* has been reported to break tolerance to luminal antigens (16), and the presence of commensal bacteria in the submucosa further triggers strong inflammatory responses (17). Intestinal damage can be reversed by treatment of mice with antibiotics prior to oral infection with *T. gondii* (14) and is reduced in mice lacking Myd88 (18) or Toll-like receptor (TLR) 9 (19, 20), suggesting that TLR recognition of microbial ligands promotes inflammation. Pathology in the gut resolves by 3 to 4 wk postinfection; however, subtle changes in the population of commensal bacteria, specifically Clostridia and *Bacteroides* species, have been reported to persist during chronic infection (21–23).

Monocytes, a major component of the mononuclear phagocytes, are constitutively generated in the bone marrow from hematopoietic stem cells (24). In response to inflammation, inflammatory monocytes ($CD11b^{+}$, $CD115^{+}$, $Ly6C^{hi}$) increase expression of the chemokine receptor 2 (CCR2), migrate from the bone marrow in response to chemokines such as MCP1 (CCL2), and reach infected or inflamed tissues via the bloodstream (25, 26). Once monocytes arrive in an inflammatory setting, their differentiation and effector functions are shaped by the local tissue environment (27, 28). During acute *T. gondii* infection, inflammatory monocytes play a dominant role in control of the parasite (8, 29) and acquire both proinflammatory and tissue-specific regulatory functions (8, 30). Prior infection with

Significance

We examined the long-term consequences of oral infection with the parasite *Toxoplasma gondii* on a model for inflammatory bowel disease caused by chemical injury of the intestinal mucosa. Despite the fact that the parasite no longer persists in the mucosal tissue, chronic infection altered the function of monocytes that emerge from the bone marrow in a preactivated state and home to sites of inflammation. The heightened inflammatory responses by infiltrating monocytes worsened the severity of tissue damage caused by chemical injury and also blunted the wound healing responses in the gut. Our findings illustrate how chronic subclinical infections can have lasting impacts on environmental triggers of inflammation.

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T. gondii leads to heightened innate immune responses by macrophages that are beneficial in imparting increased resistance to a variety of other pathogens (31, 32) and even tumors (33–36). However, with dextran sodium sulfate (DSS) treatment, inflammatory monocytes induce immunopathology in the gut by promoting inflammation (37). Hence, chronic *T. gondii* infection may enhance the severity of intestinal inflammatory syndromes through alteration of macrophage function.

To understand the effect of *T. gondii* infection on susceptibility to gut inflammation, we used treatment with DSS that induces damage to the colon and simulates colitis (38). Unlike immune deficiency models, DSS treatment does not impact resistance to infection and it provides a reproducible method to induce reversible colonic injury due to inflammation (38, 39). Here, we demonstrate that chronic infection with *T. gondii* enhances sensitivity to DSS-mediated damage and impairs healing of the epithelium. Enhanced susceptibility was driven by perpetually activated inflammatory monocytes that show up-regulated MHC class II expression in the bone marrow and that further up-regulate inflammatory markers on migration to the gut. Moreover, recruitment of these activated monocytes resulted in higher production of NO that was associated with increased intestinal damage and reduced healing in chronically infected mice after DSS treatment. These findings suggest that chronic infection with *T. gondii* may be a risk factor for more severe outcomes when inflammation is trigged by a secondary insult.

Results

Greater DSS-Mediated Intestinal Damage in Chronically Infected Mice. To test the effect of *T. gondii* infection on susceptibility to gut inflammation due to chemical injury, we treated control or chronically infected C57BL/6 mice with DSS to induce chemical injury (Fig. 1*A*). Animals were followed for weight loss and euthanized after 7 d for histological examination of the small and large intestines (Fig. 1*A*). We also analyzed the intestine for potential histological changes at 30 d postinfection, a time point when infection has resolved in the gut, although tissue cysts remain in the central nervous system and skeletal muscle (40, 41). Although parasites were readily detected by immunofluorescence staining of tissue sections at day 5 postinfection, they were not detected in the colon of the infected mice day 30 postinfection either before or after DSS treatment (*SI Appendix*, Fig. S1*A*). Tissue sections from the small and large intestines of mice from 30 d postinfection revealed normal intestinal mucosal morphologies with no signs of lesions, similar to control mice (Fig. 1 *B*, *Left*). However, in chronically infected mice after DSS treatment, the large intestine of infected mice showed more extensive damage including cellular infiltration, destruction of the crypt structure, and more extensive regions of ulcerated lesions where the architecture was severely disturbed, relative to control mice (Fig. 1 *B*, *Middle*). One notable difference was evident at the margins of lesions, wherein height of the epithelial layer from the crypt to the upper margin of the tissue was increased in control mice, suggesting healing, while this expansion of the epithelium height and wound healing response was blunted in infected mice (Fig. 1 *B*, *Right*). The average epithelium height and the density of cells per crypt in areas without lesions were also reduced in infected mice relative to control animals after DSS treatment (Fig. 1 *C* and *D*). Consistent with this reduction in cellular density, the number of $Ki67^+$ replicating cells was significantly decreased in infected mice after DSS treatment (Fig. 1*E*). Together, these results indicate chronic infection with *T. gondii* results in increased damage due to DSS treatment, likely due to impaired tissue regeneration.

Chronic *T. gondii* Infection Impairs Regenerative Stem Cells of the Colonic Mucosa. Following the damage caused by DSS, once mice are transferred to normal water the tissue heals and the crypt architecture is restored during stem-cell–dependent regeneration (42–44). To examine this recovery phase, we returned mice to normal drinking water for a 14-d recovery period followed by histological analyses (Fig. 1*A*). Following a 14-d recovery period, we detected enhanced expression of *HopX* by RNA in situ hybridization in the most distal colon of control mice (Fig. 1*F*). However, the number of *HopX*-positive stem cells were significantly reduced in infected mice (Fig. 1*F*). The recovery phase is also marked by the presence of hypertrophic crypts that contain increased numbers of proliferating cells (42, 43). Although this phenotype was evident in control animals, such elongated crypts were absent in infected mice (*SI Appendix*, Fig. S1*B*). Additionally, the number of $Ki67^+$ proliferating cells was much lower in crypts from infected mice (*SI Appendix*, Fig. S1*C*). Together, these data suggest that prior *T. gondii* infection interferes with tissue regeneration and impairs stem cell proliferation in the colonic mucosa.

