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Murine Norovirus: An Intercurrent Variable in a Mouse Model of Bacteria-Induced Inflammatory Bowel Disease

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Murine norovirus (MNV) has recently been recognized as a widely prevalent viral pathogen in mouse colonies and causes disease and mortality in mice with impaired innate immunity. We tested the hypothesis that MNV infection would alter disease course and immune responses in mice with inflammatory bowel disease (IBD). FVB.129P2-*Abcb1a*^{tm1Bor} N7 (*Mdr1a*–/–) mice develop spontaneous IBD that is accelerated by infection with *Helicobacter bilis*. As compared with controls, *Mdr1a*–/– mice coinfected with MNV4 and *H. bilis* showed greater weight loss and IBD scores indicative of severe colitis, demonstrating that MNV4 can modulate the progression of IBD. Compared with controls, mice inoculated with MNV4 alone had altered levels of serum biomarkers, and flow cytometric analysis of immune cells from MNV4-infected mice showed changes in both dendritic cell (CD11c$^+$) and other nonT cell (CD4$^-$ CD8$^-$) populations. Dendritic cells isolated from MNV4-infected mice induced higher IFNγ production by polyclonal T cells in vitro at 2 d after infection but not at later time points, indicating that MNV4 infection enhances antigen presentation by dendritic cells early after acute infection. These findings indicate that acute infection with MNV4 is immunomodulatory and alters disease progression in a mouse model of IBD.

Abbreviations: DC, dendritic cell; IBD, inflammatory bowel disease; IP, IFNγ-inducible protein; MCP, macrophage chemotactic protein; MLN, mesenteric lymph node; MNV, murine norovirus; TNF, tumor necrosis factor

The genus *Norovirus* of the family *Caliciviridae* contains a large number of single-stranded, positive-sense RNA viruses that infect vertebrates, and strains have been identified in humans, cattle, swine, and (most recently) mice.[19,29,34] Murine noroviruses (MNV) are recently recognized pathogens that can cause lethal infection in immunocompromised mice that lack innate immunity.[19] However, MNV did not cause clinical disease in wild-type mice or many other strains of immunodeficient mice, including those lacking the recombination-activating gene (*Rag*–/–) and inducible nitric oxide synthase deficient mice.[19,35,37] MNV was reported recently to be widespread in laboratory mice and may persist in immunocompetent animals, depending on the strain of MNV used.[15,16,25] Studies in *Rag*–/– mice and B-cell–deficient strains showed that the acquired immune system plays an important role in the clearance of MNV.[6,19,37] MNV has tropism for dendritic cells (DCs),[36] which are important in the presentation of antigens to T cells in draining lymph nodes and in the pathogenesis of inflammatory bowel disease (IBD). Therefore, MNV is a potential confounder for in vivo immunology studies, including murine models of IBD.

Idiopathic IBD, which encompasses both ulcerative colitis and Crohn disease, is a widely studied disorder that affects approximately 1.4 million people in the United States.[20] Although the precise cause of human IBD has not been elucidated, studies with mouse models have demonstrated that abnormal host responses of the innate and adaptive immune systems to intestinal microbiota are important in the pathogenesis of IBD.[28,38] DCs are the sentinels of the intestinal mucosal barrier and have a pivotal role in the initiation of IBD in response to microbial ligands.[39] Alterations in DC responses could lead to persistence of bacterial infection, aberrant activation of the acquired immune system, and (ultimately) tissue damage.[38]

Viral stimulation of DCs leads to activation of adaptive immune responses,[17] including effector T cells, and as demonstrated with murine coronavirus (mouse hepatitis virus), intercurrent viral infections in mice can alter the phenotype of mouse models of human disease.[10] Additional evidence suggests that intercurrent viral infection may enhance disease in human IBD patients.[12,18] Whether infection with MNV alters DC function and, therefore, influences the progression of IBD in mouse models is unclear.

Many mouse models of intestinal inflammation develop IBD that is driven by bacterial flora.[9,28] *Helicobacter* spp. have been shown to drive this process in several mouse models including IL10-deficient, SMAD3-deficient, severe combined immunodeficiency and T-cell–deficient mice.[4,5,13,23] FVB.129P2-*Abcb1a*^{tm1Bor} (*Mdr1a*–/–) mice develop spontaneous IBD that is accelerated by infection with *Helicobacter bilis*.[21,22] In this report, we tested the hypothesis that infection with MNV can modulate IBD in this mouse model of bacterial-induced disease. We demonstrate that intercurrent MNV4 infection accelerates the progression of bacte-

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rial-induced IBD in the *Mdr1a*–/– mouse and alters the immune responses in this mouse model of IBD.

Materials and Methods

Animals and infection studies. *Mdr1a*–/– (FVB.129P2-*PAbcb1a*^{tm1Bor}) mice (originally obtained from Taconic Farms, Albany, NY) were bred and housed at the University of Washington. Animals were housed in a specific pathogen-free facility in static microisolation caging (Alternative Design, Siloam Springs, AR) containing corncob bedding (The Andersons, Maumee, OH) and cotton pads. Mice were fed irradiated chow (Picolab Rodent Diet 20 number 5053, PMI Nutrition, Brentwood, MO) and autoclaved, acidified water. All supplies entering animal rooms were autoclaved or gas-sterilized and rooms were maintained at 20 to 23 °C, with a 12:12-h light:dark cycle. To prevent cross-contamination of *Helicobacter*-infected and uninfected mice, cages were changed in dedicated *Helicobacter*-infected or -uninfected changing stations. Changing stations were thoroughly disinfected (1:18:1 Clidox, Pharmacal Research Labs, Waterbury, CT) between MNV4-infected and uninfected mice. Sentinel mice (IcrTac:ICR from Taconic Farms) were tested quarterly for endo- and ectoparasites, MNV, mouse hepatitis virus, mouse parvovirus, and rotavirus and annually for *Mycoplasma pulmonis*, pneumonia virus of mice, reovirus 3, Sendai virus, and Theiler murine encephalomyelitis virus. In addition, yearly colonic fecal samples were screened (Phoenix Laboratories, Everett, WA) for *Citrobacter rodentium*, nonlactose-fermenting *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., and *Clostridium* spp. During the study period, all sentinels were negative for the listed pathogens except for sentinels on the infected racks, which seroconverted to MNV and were PCR-positive for *Helicobacter* spp.

Sex- and age (7 to 12 wk)-matched *Mdr1a*–/– mice were used in 3 different infection studies. On day 0, mice either were inoculated with 1×10^6 PFU MNV4 [a kind gift from L Riley (University of Missouri, Columbia, MO)] in 0.2 ml RAW 264.7 cell lysates clarified by centrifugation at $3000 \times g$ for 10 min or were sham-inoculated with 0.2 ml DMEM. For disease studies, mice then received *H. bilis* on day 7. Mice were euthanized by CO_2 in accordance with the AVMA Panel on Euthanasia[1] at various time points or when they developed severe diarrhea, 20% body weight loss, or loss of body condition (defined by loss of visible muscle mass), and tissue samples were taken. Mice in disease induction studies were euthanized when more than 50% of animals in a single cohort showed prominent signs of disease, including weight loss, moderate to severe dehydration (7% to 10%), and severe diarrhea (some unformed feces as well as 'sticky stools'). Mild to moderate signs of disease included hunching, mild to moderate dehydration (5% to 7%), and mild to moderate diarrhea ('sticky stools' but all formed feces). Blood samples were taken by cardiocentesis, and infection with MNV was confirmed through serology or RT-PCR of fecal samples. For positive or negative confirmation of infection with MNV4 and *Helicobacter*, feces were collected at least 7 d after inoculation or at the euthanasia endpoint. Fecal samples from both uninfected and infected mice were tested by PCR for *Helicobacter* spp. in infected mice or for absence of *Helicobacter* infection in uninfected animals, as described later. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Bacterial cultures. The strain of *H. bilis* used was a natural isolate [kindly provided by L Riley (University of Missouri, Columbia, MO)]. Organisms were streaked onto *Brucella* blood agar plates and grown under microaerobic conditions (90% N_2, 5% H_2, and 5% CO_2) in vented jars (Oxoid, Hampshire, England), and kept at 37 °C. Bacteria were harvested and inoculated into flasks containing 150 ml *Brucella* broth supplemented with 5% fetal bovine serum (Sigma Chemical, St Louis, MO). The flasks were placed on a continuous shaker and incubated for 24 h at 37 °C in microaerobic conditions. The organisms were centrifuged at $8390 \times g$ at 4 °C for 10 min. The resultant pellet was examined by Gram stain and phase microscopy for purity and morphology. Organisms were confirmed to be catalase, urease, and oxidase positive. The pellet was resuspended in *Brucella* broth and optical density was adjusted to 1.0 (OD_{600}) for an estimated 10^8 CFU/ml. Mice were inoculated by oral gavage with 0.2 ml *H. bilis* in *Brucella* broth for a dose of 2×10^7 CFU per animal.

