Contrasting immune responses mediate *Campylobacter jejuni*-induced colitis and autoimmunity

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Campylobacter jejuni is a leading cause of foodborne enteritis that has been linked to the autoimmune neuropathy, Guillain Barré syndrome (GBS). C57BL/6 interleukin (IL)-10$^{+/+}$ and congenic IL-10$^{-/-}$ mice serve as *C. jejuni* colonization and colitis models, respectively, but a mouse model for GBS is lacking. We demonstrate that IL-10$^{-/-}$ mice infected with a *C. jejuni* colitogenic human isolate had significantly upregulated type 1 and 17 but not type 2 cytokines in the colon coincident with infiltration of phagocytes, T cells and innate lymphoid cells (ILCs). Both ILC and T cells participated in interferon-γ (IFN-γ), IL-17, and IL-22 upregulation but in a time- and organ-specific manner. T cells were, however, necessary for colitis as mice depleted of Thy-1$^+$ cells were protected while neither Rag1$^{-/-}$ nor IL-10R blocked Rag1$^{-/-}$ mice developed colitis after infection. Depleting IFN-γ, IL-17, or both significantly ameliorated colitis and drove colonic responses toward type 2 cytokine and antibody induction. In contrast, *C. jejuni* GBS patient strains induced mild colitis associated with blunted type 1/17 but enhanced type 2 responses. Moreover, the type 2 but not type 1/17 antibodies cross-reacted with peripheral nerve gangliosides demonstrating autoimmunity.

INTRODUCTION

Campylobacter jejuni is a spiral, Gram-negative microaerophilic bacterium that is the second most common cause of gastroenteritis in the United States with over 2.4 million campylobacteriosis cases reported annually and many sporadic cases unreported.[1] The majority of patients ingesting *C. jejuni* in raw/undercooked meat and unpasteurized milk develop mild-to-severe gastroenteritis targeting the colon, which is debilitating but self-limiting within 7–10 days.[2,3] Histopathological manifestations include colonic crypt distortion, crypt abscesses, mucin depletion, edema of the colonic lamina propria and significant infiltration of granulocytes and mononuclear cells.[4] Lesions resolve in most patients, but campylobacteriosis can be life threatening in immune-compromised individuals with systemic spread and multi-organ damage.[5,6] Furthermore, infection with *C. jejuni* has been linked with serious autoimmune sequelae such as development or flare-up of inflammatory bowel diseases,[7] irritable bowel syndrome,[8] Reiter's arthritis,[9] and Guillain Barré syndrome (GBS).[10]

C. jejuni infection is the most common predisposing factor for developing the peripheral neuropathy GBS with 40% of US cases triggered by this bacterium.[11,12] Recently, the GBS disease burden was estimated at 3000–6000 cases per year.[13] GBS syndrome consists of at least three different subtypes including acute inflammatory demyelinating polyradiculoneuropthy, acute motor axonal neuropathy, and acute motor and sensory axonal neuropathy. Acute motor axonal neuropathy and acute motor and sensory axonal neuropathy are axonal subtypes associated with development of autoantibodies that target gangliosides on peripheral nerves; these autoantibodies are thought to result from molecular mimicry.[10] Indeed, the lipooligosaccharide of *C. jejuni* isolates from GBS patients with antecedent infections have been shown to mimic gangliosides on peripheral nerves including GM1, GD1a, and others.[10,14,15] When bound to peripheral nerves, these antibodies are expected to block nerve conduction by activation of complement and/or by cellular mechanisms.[16] At present, plasmapheresis and intravenous immunoglobulin treatment are the

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only known treatments with beneficial effect, but are effective in only 60% of GBS patients.[17] Little is known about host immunological mechanisms that lead to self-limiting gastro-intestinal disease vs. severe enteritis or neurological sequelae.

Our rationale was to utilize inbred mice deficient in interleukin-10 (IL-10) to study factors mediating the development of *C. jejuni*-induced enteritis and autoimmune manifestations. Innate and adaptive immune cells, epithelial cells, and fibroblasts can produce IL-10. It functions to desensitize antigen-presenting cells, down modulate pro-inflammatory cytokine production and suppress proliferation of mononuclear cells.[18,19] Genome-wide association analysis studies discovered single-nucleotide polymorphisms flanking the *IL10* gene as the most significant locus outside the major histocompatibility complex locus to associate with ulcerative colitis, a form of inflammatory bowel disease (IBD) affecting 8–24/10,000 individuals in the United States and Europe. Single-nucleotide polymorphisms in *IL10* also show a significant association with Crohn's disease, another form of IBD with a similar incidence.[20]

We have previously established wild-type (WT; IL-10$^{+/+}$) and IL-10$^{-/-}$ mice of various genetic backgrounds as models of *C. jejuni* colonization and colitis, respectively.[21,22] Although the IL-10$^{+/+}$ mice of C57BL/6, C3H/HeJ, and non-obese diabetic background were stably colonized with *C. jejuni* (strain NCTC11168) for 35 days after oral inoculation without any adverse clinical or histopathological effects, the IL-10$^{-/-}$ mice of these three genetic backgrounds developed typhlocolitis (inflammation of cecum and colon).[22] Thus, the enteritis model of oral inoculation of IL-10$^{-/-}$ mice with *C. jejuni* essentially involves combining the most strongly associated pathway for susceptibility to IBD (*IL10*) with the most common causative bacterium for colitis (*C. jejuni*) through the natural route of infection. The histopathological features of colitis in IL-10$^{-/-}$ mice also replicate the histopathological features of *C. jejuni*-associated colitis in humans,[4,21] including *C. jejuni* invasion of the colonic epithelium followed by ulceration, necrosis, and neutrophilic exudates, infiltration of mononuclear and poly-morphonuclear cells into the colonic lamina propria and occasionally the muscularis, and crypt distension with abscesses and edema most prominent in the submucosa. These effects were dose independent as the dose range of 10^2–10^{10} colony-forming unit per mouse produced similar levels of pathology.[21,23] Furthermore, C57BL/6 IL-10$^{-/-}$ mice inoculated with *C. jejuni* strains obtained from human GBS patients were colonized, but developed little or no colitis.[24] Recent studies have revealed the importance of diet,[25] pattern recognition receptors (Toll-like receptor 2, 4, and 9)[26] and particular signaling molecules (nuclear factor-κB, mammalian target of rapamycin, and phosphatidylinositol 3 kinase-γ)[27,28] in *C. jejuni* colonization and induced pathology in WT or gnotobiotic IL-10$^{-/-}$ mouse models. However, the role of inflammatory mediators—particularly lymphocytes and their secreted cytokines—has not been established *in vivo*. We hypothesized that differential cytokine responses mediated by lymphocytes in the colon are responsible for *C. jejuni*-induced

colitis, protection from colitis, and initiation of autoimmune sequelae in the IL-10$^{-/-}$ murine host. In humans, autoreactive immunoglobulin G1 (IgG1) is the commonly associated antibody subtype after *C. jejuni* infection and enhanced IgG1 titers also associate with enhanced severity and a poor long-term prognosis for GBS cases.[29] As IgG1 isotype classically requires T$_H$2-mediated class switching, we further hypothesized that a *C. jejuni*-specific T$_H$2 response generated by the GBS but not the colitogenic strains will lead to induction of autoreactive IgG1.

RESULTS

C. jejuni induced a mixed type 1 and type 17 cytokine and cellular response in IL-10$^{-/-}$ mice

All mice infected with *C. jejuni* strain 11168 were colonized, with 40–90% of them exhibiting premature mortality.[24,25] Furthermore, colon histopathology scores were significantly enhanced compared with the Trypticase Soy Broth (TSB) inoculated controls.[24,25] To assess the type of inflammatory reaction(s) *C. jejuni* induces to cause colitis, C57BL/6 IL-10$^{-/-}$ (IL-10$^{-/-}$) mice were orally infected with 10^9 colony-forming unit of *C. jejuni* 11168 or TSB-sham and observed for clinical signs of enteritis. Mice were killed upon showing a severe enteric disease end point or at day 35 after inoculation. Tissues were collected during necropsy and analyzed simultaneously. When colon protein extracts were analyzed, primary/innate cytokines (IL-1β and IL-6), type 1 (interferon (IFN)-γ) and type 17 (IL-17A and IL-22), but not type 2 (IL-4) cytokines were significantly increased in the infected group (**Figure 1a**). Similar to the colon, plasma IFN-γ, IL-17A, and IL-6 were increased significantly in the infected group (**Figure 1c**), whereas other cytokine were not detectable. The numbers of macrophages (F4/80$^+$) and T cells (CD3$^+$) infiltrating the lamina propria as quantified by immunohistochemistry were significantly increased in the infected group (**Figure 1b**). These data suggest that *C. jejuni* infection induces a mixed type 1 and type 17 response in infected IL-10$^{-/-}$ mice. Furthermore, there were consistently high correlations between histopathology score and colonic IFN-γ, IL-1β, IL-6, IL-17A, and IL-22 levels (Spearman rank correlation factor, $r_s = 0.7$–0.9, **Supplementary Table 1** online).

