Animal Model

Helicobacter bilis Infection Accelerates and *H. hepaticus* Infection Delays the Development of Colitis in Multiple Drug Resistance-Deficient (*mdr1a*–/–) Mice

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***mdr1a*-deficient mice lack P-glycoprotein and spontaneously develop colitis with age. *Helicobacter* spp. are gram-negative organisms that have been associated with colitis in certain mouse strains, but *Helicobacter* spp. have been excluded as contributing to the spontaneous colitis that develops in *mdr1a*–/– mice. We wished to determine whether infection with either *H. bilis* or *H. hepaticus* would accelerate the development of inflammatory bowel disease (IBD) in *mdr1a*–/– mice. We found that *H. bilis* infection induced diarrhea, weight loss, and IBD in *mdr1a*–/– mice within 6 to 17 weeks post-inoculation and before the expected onset of spontaneous IBD. Histopathology of *H. bilis*-induced IBD included crypt hyperplasia, inflammatory cell infiltrates, crypt abscesses, and obliteration of normal gut architecture. Reverse transcription-polymerase chain reaction and Taqman analysis from colonic tissue showed increased transcripts for interferon- γ and interleukin-10 from *H. bilis*-infected colitic *mdr1a*–/– mice. Additionally, mesenteric lymph nodes had increased cellularity with expansion of CD4⁺ and CD8⁺ T cells and B cells and increased proliferation to soluble *H. bilis* antigens with elaboration of interferon- γ , tumor necrosis factor- α and interleukin-10. In contrast, *H. hepaticus* infection of *mdr1a*–/– mice did not accelerate disease but rather delayed the onset of spontaneous colitis which was milder in severity. *mdr1a*–/– mice infected with *Helicobacter* spp. may provide a useful tool to explore the pathogenesis of microbial-induced**

IBD in a model with a presumed epithelial cell “barrier” defect. (Am J Pathol 2002, 160:739–751)

Inflammatory bowel disease (IBD) is thought to result from an aberrant immune response in the intestine, a response potentially triggered by one or more commensal microorganisms.¹ T-cell adoptive transfer studies,^{2–4} as well as genetically modified mouse models^{5–9} suggest that abnormal T cell regulation and/or T cell-cytokine disturbances contribute to IBD pathogenesis.¹⁰ Previously, we have shown that *mdr1a*–/– mice spontaneously develop colitis with age, but they differ from some of the other IBD-susceptible mouse models that have been described. These mice are deficient in the *mdr1a* gene which codes for P-glycoprotein, a membrane efflux pump, expressed in several cell types including intestinal epithelial cells.^{11,12} *mdr1a*–/– mice are immunologically normal¹³ and develop spontaneous colitis presumably due to an intestinal epithelial “barrier” defect.

Helicobacter are microaerophilic gram-negative spiral bacterial organisms¹⁴ that have been associated with hepatitis,¹⁵ hepatocellular carcinoma,^{16,17} and IBD in rodents.¹⁸ Several species of *Helicobacter* infecting rodents have been described,^{19,20} including *H. hepaticus*²¹ and *H. bilis*,^{19,22,23} which have been shown to colonize the liver and intestine and elicit IBD in immunodeficient mice and rats.²⁴ Experimental infection with either *H. bilis* or *H. hepaticus* induces IBD in mice lacking T and B lymphocytes (SCID),^{22,23,25} in mice with cytokine dysregulation (IL-10)²⁶ and in mice with abnormal T cell receptor repertoires (TCR α –/–).²⁷ To better understand how bacteria “trigger” IBD in a genetically susceptible host, we infected *mdr1a*–/– mice with *Helicobacter* spp. We found that *H. bilis*

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accelerated development of IBD in *mdr1a*^{-/-} mice while *H. hepaticus* delayed development of spontaneous disease.