Pathology. At necropsy, colitis was defined by the combination of enlargement of the colon or cecum (or both) by thickened or edematous walls with reduction or loss of formed feces and variable involvement of the mesentery. Severity was scored subjectively based on the degree of enlargement or thickening of the colon. Colons thickened to 2 times their normal diameter with soft, ill-formed fecal pellets were considered to have mild colitis. Colons thickened to 3 to 4 times their normal diameter with reduction or loss of fecal contents were considered to have severe colitis. Tissue sampling, processing, and histologic examination were performed as described previously, with additional sampling of mesenteric lymph node (MLN) and ileum.[4] Briefly, MLN, ileum, cecum, colon, and rectum were fixed in 10% buffered formalin. The colon was prepared in a 'Swiss roll' technique to evaluate the entirety of the proximal, middle, and distal colon on the same section. The tissues were processed routinely, embedded in paraffin, sectioned at 4 to 5 µm, and stained with hematoxylin and eosin. Tissues were scored for inflammation and dysplasia by a pathologist blinded to the treatment groups. The cecum and proximal, middle, and distal colon from each mouse were scored on severity of mucosal epithelial changes, degree of inflammation, and extent of pathology. The total inflammation score (IBD score) for each mouse was derived by summing the scores from the individual segments (maximal score, 64) and subsequently, the mean was derived for each treatment group. The ileum was scored in a similar manner as the colon. The MLN, lymphatics, and colonic mesentery were scored separately for degree and extent of inflammation (Table 1), and the mean was derived for each treatment group.

Fecal DNA and RNA extraction, MNV RT-PCR, and *Helicobacter* spp. PCR. DNA or RNA was isolated from an unquantified amount of feces. The samples for RNA extraction underwent guanidium thiocyanate–phenol–chloroform extraction as previously described.[7] DNA was extracted by using a QIAamp DNA mini kit (Qiagen, Valencia, CA).

RT-PCR for MNV was performed (with slight modification) as previously described by using primers that can also detect MNV1.[16] Briefly, the RNA was amplified by using the OneStep RT-PCR Kit (Qiagen). The RT-PCR mixture was heated at 50 °C for 30 min, then for 95 °C for 15 min followed by 37 cycles comprised of 94 °C for 1 min, 59 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

PCR for *H. bilis* and *Helicobacter* spp. was performed as previously described,[22] except for modified cycling conditions. For *H. bilis*, 40 cycles of 30 s at 94 °C, 30 s at 63 °C and 3 min at 68 °C

Table 1. Scoring system for histologic evaluation of MLN and lymphatic lesions

Inflammation	Extent 1	Extent 2
0: No lesions; MLN show no reactive hyperplasia	0: No lymphatics affected in any manner	0: Normal
1: (Mild) Thickening of lymphatic wall with inflammatory cells, proliferative endothelium, less than 2x normal size	1: <5% of lymphatics affected in any manner	1: <5% affected by most severe change
2: (Moderate) Increase in size (3–4x normal) with necrosis or apoptosis; MLN show mild to moderate reactive hyperplasia	2: 6% to 30% of lymphatics affected in any manner	2: 6% to 30% affected by most severe change
3: (Marked) Increase in size (5x normal) with necrosis or apoptosis and intraluminal debris or inflammatory cells, mild extension into adjacent tissues; MLN show marked hyperplasia	3: 31% to 59% of lymphatics affected in any manner	3: 31% to 59% affected by most severe change
4: (Severe) Grade 3 plus moderate to severe extension into adjacent mesenteric fat	4: >60% of lymphatics affected in any manner	4: >60% affected by most severe change

Total inflammation score = inflammation + extent 1 + extent 2. Scoring system adapted from reference 4.

were performed, followed by final extension at 72 °C for 7 min. For *Helicobacter* spp., PCR mixtures were heated to 95 °C for 5 min and 40 cycles of 5 s at 95 °C, 2 s at 58 °C, and 1 min at 72 °C were performed, followed by final extension at 72 °C for 7 min. The RT-PCR and PCR products were separated on 1% agarose electrophorectic gels containing ethidium bromide and visualized under UV light.

Evaluation of serum cytokines and chemokines. Serum stored at –80 °C was assayed in duplicate on an automated plate reader (model 100 IS, Luminex, Austin, TX) by using commercially available reagents (LINCOplex Mouse Cytokine–Chemokine Panel, Millipore, Billerica, MA) and antibodies to IL2, IL6, IL12p70, IL17, IFNγ, IFNγ-inducible protein 10 (IP10), macrophage chemotactic protein (MCP) 1, and TNFα. Data were analyzed by using the Data Interpretation Application (Luminex). Values that were less than 0 are noted as undetectable and classified as 0 for data analysis purposes. Samples with insufficient bead counts (<50 per well) also were excluded from analysis.

Flow cytometric analysis. For flow cytometric analysis of mononuclear cells, MLN and spleens were prepared and stained with antibodies as described previously.[3] All antibodies were purchased from BD Biosciences (San Jose, CA) unless otherwise indicated. Cells (1 to 2×10^6 cells per stain) were stained with combinations of the following antibodies: antiB220–FITC (clone RA3-6B2), antiCD4–APC (clone RM4-5), antiCD4–PerCpCy5.5 (clone RM4-5), antiCD8–APC–Cy7 (clone 53-6.7), antiCD8–FITC (clone 53-6.7), antiCD11b–biotin (clone M1/70), antiCD11c–APC (clone HL-3), antiCD11c–FITC (clone HL-3), antiCD11c–PE (clone HL-3), antiCD40–APC (clone 1C10, eBioscience, San Diego, CA), antiCD44–PE–Cy5 (clone IM7), antiCD45RB–FITC (clone 16A), antiCD86–biotin (clone GL1), antiF4/80–PE (clone BM8, Caltag–Invitrogen, Carlsbad, CA), antiIA/E–PE (clone M5/114.15.2), antiTCR–biotin (clone H57-597), or antiTCR–PE (clone H57-597). Secondary staining reagents were streptavidin-conjugated Alexa Fluor 488 (Molecular Probes–Invitrogen), PE–Cy5, and PE–Cy7. Samples were run on a FACScan or FACSCanto instrument (BD Biosciences). Data were analyzed by using FlowJo (Tree Star, Ashland, OR).

For analysis of DC subsets and cell surface markers in MLN, MLN from individual mice underwent mild collagenase digestion (0.18 Wünsch U/ml Liberase RI, Roche, Indianapolis, IN) in the presence of 15 µg/ml DNase I (grade II, Roche) diluted in RPMI medium (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES for 45 min at 37 °C with intermittent mixing in a total volume of 1 ml. Digestion was stopped by the addition of this media also containing 10% FCS and 10 mM EDTA. Red blood cells were lysed, and the sample was filtered through 40-µm nylon mesh. DCs were stained and analyzed as described earlier.