IgG subtype class switching in activated B cells is modulated by type 1, type 2, and type 17 cytokines. It has been long established that IFN-γ induces isotype class switching to IgG2a (or IgG2c, an isoform of IgG2a in C57BL/6 background) and IgG3, whereas IL-4 primarily induces IgG1 class switching.[30–32] IL-17 was shown to be necessary for autoreactive IgG2b (but not IgG1 or IgG2a) antibodies in an experimental autoimmune myasthenia gravis model.[33] Therefore, antibody responses can inform the nature of *C. jejuni*-specific cytokine responses. Consistent with the upregulation of a type 1 and type 17 but not type 2 response by colon and plasma cytokine analysis, plasma levels of *C. jejuni*-specific IgG2b (published previously),[24] IgG2c and IgG3 but not IgG1 were significantly enhanced in infected mice (**Figure 1d**). Therefore, plasma IgG analysis reinforced our findings of a mixed type 1 and type 17 response, and also demonstrated the *C. jejuni* specificity of the response.
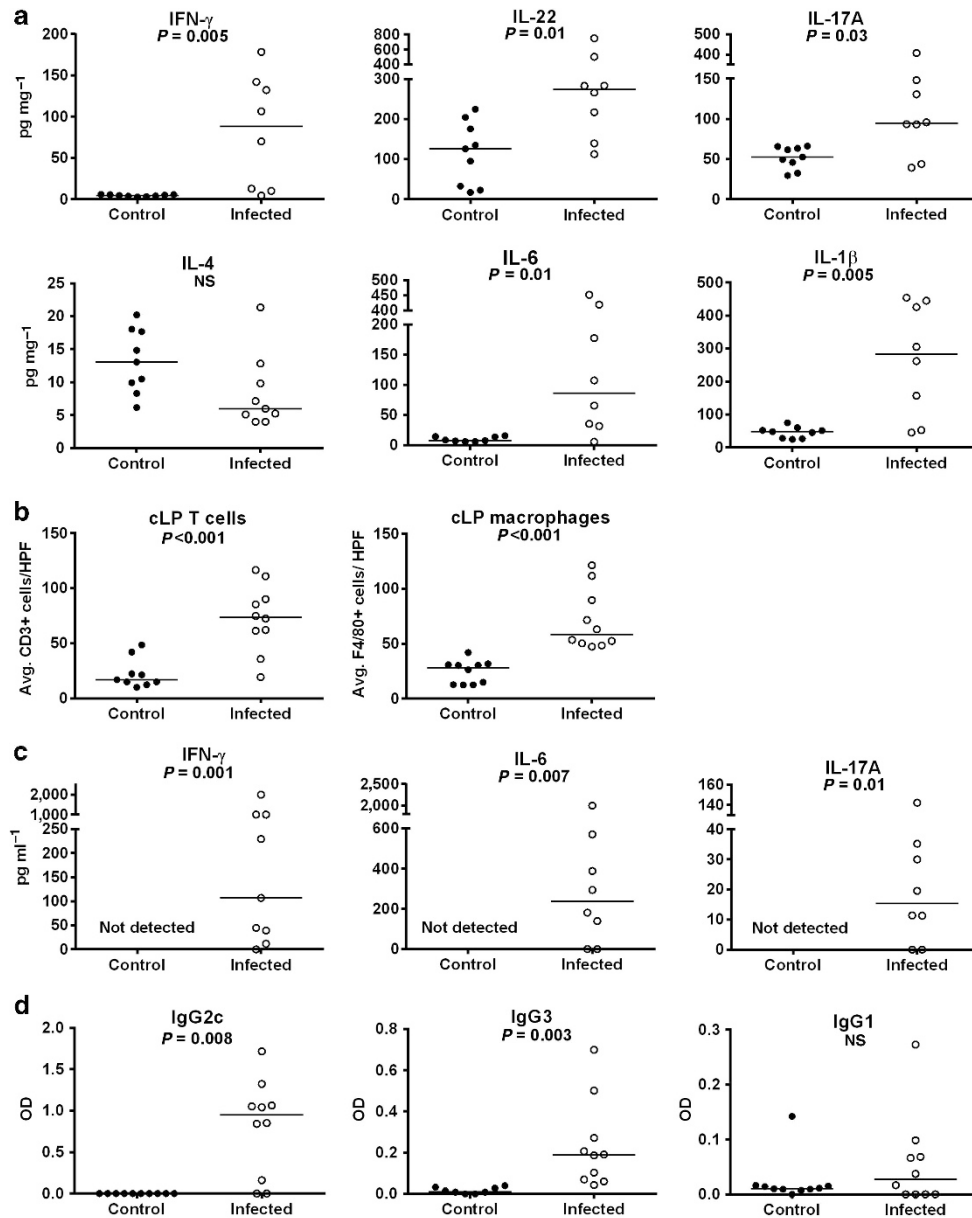
Figure 1 End point cytokine, antibody, and colonic cellular infiltration analysis. IL-10$^{-/-}$ mice were inoculated with *C. jejuni* 11168 or TSB and killed at humane end point or 35 days after inoculation. (**a**) Colon homogenate ELISA. IL-12p70, tumor necrosis factor (TNF)-α and IL-17F were only detected in colons of 20% of the mice and are not shown. (**b**) Colon CD3 and F4/80 immunohistochemistry, at the time of necropsy, the ileocecocolic junction (junction of ileum, cecum, and colon) was fixed in formalin and embedded in paraffin. Its sections were stained for CD3 or F4/80 and the number of positively staining cells in the colonic lamina propria were quantified in 10 random HPFs. (**c**) Plasma cytokine ELISA. Other cytokines from **Figure 1a** were not detectable in majority of mice from infected/control group, and are not shown. (**d**) Plasma *C. jejuni*-specific antibody analysis. Anti-*C. jejuni* IgM and IgA was detectable but did not change significantly and is not shown. Data represented are one of three independent experiments with 8–10 mice per group. Bar indicates the median, Mann–Whitney *U*-test. ELISA, enzyme-linked immunosorbent assay; HPF, high-powered field; IFN, interferon; IL, interleukin; NS, not significant; OD, optical density; TSB, Trypticase Soy Broth.

We further analyzed the time course of cellular and cytokine changes in a kinetic study wherein mice were killed at days 4, 7, and 11 after *C. jejuni* inoculation. Day 11 was chosen as the end point to avoid the need for premature killing in the infected mice. Histopathology scores evaluated on hematoxylin and eosin–stained sections of the ileocecocolic junctions increased with time, reaching significance at days 7 and 11 (**Figure 2a**). On day 7, histological changes were moderate exhibiting slight epithelial hyperplasia and diffuse or patchy infiltration of mononuclear and polymorphonuclear cells in the colonic and cecal lamina propria that sometimes extended into the muscularis. On day 11, histological changes were more intense with marked increase in mononuclear and polymorphonuclear cells in the lamina propria and the muscularis. The epithelium was ulcerated along with crypt dysplasia and abscess formation. Neutrophilic exudates were also apparent
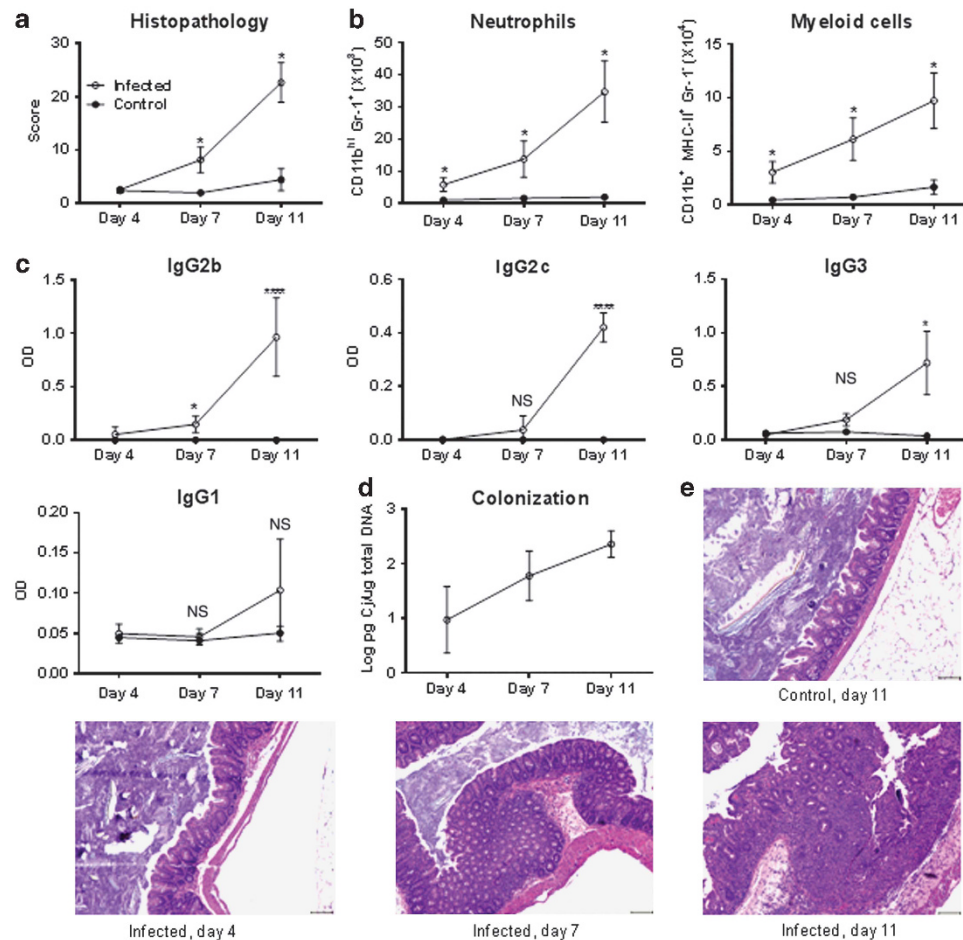
Figure 2 Temporal analysis of colon leukocyte and *C. jejuni*-specific plasma antibody. Interleukin (IL)-10$^{-/-}$ mice were inoculated with *C. jejuni* or Trypticase Soy Broth (TSB), and killed at indicated days after inoculation. At the time of necropsy, the ileocecocolic junction (junction of ileum, cecum, and colon) was fixed in formalin and embedded in paraffin. (**a**) Hematoxylin and eosin (H&E) sections were scored in blinded manner. (**b**) Colon leukocytes were prepared and analyzed for indicated populations by flow cytometry. Dead/dying cells were excluded on the basis of forward and side scatter. All cells were gated on CD19-gate. (**c**) Mice were bled at time of necropsy, and serum was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. Anti-*C. jejuni* IgM and IgA was detectable but did not change significantly for any time point and is not shown. (**d**) *C. jejuni* colonization was measured in the feces at the time of necropsy by quantitative-PCR. (**e**) Representative photomicrographs from H&E-stained proximal colon sections. Data are represented as mean ± s.e.m.; two independent experiments; 5–8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Kruskal–Wallis test and Dunn's *post test*. OD, optical density.

in the lumen. The numbers of infiltrating neutrophils (CD11b^{hi}Gr-1^{+}) and inflammatory myeloid cells (CD11b^{+}MHCII^{+}Gr-1^{-}) were significantly increased at the earliest time point examined (day 4) and continued to increase until the end of the experiment (day 11), as assessed by flow cytometry (**Figure 2b**). *C. jejuni*-specific IgG2b levels were increased significantly at days 7 and 11 after inoculation, whereas levels of IgG2c and IgG3 were found to be significantly enhanced on day 11. IgG1 levels (type 2 dependent) were not significantly different at any time point after inoculation (**Figure 2c**). Therefore, *C. jejuni*-specific IgG responses corroborate type 1 and type 17 cytokine responses. Also, as ascertained by quantitative-PCR for *C. jejuni*-specific *gyrA* in fecal DNA (**Figure 2d**), the degree of colonization did increase with time. However, it cannot be determined at this stage if increase in colonization is a cause of and/or a consequence of inflammation. Remarkably, levels of IFN-γ,

IL-17A, and IL-22 were increased in draining lymph nodes as early as day 4, and continued to rise until day 11 (**Figure 3a**). However, in the colon, an increase in IFN-γ production was not significant until day 7 and continued to rise at day 11. IL-17, IL-22, tumor necrosis factor-α, and monocyte chemotactic protein-1 were also highest at day 11 in both organs (**Figure 3b**). This continuous increase in the levels of pro-inflammatory cytokines from colon and lymph nodes reflects the continuous increase in the number of colon infiltrating neutrophils and inflammatory myeloid cells.