Chronic *T. gondii* Infection Did Not Alter Host Microbiota Composition. Given that acute *T. gondii* infection results in microbial dysbiosis in the gut (14), we examined whether changes in the microbiota also occur during chronic infection and following DSS treatment (Fig. 2*A*). There was no significant difference in diversity, richness, or phylogenetic composition of the microbiota between the control and infected mice either at day 0 (*SI Appendix*, Fig. S2 *A–C*) or at day 30 postinfection (Fig. 2 *B–D*). In contrast to published studies (21–23), we did not observe increases in the bacterial taxa Bacteroidetes, Verrucomicrobia, Clostridia or decreases in Lactobacillales in the infected mice at day 30 postinfection (*SI Appendix*, Fig. S2 *D–G*). We next compared the microbiota composition in control and infected mice after DSS treatment. Consistent with previous studies (45, 46), both control and infected animals showed significant changes in the microbial composition after exposure to DSS (*SI Appendix*, Fig. S2 *H–M*). However, there was no significant difference in the microbiome in control and infected mice after DSS treatment (Fig. 2 *E–G*). Together, these finding suggest that differences in the microbiota composition do not play a major role in the increased severity of DSS treatment in chronically *T. gondii*–infected mice.

DSS Treatment Enhances Recruitment of Leukocyte Subsets to the Gut. One potential explanation for greater damage in *T. gondii*–infected mice after DSS treatment would be inflammation driven by recruitment of immune cells to the site of infection. To identify the specific cell populations associated with gut pathology, we investigated the composition of immune cells present in the lamina propria of the large and small intestine of control or *T. gondii*–infected mice after DSS treatment by flow cytometry. Notably, the frequency of inflammatory monocytes ($CD11b^+Ly6C^+$) and neutrophils ($CD11b^+Ly6G^+$) increased significantly after DSS treatment in the lamina propria of the large intestine of both control and infected mice (Fig. 3*A* and *SI Appendix*, Fig. S3*A*). The increase in recruitment of monocytes and neutrophils seen in the large intestine was not observed in the small intestine (*SI Appendix*, Fig. S3 *J* and *K*), consistent with the major site of damage caused by DSS being confined to the large intestine.

We did not observe any significant differences in the frequency of resident tissue macrophages ($CD11b^+$ $CD64^+$) nor $CD103^+$ dendritic cells in the large intestine of infected mice compared to control mice after DSS treatment (*SI Appendix*, Fig. S3 *C–E*). However, there were significantly more $CD4^+$ T cells in the large intestine of infected mice as compared to control mice, irrespective of DSS treatment (*SI Appendix*, Fig. S3*F*). In the large intestine, the elevated frequency of $CD4^+$ T cells in infected mice was accompanied by elevated intracellular IFN-γ staining irrespective of DSS treatment, although elevated IFN-γ staining was only significantly higher in $CD8^+$ T cells of mice treated with DSS (Fig. 3 *B* and *C*). IFN-γ secretion by CD4 and CD8 T cells remained similar in the small intestine after DSS treatment, consistent with the fact that this treatment largely damages the colon (*SI Appendix*, Fig. S3 *L* and *M*). Importantly, we did not observe any decrease in $Foxp3^+$ Treg cells in infected mice either without or with DSS treatment