IFNγ production assays. $CD4^+$ T cells were purified from MLN using negative selection ($CD4^+$ T Cell Isolation Kit, Miltenyi Biotec, Auburn, CA), and DCs were purified by using antiCD11c-coated microbeads (Miltenyi Biotec) followed by automated magnetic cell sorting (AutoMACs, Miltenyi Biotec). MLN DCs were isolated from collagenase-treated tissues. Experiments were conducted with DCs pooled from 4 to 5 mice per group and T cells from 3 to 5 mice. Cells were counted and purity was checked by flow cytometric analysis using antiCD11c or antiCD4 and antiTCRβ antibody staining. For DCs, purity exceeded 90% in all but 2 experiments, in which purity was 84% and 77%. For T cells, purity always exceeded 93%. Purified cells (1×10^5 $CD4^+$ T cells/well and 1×10^4 DCs/well) were cultured at 37 °C and 5% CO_2 in 96-well flat-bottomed plates in a total volume of 0.2 ml RPMI supplemented with 10% FCS, 1× nonessential amino acids (Irvine Scientific, Santa Ana, CA), 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were cultured in medium alone with various concentrations of *H. bilis* antigen for 72 h. *H. bilis* antigen was prepared as previously described.[22] Supernatants were harvested and stored at –20 °C until ELISAs were performed. IFNγ levels in supernatants were assayed in 96-well plates (Microlon 600-High Bind, Greiner Bio-One, Monroe, NC) by using a commercially available kit (OptEIA Mouse IFNγ Kit, BD Biosciences) according to the manufacturer's directions. Substrate for ELISA was TMB Substrate Reagent Set (BD Biosciences). Optical densities were measured at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Serology. Blood samples were taken by cardiocentesis at the time of necropsy. Sera were frozen and stored at -80 °C until processed. Antibodies to MNV were detected by microsphere-based

serologic multiplexed fluorescent immunoassay, with secondary testing by indirect fluorescent antibody at the University of Missouri Research Animal Diagnostic Laboratory (Columbia, MO) or at the Department of Comparative Medicine, University of Washington.

Statistics. For ELISA studies, data were normalized and merged across experiments according to average maximal response for individual experiments. To classify total histopathology scores into severe or mild disease groups, a nearest-neighbor cluster analysis using squared Euclidean distances was done with SPSS (version 16, SPSS, Chicago, IL). Other statistical analyses were performed using GraphPad Prism 3.02 or 5 (GraphPad Software, San Diego, CA). The significance level (α) was set at 0.05, and 2-sample unpaired *t* test with Welch correction (not assuming equal variance) was used to compare mean values. Data are presented as mean ± SEM. Differences in colonic lymphangitis scores were analyzed by using the Mann–Whitney test. Gross necropsy results and frequency of MLN lymphangitis or severe colitis were analyzed with contingency tables, and a chi-square statistic was calculated. Except where indicated, the data presented are the combination of at least 2 independent experiments.

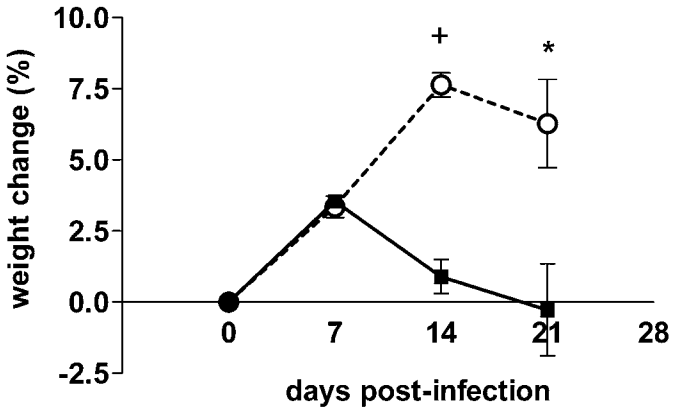

Figure 1. Percentage weight change of *Mdr1a-/-* mice infected with MNV and *H. bilis* (solid squares, n = 21) or *H. bilis* alone (open circles, n = 20) over 3 wk of infection. MNV infection at day 0; *H. bilis* infection at day 7. Coinfected animals gained significantly less weight by 2 wk after infection with MNV (+, $P = 0.004$) and had lost significantly more weight by week 3 (*, $P = 0.038$). Data are from 2 independent experiments; error bars represent SEMs.

Results

MNV infection accelerates weight loss and exacerbates IBD progression in *Mdr1a–/–* mice. Because MNV is associated with histopathologic changes in the intestine in immunocompetent hosts,[26] we hypothesized that MNV4 would alter the progression of inflammatory bowel disease in the *Mdr1a–/–* mouse. Male *Mdr1a–/–* mice were inoculated with either MNV4 or broth on day 0. On day 7 after inoculation, both groups received *H. bilis*, which has been shown to accelerate and synchronize the development of IBD in this model.[22] Compared with controls, *Mdr1a–/–* mice coinfected with MNV4 and *H. bilis* began to develop diarrhea and weight loss earlier during the experiment (Figure 1). By day 21 after infection, more than 50% of animals in the coinfected cohorts were showing marked signs of disease, whereas the singly infected mice showed only mild to moderate signs of disease at this time point. By the final week of the study, *Mdr1a–/–* mice coinfected with MNV4 and *H. bilis* lost significantly ($P = 0.038$) more weight than controls (Figure 1), and more mice had gross evidence of severe colitis at necropsy (Figure 2 A, B). Gross observations at necropsy showed that 80.9% of the mice coinfected with MNV4 and *H. bilis* had evidence of colitis compared with 55% in animals infected with *H. bilis* alone [$\chi^2(1, n = 41)=3.19$, $P = 0.03$).

Histology correlated well with gross observations, and a higher ($P = 0.02$) percentage of coinfected mice had severe colitis characterized by dense inflammatory cell infiltration and hyperplasia, crypt abscessation and mucosal erosion, compared with animals infected with *H. bilis* alone (Figure 3 and 4). The average IBD score for coinfected animals was 47.1 ± 3.9 (n = 21), whereas singly infected animals had an average IBD score of 32.8 ± 5.7 (n = 20).

Because MNV replicates in the MLN and distal ileum,[15,30] we examined these tissues as well. No differences were seen in the ileal inflammation scores (data not shown). However, the MLN and associated lymphatics showed moderate to marked reactive lymphoid hyperplasia more consistently in coinfected animals than singly infected animals, and the average MLN score was significantly ($P = 0.02$) higher in coinfected animals (6.8 ± 1.1 versus 2.8 ± 1.6). In some animals, the mesentery attached to the colon and surrounding the mesenteric lymph nodes was hypercellular and edematous (Figure 5 B through D). There, the lymphatics, and to a lesser extent the veins, were thickened by primarily lymphohistiocytic inflammation, with rarer neutrophils and necrotic or apoptotic debris (Figure 5 C, D). The endothelia of these affected vessels were plump and reactive, the lumens were dilated moderately and contained abundant proteinaceous lymph with few viable and degenerate inflammatory cells. Rarely, vessel architecture was obliterated with lumen occlusion by inflammatory cells, with extension of the inflammation into the surrounding mesentery. The degree of lymphangitis was scored by using a numerical severity system similar to that used for IBD (Table 1). Lymphangitis was seen in the colonic mesentery more frequently in coinfected animals than in singly infected animals [77% versus 22%, $\chi^2(1, n = 18) = 5.59$, $P = 0.01$]. In addition, lymphangitis scores reflected the overall impression that IBD in sick coinfected animals was more severe than IBD in sick singly infected animals. Of the animals with severe IBD scores, coinfected animals had increased lymphangitis scores. This difference was statistically significant ($P = 0.03$) if a single outlying data point demonstrating severe IBD and a lymphangitis score of 0 was removed from the analysis; results were marginally nonsignificant ($P = 0.06$) if this data point was included (Figure 6). Together, these data indicate that coinfected animals have an earlier progression of IBD and more severe disease. In addition, the effects of MNV4 infection are not limited to the gastrointestinal tract but extend into draining lymphoid tissues in the colonic mesentery.