Both innate lymphocytes and T cells contribute to type 1 (IFN-γ) and type 17 (IL-17 and IL-22) cytokine production in an organ, time and cell type-specific manner

C. jejuni-induced colitis is elicited upon infiltration of mononuclear cells and neutrophils in the colon, both in humans and IL-10$^{-/-}$ mice.[4,21] These mononuclear cells can belong to
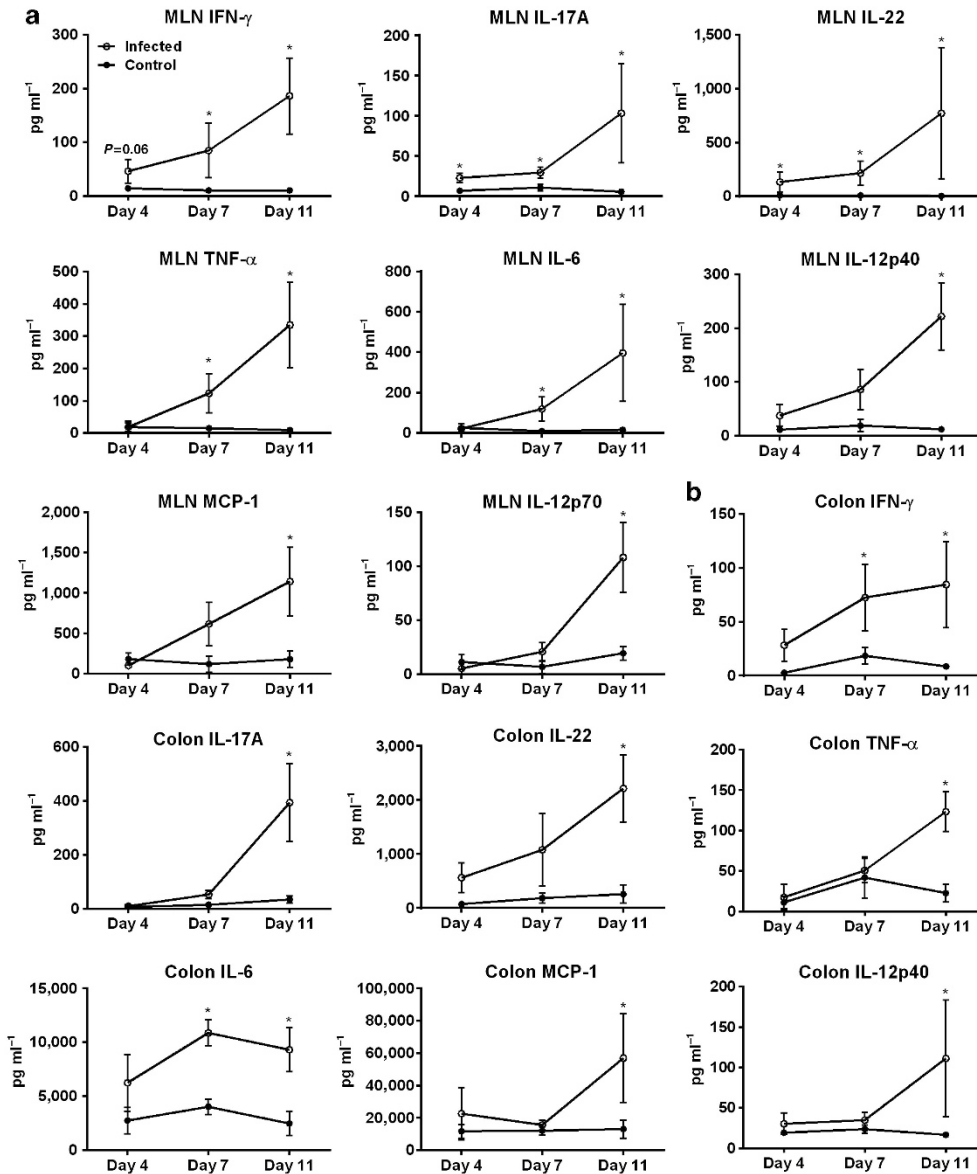
Figure 3 Temporal cytokine analysis of colon and MLN. IL-10$^{-/-}$ mice were inoculated with *C. jejuni* (open circles) or Trypticase Soy Broth (TSB; filled circles) and killed at indicated day after inoculation. (**a**) In all, 5 mm of proximal colon or (**b**) 2.5×10^6 red blood cell-depleted cells from mesenteric lymph node (MLN) were incubated in 0.5 ml tissue culture media for 48 h. Supernatant media was clarified and analyzed for indicated cytokines, as well as interleukin (IL)-1β, IL-4, IL-23, and IL-13, which were not detectable. IL-12p70 was detectable from MLN but not the colon. Data are represented as mean ± s.e.m.; two independent experiments; 5–8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Kruskal–Wallis test and Dunn's *post test*.

myeloid or lymphoid lineages. Lymphoid cells can further be categorized into adaptive lymphocytes—B cells (CD3$^-$ CD19$^+$) and T cells (CD3$^+$CD19$^-$), and Rag-independent innate lymphocytes. Mucosal innate lymphocytes can further be divided into natural killer (NK) cells (CD19$^-$NKp46$^+$) and relatively novel innate lymphoid cells (ILCs) (Thy1^{hi}Lin$^-$) where Lin has the following markers (CD3$^-$CD19$^-$NKp46$^-$ Gr-1$^-$MHC-II$^-$CD11b$^-$). T cells, NK cells, and ILCs have been shown to be the major producers of IFN-γ, IL-17, and IL-22 in a number of inflammatory diseases.[34–36] Therefore, in a kinetic study, we evaluated the relative contribution of T-cell subsets, NK cells, and ILCs to the increase in production of

IL-17, IL-22, and IFN-γ in the colon and mesenteric lymph nodes (MLNs). In both organs, there were significant increases in the absolute number of T cells (CD3$^+$CD19$^-$) and NK cells at days 7 and/or 11, whereas ILCs were enhanced at days 4 and 7 but decreased to the level of controls by day 11 (**Figures 4a–c** and **5a–c**). The maintenance of elevated numbers of T cells and NK cells and basal levels of ILCs was also confirmed in a separate experiment at a later time point of day 21 (data not shown). Within the T-cell compartment, the proportion of CD4$^+$ T$_H$ cells doubled in the colon by day 11, whereas the proportion of γδ$^+$ T cells was unchanged during the course of the experiment (**Figure 4a**).
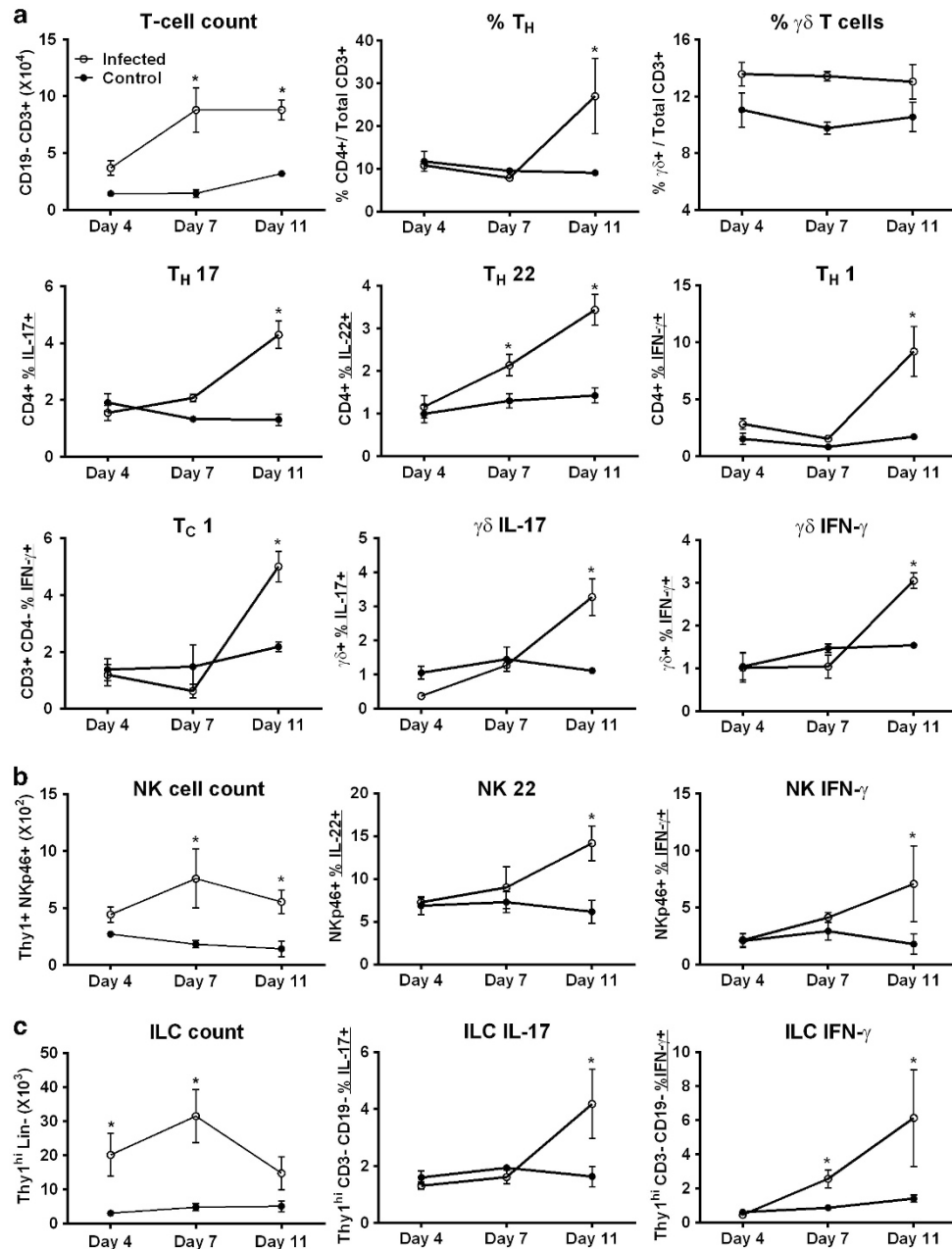
Figure 4 Colon cytokine-lymphocyte subset analysis. Interleukin (IL)-10$^{-/-}$ mice were inoculated with Trypticase Soy Broth (TSB) or *C. jejuni* and killed at indicated day after inoculation. Single-cell suspension was prepared from colon. T cells (**a**), innate lymphoid cells (ILCs) (**c**), and natural killer (NK) cells (**b**) were analyzed for interferon (IFN)-γ and IL-17 or IFN-γ and IL-22 production by intracellular cytokine staining and flow cytometry following brief restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin in presence of brefeldin A. Double positives were relatively rare and data presented here represent total positive. Dead/dying cells were excluded on the basis of forward and side scatter. Data are represented as mean ± s.e.m.; two independent experiments with 5–8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Kruskal–Wallis test and Dunn's *post test*. Proportion of IL-17 + or IL-22 + cells from CD3 + CD4-cells (which are likely CD8 +) was not significantly different at any time point, and is not shown.