Materials and Methods

Animals

mdr1a^{-/-} and FVB+/+ (FVB) mice were obtained from Taconic Farms (Albany, NY). To determine whether the absence of another membrane transporter was important in a bacterial-induced IBD, we also infected multidrug resistance associated protein null (*mrp*^{-/-}) mice (Taconic Farms) with *H. bilis*. For all studies reported, we used 3- to 6-week-old, female FVB (*n* = 85), *mdr1a*^{-/-} (*n* = 129), and *mrp*^{-/-} (*n* = 20) mice. Foundation stock for all of these strains are Cesarean-derived and populated with an altered Schaedler's cocktail; expansion stock are maintained under specific pathogen-free (SPF) conditions before being shipped. All mice were certified free of *Helicobacter* spp. by the vendor and re-tested on site before each experiment. Cohorts of mice used for experiments were housed at Immunex Corporation (IMNX) and at the University of Washington (UW). Animals were housed in an SPF environment in polycarbonate microisolator cages containing Bed-O-Cob (Andersons, Maumee, OH) and a nestlet. Mice were fed irradiated Picolab Rodent Diet 20 5053 (PMI Nutrition International, Brentwood, MO) and autoclaved, acidified water. All supplies entering animal rooms were autoclaved and rooms were maintained at 70–74°F, 45 to 55% humidity, with 28 air changes/hour 12/12-hour light/dark cycle. To prevent cross contamination of uninfected mice and infected mice, animals were housed in separate cubicles within the same room and cages changed in "uninfected" or "infected" laminar flow hoods (UW) or, uninfected mice and infected animals were housed in separate rooms (IMNX). Sentinel mice were tested quarterly for endo- and ectoparasites, mouse hepatitis virus, mouse parvovirus, and rotavirus and annually for *Mycoplasma pulmonis*, pneumonia virus of mice, reovirus-3, Sendai virus, and Theiler's murine encephalomyelitis virus. Also, quarterly sterile colon samples were screened for *Citrobacter rodentium*, non-lactose fermenting *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., and *Clostridium* spp. (Phoenix Laboratories, Seattle, WA). All animal procedures were

approved by the UW Animal Care and Use Committee and the IMNX Animal Care and Use Committee.

Experimental Design

Before inoculation, mice were determined to be negative for *Helicobacter* spp. by fecal polymerase chain reaction (PCR). *mdr1a*^{-/-} and FVB mice were given *H. bilis* or *H. hepaticus* or broth while *mrp*^{-/-} mice were given *H. bilis* or broth. *Helicobacter* species-specific fecal PCR was done on pooled cage samples taken every 4 to 8 weeks until the end of the study. Mice were given *Brucella* broth alone or *H. hepaticus* or *H. bilis* by oral gavage 3 times over a 1-week period. Mice were infected with 2×10^7 CFU of *H. bilis* or *H. hepaticus* in 0.2 ml volume on each occasion. Mice were weighed weekly and monitored for weight loss, dehydration, and diarrhea. Mice were euthanized by CO₂ or cervical dislocation when they developed diarrhea or 20% body weight loss and samples were taken for histopathology, RNA isolation, or lymphocyte phenotype and proliferation analysis. Fecal samples from both uninfected and infected mice were tested by PCR for cross-contamination with the other *Helicobacter* spp. in infected mice or for absence of *Helicobacter* infection in uninfected animals.

Bacterial Cultures

H. bilis was a natural isolate and kindly provided by L. Riley (University of Missouri, Columbia, MO) and *H. hepaticus* was obtained from the American Type Culture Collection (ATCC 51448). Organisms were streaked onto *Brucella* blood agar plates and grown under microaerobic conditions (90% N₂, 5% H₂, and 5% CO₂) in vented jars (Oxoid, Hampshire, England), and kept at 37°C. Bacteria were harvested and inoculated into flasks containing 150 ml of *Brucella* broth supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). The flasks were placed on a continuous shaker and incubated for 24 to 48 hours at 37°C in microaerobic conditions. The organisms were centrifuged at 10,000 rpm at 4°C for 20 minutes. The resultant pellet was examined by gram stain and phase microscopy for purity, morphology, and motility. Organisms were confirmed to be catalase, urease