To confirm infection with MNV and ensure that these differences were not due to previous exposure to MNV, all animals in infection studies were serologically surveyed for antibodies to MNV at the study endpoint. Mice experimentally infected with MNV4 seroconverted as early as 3 wk after infection, and uninfected mice were seronegative throughout the studies. Fecal PCR confirmed that all *H. bilis*-infected animals were positive for *H. bilis* sequences, whereas all negative animals remained negative by PCR for *Helicobacter* spp.

Disease severity in MNV4- and *H. bilis*-infected *Mdr1a–/–* mice correlates with serum biomarker levels. Because MNV causes

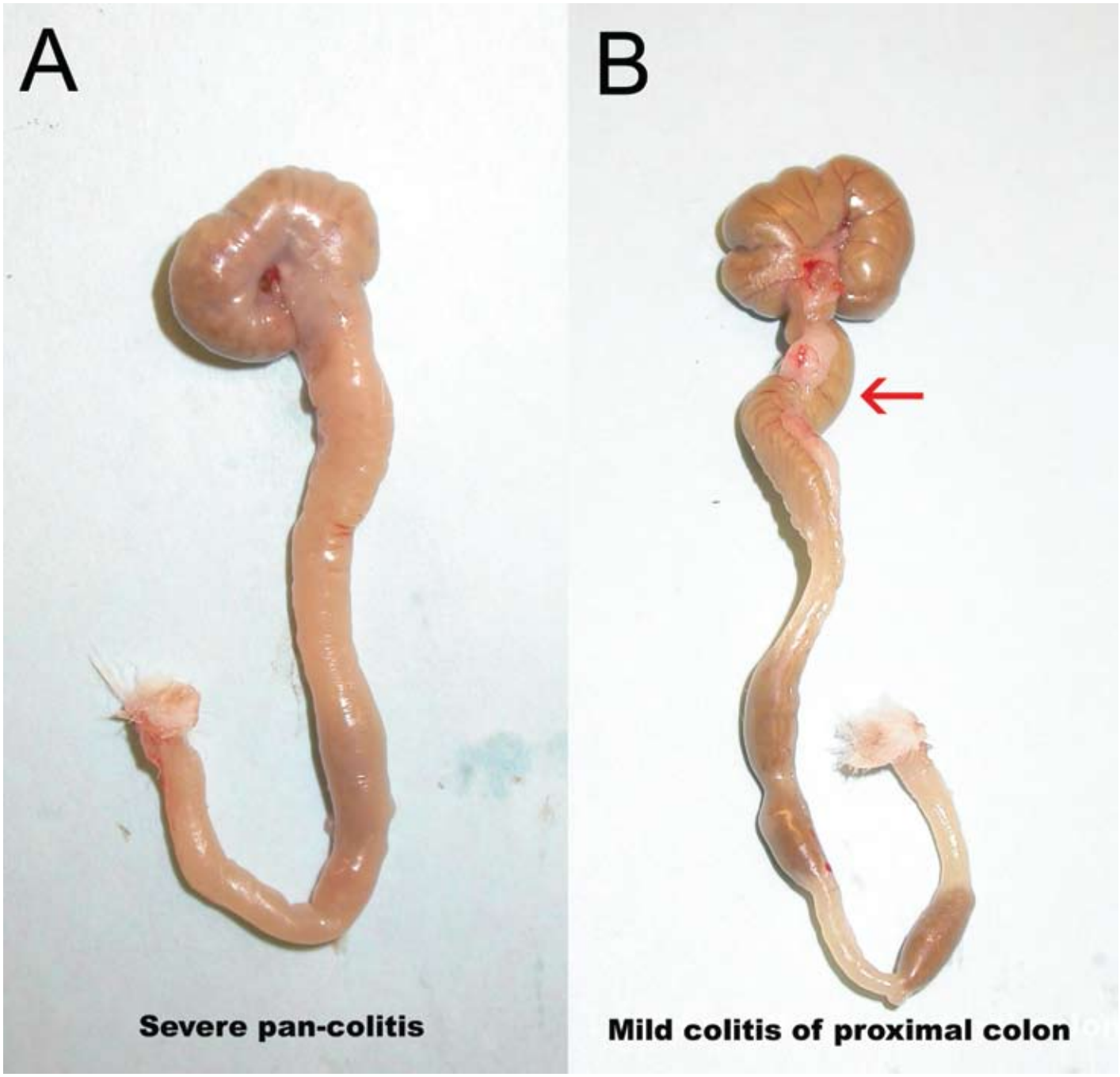

Figure 2. Mild versus severe colitis. (A) Representative colon and cecum from a colitic *Mdr1a-/-* mouse infected with MNV and *H. bilis*. Colon is thickened and edematous (IBD score of 52: severe colitis). (B) Representative colon and cecum from an *Mdr1a-/-* mouse infected with *H. bilis*. Proximal colon has mild thickening (arrow), whereas the mid- to distal colon appears normal (IBD score of 24, mild colitis). Normal fecal pellets are present in the mid- to distal colon.

disease in several strains of immunodeficient mice and can infect DCs and macrophages in vitro and in vivo,[26,35,36] we were interested in characterizing the immune response generated by MNV. Specific serum cytokines and chemokine levels reflect immune responses in vivo and correlate with disease progression in *Mdr1a*–/– mice;[31] we therefore examined the levels of several serum biomarkers—MCP1, IL2, IL6, IFNγ, IL12p70, IP10, IL17, and TNFα—in coinfected and singly infected mice at the disease endpoint. Consistent with previous studies,[31] in both coinfected and singly-infected mice, serum levels of IP10 positively correlated with histologic scores (Figure 7 A). Mice coinfected with MNV4 had increased average IP10 levels overall (3347 ± 459.80, n = 20 versus 1906 ± 442.70, n = 15; $P = 0.015$), consistent with the increased percentage of coinfected mice with severe disease. Serum levels of IL6 and TNFα positively correlated with disease (Figure 7 B, C), although coinfected mice did not show significant

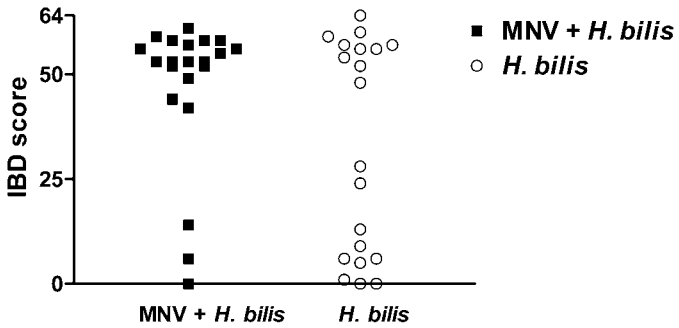

Figure 3. At 3 wk after infection, coinfected animals had significantly higher average IBD scores [47.1 ± 3.9 (n=21) versus 32.8 ± 5.7 (n = 20); $P = 0.02$), and the percentage of mice with severe colitis was significantly higher in the group coinfected with MNV4 and *H. bilis* (86% versus 48%; $P = 0.0040$). Histology was scored by a pathologist blinded to the treatment groups. Scores were categorized into mild and severe colitis groups by using a nearest-neighbor cluster analysis (see Materials and Methods). Data are from 2 independent experiments.

changes in average serum levels of either IL6 (141.4 ± 35.13 n = 19 versus 85.42 ± 17.28, n = 18, $P = 0.165$) or TNFα (42.08 ± 8.39, n = 8 versus 22.34± 6.06, n = 8, $P = 0.077$). However, for TNFα, the slope of the linear regression line was greater in the coinfected group versus the singly infected group (Figure 7 C), suggesting that TNFα is associated with the severe disease noted in coinfected animals. Serum levels of IL17, MCP1, IL2, IFNγ, and IL12p70 did not correlate with severity of disease, and average levels of these biomarkers did not differ between groups (data not shown).