The proportion of the IFN-γ^+ cells was increased, in T_H, T_C, $\gamma\delta$ T, and NK cell compartments on day 11 after infection in both organs (**Figures 4a, b** and **5a, b**). The proportion of IL-17$^+$ ILCs (ILC17) increased as early as day 4 in the MLN (**Figure 5c**), but decreased to control levels at day 7 while the proportion of IL-17$^+$ T_H cells increased on days 7 and 11 (**Figure 5a**). This pattern of IL-17 production initially from

ILCs and subsequently by T cells correlated with the kinetics of infiltration of these cells in the colon and MLN. In contrast, the proportion of ILC17 in the colon did not increase until day 11 (**Figure 4c**), at which time the total number of these cells had decreased to basal level. This demonstrates a selective organ-specific activation of this cell type. Furthermore, increases in IL-22$^+$ T_H cells and NK cells were observed at
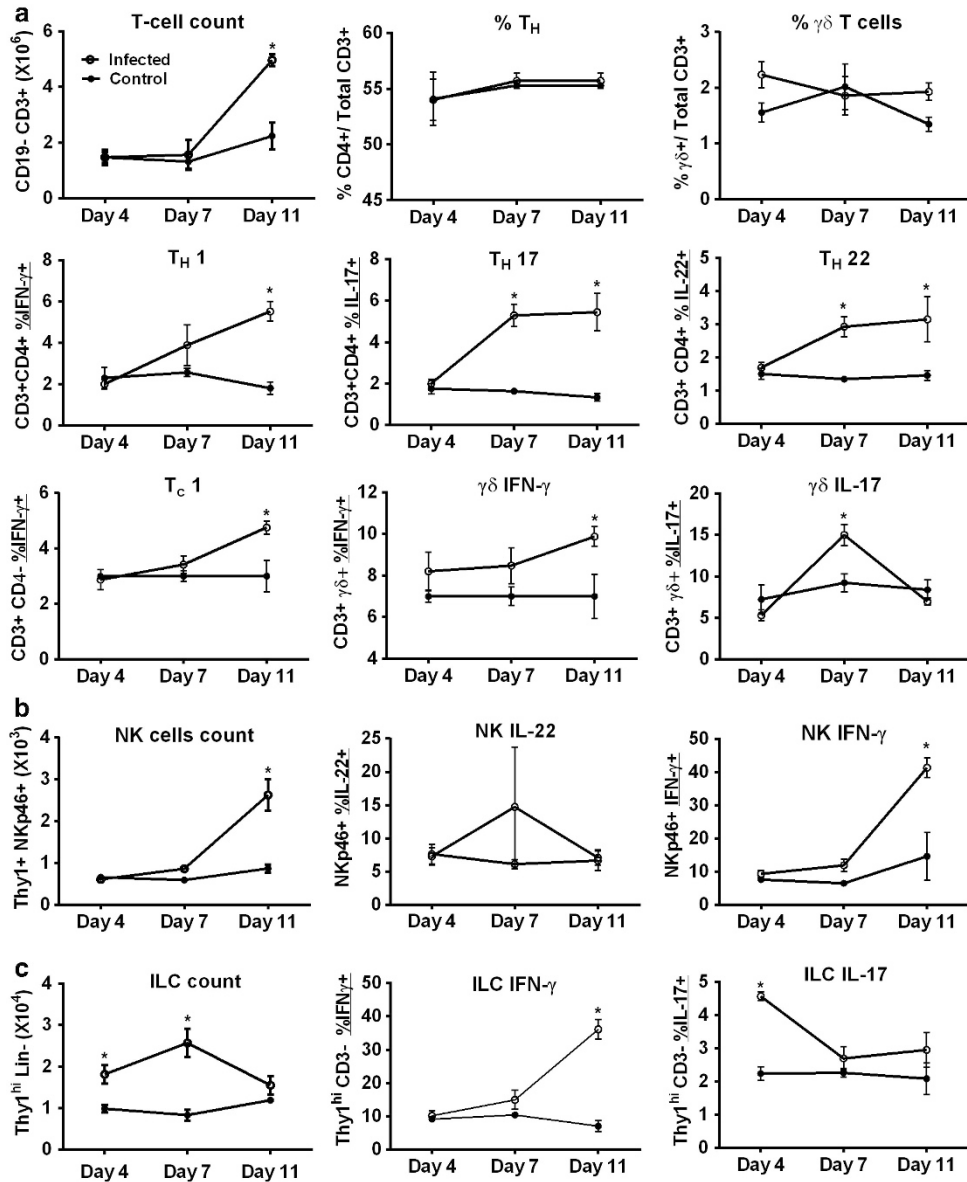
Figure 5 Mesenteric lymph node cytokine-lymphocyte subset analysis. Interleukin (IL)-10$^{-/-}$ mice were inoculated with Trypticase Soy Broth (TSB) or *C. jejuni* and killed at indicated day after inoculation. Single-cell suspension was prepared from mesenteric lymph node (MLN). T cells (**a**), innate lymphoid cells (ILCs) (**c**), and natural killer (NK) cells (**b**) were analyzed for interferon (IFN)-γ, IL-17, or IL-22 production by intracellular cytokine staining and flow cytometry following brief restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin in presence of brefeldin A. Data are represented as mean ± s.e.m.; two independent experiments with 5–8 mice per group per time point. Infected group for each time point was compared with control group pooled for each time points by Mann–Whitney *U*-test. Proportion of IL-17 + or IL-22 + cells from CD3 + CD4–cells (which are likely CD8 +) was not significantly different at any time point, and is not shown.

days 7 and/or 11 in both the colon and MLN (**Figures 4a, b** and **5a, b**). Therefore, a time and cell type-specific type 1 and type 17 response was observed in the colon and MLNs of *C. jejuni* challenged IL-10$^{-/-}$ mice.

Thy-1$^+$ lymphocytes are necessary for *C. jejuni*-mediated colitis

Thy-1 (CD90) is a cell surface marker expressed by both innate lymphocytes and T cells and consequently Thy-1-depleting antibody was utilized to deplete these cells in the mouse model. Infected and Thy-1-depleted IL-10$^{-/-}$ mice had significantly

lower histopathology scores (**Figure 6a**) as well as decreased numbers of neutrophils, inflammatory myeloid cells, T cells, and DC infiltration of colonic tissues (**Figure 6b**) as compared with mice that were infected but given control antibody injections (11168 + CIgG). In fact, the infected and depleted group (11168 + α-Thy-1) had numbers similar to the uninfected group (TSB + α-Thy-1). Out of 10 mice in the positive control group (11168 + CIgG group), two mice had to be killed early (day 9 and 21 post inoculation (p.i.)) because they reached the threshold of clinical signs. Their tissues were collected during necropsy and appropriately fixed or frozen and
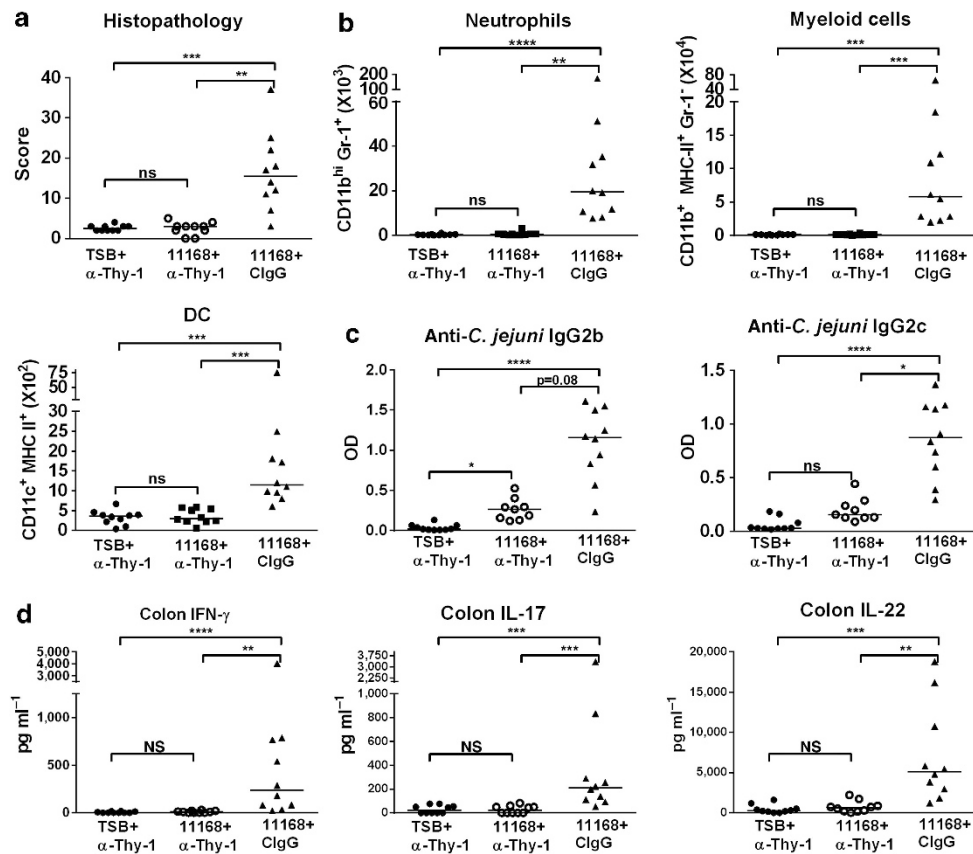
Figure 6 Role of Thy-1$^+$ lymphocytes in *C. jejuni*-mediated colitis. Interleukin (IL)-10$^{-/-}$ mice were orally inoculated with *C. jejuni* or Trypticase Soy Broth (TSB) and injected with α-Thy-1 or CIgG twice weekly starting 3 days before inoculation, and killed at days 23–24 after inoculation. At the time of necropsy, the ileocecocolic junction (junction of ileum, cecum, and colon) was fixed in formalin and embedded in paraffin. (**a**) Hematoxylin and eosin (H&E) sections were scored in a blinded manner. (**b**) Colon leukocytes were prepared and analyzed for indicated populations by flow cytometry. (**c**) Mice were bled at time of necropsy, and plasma was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. (**d**) In all, 5 mm of proximal colon was washed and incubated in 0.5 ml tissue culture media for 48 h. Supernatant media was clarified and analyzed for indicated cytokines, as well as other cytokines from **Figure 1**, which were not significantly different. Bar indicates the median, $n = 10$ mice per group. Kruskal–Wallis test followed by Dunn's *post test*. ****$P \leqslant 0.0001$. OD, optical density.