Table 1. Conditions for Fecal PCR Reactions

Primer	Sequence (5' to 3')	PCR condition
<i>H. genus-specific</i> H276f H676r	CTA TGA CGG GTA TCC GGC ATT CCA CCT ACC TCT CCC A	PCR mixtures were heated at 94°C for 7 min, followed by 35 cycles, each comprising 45 s at 94°C, 1 min at 53°C, 2 min at 72°C, and final extension at 72°C for 8 min was performed. PCR mixtures were first heated at 94°C for 5 min, then cooled to 61°C, at this time 3 U of Amplitherm DNA polymerase and 5 µl enhancer were added, followed by 35 cycles, each comprising 1 min at 94°C, 2 1/4 min at 61°C, 2.5 min at 72°C, and final extension at 72°C for 7 min was performed. PCR mixtures were first heated at 94°C for 5 min, then cooled to 56°C, at this time 3 U of Amplitherm DNA polymerase and 5 µl enhancer were added, followed by 33 cycles, each comprising 1 min at 94°C, 1.5 min at 61°C, 2.3 min at 72°C, and final extension at 72°C for 5 min 40 s was performed.
<i>H. hepaticus-specific</i> B38 B39	GCA TTT GAA ACT GTT ACT CTG CTG TTT TCA AGC TCC CC	
<i>H. bilis-specific</i> C62 C12	AGA ACT GCA TTT GAA ACT ACT TT GGT ATT GCA TCT CTT TGT ATG T	

Table 2. Scoring System for Histopathological Evaluation of IBD Lesions

Mucosa	Inflammation	Extent*
0, no significant lesions	0, None	0, no significant lesions
1, mild hyperplasia	1, mild inflammation limited to mucosa	1, ≤1%
2, moderate hyperplasia	2, moderate inflammation in mucosa + submucosa	2, ~30%
3, severe hyperplasia, crypt branching or aberrant formation, crypt herniation into submucosa	3, severe inflammation with obliteration of normal architecture, superficial erosions and/or crypt abscesses	3, ~60%
	4, severe inflammation with obliteration of normal architecture, superficial erosions and/or crypt abscesses plus mucosal ulceration	4, ≥60%

*Extent 1, % of intestine affected in any manner; Extent 2, % of intestine affected by the most severe score.

and oxidase positive. The pellet was resuspended in *Brucella* broth and optical density was adjusted to 1.0 (OD₆₀₀) for an estimated 10⁸ CFU/ml.

Generic *Helicobacter* and Specific *H. hepaticus* and *H. bilis* PCR

Fecal samples were analyzed for *Helicobacter* spp., *H. hepaticus*, and *H. bilis* as described previously²⁸⁻³⁰ with slight modifications. For generic *Helicobacter* PCR, 5 µl of unquantitated fecal DNA was used as template, and for *H. hepaticus* and *H. bilis* PCR, 10 µl of unquantitated fecal DNA was used as template for all samples. Primers, their sequences, and PCR conditions used are summarized in Table 1.

Pathology

Liver, MLN, cecum, colon, and rectum were fixed in 10% buffered formalin. The colon was prepared in a "Swiss roll" technique³¹ to evaluate the entirety of the proximal, middle, and distal colon on the same section. The tissues were routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Tissue sections were coded to blind the pathologist (K.W.) to the infection status of the animal. The cecum, proximal colon, middle and distal colon, and rectum from each mouse were scored on severity of mucosal epithelial changes, degree of inflammation and extent of pathology (Table 2). The segment score was derived by summing the severity scores [segment score = mucosal score + inflammation score + extent of intestine affected in any manner (Extent 1) + extent of intestine affected at level 3 or 4 (Extent 2)].²⁶ The total score for each mouse was derived by summing the scores from the individual segments and subsequently, the mean was derived for each treatment group.