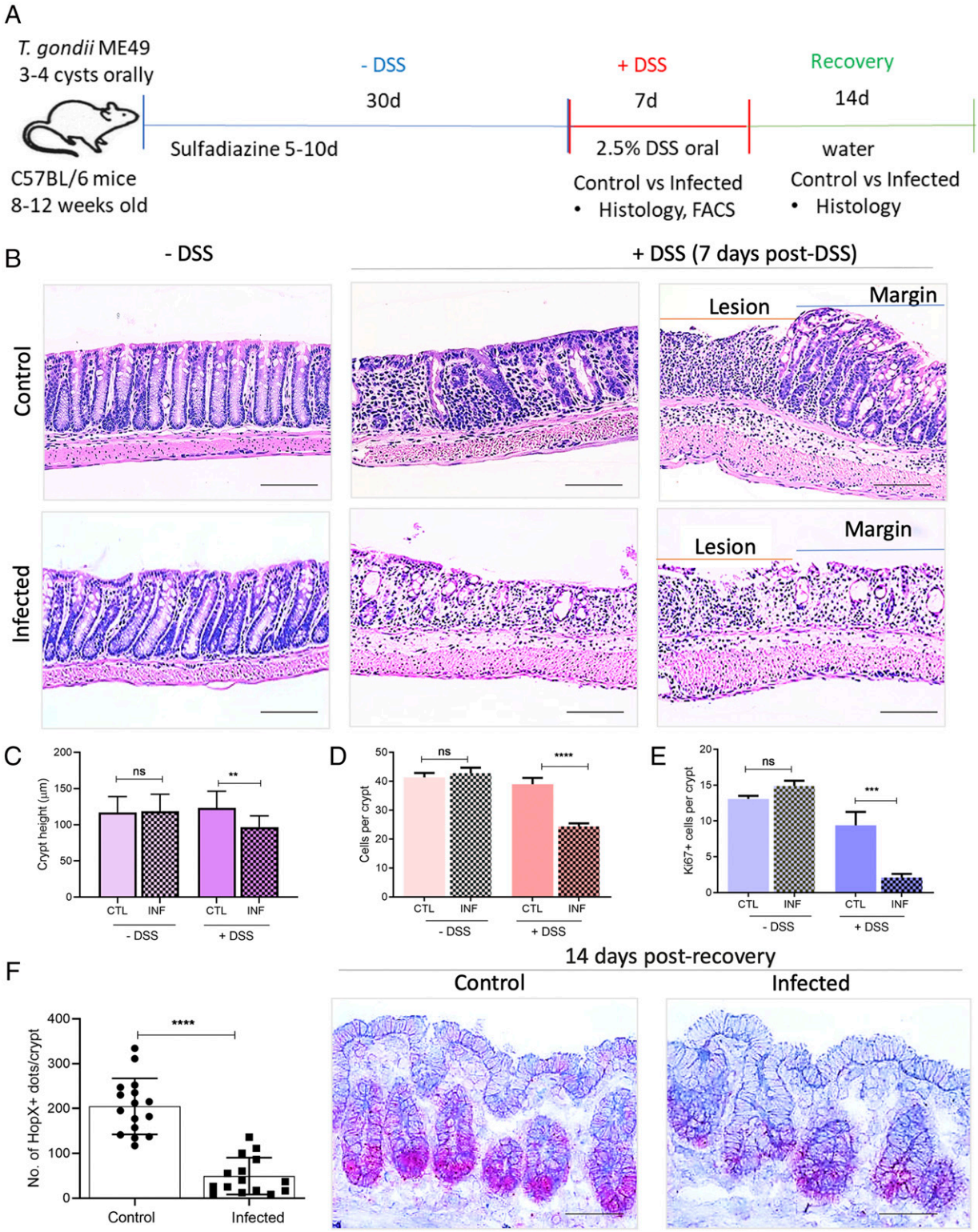

Fig. 1. Greater DSS-mediated intestinal damage in mice chronically infected with *T. gondii*. (*A*) Experimental design for DSS treatment and recovery phase for control and chronically infected mice. (*B*) Representative images and (*C–E*) graphical summary of H&E and Ki67-stained sections of colon in control and *T. gondii*–infected mice before and after DSS treatment. (*F*) Graphical summary and representative images of expression of stem cell marker, HopX by RNA in situ hybridization. Statistics: (*C–E*) Mean ± SEM. Four independent experiments, $n = 12$ female and 4 male mice per group. One-way ANOVA with Sidak's multiple-comparison test $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant. (*F*) Data were pooled from two independent experiments, $n = 3$ to 6 female mice per group. Unpaired Student's *t* test was used for comparison $^{****}P < 0.0001$. (Scale bars, 20 µm.) Please also see *SI Appendix*, Fig. S1.

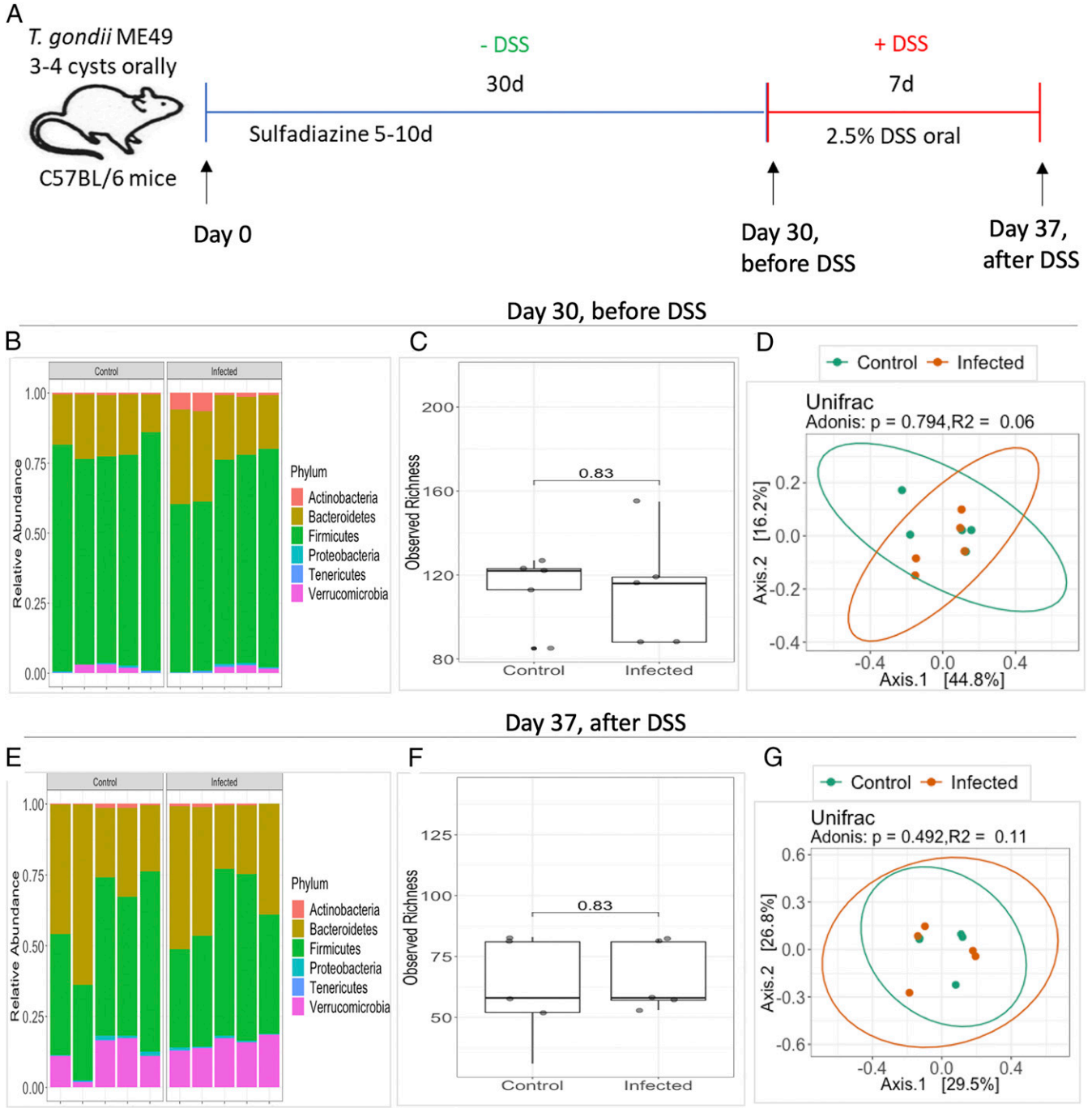

Fig. 2. Chronic *T. gondii* infection did not alter host microbiota composition. (*A*) Experimental design for DSS treatment and collection of samples for microbiota analysis for control and chronically infected mice. Phylum level composition, alpha diversity, and beta diversity plots in control and infected mice at day 30 postinfection and before DSS treatment (*B–D*) and at day 37 postinfection after DSS treatment (*E–G*). Statistics: (*C* and *F*) Two independent experiments, $n = 2$ to 3 female mice per group. Alpha diversity was computed using a nonparametric Wilcoxon test. (*D* and *G*) Beta diversity was computed using uniFrac distance. Please also see *SI Appendix*, Fig. S2.

(*SI Appendix*, Fig. S3*H*), unlike previous reports showing these cells are depleted during acute infection (16). We also did not observe any significant differences in the frequency of effector memory T cells ($CD44^{hi}$ $CD62L^{lo}$ $CD4^{+}$) in the large intestine between infected and control mice after DSS treatment (*SI Appendix*, Fig. S3*I*).