analyzed alongside the other samples after the end of the experiment (day 25 p.i.). As T cells are necessary for IgG isotype class switching in B cells, Thy-1-depleted and *C. jejuni* 11168-infected mice had lower levels of *C. jejuni*-specific IgG2b, IgG2c, and IgG3 in the plasma as compared with the 11168 + CIgG group (**Figure 6c** and data not shown). Furthermore, colonic levels of IFN-γ, IL-17, and IL-22 were decreased to basal levels in Thy1-depleted infected mice reinforcing that Thy1$^+$ cells are the major producers of these cytokines (**Figure 6d**). The success of depleting Thy-1-positive cells with antibody treatments was confirmed in the blood and colon by flow cytometry (**Supplementary Figure S1B** online).

Innate immunity is insufficient to mediate severe *C. jejuni*-induced colitis

To assess if innate immunity is sufficient for *C. jejuni*-mediated colitis, Rag1$^{-/-}$ mice on the C57BL/6 background were challenged with *C. jejuni* alongside C57BL/6 wt mice that serve as a known positive colonization control. Although all infected mice were colonized, neither group developed any clinical signs of colitis, and did not have differences in the histopathology scores (**Figure 7a**) or the numbers of colonic neutrophils,

inflammatory myeloid cells, or dendritic cells (data not shown) at days 32–35 p.i. as assessed by flow cytometry. To determine if IL-10 was responsible for lack of inflammation in Rag1$^{-/-}$ mice, they were given IL-10R blocking antibody after infection. Even after IL-10R blocking, Rag1$^{-/-}$ mice did not develop any clinical signs of colitis, and had no increase in histopathology scores (**Figure 7b**) or numbers of phagocytes in the colon (data not shown) as compared with TSB + α-IL-10R or 11168 + CIgG-treated groups. C57BL/6 wt mice did develop *C. jejuni*-specific antibody responses similar in nature to that of IL-10$^{-/-}$ mice with significant increases in the IgG2b, IgG2c, and IgG3 isotypes and with no difference in IgG1, IgM, or IgA (**Figure 7c**), demonstrating that the nature of *C. jejuni* antibody responses are independent of IL-10. Taken together, these data, along with the requirement of Thy-1$^+$ cells to produce colitis, show that T cells are necessary for severe *C. jejuni*-induced colitis. *C. jejuni* colonization correlated with colitis induction in as much as the mouse genetic background that supports inflammation (IL-10$^{-/-}$) had higher colonization extent by the end of the experiment than the mouse genotypes/treatments that did not (BL/6wt or Rag1$^{-/-}$ or Rag1$^{-/-}$ IL-10R blocked mice; **Figure 7d**).
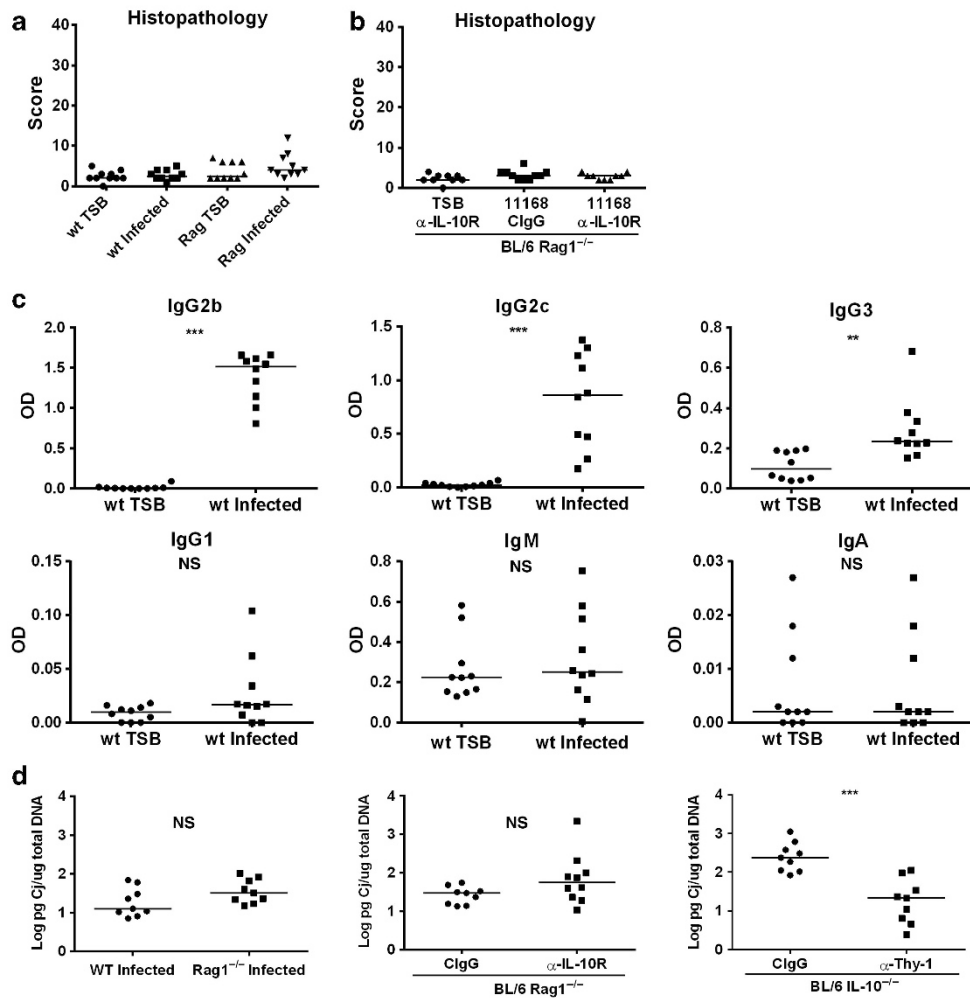
Figure 7 Innate immunity is insufficient to induce colitis after *C. jejuni* infection. (**a**) C57BL/6 wt or Rag1$^{-/-}$ mice were orally inoculated with *C. jejuni* or Trypticase Soy Broth (TSB) and killed at days 32–34 after inoculation. (**b**) Rag1$^{-/-}$ mice were injected with α-IL-10R or CIgG after infection. At the time of necropsy, the ileocecocolic junction (junction of ileum, cecum, and colon) was fixed in formalin and embedded in paraffin. Hematoxylin and eosin (H&E) sections were scored in a blinded manner. (**c**) Mice were bled at time of necropsy, and plasma was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. (**d**) *C. jejuni* colonization was measured in the feces at the time of necropsy by quantitative-PCR. Data are represented as median with 9–10 mice per group. Kruskal–Wallis test followed by Dunn's *post test*. OD, optical density.

Both IFN-γ and IL-17 participate in *C. jejuni*-induced colitis

To account for the relative contribution of IFN-γ and IL-17 in *C. jejuni*-mediated pathology, one or both were depleted during the course of the disease by intra-peritoneal injection of neutralizing antibodies. Results showed that both of these cytokines have a positive role in mediating colitis because depleting either IFN-γ or IL-17 or both led to significant decreases in histopathology scores (**Figure 8a**). Furthermore, depleting IFN-γ or IL-17 significantly decreased or trended toward decreasing the extent of colonic infiltration of neutrophils, inflammatory myeloid cells, and T cells, whereas depleting both had a significant effect (**Figure 8b**). The number of colon infiltrating ILCs and the proportion of CD4$^+$ T$_H$ and γδ$^+$ T cells were unchanged between all groups (data not shown). The number of NK cells, however, decreased in the IFN-γ-depleted groups, suggesting a positive feedback mechanism (**Figure 8b**). Notably, depleting IFN-γ and IL-17

pushed the response toward type 2 cytokine and antibody induction. IFN-γ-depleted groups had decreased levels of *C. jejuni*-specific plasma IgG2c but increased plasma IgG1 (**Figure 8c**) along with modestly increased levels of IL-4 and IL-13 in the proximal colon (**Figure 8d** and data not shown). IL-17-depleted groups had decreased IgG2b and increased IgM (**Figure 8c**). The double depleted group also had increased levels of *C. jejuni*-specific IgA (**Figure 8c**). Thus, depleting IFN-γ and IL-17 after infection with the colitogenic 11168 prevented colitis and shifted the immune response toward type 2 cytokines and antibodies.