Antibodies

The following antibodies were used for flow cytometric analyses: rat IgG2a κ (R35-95), anti-CD45R/B220 (RA3-6B2), anti-Mac-1/CD11b (M1/70), anti-NK1.1 (PK136), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-TCRαβ (H57-597), anti-TCRγδ (GL3), anti-IgD (11-26c.2a), anti-IgM (G155-

228). All mAbs were purchased from Pharmingen, San Diego, CA. The following antibodies were used for antigen-specific serum antibody analyses: anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, anti-IgA, anti-IgG unlabeled and human adsorbed HRP-labeled antibodies were all purchased from Southern Biotechnology Associates, Inc., Birmingham, AL. The following antibodies were used for cytokine analyses: anti-IFN-γ (R4-6A2 and XMG1.2), anti-IL-4 (11B11 and BVD6-24G2), anti-IL-10 (JES5-2A5 and SXC-1) unlabeled and biotin-labeled antibodies were all purchased from Pharmingen, San Diego, CA. TNF-α ELISA kits were purchased from R & D Systems, Minneapolis, MN.

Preparation of *Helicobacter* Antigen: *Helicobacter* Antigens Were Prepared from Cultures of *H. bilis* or *H. hepaticus*

The organisms were harvested as for inoculation, washed in PBS, and sonicated at 4°C for 2 minutes, using 50% duty cycle with output setting 7 (Branson Ultrasonics, Danbury, CT). Lysed suspensions were centrifuged at 10,000 × *g* at 4°C for 20 minutes and the supernatant was 0.22 µm filtered. Protein content was determined by BCA protein assay (Pierce, Rockford, IL) and endotoxin levels were determined by LAL assays. Supernatants were frozen at -20°C until use.

Cell Preparation

MLN or spleens were collected in cold HBSS, 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/L glutamine. Single cell suspensions were prepared by teasing tissues apart in collection medium, followed by filtering through nylon mesh. Cells were washed and resuspended in tissue culture medium (RPMI, 5% FBS, PSG as above, 1 mmol/L sodium pyruvate, 0.1 mmol/L non-essential amino acids, 50 µmol/L β-mercaptoethanol, and 20 mmol/L HEPES) at 10⁷ cells/ml. Prior to final wash, RBCs from spleen were lysed for 5 minutes at room temperature in Tris-ammonium chloride, pH 7.6. Experiments were performed with lymphocyte suspensions pooled from 2 to 4 mice per group.

Table 3. Pathology of Large Bowel Regions in Uninfected *mdr1a*^{-/-} Mice with Spontaneous IBD and FVB Mice

Mouse	Age at necropsy	Cecum	Proximal colon	Middle colon	Distal colon	Rectum	Total score
<i>mdr1a</i> ^{-/-}	4 mo	9 (6–13)	14 (13–15)	14 (12–15)	14 (12–15)	14 (13–15)	65 (62–70)
FVB ^{+/+}	4 mo	0	0	0	0	0	0

Scores are means and ranges derived from 10 mice in each group; maximal score for individual regions is 15 and 75 for total score.

FACs Staining

All FACS staining was performed in covered ice buckets. Cells ($0.25\text{--}1 \times 10^5$ cells/stain) were washed in PBS-FBS (0.1%) then nonspecific binding sites were blocked by incubating the cells for 10 minutes in PBS-FBS with 2% mouse serum, 10% goat serum, and 10 $\mu\text{g}/\text{ml}$ anti-Fc receptor II (2.4G2), followed by another wash in PBS-FBS. Cells were incubated with fluorochrome-labeled antibodies for 30 minutes, washed twice, resuspended in PBS-FBS with 0.5% paraformaldehyde, and stored at 4°C until analysis on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Lymphocyte Proliferation