MNV4 infection in *Mdr1a–/–* mice is associated with changes in serum biomarkers early after infection. To address whether MNV4 alone was capable of altering serum biomarker levels, mice were inoculated with either MNV4 or broth, and serum was taken at 2 (n = 10), 7 (n = 9), and 28 (n = 4 or 5) d after inoculation. At 2 and 28 d after inoculation, both groups had similar levels of all serum cytokines and chemokines examined (data not shown). However, at 7 d after inoculation, MNV4-infected animals displayed significantly decreased levels of IFNγ (MNV-infected: 1.4 ± 1.3 pg/ml, n = 8; broth-inoculated: 12.5 ± 2.1pg/ml, n = 8, $P < 0.001$) and IL12p70 (MNV-infected: 75.9 ± 2.2 pg/ml, n = 8; broth-inoculated: 109.7 ± 7.5 pg/ml, n = 8, $P < 0.001$). Because IL12 secretion leads to IFNγ production and a Th1 response,[32] we expected that a decrease in IL12p70 would be accompanied by a concurrent decrease in IFNγ. Levels of IP10, IL2, IL6, MCP1, and TNFα were not significantly different at any of the time points examined. Overall, these data suggest a trend toward decreased serum levels of type 1 cytokines (IL12p70, IFNγ) after MNV4 infection in *Mdr1a–/–* mice.

MNV4-infected *Mdr1a–/–* mice have alterations in immune cell populations. Viral infection leads to an initial cytokine–chemokine response that directs secondary immune responses and may influence immune cell proliferation and activity.[40] Considering the altered levels of these proinflammatory serum cytokines and chemokines seen in our studies, we hypothesized that MNV infection would also be associated with changes in cell populations in the MLN and spleen. We analyzed the cellularity and expression of cell surface markers on immune cells in the MLN and spleens of *Mdr1a–/–* mice at 2, 7, and 28 d after infection with MNV. When compared with broth-treated animals, splenic cellularity of MNV-infected mice was increased at day 7 after infection (MNV-infected: $91.4 \times 10^6 \pm 8.0$, n = 10; broth-inoculated: $63.3 \times 10^6 \pm 6.6$, n = 11, $P < 0.01$). Associated with the increase in splenic cellularity in MNV-infected mice compared with controls at day 7 after infection, the percentage of $CD4^+$ cells decreased (MNV-infected: 39.2% ± 3.6%, n = 9; broth-inoculated: 45.0% ± 2.2%, n = 8, $P < 0.001$), the majority of which are T cells, although the absolute numbers of these cells did not change (data not shown). Accordingly, both the percentage and absolute number of nonT cells ($CD4^-CD8^-$) increased at day 7 (MNV-infected: $37.6 \times 10^6 \pm 12.2$, n = 10; broth-inoculated: $22.7 \times 10^6 \pm 7.9$, n = 8, $P < 0.01$), and absolute numbers of this population remained increased to day 28 (MNV-infected: $18.8 \times 10^6 \pm 3.4$, n = 5; broth-inoculated: $11.6 \times 10^6 \pm 2.2$, n = 5, $P < 0.01$). The cell subtypes included in this population require further characterization. Interestingly, there were also more splenic $CD11c^+$ cells (DCs) in MNV4-infected spleens (MNV-infected: $3.6 \times 10^6 \pm 0.9$, n = 10; broth-inoculated: $2.0 \times 10^6 \pm 0.5$, n = 11, $P < 0.001$) compared with those in broth-inoculated animals at day 7; this result may primarily be due to an increase in percentage and absolute numbers of $CD11c^{low}$ (plasmacytoid DCs) cells in these animals (MNV-infected: $3.1 \times 10^6 \pm 0.9$, n = 5; broth-inoculated: $1.8 \times 10^6 \pm 0.5$, n = 5, $P < 0.01$). This increase was not seen at day 28. Surprisingly, there were few detectable differences in MLN populations except increased $CD11c^+$ cells at day 28 (MNV-infected: $9.4 \times 10^5 \pm 2.5$, n = 10; broth-inoculated: $5.8 \times 10^5 \pm 1.7$, n = 10, $P < 0.05$); this change also was associated with increased percentage and numbers of $CD11c^{low}$ cells (MNV-infected: $6.4 \times 10^6 \pm 2.3$, n = 10; broth-inoculated, $3.6 \times 10^6 \pm 0.7$, n = 10, $P < 0.05$).

We were unable to detect changes in $CD11b^+$, $CD8^+$ cell populations in either MLN or spleen, and there were no detectable differences in the costimulatory molecules CD40, CD80, and CD86 on $CD11c^+$ or $CD11b^+$ cells (macrophages) at any time point (data not shown). Because previous authors saw increases of $F4/80^+$ cells (macrophages) and $B220^+$ cells in the spleen at 72 h after infection[26], we examined F4/80 and B220 cell populations in the spleen at 2 d after infection and saw no difference between MNV4- and broth-inoculated *Mdr1a–/–* mice (data not shown). We also examined CD45RB and CD44 on T cells at 16 d after inoculation with MNV4 or broth in *Mdr1a–/–* mice that were coinfected with *H. bilis* at day 7 after infection and did not see alterations in these T cell activation markers at this time point (data not shown).

MNV4 infection does not alter polyclonal T cell IFNγ responses. Although we did not discern changes in specific activation markers on immune cells through flow cytometric analysis, we wanted to determine whether the *H. bilis*-specific T cell cytokine release was altered in *Mdr1a–/–* mice coinfected with MNV4 and *H. bilis* because IBD has been associated with T cell activation and skewing toward a Th1 response.[9,13] In our laboratory, we have found that IFNγ production by *H. bilis*-specific T cells is detectable 9 d after infection with *H. bilis* (data not shown). Mice were inoculated with either MNV4 (n = 5) or broth (n = 5) at day 0, after which both groups were infected with *H. bilis* 7 d later. We isolated MLN T cells from these mice 16 d after infection with MNV4 (9 d after infection with *H. bilis*) and incubated these polyclonal T cells with purified splenic DCs, to serve as antigen-presenting cells, in the presence of *H. bilis* antigen in vitro for 72 h. The antigen-presenting cells were isolated from animals free of both MNV and *H. bilis*. No significant difference in IFNγ production was detected in T cells isolated from *Mdr1a–/–* mice coinfected with MNV4 and *H. bilis* and those from mice infected with *H. bilis* alone (data not shown).