Infection with GBS-associated *C. jejuni* strains induces type 2 immunity that is protective for colitis but leads to autoimmunity

Having observed a type 2 response in 11168-infected IL-10$^{-/-}$ mice after IL-17 and IFN-γ neutralization, we hypothesized
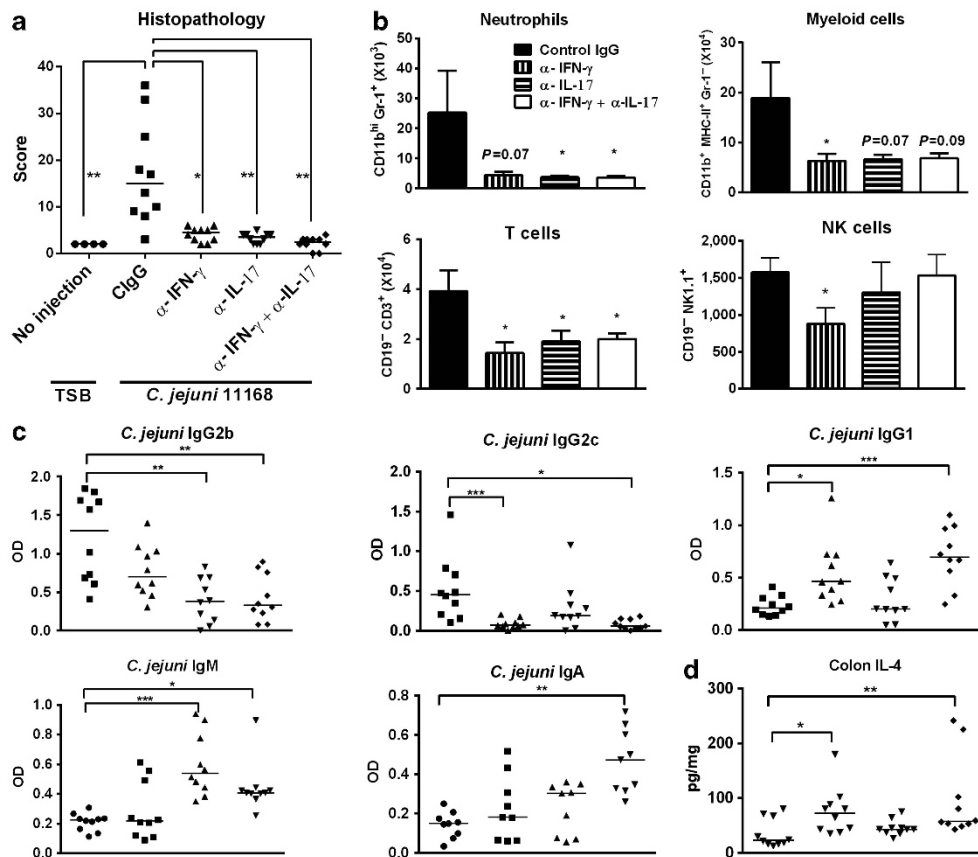
Figure 8 Both interferon (IFN)-γ and interleukin-17 (IL-17) are involved in *C. jejuni*-mediated colitis and humoral responses. IL-10$^{-/-}$ mice were orally inoculated with *C. jejuni* and killed at days 21–22 after inoculation. IFN-γ, IL-17, or both were neutralized by intraperitoneal injection of neutralizing antibodies twice a week for 3 weeks, starting on the day of inoculation. (**a**) Hematoxylin and eosin (H&E) sections were scored in a blinded manner. (**b**) Colon leukocytes were prepared and analyzed for indicated populations by flow cytometry. (**c**) Mice were bled at time of necropsy, and plasma was analyzed for indicated antibody subtypes reactive against *C. jejuni* antigen. (**d**) Level of IL-4 was measured in the proximal colon homogenate by enzyme-linked immunosorbent assay (ELISA). Data are represented as medians or mean ± s.e.m., with 10 mice per group. Kruskal–Wallis test followed by Dunn's *post test*. Only comparisons with CIgG group are shown. OD, optical density.

that the non-colitogenic GBS strains, 260.94 and HB93-13, also induce a blunted type 1/17 but enhanced type 2 response in the IL-10$^{-/-}$ mouse. Indeed, the GBS strains induced significantly blunted type 1/17 mediators (IFN-γ, IL-17, IL-22, IL-6, and T-bet; **Figure 9a** and data not shown) locally in the colon and systemically in the plasma (data not shown) when compared with the colitogenic strains 11168, CF93-6 (data shown) and CG8421 (data not shown). *C. jejuni*-specific IgGb (published previously),[24] IgG2c and IgG3 responses by the GBS strains were also trending toward a decrease as compared with the colitogenic strains (**Figure 9b**). In contrast, the GBS strains significantly enhanced the *C. jejuni*-specific type 2 mediators in the colon (IL-4, IL-13, and Gata-3; **Figure 9a**) and antibody (IgG1) responses (**Figure 9b**) in the plasma. Remarkably, type 2 but not type 1/17 autoantibodies were detected in plasma of *C. jejuni* GBS strain challenged mice. These IgG1 subclass autoantibodies reacted against the peripheral nerve gangliosides, GM1 and GD1a, in an enzyme-linked immunosorbent assay (ELISA) format (**Figure 9c** and **Supplementary Figure S2**). Molecular mimicry along with cytokine milieu was essential for autoimmunity because only the GBS

strains induced autoantibodies. Challenge infections with *C. jejuni* 11168 even in type 2 inducing conditions, that is, after depletion of IFN-γ and IL-17 (from **Figure 8c**), did lead to type 2 antibody induction against the *C. jejuni* antigen, but these antibodies failed to cross-react with the nerve ganglioside autoantigens (data not shown). The GBS strains colonized stably to the same extent as the colitogenic 11168 strain at the early time point of day 4 (from **Figure 2d**), but had lower fecal colonization at the later time points of day 11 (from **Figure 2d**) or day 16 (**Figure 9d**). Therefore, 11168 has enhanced colonization prowess that may be related to its enhanced colitogenic ability. For the GBS strain HB93-13, all stably colonized mice (8 out of 10), but none of the uncolonized mice (2 out of 10) tested positive for autoantibodies against GD1a, suggesting stable colonization as essential for inducing significant autoantibody responses.

The blunted type 1 responses elicited by the GBS strain HB93-13 were also confirmed *ex vivo* by measuring secreted cytokines from WT mouse splenocytes challenged with colitogenic 11168 and CF93-6, or GBS-associated HB93-13 and 260.94 strains using a gentamicin killing assay. At 72 h after
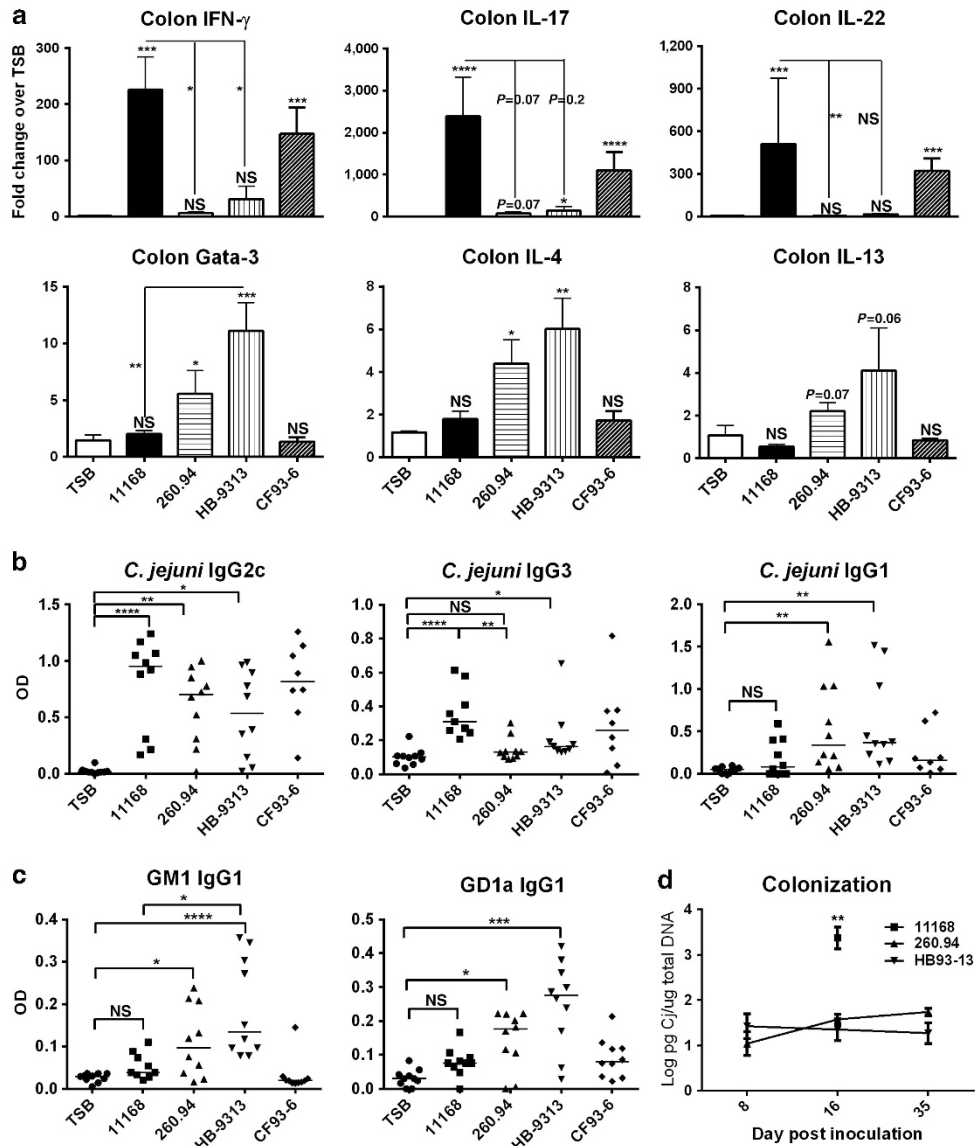
Figure 9 Strain-dependent colitis and autoimmunity. IL-10$^{-/-}$ mice were orally inoculated with given *C. jejuni* strains and killed at day 35 after inoculation, or earlier if requiring killing. (**a**) Expression of indicated mediators in the colon by real-time PCR. (**b**) Antibody subtypes reactive against *C. jejuni* antigen. (**c**) Peripheral nerve gangliosides autoantigens by ELISA. (**d**) *C. jejuni* colonization was measured in the feces at the indicated days after inoculation for the GBS strains and the time of necropsy for all strains by quantitative-PCR. Data are represented as median or mean ± s.e.m., with 10 mice per group. Kruskal–Wallis test followed by Dunn's *post test*. No other comparisons were significant. ELISA, enzyme-linked immunosorbent assay; GBS, Guillain Barré syndrome; IFN, interferon; IL, interleukin; TSB, Trypticase Soy Broth. OD, optical density.

challenge, GBS strains produced significantly less IFN-γ, TNF-α, IL-22, and IL-10 than either of the two colitogenic strains, consistent with the *in vivo* data (**Figure 10a**). However, they induced similar IL-12p40 and more IL-6, which points toward differential innate immune activation and/or T-cell maturation downstream of $T_H1/T_H17/T_H2$ pathways. To evaluate IL-12p70 induction by the colitogenic/GBS strains, adherent splenocytes alone were challenged individually (**Figure 10b**). Consistent with enhanced IFN-γ production by colitogenic strains, higher IL-12p70 was produced by them when compared with the GBS strains. In contrast, the GBS strains produced more IL-6 and IL-10. As invasion is likely an important feature of *Campylobacter*-mediated enteritis,

epithelial invasion by gentamicin killing assay was performed using the young adult mouse epithelial cell line (**Figure 10c**). Although the non-colitogenic GBS strain 260.94 invaded to a slightly lower extent as the colitogenic 11168, the other GBS strain HB93-13 invaded significantly more. Therefore, different outcomes to infection by colitogenic vs. GBS strains cannot be explained by differences in epithelial cell invasion but rather by the type 1/17 or type 2 induction by individual strains.