Lymphocyte suspensions at a density of 10^5 cells/well were cultured in 96-well flat-bottomed plates in a total volume of 0.2 ml/well at 37°C and 5% CO_2 . All cultures were performed in triplicate. Cells were cultured in medium alone with various concentrations of *Helicobacter* antigens (Ags), LPS (*E. coli* 0127:B8, Difco Laboratories, Detroit, MI) at the highest concentration determined for the Ag preparation and 1 $\mu\text{g}/\text{ml}$ solid phase anti-CD3 (mAb 500A2). Proliferative responses were measured by [^3H]thymidine incorporation (1 $\mu\text{Ci}/\text{well}$ [^3H]thymidine, NEN, Boston, MA) during the final 6 hours of a 72-hour incubation. The amount of radioactivity incorporated into DNA was measured using a Matrix-96 cell harvester (Inotech, Lansing, MI) and a direct β counter (Packard, Meridian, CT). The data are reported as the mean counts $\pm$ SD per minute of triplicate wells.

Cytokine Analysis by ELISA

Lymphocytes were cultured exactly as for proliferation assays. All cultures were performed in triplicate. After 48 or 72 hours, plates were centrifuged at 1000 rpm for 10 minutes at 4°C and culture supernatants were transferred to round-bottomed 96-well plates. Samples were stored at -20°C until assays were performed. Levels of soluble mIFN- γ , mTNF- α , mIL-10, and mIL-4 were determined by ELISA. Purified capture antibodies were coated overnight at 4°C onto 96-well ELISA plates (Maxisorp, Nunc, Naperville, IL) at a concentration of 1–2 $\mu\text{g}/\text{ml}$ in 0.1 mol/L NaHCO_3 buffer, pH 8.5. Plates were washed four times with PBS/0.1% Tween-20 (PBST) and nonspecific binding sites were blocked with 10% FBS in PBS for 2 hours at room temperature. Plates were washed four times with PBST, standard dilutions and samples were loaded either neat (mIL-10, mIL-4, mTNF- α) or diluted 1:3 (mIFN- γ) with blocking buffer and incubated overnight at 4°C. Plates

were washed four times with PBST, and biotinylated antibodies specific for mouse cytokines, diluted to 1 $\mu\text{g}/\text{ml}$ with 10% FBS in PBS were added. The plates were allowed to incubate for 2 hours at room temperature, then were washed six times. Streptavidin-horseradish peroxidase (SA-HRP) (Zymed Laboratories Inc., South San Francisco, CA) was added and the plates incubated for 1 hour at room temperature followed by six washes with PBST. Enzyme activity was detected with 3,3',5,5'-tetramethyl-benzidine (TMB) substrate (Kirkegaard & Perry, Inc., Gaithersburg, MD) and development stopped with 1 N phosphoric acid. Optical densities were measured at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA) and protein concentrations were determined by using the Deltasoft Program (BioMetallics Incorporated, Princeton, NJ).

Analysis of Ag-Specific Serum Antibodies

Blood samples were obtained via cardiac puncture at the same time tissues were collected for analyses. Resulting sera samples were stored at -20°C until assays were performed. Determination of *Helicobacter*-specific antibodies in sera was performed by ELISA. *Helicobacter* antigens were coated overnight at 4°C onto 96-well Maxisorp ELISA plates at a concentration of 8 $\mu\text{g}/\text{ml}$ in carbonate buffer, pH 9.5. Plates were washed six times with PBST and nonspecific binding sites were blocked with 10% FBS in PBS for 1 hour at room temperature. Plates were washed six times with PBST, samples were diluted with blocking buffer and incubated for one hour at room temperature, followed by six more washes. Peroxidase-conjugated goat antibodies specific for mouse immunoglobulins were diluted with 50% goat serum in PBS, allowed to incubate for 1 hour at room temperature, and the plates then washed six times. Enzyme activity detection was performed as described above.

RNA and cDNA Preparation from Colon Tissue Samples

Fecal contents were removed and the colon was homogenized in guanidinium isothiocyanate buffer (4.5 mol/L guanidinium isothiocyanate, 50 mmol/L sodium citrate, and 0.5% (w/v) sodium sarcosyl) containing 2% 2-ME (Life Technologies). Two to four colons per group were pooled and total RNA was isolated over a cesium chloride cushion and subjected to phenol chloroform extraction. cDNA was prepared from 5 μg of isolated RNA which was reverse transcribed with Superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in the

presence of 0.3 μ g of random primers, 5X RT buffer, 0.1 mol/L DTT, 10 mmol/L dNTPs, and water.