Monocytes Promote DSS-Mediated Intestinal Damage in Mice Chronically Infected with *T. gondii*. Because we found $Ly6C^{+}$ monocytes to be the dominant immune cell population infiltrating the large intestine lamina propria after DSS treatment, we hypothesized $Ly6C^{+}$ monocytes could be responsible for the enhanced gut pathology observed in chronically infected mice. To test this hypothesis, we depleted $Ly6C^{hi}$ inflammatory monocytes by intraperitoneally injecting a monoclonal antibody that removes $CCR2^{+}$ cells, starting at day 3 after DSS exposure daily for a period of 7 d (47, 48) (Fig. 3*D*). Flow cytometry analysis from antibody-treated mice confirmed the depletion of inflammatory monocytes in the large intestine (Fig. 3*E*). Both control and infected mice challenged with

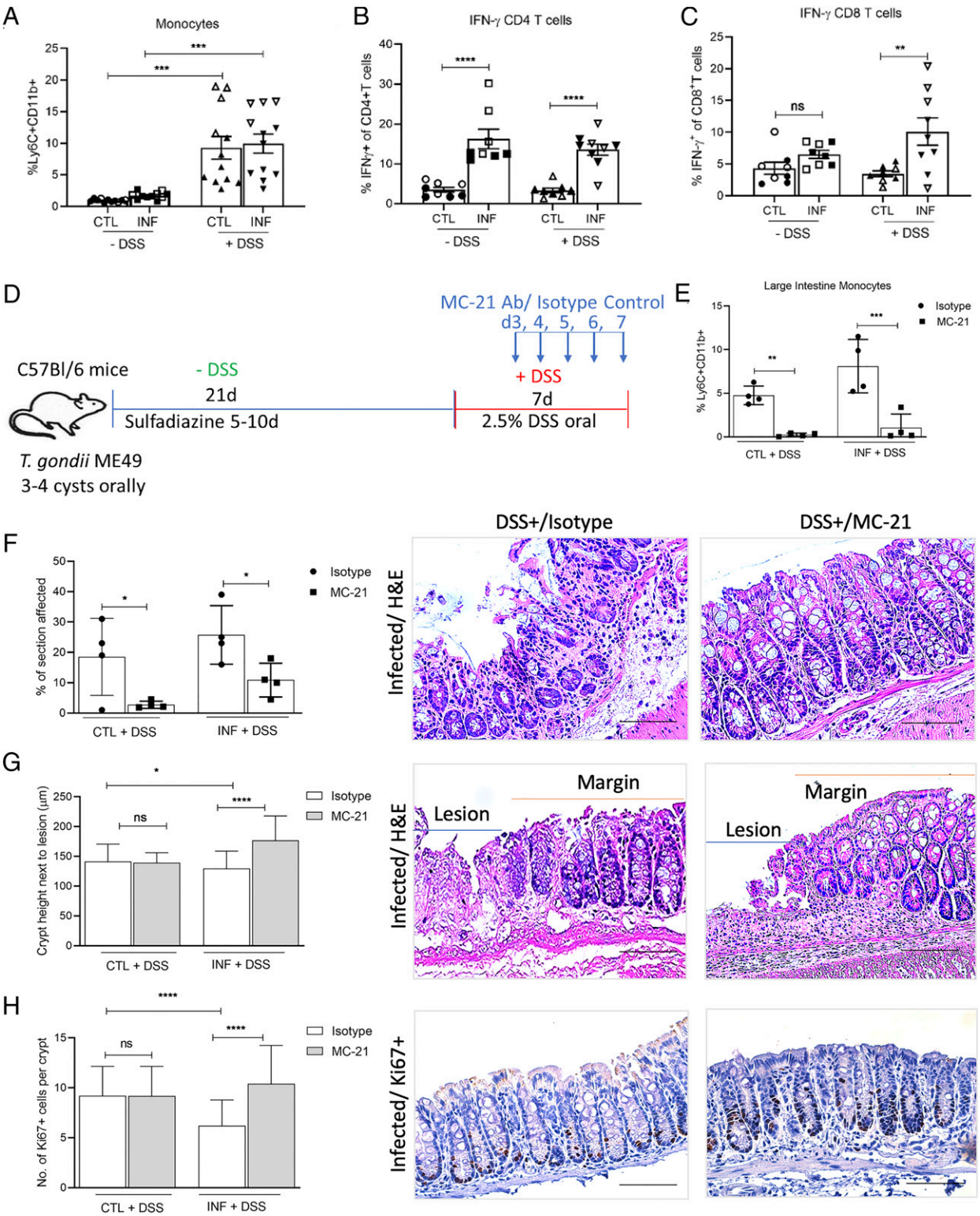

Fig. 3. Monocytes promote DSS-mediated intestinal damage in mice chronically infected with *T. gondii*. (*A*–*C*) Frequency of monocytes, IFN-γ producing $CD4^+$ and $CD8^+$ T cells in the large intestine lamina propria of control (CTL) and *T. gondii*–infected mice analyzed by flow cytometry. −DSS = before administration of DSS, +DSS = final day of treatment. Means ± SD from three independent experiments (*A*) or one experiment (*B* and *C*), $n = 4$ to 5 mice per group (open symbol, male mice; closed symbol, female mice). Statistics: One-way ANOVA analysis with Sidak's multiple-comparison test $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant. (*D*) Experimental design for depletion of inflammatory monocytes using anti-CCR2 (MC-21) mAb vs. isotype control (Iso). (*E*) Frequency of $Ly6C^{hi}$ inflammatory monocytes in the large intestine after DSS treatment with MC-21 mAb vs. isotype control. (*F*–*H*) Graphical summary and histological analysis of (*F*) percent of section affected, (*G*) crypt height adjacent to lesion, and (*H*) number of $Ki67^+$ cells in the colon. Mean ± SD. Data from one experiment, $n = 4$ female mice per group. Statistics: (*E* and *H*) One-way ANOVA with Sidak's multiple comparison $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (*G*) Kruskal–Wallis with Dunn's multiple comparison $^{*}P < 0.05$, $^{****}P < 0.0001$, ns, not significant. (Scale bars, 20 μm.)