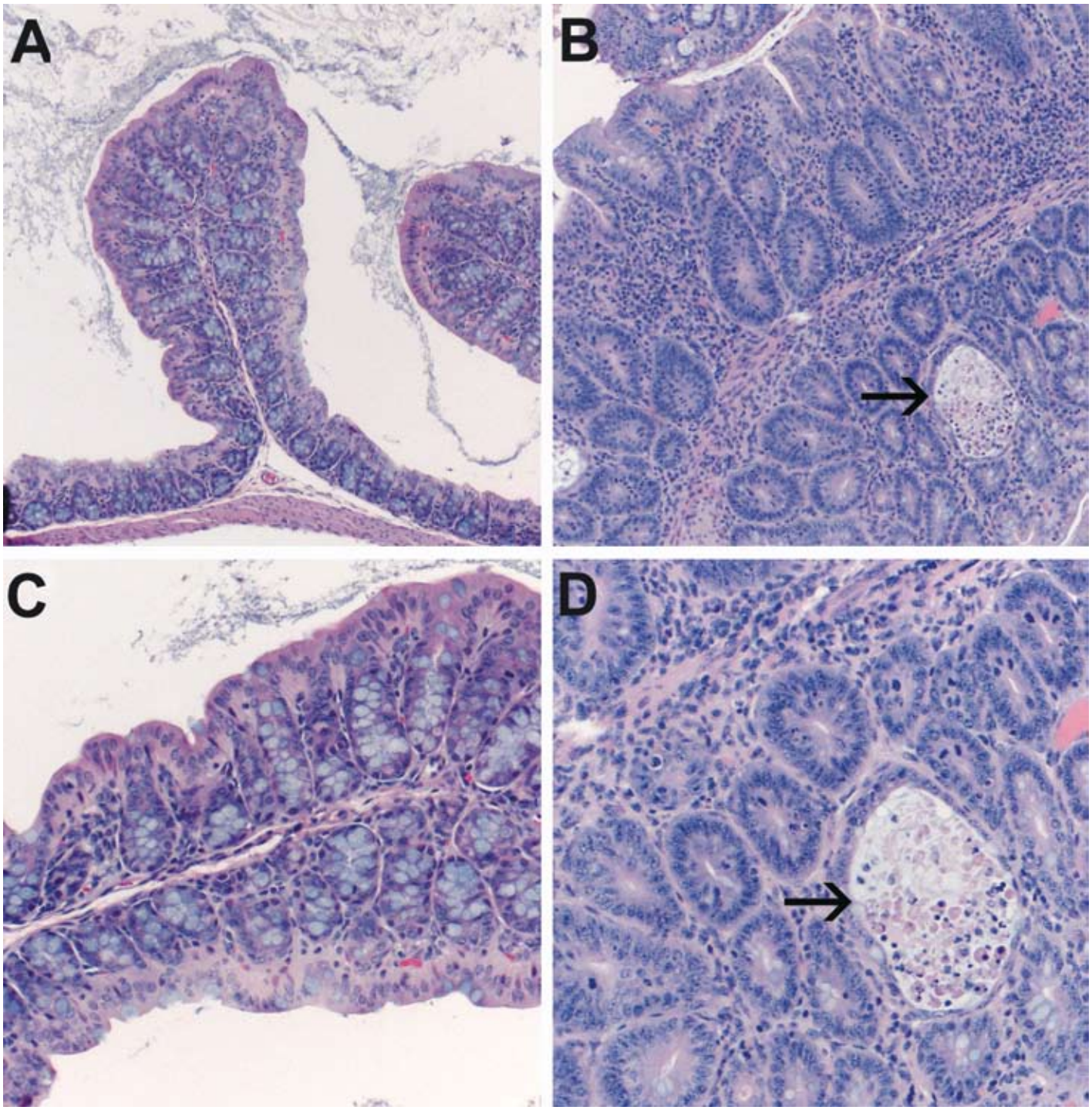

Figure 4. Hematoxylin- and eosin-stained sections of proximal colon from unaffected (A, C) and colitic (B, D) *Mdr1a-/-* mice. (A) Normal proximal colonic mucosa from a mouse infected with *H. bilis* only (IBD score of 6). (B) Proximal colon from mouse coinfected with MNV4 and *H. bilis* (IBD score of 61) demonstrating severe proliferative and lymphohistiocytic colitis. (C) Higher power view of the same section as in panel A. Note the regular short glands and abundant goblet cells. (D) Higher power view of the same section as in panel B. Note the thickened mucosa, abundant mitotic figures, loss of goblet cells, and crypt abscess (arrows). Original magnification, ×10 (A, B); ×20 (C, D).

MNV4 infection enhances antigen presentation by DCs in vitro. Because MNV infects DCs and macrophages both in vitro and in vivo,[26,35,36] we hypothesized that MNV4 would interfere with DC function. Therefore, we analyzed the ability of MLN DCs from *Mdr1a–/–* mice to stimulate IFNγ production by polyclonal T cells at various time points after MNV4 infection. Purified MLN DCs from mice that received either MNV4 or broth were isolated at 2, 7, and 28 d after infection and incubated with *H. bilis*-primed

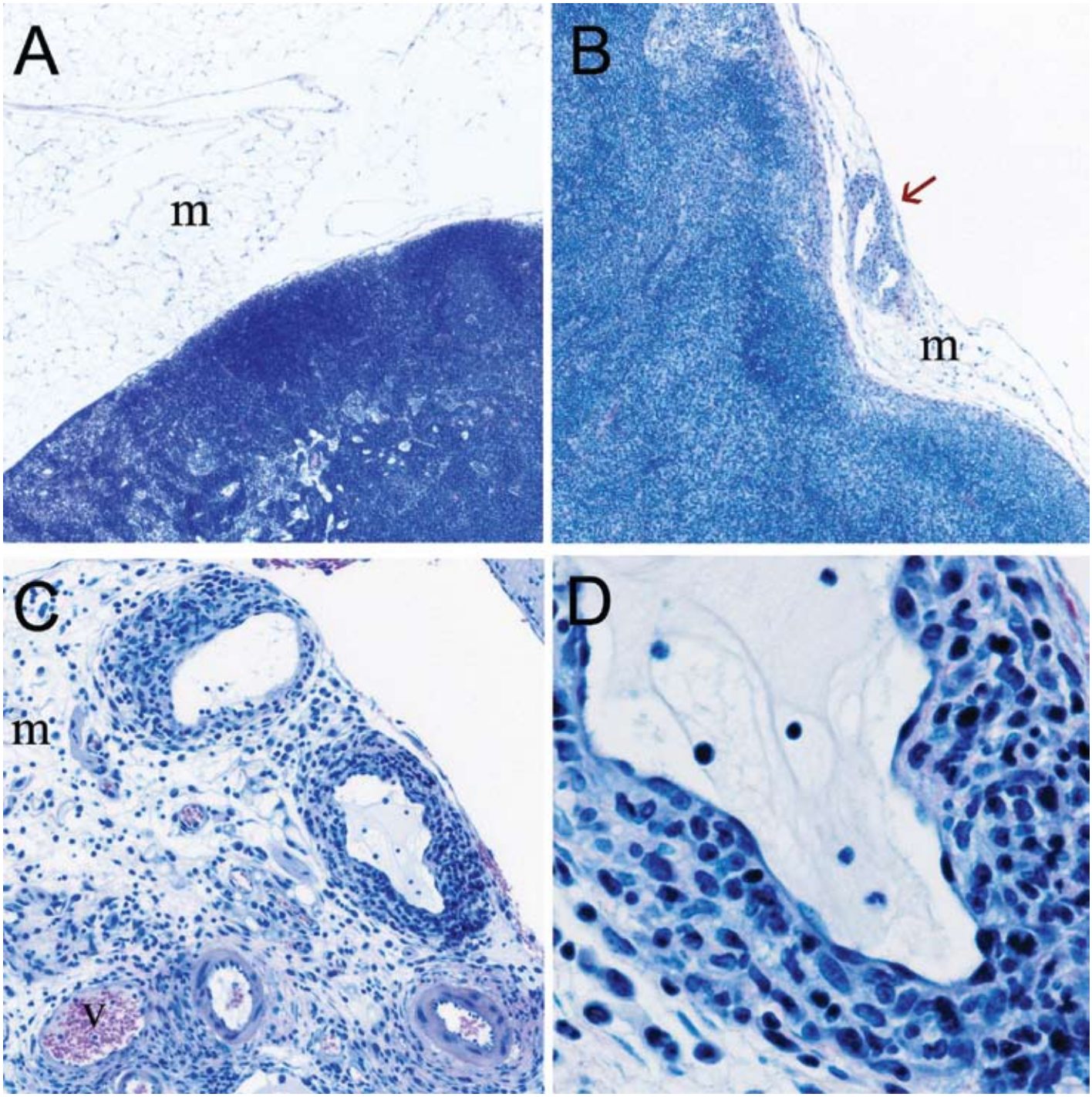

Figure 5. Representative images of hematoxylin- and eosin-stained tissues from mice infected with *H. bilis* only (A, B) and mice coinfected with MNV4 and *H. bilis* (C, D). (A) MLN and attached mesentery (m) from a mouse with an IBD score of 0 and lymphangitis score of 0. The mesentery is within normal limits. Original magnification, ×4. (B) MLN from a mouse infected with *H. bilis* only with an IBD score of 56 and lymphangitis score of 13. The mesentery (m) has mild focally extensive increase in cellularity surrounding lymphatics and veins (arrow). Original magnification, ×4. (C) Colonic mesentery from a coinfected mouse with an IBD score of 53 and lymphangitis score of 21. The mesentery is hypercellular and edematous with marked lymphangitis. Note the perivascular inflammation surrounding a vein (v) and arteries that have mild to moderate medial smooth muscle hypertrophy. Original magnification, ×20. (D) Higher power view of section in panel C. Note the lymphohistiocytic lymphangitis and moderate ectasia. Original magnification, ×40.