DISCUSSION

In this study, we show that *C. jejuni* 11168—a known colitogenic strain—induced a mixed type 1 and 17 cytokine
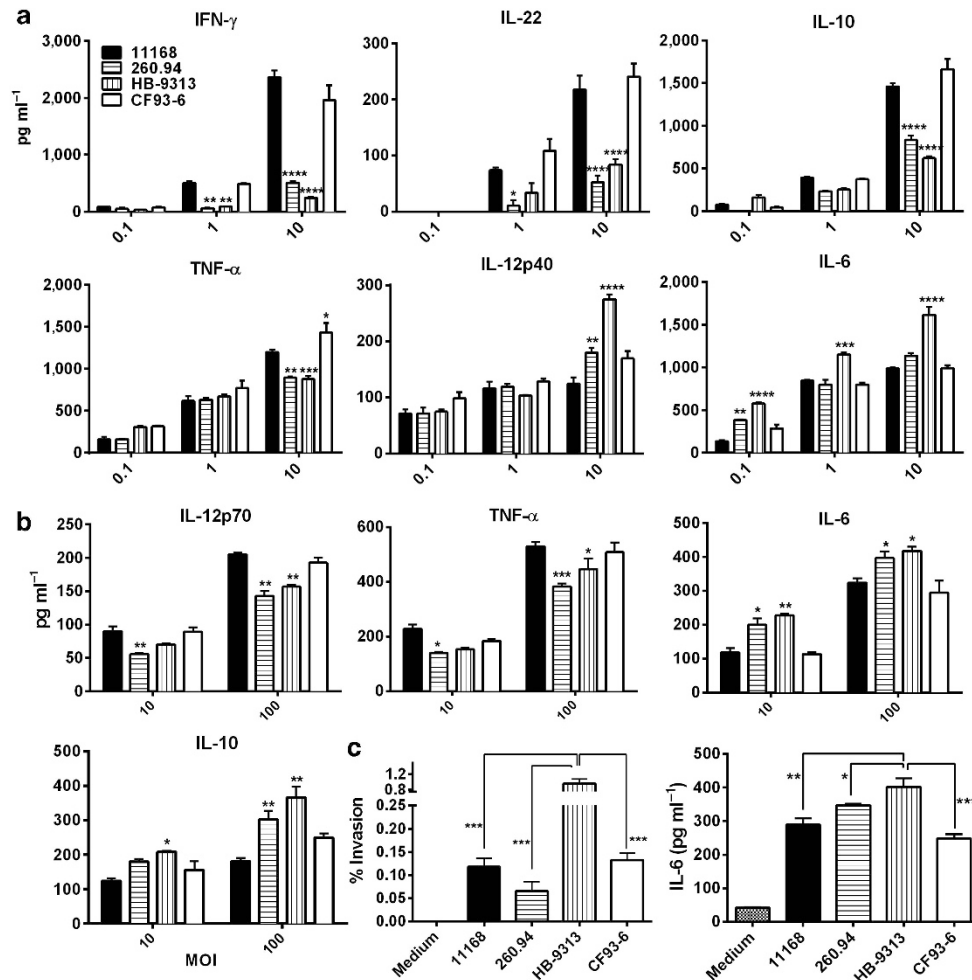
Figure 10 Whole splenocyte and epithelial cell challenge by gentamicin killing assay. Whole splenocytes (**a**) or adherent splenocytes (**b**) from naive C57BL/6 wild-type (wt) mouse were challenged with indicated strains and MOIs in antibiotic-free R10 media. Gentamicin was added after one hour, and cultures were further incubated for 72 h for whole or 24 h for adherent splenocytes, upon which supernatants were clarified and analyzed for indicated cytokines by ELISA. IL-17, IL-4, and IL-23 were not detectable with whole splenocytes while IL-12p70 was only detectable with adherent cell culture. (**c**) Invasion and IL-6 elicitation by the indicated strains was assessed on young adult mouse colon (YAMC) cells at MOI of 100. Data represent mean ± s.e.m. of three wells and analyzed by two-way analysis of variance (ANOVA) for splenocytes or one-way ANOVA for epithelial cells followed by Bonferonni's *post test*. Only comparisons with respect to 11168 for splenocytes or HB93-13 for epithelial cells are shown. One of three independent replicates is shown. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; MOI, multiplicity of infection; TNF, tumor necrosis factor. ****$P \leqslant 0.001$.

response in the colon and the draining MLNs of the C57BL/6 IL-10$^{-/-}$ mouse colitis disease model. This is consistent with the observations made by Edwards *et al*, which demonstrated that the induction of type 1 and 17 responses from *C. jejuni* 11168H challenged human colon explants and peripheral blood-derived cells *ex vivo*, along with a novel role for IL-17 in reducing intracellular survival in intestinal epithelial cells.[37] In our study, these cytokines orchestrated infiltration of neutrophils, macrophages, T cells, NK cells, and ILCs in the colonic mucosa and sub-mucosa that closely resembled lesions seen in patients with *Campylobacter*-induced enteritis. In our model, ILCs were already increased significantly at day 4, peaked at day 7, and decreased to background level by day 11. Increases in T cells and NK cells did not reach significance until days 7 or 11, respectively. By day 4 after infection, the amount of secreted IFN-γ, IL-17, and IL-22 from the MLNs was already increased two- to threefold, which indicated innate sources for these

cytokines since this response is too early for adaptive immunity to arise. We also found that innate lymphocytes as well as T cells participated in the upregulation of IFN-γ, IL-17, and IL-22, but in a time- and organ-specific manner. Mirroring the kinetics of their infiltration in the colon, ILCs upregulated IL-17 as early as day 4 in the lymph nodes that switched to an IFN-γ response by day 7 at which time IFN-γ$^+$ and IL-17$^+$ cells were also increased in the T$_H$ and γδ cell compartments. As only adaptive immunity can be antigen specific, it is tempting to speculate that early upregulation of ILCs followed by their down-regulation is a mechanism of ensuring an acute response to infection while preventing nonspecific inflammatory responses that predispose to autoimmunity. However, in the colon, the cytokine production profile from the ILCs over time is similar to that of T cells, and further studies are needed to determine their functional discrepancy in the two organs. Neutrophil and inflammatory myeloid cell numbers in the colon were

enhanced significantly at day 4 and continued to increase until the later time points (e.g., day 11) after infection. This was another reflection of the continuous significant increases in pro-inflammatory factors from the colon and MLNs by a combination of nonspecific ILCs and adaptive T cells that maintain and specify the responses.

It should be recognized that although ILCs participated in *C. jejuni* colitis, the innate immune system was unable to induce full blown colitis. Neither Rag1$^{-/-}$ nor IL-10R blocked Rag1$^{-/-}$ mice developed colitis after *C. jejuni* inoculation. However, Thy-1^{+} lymphocytes were necessary for the colitogenic response as significantly decreased pathology was observed in adaptive immune competent IL-10$^{-/-}$ mice that were depleted for Thy-1^{+} cells. These findings demonstrated that T cells were necessary for *C. jejuni* to elicit colitis. In contrast, Jobin *et al.* have very recently shown that germ-free Rag2 IL-10 double KO mice of the 129/SvEv background develop colitis after *C. jejuni* 11168 inoculation.[28] The difference in outcomes may be attributable to differences in the host genetics (C57BL/6 vs. 129/SvEv) and/or protective effect of host gut microbiota on degree of colonization and immune stimulation. Jobin *et al.* have also recently shown that depletion of CD4^{+} cells does not affect the pathology in *C. jejuni*-infected gnotobiotic C57BL/6 IL-10$^{-/-}$ mice.[27] This observation is complicated by the fact that in addition to immature thymocytes and mature T_H cells, CD4 is also expressed by macrophages and therefore CD4 depletion cannot be used as a specific tool for T_H cell depletion. Nevertheless, it does suggest a pro-inflammatory role of other lymphocytic populations in *C. jejuni* colitis and, in our model, we indeed observed the increase in numbers of IFN-γ^{+} and IL-17^{+} cells in non-T_H cell compartments, including $\gamma\delta$ and CD4^{-}(CD8^{+}) cells. ILCs can also be further divided into lymphoid tissue inducer cells (Lin^{-}Thy1^{hi}CD4^{+}) and LTi-like cells (Lin^{-}Thy-1^{hi}Sca-1^{+}) that have been shown to perform both pro- and anti-inflammatory functions.[38–40] Early IL-22 production from LTi's in the colon of *Citrobacter rodentium*-infected BL/6 IL-10$^{+/+}$ mice was shown to be protective,[41] whereas the production of IFN-γ and IL-17 from LTi-like cells in the colons of *Helicobacter hepaticus*-infected Rag.129 mice was shown to be pro-inflammatory.[42] Further studies will be necessary to determine the exact role of these subsets in *C. jejuni*-mediated colon pathology.

We have shown that enhanced IFN-γ and IL-17 cytokine secretion induces lesions in *C. jejuni*-induced colitis. Neutralizing either or both of these cytokines prevented the overt histopathological changes associated with the disease, including the infiltration of neutrophils, inflammatory myeloid cells, and T cells into the colon. It was notable that neutralizing these cytokines shifted the immune response toward type 2 cytokines (IL-4 and IL-13) and antibody responses (IgG1), alongside increases in *C. jejuni*-specific IgM. The non-colitogenic GBS strains also induced similarly enhanced type 2 and blunted type 1/17 responses, suggesting that *C. jejuni*-mediated colitis can be explained by the balance between type 1/17 and type 2 responses. This may also suggest the mechanism underlying the

development of autoantibodies and autoimmune diseases after treatment with tumor necrosis factor-α blockers in humans.[43–45] It remains to be seen if blocking the type 2 cytokines in the mouse model could lead to induction of colitis thus diverting the expected autoimmune outcomes after challenge with the non-colitogenic GBS patient strains.