DSS and depleted of Ly6C$^+$ monocytes exhibited less intestinal damage, with the reversal in damage indicated by quantitative histological assessment of the percent of sections occupied by lesions (Fig. 3*F*). Infected mice showed significantly decreased crypt height and number of proliferating Ki67$^+$ cells and depletion of inflammatory monocytes specifically enhanced the crypt epithelium height at the margins of lesions (Fig. 3*G*) and increased the number of proliferating Ki67$^+$ cells (Fig. 3*H*). DSS-challenged mice depleted of Ly6C^{hi} blood monocytes exhibited milder features of colitis as evaluated by stool consistency, presence of blood in the stool, and length of the colon. Collectively, these findings suggest a pathogenic role for inflammatory monocytes in an acute experimental colitis model that is further exacerbated in chronically *T. gondii*–infected mice.

Chronic *T. gondii* Infection Activates Monocytes in Bone Marrow and Enhances Inflammation in Large Intestine after DSS Treatment. To evaluate the activation status of monocytes, we examined the levels of MHC class II on CD11b$^+$ Ly6C$^+$ monocytes in the bone marrow by flow cytometry and found these were significantly higher in chronically infected mice than control mice, suggesting that they are activated before they exit the bone marrow (Fig. 4 *A* and *B*). When we evaluated levels of MHC class II on CD11b$^+$ Ly6C$^+$ monocytes in the blood and large intestine lamina propria, we found that this difference between chronically infected and control mice progressively increased from the bone marrow to the circulation, peaking at levels that were ∼10-fold higher in the intestine (Fig. 4 *A*–*D*). Together, these findings suggest that prior exposure to *T. gondii* infection changes the activation state of the monocytes even before they exit the bone marrow, and these responses are heightened when they reach sites of inflammation.

We next questioned whether monocytes in the bone marrow of infected mice also showed changes in other inflammatory cytokines and if these changes would be further enhanced following recruitment to the lamina propria following DSS treatment. To test this possibility, we sorted inflammatory monocytes (*SI Appendix*, Fig. S4*A*) and used qRT-PCR to analyze expression of *Tnfa*, *Il1b*, *Il6*, *Il10*, *Il23*, *Nos2*, and *Ptgs2*. We observed that after DSS treatment, cytokine expression by Ly6C^{hi} inflammatory monocytes in the bone marrow of infected mice were not significantly different from the control mice (*SI Appendix*, Fig. S4*B*). The differences in *Tnfa* between infected and control animals treated with DSS increased to 2.6-fold when monocytes entered the gut, although this difference did not achieve significance (Fig. 4*E*). More importantly, there was a highly significant 8.9-fold increase in the expression *of Nos2* produced by inflammatory monocytes in the lamina propria of infected mice relative to control mice treated with DSS (Fig. 4*E*). These results suggest that inflammatory mediators such as *Tnfa* and *Nos2* contribute to enhanced intestinal damage in *T. gondii*–infected mice after DSS treatment.

Blocking iNOS Protects against DSS-Mediated Damage in Mice Chronically Infected with *T. gondii*. To confirm the enhanced transcriptional response seen in monocytes, we also examined the expression of iNOS at the protein level using immunohistochemical staining of tissues. Histological analysis revealed that there were significantly more inflammatory monocytes, and higher numbers of iNOS-positive cells in the lamina propria of infected mice treated with DSS (Fig. 5 *A* and *B*, *Left*). Moreover, iNOS staining occurred in regions where inflammatory monocytes clustered, suggesting that staining was largely due to expression in monocytes (Fig. 5 *A* and *B*, *Left*). Expression of iNOS was reduced to almost negligible levels in infected mice treated with monoclonal antibody MC-21 to deplete inflammatory monocytes (Fig. 5 *A* and *B*, *Right*). This result suggests that the enhanced production of iNOS by inflammatory monocytes may contribute directly to the tissue damage seen in the intestine of infected mice treated with DSS.

To evaluate whether iNOS or TNF-α were responsible for intestinal pathology, we examined the effect of their selective removal on the response to DSS treatment. For these studies, we either used *Nos2*$^{-/-}$ mice or blocked TNF-α by intraperitoneal injection of a neutralizing monoclonal antibody (*SI Appendix*, Fig. S5 *A* and *B*). Blocking of TNF-α did not protect mice from DSS-induced pathology (*SI Appendix*, Fig. S5*C*). In contrast, control and infected *Nos2*$^{-/-}$ mice challenged with DSS exhibited less intestinal damage (*SI Appendix*, Fig. 5 *C*–*E*). This reversal in damage was seen by quantitative histological assessment of the percent of sections occupied by lesions (Fig. 5*C*) and in increased crypt height (Fig. 5*D*). Additionally, the decrease in the number of Ki67$^+$ replicating cells seen in infected mice treated with DSS was almost fully reversed by iNOS depletion (Fig. 5*E*). Furthermore, in DSS-treated *Nos2*$^{-/-}$ mice, there was a marked improvement in gross pathology as shown by increased colon length, reduced blood in the stool, and improved stool consistency. Collectively, these findings suggest that increase in iNOS expression is responsible for the enhanced intestinal damage in mice chronically infected with *T. gondii* and treated with DSS.

Discussion

Although prior studies have shown that acute *T. gondii* infection in mice causes dysbiosis and ileitis, these symptoms rapidly resolve, leaving it unclear whether infection has lasting effects on mucosal responses. Using a model for chemically induced colitis, we show that chronically infected mice show greater damage and impaired wound healing following treatment with DSS. Enhanced susceptibility to DSS was not related to alterations in the microbiota, which returned to baseline in chronically infected mice, and which was similarly affected by DSS treatment in control and infected animals. Enhanced susceptibility was also not related to changes in regulatory T cells (Tregs) that were unaltered in chronically infected mice. Rather, chronically infected mice showed persistently elevated levels of IFN-γ in CD4$^+$ and CD8$^+$ T cells and systemic activation of inflammatory monocytes that migrate to the intestine and produce inflammatory mediators. Blocking monocyte recruitment or use of *Nos2*$^{-/-}$ mice that lack inducible nitric oxide synthase, protected chronically infected animals from DSS-associated intestinal damage. Together, our work uncovers a mechanism for persistent monocyte activation during chronic *T. gondii* infection that may increase the risk of inflammatory diseases triggered by secondary stimuli.