$CD4^+$ T cells and increasing concentrations of *H. bilis* antigen, after which production of IFNγ was measured. At 2 d after infection, DCs harvested from mice infected with MNV4 were able to stimulate increased IFNγ production compared with DCs harvested

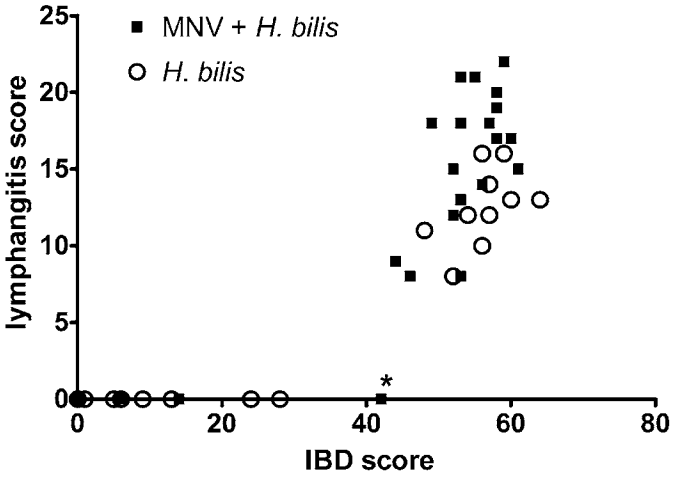

Figure 6. Lymphangitis is more severe in mice coinfected with MNV4 and *H. bilis*. Mesentery attached to the colon and surrounding mesenteric lymph nodes was graded according to the scale outlined in Table 1. Scores of the mesentery surrounding the cecum and proximal colon were summed and plotted against IBD scores. Lymphangitis scores differ significantly ($P = 0.009$) between groups. In addition, lymphangitis scores differed between coinfected and singly infected groups when only animals with severe IBD were compared (excluding asterisked outlier: $P = 0.03$; including indicated outlier, $P = 0.06$), indicating that IBD in coinfected animals is more severe.

from uninfected mice, at several concentrations of *H. bilis* antigen (Figure 8). This response did not occur at 7 d after infection but was noted at 28 d after infection at the 1-μg/ml dose of *H. bilis* antigen. No clinical disease was noted in any of the animals infected with MNV4 alone throughout the experiment.

Discussion

Because MNV is a prevalent virus in research mouse colonies,[16] an important question is how this virus would affect mouse models of human disease. MNV can infect DCs and macrophages and therefore may alter immune responses, suggesting it has the potential to affect mouse models involving inflammation and other immune responses. To our knowledge, this report is the first that directly tests the effect of MNV4 on a mouse model of IBD.

Results of our studies indicate that MNV4 is an intercurrent variable in a bacteria-induced immunocompetent mouse model of IBD. MNV4 accelerated weight loss and development of IBD in *Mdr1a*–/– mice coinfected with both MNV and *H. bilis*. Severe IBD correlated with lymphangitis and vasculitis in coinfected animals, indicating that MNV infection resulted in more severe disease overall in *Mdr1a*–/– mice. The severe lymphangitis and vasculitis seen in the colonic mesentery MLN may be due to either direct cytopathic effects of the virus, an immune response to the virus that is localized in these regions, or, more likely, an overall more severe nonspecific inflammatory response. Although we did not see clinical signs of inflammation in *Mdr1a*–/– mice that were singly infected with MNV4 as long as 34 d after infection, MNV has been associated with subclinical histopathologic changes, including increased granulocytes in the intestine at 24 h after infection in experimentally infected immunocompetent mice[26] and hepatitis in naturally infected immunodeficient mice.[35] Consistent with these observations, we here demonstrate that MNV4 infections induce alterations in immune cell populations, cytokines

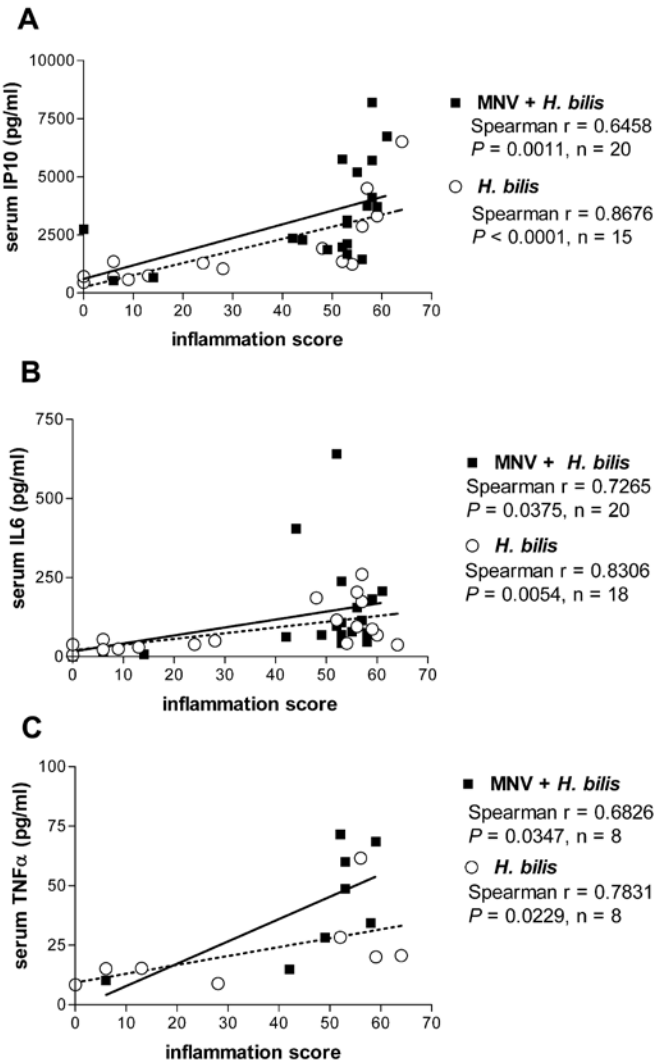

Figure 7. Serum cytokine levels are altered in *Mdr1a-/-* mice coinfected with MNV4 and *H. bilis* (solid line) compared with mice mouse infected with *H. bilis* only (dotted line). The Spearman rank test shows that serum (A) IP10, (B) IL6, and (C) TNFα levels positively correlate with IBD scores at 3 wk after infection in both singly infected and coinfected mice. The slope of the TNFα linear regression line is significantly ($P < 0.01$) greater in coinfected animals (0.94 ± 0.41, n = 8) than in singly infected mice (0.37 ± 0.22, n = 8).

associated with immune function, and in vitro functional assays of various immune cells—these results may explain the altered disease phenotype in our IBD model.