Cytokine Analysis by Polymerase Chain Reaction

IL-4, IL-10, and IFN- γ mRNA was analyzed using the Mouse TH1/TH2 Switch cytokine kit (Biosource International, Camarillo, CA). PCR reaction tubes contained 5 μ l of cDNA and reagents provided by the kit. The following PCR profile was used for amplification: 96°C for 1 minute, followed by two cycles of 96°C for 1 minute and 59°C for 4 minutes, then 30 cycles of 94°C for 1 minute and 59°C for 2.5 minutes, and a final cycle of 70° for 10 minutes. PCR product and 1 μ l of loading buffer were loaded onto a 1.0-mm, 15-well, 10% TBE precast gel (Novex, San Diego, CA) and run at 126 V. The gel was stained with ethidium bromide and photographed.

Reverse Transcription and Real-Time Quantitative PCR

cDNA was synthesized from 5 μ g of DNA-free treated (AMBION, Austin, TX) total RNA using mixture of 125 ng of oligodT and 37.5 ng of random hexamers and Superscript II reverse transcriptase (Lifetech, Gaithersburg, MD) per manufacturer's instructions. Real-time quantitative PCR analyses were performed starting with 250 ng and 125 ng of reverse transcribed total RNA with 10 pmol of both sense and antisense primers in a final volume of 25 μ l using the SYBR Green PCR Core Reagents on the GeneAmp5700 Sequence Detection System. PCR reactions were cycled 95°C for 10 minutes followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. PCR reactions were also subjected to a heat dissociation protocol. Real-time PCR primers were design with Primer Express (PE Biosystems) software (IFN- γ : 5'-CTC ATC CGA GTG GTC CA-3' and 5'-AGC AGC GAC TCC TTT TC-3'; IL-10: 5'-TTC CAA ATC CTC GGC ATA ATG-3' and 5'-CCA GCG TCG TGA TTA GC-3' and HPRT: 5'-TTC CAA ATC CTC GGC ATA ATG -3' and 5'-GTC CCA GCG TCG TGA TTA GC-3') A standard curve for each gene (HPRT, IFN- γ , and IL-10) was constructed whose concentration was based on the equivalent of the reverse transcribed total RNA input. Following normalization of the cDNA concentrations to HPRT, relative concentration of IFN- γ and IL-10 was calculated for each experimental sample. The data are expressed by showing relative

Figure 1. *mdr1a*^{-/-} mice develop severe, diffuse intestinal inflammation in all parts of the large bowel, while FVB controls show no evidence of IBD. All mice are *Helicobacter* spp. negative and 4 months of age. Spontaneous IBD in the distal colon and rectum (**A** and **E**) of *mdr1a*^{-/-} mice was characterized by epithelial cell hyperplasia, crypt branching, crypt abscesses (not shown), and inflammatory cell infiltrate into the mucosa and submucosa. Significant IBD also occurred in the cecum (**C**) of *mdr1a*^{-/-} mice, although the lesions were milder than that seen in the colon. Interestingly, squamous metaplasia of the rectal mucosa was common (**arrow** in **E**). Mucosal ulceration was found throughout the colon, but was infrequently seen in the cecum. Ulcers in the middle colon (**G**) and rectum (**arrow** in **H**) are shown here. Age-matched, FVB control mice showed no evidence of intestinal inflammation in the colon (**B**) cecum (**D**), or rectum (**F**). Hematoxylin & eosin; original magnifications: **A-D**, **G**, **H**, $\times 10$; **E**, **F**, $\times 4$.