Oral administration of DSS to mice induces damage and ulceration of the colonic mucosa providing a model for colitis (38). We used the DSS model to examine the effects of prior *T. gondii* infection on the inflammatory response to this chemical irritant that results in intestinal epithelial damage. Because acute infection with *T. gondii* can cause ileitis (49), we examined mice during chronic infection when damage to the tissue is no longer apparent and crypt height, cell density, and replication crypt cells have returned to normal. Despite normal tissue architecture, and an absence of residual parasite infection in the gut, chronically infected mice exposed to DSS developed more severe tissue damage than control animals. This was evident by the extent of the tissue that was affected by lesions, although the severity of the areas affected was similar. Additionally, the wound healing response that occurs by regeneration of epithelium at the margins of the lesions after removal of DSS was impaired in chronically infected mice. Recent studies reveal that following DSS treatment, a population of fetal-like HopX$^+$ stem cells within hypertrophic crypts undergo expansion and contribute to restoration of the epithelium and crypt architecture (43). We observed decreased expression of HopX during recovery from DSS in the infected mice compared to the control mice, consistent with decreased cell proliferation and impaired tissue regeneration. Our results indicate that recovery from DSS is impaired in infected animals, likely due to compromised development of HopX$^+$ stem cells and their

diminished ability to repair the injured epithelium. Separate studies have shown that decreased numbers of colonic Lgr5^{+} stem cells, either due to infection with *Clostridioides difficile* or due to overproduction of Th1 or Th2 cytokines, can increase susceptibility to subsequent infections or disease recurrence (50, 51). However, these models involve active infection that impairs stem cell homeostatic functions that disrupt development of intestinal cell lineages. In contrast, chronic infection with *T. gondii* appears not to alter normal crypt function nor affect epithelial development, but rather impairs a specific subset of HopX^{+} stem cells that emerge during wound healing.

Previous studies have reported slightly increased levels of Bacteroidetes, Verrucomicrobia, and Clostridia and decreased Lactobacillales in chronically *T. gondii*–infected mice (21–23). However, in our study the microbial communities were not significantly different between control and chronically infected mice at day 30 postinfection. The differences in our findings may result from colony differences or the severity of infection between experimental models. Exposure to DSS results in microbial dysbiosis and promotes expansion of *Bacteroides*, *Clostridium*, *Enterobacteriaceae*, and *Bacteroidaceae* (45, 46). Consistent with these reports, we observed altered microbiota in DSS-treated mice; however, these changes were similar in the diversity, richness, or phylogenetic composition of the microbiota between control and infected mice. Although we observed more iNOS expression in infected mice, it did not support the expansion of *Enterobacteriaceae* (Proteobacteria) in response to DSS, in contrast to previous findings that generation of NO drives dysbiosis during acute infection (52). Collectively, these findings indicate that the enhanced susceptibility of infected animals to DSS-mediated colitis is likely due to