Increasing evidence indicates that DCs, including plasmacytoid DCs, are important in the regulation of tolerance in the gut.[11] Plasmacytoid DCs ($CD11c^{low}$) are important mucosal regulators involved in innate responses to viral infection and major producers of type 1 interferons.[2,17,40] Our data illustrate that MNV4 infection results in alterations in immune cell populations, including changes in $CD11c^{low}$ cells, that varied depending on how long the MNV4 infection had been present. These changes were not restricted to the draining lymph nodes of the intestine but occurred in the spleen as well. Early in infection (2 d after infection), we did not detect significant changes in cell populations in either the MLN or spleen, a finding that may be related to high mouse-to-

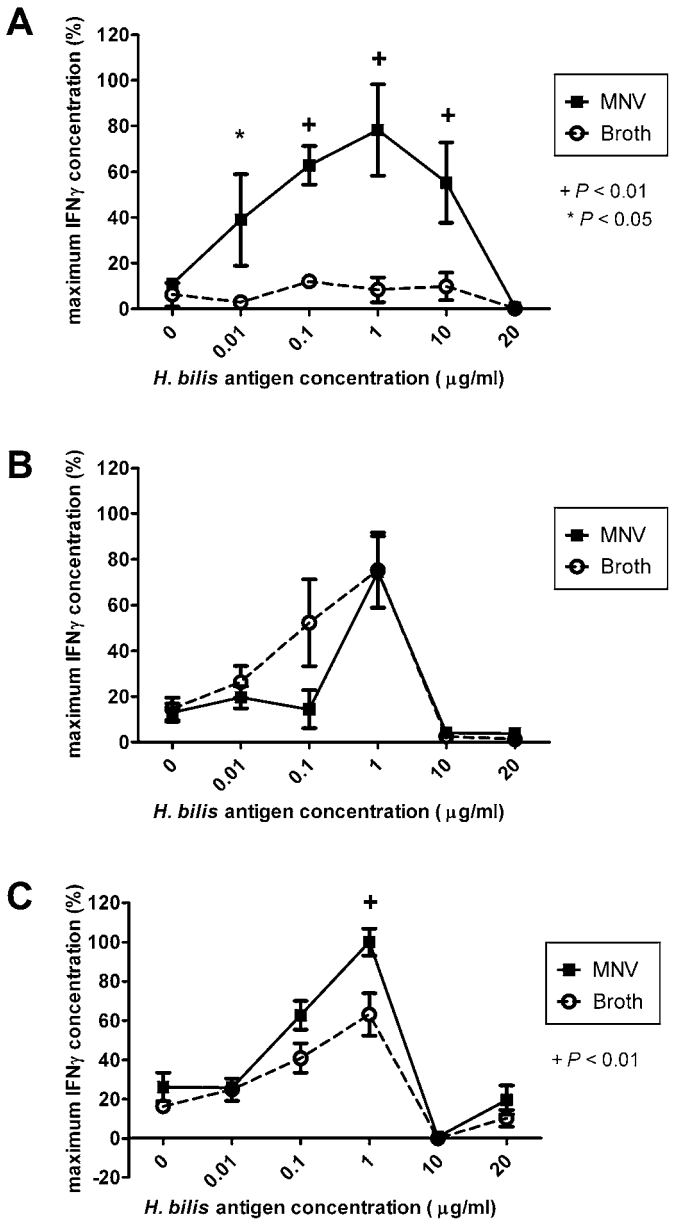

Figure 8. MLN DCs isolated from mice infected with MNV were incubated for 72 h in vitro with *H. bilis*-primed polyclonal T cells and *H. bilis* antigen at various concentrations. (A) At 2 d after infection, DCs from infected mice show greater stimulatory capacity than do those from sham-infected animals. The IFNγ response in MNV-infected animals is significantly greater at several antigen doses: 0.01 μg ($P < 0.01$), 0.1 μg ($P < 0.01$), 1 μg ($P < 0.001$), and 10 μg ($P < 0.05$). Data presented are the average of 3 independent experiments. (B) At 7 d after infection, response to *H. bilis* antigen is not significantly different at any antigen concentration. Data presented are the average of 2 independent experiments. (C) At 28 d after infection, response to *H. bilis* antigen is statistically different at 1 μg but not at other points. Data are the average of 2 independent experiments, normalized to maximal T-cell response.

mouse variability at this early time point after infection. However, at 1 wk after infection, overall splenic cellularity increased associated with modest increases in the $CD11c^{low}$ and overall nonT-cell populations. These results agree with previous findings[26] that showed a small increase in $CD11c^{+}$ cells after MNV1 infection at 72 h after infection in 129 mice. It is of interest that we did not appreciate increased staining for F480 or B220 at 2 d after infection in the spleen, in contrast to previous findings at 3 d after infection.[26] This result may reflect a difference between the FVB.129P2-*PAbcb1a*^{tm1Bor} (*Mdr1a–/–*) strain and the 129S6/SvEvTac strain used in previous experiments, the timing of analysis after infection, or in the pathogenesis of MNV strains, because MNV4 is genetically and biologically distinct from other strains of MNV.[14,25,30]

DCs were altered functionally by MNV4 infection. DCs isolated from MNV4-infected mice early after infection stimulated enhanced IFNγ production by T cells in vitro, although these changes did not persist to 7 d after infection. Although the ability of DCs to present antigen to T cells changed at day 2 after infection, the dendritic cell activation markers CD80 or CD86 did not change at any time point. However, other potential markers of DC activation exist and should be explored in future studies. We speculate that changes in the ability of DCs to stimulate T cells to produce proinflammatory cytokines at early time points, in combination with changes in DC cell populations, may contribute to the exacerbation of IBD seen in the *Mdr1a–/–* model.

In response to viral infection, the innate immune system initiates a cytokine–chemokine cascade that influences the degree of inflammation and progression of disease. Our data indicate that proinflammatory cytokine and chemokine expression is altered after MNV4 infection, with concurrent changes in immune cell populations. Serum levels of IP10 and TNFα correlated with IBD scores and were differentially affected in coinfected versus singly infected animals, whereas serum IL6 only correlated with disease. TNFα is a general proinflammatory cytokine made by a number of cells and the increases in this cytokine may reflect the increased lymphangitis–vasculitis and associated inflammation seen in the coinfected animals. IP10 is a CXC chemokine that is induced on several cell types in response to IFNγ and other inflammatory mediators and has been shown to be upregulated in ulcerative colitis patients.[33] Serum levels of 2 other inflammatory cytokines, IFNγ and IL12p70, were decreased on day 7 after infection with MNV alone. This decrease could be associated with a cytopathic effect of the virus on DCs (key producers of IFNγ and IL12). Alternatively, production of these cytokines may have peaked at an earlier time point and then waned, perhaps due to type 1 interferons. A large increase in type 1 interferons, produced early in inflammatory responses, decreases production of IL12 and IFNγ in a feedback loop.[8] We did not evaluate levels of type 1 interferons in this study, although that would be interesting for future studies. In addition, most chemokines and cytokines act locally, so evaluation of tissue levels of these molecules may offer additional clues to the pathophysiology of MNV infection.

Fecal–oral transmission of MNV has been established,[27] and maintaining MNV infections within groups without cross-contamination is essential in studies like those described here. Because we were concerned about the possibility of exposing our uninfected colony mice to MNV4, sentinel serologic surveillance during the study periods was performed monthly for MNV. Dirty bedding sentinels are effective at identifying MNV infection.[24,27] However, the initial sentinel mice did not seroconvert by 12 wk after being placed on the infected rack (data not shown). This result was likely due to insufficient transfer of contaminated bedding into the sentinel cage from the infected mice. After the husbandry technician was retrained to place bedding that specifically

contained fecal material into the sentinel cages, the next set of sentinels seroconverted within 8 wk from the start of dirty bedding transfer from infected cages. None of the sentinels on our MNV4-negative racks tested positive during the study periods (data not shown) To date, we have not detected cross-contamination of MNV from infected to uninfected mice on the same rack or row (data not shown) in our mouse room. This finding suggests that our current specific-pathogen-free techniques are adequate to contain this virus.

MNV is a prevalent and endemic virus in research mouse colonies[16,27] in the United States. At the University of Washington, 58% of selected sentinels tested were seropositive (data not shown). Our studies suggest that MNV4 infection can modulate a mouse model of IBD and is therefore an undesirable variable. The effects of MNV on models may occur early after infection and may be short-lived, but the time of introduction and exposure of a particular mouse to MNV may not be known during natural transmission. Endemic infections may have a different pathogenesis than acute infections, so it is crucial that future studies include investigations into control and prevention along with identification of the effects of chronic, persistent infections. Given the overt effects of MNV on several strains of immunodeficient mice and the subclinical effects on immunocompetent animals such as the *Mdr1a−/−* mouse, researchers should give serious consideration to excluding this organism from studies using immunodeficient strains of mice or those studies involving immunologic responses.

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