GBS, especially the acute motor axonal neuropathy form, has most commonly been associated with antecedent *C. jejuni* infection.[17] It has also been published that antecedent gastrointestinal symptoms are only observed in 50% of GBS patients that are serologically positive for *C. jejuni*.[46,47] This suggests that there is no clear cut association between extent of colitis and GBS in humans and at least half of the human GBS cases mirror our mouse model in that infection with GBS strains leads to autoantibodies without antecedent diarrhea. Furthermore, identical to what has been observed in human GBS patients, autoreactive antibodies exclusively of the IgG1 isotype were found in mice infected with the GBS strains but not the colitogenic strains. Molecular mimicry and not just the cytokine milieu is essential for autoantibody development because the colitogenic non-GBS strain *C. jejuni* 11168 failed to induce autoantibodies even when the immune response was biased toward type 2 after depleting IFN-γ and IL-17. The non-colitogenic GBS strains were colonized to a similar extent as the colitogenic 11168 strain, but only at the early time point (day 4). Colonization with *C. jejuni* 11168 was higher at later time points (days 11–16). Therefore, 11168 has enhanced colonization prowess that may be related to its enhanced colitogenic ability and type 1/17 over type 2 maturation. However, there is still proof of strain-intrinsic but colonization-independent bias of type 1/17 maturation from *ex vivo* experiments. Stable colonization was nevertheless necessary to develop autoantibodies because two of the mice infected with the GBS strain HB93-13 strain that were not colonized failed to develop a significant autoantibody level when compared with their eight colonized group mates. Consistent with our *in vivo* findings, Bax *et al.* have shown *in vitro* that lipooligosaccharide purified from GBS strains induced blunted T_H1 and enhanced T_H2 response when compared with lipooligosaccharide from non-GBS strains.[48] The T_H2 response depended on the nature of sialylation of the lipooligosaccharide and correlated with binding to Sialoadhesin, a DC and macrophage cell surface receptor. The role of Sialoadhesin in *C. jejuni* phagocytosis and primary IFN induction has also been established.[49,50] These results suggest that surface modification of the *C. jejuni* GBS strains have an important role in development of autoimmunity, but more work is needed to determine the relevant ligand-receptor and downstream interactions in this model. Future studies are underway to determine the pathological and histological consequences of developing these autoantibodies, and the innate immune mechanism of differential T-cell responses by colitogenic vs. GBS strains. It is imperative to understand the host factors and bacterial ligands that are responsible for GBS and IBD in order to develop the needed novel therapeutic interventions for these conditions.

METHODS

Mice. C57BL/6J WT, BL/6.129P2-IL-10[tm1Cgn]/J (IL-10^{−/−}), and B6.129S7-Rag1[tm1Mom]/J (Rag1^{−/−}) mice were purchased from the Jackson Laboratory (Bar Harbor, MA) and bred in a specific pathogen-free breeding colony. Mice at 8–12 weeks of age were orally inoculated with TSB-vehicle control or 10[9] colony-forming unit of *C. jejuni* 11168 in 0.2 ml TSB, as described previously.[21] All animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee. Colonization by *C. jejuni* was confirmed and hematoxylin and eosin–stained sections of the ileocecocolic junction were scored as described previously.[21]

Antibodies for *in vivo* neutralization. Anti-IFN-γ (XMG1.2), α-IL-10R (1B1.3A), and α-Thy-1 (30H12) was purchased from Bio-X-Cell (West Lebanon, NH), and α-IL-17 (ebioMM17F3) was purchased from eBiosciences (San Diego, CA). XMG1.2 and ebioMM17F3 injected with 500 and 200 μg intraperitoneally, respectively, per mouse twice weekly, starting on the day of inoculation. α-IL-10R (1B1.3A) was injected with 1 mg per mouse intraperitoneally at the time of inoculation, and weekly thereafter. α-Thy-1.2 (30H12) was injected with 250 μg per mouse intraperitoneally twice a week, starting 3 days before inoculation. Rat or mouse IgG (Jackson ImmunoResearch, West Grove, PA) were used as controls where appropriate.

Immunohistochemical analysis of mononuclear cells. In all, 5 μm serial sections were cut, deparaffinized in two changes of xylene, and rehydrated in graded alcohol series. Slides were incubated in 1% H_2O_2 in tris-buffered saline and boiled for 15 min in citrate buffer (Vector Laboratories, Burlingame, CA) for CD3 or incubated at 37 °C in Proteinase K (Genemed Biotechnologies, South San Francisco, CA) for F4/80. Thereafter, sections were blocked for 1–3 h with 1% bovine serum albumin + 1.5% goat serum in tris-buffered saline and then incubated with respective primary antibody (1:500 for CD3ε, 1:50 for F4/80) at 4 °C overnight. Slides were then stained with the Vectastain ABC kit (Vector Laboratories) for CD3 or rat on mouse HRP polymer kit (Biocare Medical, Concord, CA) for F4/80 according to the manufacturer's instructions. Nonspecific IgG (Vector Laboratories) for CD3 or irrelevant isotype control (RTK4530, Biolegend, San Diego, CA) for F4/80 were used as negative controls. From each section, 10 non-overlapping ×400 magnification fields were chosen randomly, photographed, and positively staining cells were counted using the ImageJ cell counter (NIH, Bethesda, MD).

Preparation of colonic lamina propria leukocytes. Lamina propria leukocytes were isolated as previously described.[51,52] Briefly, for removal of epithelial cells, the colon was washed; cut into small pieces, and then the pieces were incubated with calcium- and magnesium-free Hank's balanced salt solution supplemented with 5% fetal bovine serum (FBS) and 5 mM EDTA (Sigma-Aldrich, St Louis, MO) at 100 r.p.m. at 37 °C for 30 min. The tissues were then incubated with RPMI 1640 containing 10% FBS and 0.5 mg ml^{−1} collagenase type IV and 0.5 mg ml DNaseI (Sigma-Aldrich) for 1 h at 37 °C with shaking at 150 r.p.m. The liberated cells were collected by passage through a 70 μm nylon mesh. The isolated cells were separated on a 40/80% discontinuous Percoll gradient (GE Bioscience, Pittsburgh, PA).

Flow cytometry. The following monoclonal antibodies (eBiosciences or Biolegend) were used: anti-CD3 (clone 145–2C11), anti-CD4 (clone RM 4–5), CD8 (clone 53–67); anti-TCR γδ (clone GL3, CD19 (clone 1D3); anti-NKp46 (clone 29A1.4); anti-CD11b (clone M1/70), anti-Gr1 (clone RB6-8C5), anti-CD90.2 (clone 53-2.1), and anti-CD16/CD32 (clone 2.4G2). The cells were preincubated for 20 min with anti-CD16/CD32 to block Fc receptors, then washed and labeled with appropriate mixture of antibodies or isotype-matched controls for 30 min, centrifuged at 650 g, and resuspended in fluorescence-activated cell sorting buffer. To exclude dead/dying leukocytes were gated according to forward and side scatter. For intracellular cytokine staining, cells were restimulated for 4 h with cell stimulation cocktail (eBiosciences) and fixed and permeabilized using fixation and permeabilization solution (eBiosciences). All cells were analyzed on a LSRII flow cytometer (BD Biosciences, San Jose, CA) using FlowJo software (Tree Star, Ashland, OR).

Enzyme-linked immunosorbent assay. All cytokines were measured according to the manufacturer's protocol (Ready-Set-Go ELISA kits, eBiosciences). To prepare protein extracts from colon, flash frozen tissue was homogenized with 0.5 ml Hank's balanced salt solution 1% Triton X-100 with the mini protease inhibitor cocktail (Roche, Indianapolis, IN). The homogenates were centrifuged at 12,000 g for 30 min at 4 °C, and the supernatants were collected for ELISA. For *ex vivo* culture, red blood cell-depleted MLN cells at 5 × 10^{6} cells ml^{−1} or 0.5 cm of proximal colon in 0.5 ml of RPMI 1640 supplemented with 10% FBS and 100 μg ml^{−1} penicillin G, 10 μg ml^{−1} of streptomycin, and gentamicin and 5 μg ml^{−1} amphotericin B were incubated at 37 °C for 48 h. Supernatants were clarified and stored at −70 °C. *C. jejuni*-specific antibody ELISA has been described previously.[21] The following plasma dilutions were used: 1:10 for IgA; 1:50 for IgG1, IgG2c, and IgG3; 1:100 for IgM; and 1:400 for IgG2b. Only absorbance values > 2 s.d. away from mean of negative control were considered positive. GM1 (Sigma-Aldrich) and GD1a (USBio, Salem, MA) were used at 2 and 20 μg ml^{−1}, respectively, and handled similarly.

Quantitative and reverse transcriptase PCR. RNA was extracted from flash frozen proximal colon samples using RNaeasy plus extraction kit (Qiagen, Valencia, CA). Complementary DNA was subsequently synthesized by GoScript Reverse Transcriptase kit (Promega, Madison, WI). Real-time PCR was performed for the target and *hprt* genes using Quantifast primers and probe assay (Qiagen) in ABI 7500 PCR machine (Applied Biosystems, Grand Island, NY) or iQ5 iCycler (Bio-Rad, Hercules, CA). For *C. jejuni* DNA estimation in feces, DNA was extracted as described previously.[21] In all, 50 ng of fecal DNA was used in quantitative-PCR reaction with *C. jejuni*-specific *gyr*A primers and iQ SYBR green supermix. *C. jejuni* DNA in fecal DNA was estimated by running a parallel standard curve of pure *C. jejuni* DNA.

Splenocyte challenge by gentamicin killing assay. Red blood cell-depleted splenocytes from naive C57BL/6 wt mouse (10[6] cells ml^{−1}) were plated in antibiotic-free R10 medium and challenged with indicated *C. jejuni* strains at multiplicity of infection of 0.1, 1, or 10. One hour after infection, gentamicin (250 μg ml^{−1}) was added to all the wells to kill extracellular bacteria. Supernatants were collected after 72 h for cytokine measurement by ELISA. To obtain adherent cells, splenocytes were plated at 10[7] cells ml^{−1} for 90 min upon which the non-adherent cells were washed off.

Epithelial challenge by gentamicin killing assay. In all, 1.5 × 10^{6} young adult mouse colon cells were grown in permissive media (RPMI 1640 with 5% FBS, ITS (insulin, transferrin, selenium) and 5 IU ml^{−1} IFN-γ) in 24-well plates at 33 °C and 5% CO_2. At 80% confluence, media is changed to ITS and IFN-γ-free RPMI 1640 with 5% FBS and incubated for 18 h at 37 °C. *C. jejuni* is then added at multiplicity of infection of 100 followed by 2-h incubation and three washes in PBS. For measuring invasion, cells are further incubated for 1 h with 250 μg ml^{−1} gentamicin, washed in PBS, lysed in 0.1% Triton X-100, and released bacteria is enumerated by serial dilution. For cytokine measurement, cells are incubated for further 24 h in media containing gentamicin and supernatant is clarified. Sensitivity of all strains to this concentration of gentamicin was also confirmed.

Statistical analysis. All statistical tests were performed in Prism 6.0 (GraphPad Software, La Jolla, CA) and described in figure legends. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ****$P < 0.0001$; NS, not significant.

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

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