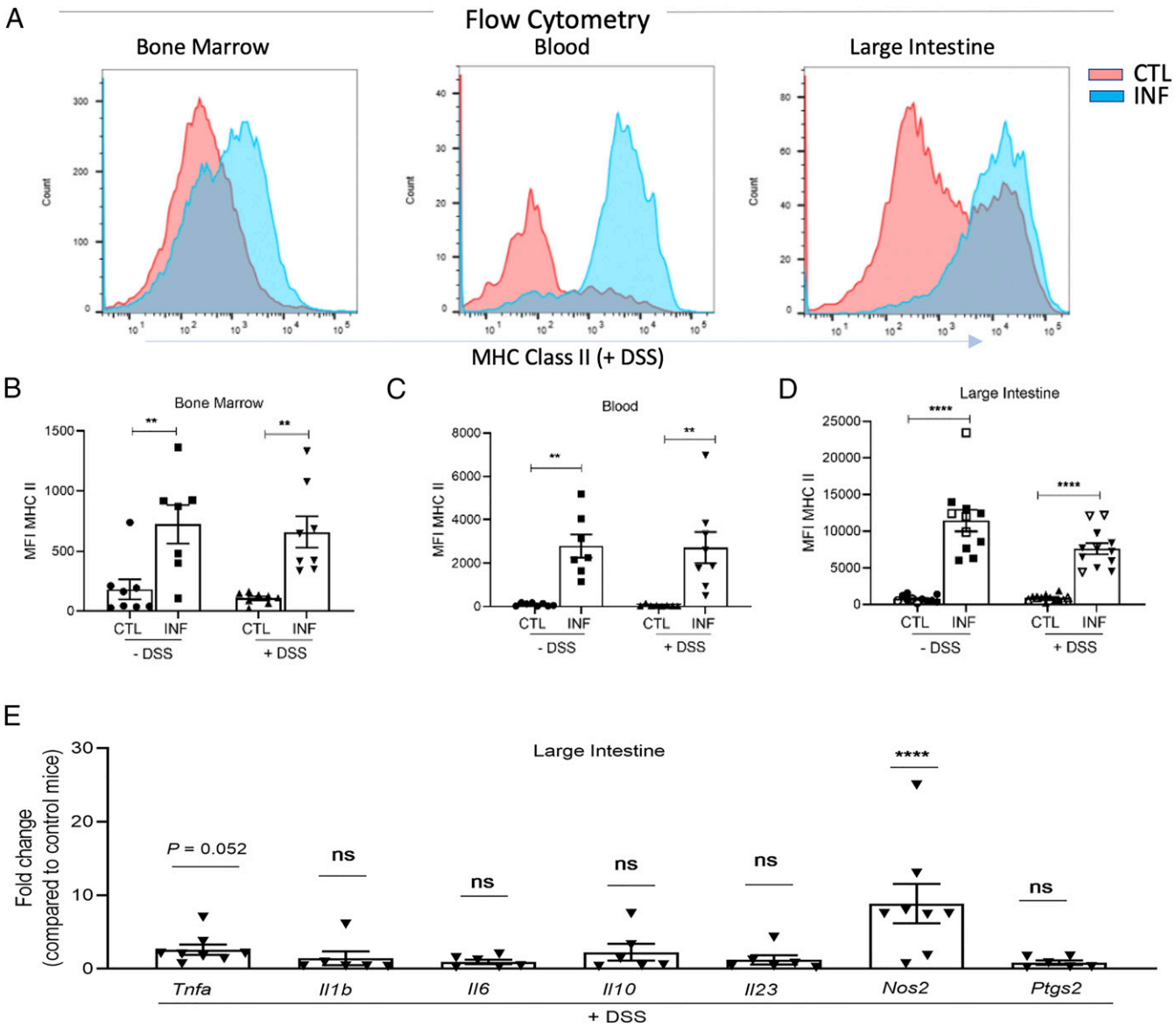

Fig. 4. Chronic *T. gondii* infection activates monocytes in bone marrow and enhances inflammation in large intestine after DSS treatment. (*A–D*) Mean fluorescence intensity of MHC class II on Ly6C^{hi} inflammatory monocytes in the bone marrow, blood, and large intestine of control (CTL) and *T. gondii*–infected mice before and after DSS treatment. (*E*) qRT-PCR analysis of mRNA levels of indicated cytokines in Ly6C^{hi} inflammatory monocytes in the large intestine of *T. gondii*–infected mice before and after DSS treatment. Mean ± SEM compared to average values of control samples from large intestine. Data were pooled from two or three independent experiments, $n = 3$ to 4 female mice per group. One-way ANOVA with Sidak's multiple comparison $^{**}P < 0.01$, $^{****}P < 0.0001$, ns, not significant. Please also see *SI Appendix*, Fig. S4.

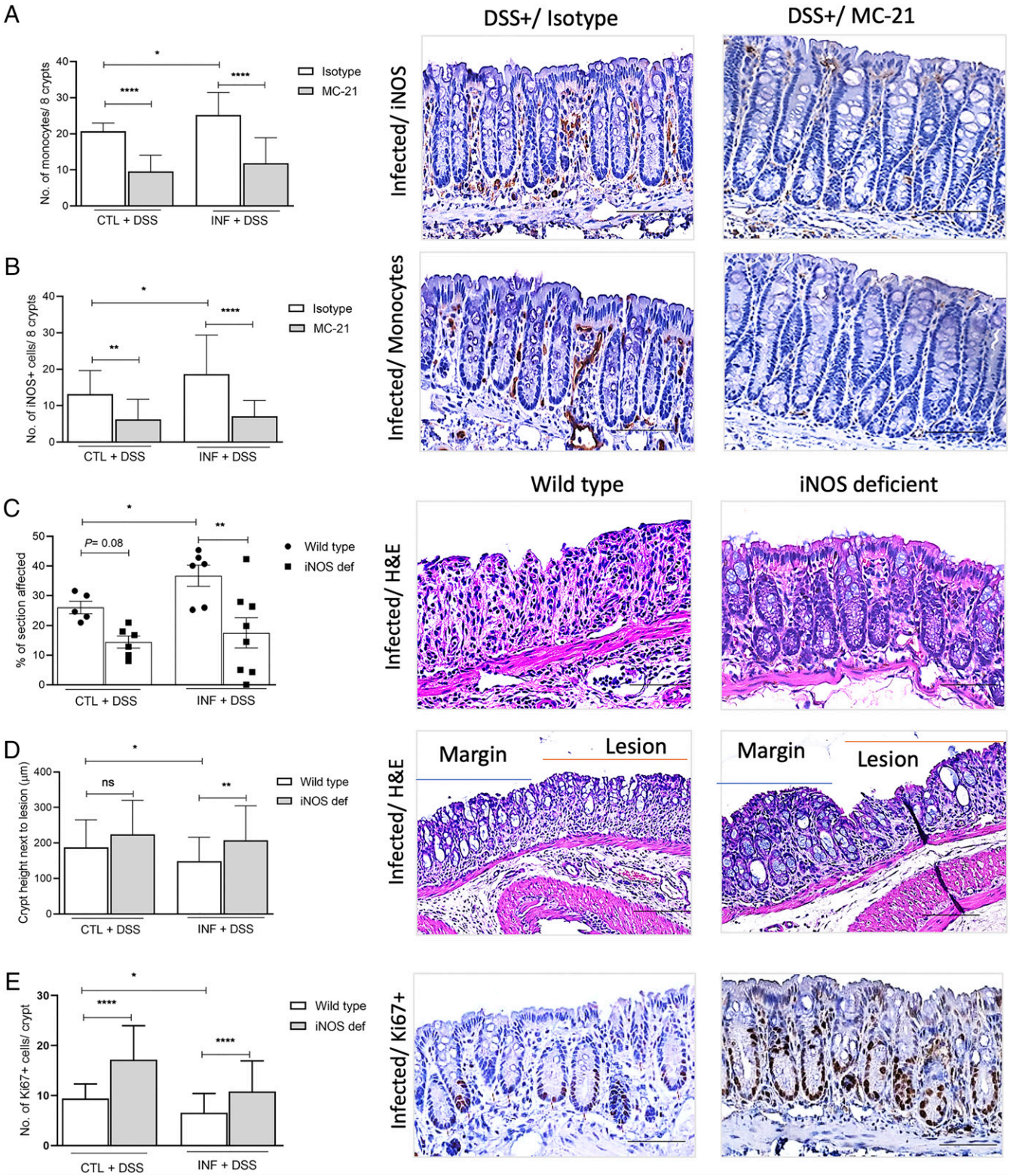

Fig. 5. Blocking iNOS protects against DSS-mediated damage in chronically infected mice with *T. gondii*. (*A* and *B*) Depletion of inflammatory monocytes in chronically infected mice treated with DSS using MC-21 mAb as in Fig. 3*D*. Immunohistochemical staining of serial 5-µm sections for Ly6C$^+$ monocytes (rat anti-Ly6C) or iNOS (anti-iNOS mouse mAb) in the colon of control and *T. gondii*–infected mice. Data from one experiment, $n = 4$ female mice per group. Mean ± SD. One-way ANOVA analysis with Sidak's multiple comparison $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. (*C*–*E*) Histological analysis of the colon of control and *T. gondii*–infected, wild-type vs. iNOS-deficient mice after DSS treatment. Graphical summary and representative images of (*C*) percent of section affected, (*D*) crypt height, and (*E*) number of Ki67$^+$ cells in the colon. Data were pooled from two independent experiments, $n = 3$ to 4 female mice per group. Mean ± SEM. Statistics: (*C*) One-way ANOVA with Sidak's multiple comparison $^*P < 0.05$, $^{**}P < 0.01$. (*D* and *E*) Kruskal–Wallis test with Dunn's multiple comparison was performed $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, ns, not significant. (Scale bars, 20 µm.) Please also see *SI Appendix*, Fig. S5.

differences in the immune response, rather than being driven by the change in the microbiota. However, followup studies are required to completely rule out the role of the microbiota in aggravating the pathology, as a recent study demonstrated that intestinal inflammation can dramatically alter gene transcription by commensal bacteria without altering the microbiota composition (53).

Chronically *T. gondii*–infected mice showed an elevated expression of IFN-γ by $CD4^+$ and $CD8^+$ T cells isolated from the lamina propria, likely as a consequence of the ongoing chronic infection releasing *T. gondii* antigens from semidormant tissue cysts that persist in muscle and brain (54). We did not observe reduction of Treg cells in the lamina propria as previously reported following acute infection (16), indicating that the heightened sensitivity to DSS seen in chronically infected animals is unlikely to be due to loss of tolerance. Rather, the enhanced intestinal damage in chronically infected mice treated with DSS was associated with recruitment of $Ly6C^+$ monocytes that exit the bone marrow and home to sites of inflammation. Depletion of $Ly6C^{hi}$ monocytes reduced damage to the gut caused by DSS in both control and *T. gondii*–infected animals, consistent with a previous study that reported inflammatory monocytes are responsible for colonic inflammation during acute colitis caused by DSS (37).The lack of complete protection by monocyte blockade suggests that $CD4^+$ and $CD8^+$ T cells may also play a more direct role in the exacerbation of DSS-mediated colitis due to detrimental effects of IFN-γ on epithelial barrier function (55–58). Alternatively, the high level of neutrophil recruitment could also contribute to pathology as has been reported previously in other models of inflammatory bowel disease (59, 60).

Interestingly, we observed high levels of MHC class II on $Ly6C^{hi}$ monocytes in the bone marrow of infected mice prior to DSS treatment, indicating they exist in a preactivated state, likely due to elevated levels of circulating IFN-γ. Similarly, during acute infection, production of IFN-γ by NK cells in the bone marrow primes monocytes to develop heightened antimicrobial and inflammatory responses even before they reach inflamed tissues (61). In our study, the expression of MHC class II and other makers of inflammation increased as monocytes migrated to the blood and lamina propria, consistent with further imprinting once they reach sites of inflammation (27, 28). The heightened activation status of monocytes in chronically *T. gondii*–infected mice has been shown to be protective against heterologous challenge (31, 62, 63). Heightened innate response due to prior infection has been termed trained immunity, which involves both epigenetic and metabolic reprogramming that can occur in the absence of ongoing infection due to stimulation with strong agonists (64–66).

$Ly6C^{hi}$ monocytes that emerge from the bone marrow in chronically *T. gondii*–infected mice are partially activated and show elevated MHC class II and TNF-α expression. Previous studies in mice and human have reported that blocking TNF-α ameliorates IBD symptoms (67, 68). However, neutralization of TNF-α did not decrease colitis severity in *T. gondii* chronically infected mice studied here, suggesting it is not the basis for the observed pathology. Additionally, $Ly6C^{hi}$ monocytes in the lamina propria of DSS-treated mice up-regulated iNOS in *T. gondii* chronically infected mice to an even greater extent than noninfected mice. Previous findings have implicated iNOS and production of NO as responsible for colonic damage caused by DSS (69). Consistent with this finding, we observed that uninfected iNOS knockout mice ($Nos2^{-/-}$) were protected from DSS-associated epithelial damage. Importantly, lack of iNOS also reversed the enhanced damage due to DSS treatment seen in *T. gondii*–infected animals. These findings suggest that enhanced production of NO by inflammatory monocytes results in damage to colonic epithelial cells and impairs $HopX^+$ stem cells needed for regeneration. Consistent with such a mechanism, elevated NO production has been shown to cause DNA damage to colonic epithelial cells in vitro (70), and several studies have shown overexpression of iNOS and elevated concentrations of NO in mucosa of patients with IBD (71, 72). Hence similar mechanisms to those described here involving monocytes and inflammatory mediators, including NO, may be important in human intestinal diseases characterized by hyperinflammation.

Together, our study demonstrates that chronic infection with *T. gondii* leads to heightened activation of monocytes that further up-regulate antimicrobial and inflammatory responses once they reach inflamed tissue sites. Although such heightened innate immunity contributes to protection, our findings demonstrate that such responses not always beneficial as preactivated monocytes also increased tissue damage in response to DSS. Similarly, enhanced trained immunity has been associated with detrimental outcomes for patients suffering from diseases associated with inflammation (73). Hence prior exposure to *T. gondii*, and possibly other chronic infections, may exacerbate inflammatory responses either to environmental irritants, underlying genetic susceptibilities, or other enteric infections that are implicated in intestinal diseases such as sepsis (6), celiac disease (74), and autoimmunity (5).

Materials and Methods

Mice. Animal studies were conducted according to the US Public Health Service policy on human care and use of laboratory animals. Animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care as approved by the Division of Comparative Medicine, Washington University.

Chronic Infection and ELISA for Monitoring Infection. C57BL6/J and iNos knockout mice (strain: B6.129P2-*Nos2*^{tm1Lau}/J) were purchased from The Jackson Laboratory. Mice were infected with ME49 *T. gondii* type II strain and chronic infection (day 30 postinfection) confirmed by ELISA, as described previously (75). Mice were given 2.5% DSS in drinking water for 7 d, followed by a 14-d recovery period. Bone marrow and blood and tissue samples were harvested from animals. Histological analyses were performed as described in *SI Appendix* and Table S1. Fluorescence-activated cell sorting analysis was performed as described previously (76, 77) and further detailed in *SI Appendix*, Table S1.

Quantitative RT-PCR. RNA samples were used to generate cDNA, and real-time PCR amplification, performed using SYBR Green (primers are listed in *SI Appendix*, Table S2), was analyzed using QuantStudio Design and Analysis Software (Applied Biosystems). The expression of target mRNA was calculated and normalized to the expression of the housekeeping gene β-actin using the 2(-ΔΔCT) method (78).

16S rRNA Sequencing and Analysis. PCR amplification of the V4 region of 16S rRNA was performed as described previously (79) and further detailed in *SI Appendix*. Data were analyzed using the dada2 R package; reads were assigned taxonomy (80); and ecological analyses were performed using PhyloSeq and additional R packages (81). Sequencing data are available from the European Nucleotide Archive accession no. PRJEB43614.

Quantification and Statistical Analysis. All analyses were done using GraphPad Prism 8.2.1 software as further defined in *SI Appendix*.

Data Availability. 16S RNA sequence data have been deposited in the European Nucleotide Archive (PRJEB43614) (82).

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