Figure 2. Growth curves (% change in body weight) of *H. bilis*-infected (A) and *H. hepaticus*-infected (C) *mdr1a*^{-/-} mice and their uninfected controls or *H. bilis*-infected (B) and *H. hepaticus*-infected (D) FVB mice with their uninfected controls. Asterisks indicate points of statistical significance (1A: $P = 0.002$ and 0.04 respectively; 1C: $P = 0.0004$, 0.02 , 0.004 , and 0.003 , respectively); Student's *t* test.

concentration of experimental sample to the standard sample.

Statistical Analyses

Differences between mean total pathology scores and body weight in uninfected and *H. bilis*-infected or *H. hepaticus*-infected *mdr1a*^{-/-} mice were evaluated by one way analysis of variance and Student's *t*-test; significance was set at $P \leq 0.05$ (SigmaStat; Jandel Corp., San Rafael, CA).

Results

Spontaneous IBD in *mdr1a*^{-/-} Mice

mdr1a^{-/-} mice with spontaneous IBD were assessed using our pathology scoring system to determine the degree and extent of disease in mice free of *Helicobacter* infection. At 4 months of age, *mdr1a*^{-/-} mice had severe IBD in all portions of the large bowel (Table 3) (Figure 1). In the colon and rectum, the disease was characterized by diffuse epithelial cell hyperplasia, goblet cell loss, and a chronic inflammatory cell infiltrate in the mucosa, submucosa, and occasionally was transmural. The infiltrate was comprised predominantly of macrophages and lymphocytes, with fewer neutrophils (Figure 1A). Intestinal

crypts were often dilated and filled with cellular debris (crypt abscesses) while occasional crypt branching and herniation were also seen (Figure 1A). Interestingly, squamous metaplasia of the rectal epithelium (Figure 1E) was found in all mice. Although significant chronic IBD occurred in the cecum of *mdr1a*^{-/-} mice (Figure 1C), the cecum was involved to a lesser degree and lesions were segmental rather than diffuse as compared to the colon (Figure 1A). While mice showed mucosal ulceration throughout the colon (Figure 1, G and H), cecal ulcers were infrequent. Wild-type FVB mice of similar ages showed no evidence of IBD (Figure 1, B, D, and F).

H. bilis Accelerates Development of IBD in *mdr1a*^{-/-} Mice

Mdr1a^{-/-} mice infected with *H. bilis* at 3 to 4 weeks of age had evidence of diarrhea beginning 3 weeks post-infection (PI) and showed altered weight gains as compared to uninfected *mdr1a*^{-/-} mice (Figure 2A). In contrast, uninfected and *Helicobacter*-infected FVB mice showed no evidence of diarrhea or differences in body weight curves (Figure 2, B and D) and subsequent pathological analysis revealed no evidence of IBD (Tables 4 and 5) (Figure 3B). In *H. bilis*-infected *mdr1a*^{-/-} mice, lesions were found from cecum to rectum and these were more severe than the minimal disease seen in uninfected *mdr1a*^{-/-} mice within the time frame of this study (8 weeks). This was reflected in higher mean total histopathology scores in *H. bilis*-infected *mdr1a*^{-/-} mice (59) than in uninfected *mdr1a*^{-/-} mice (4) (Table 4) (Figure 3). Specifically, *H. bilis*-induced IBD in *mdr1a*^{-/-} mice was characterized by severe chronic-active inflammation with pronounced accumulations of lymphocytes, macrophage, and neutrophils within the lamina propria (Figure 3, A and C). However, only occasionally did the inflammatory infiltrates extend into the submucosa and tunica muscularis. Changes to the colonic mucosa ranged from severe epithelial hyperplasia to loss of crypts and attenuation of the overlying epithelium. Ulcers were rarely seen. Crypt abscesses were present (Figure 3A); however, no crypt herniation or branching were seen. Lesions were similar in the cecum (Figure 3C) but the presence of crypt abscesses was significantly less than that seen in the colon. An additional infection study was done using larger numbers of *mdr1a*^{-/-} mice (Table 6). By 16 weeks PI, 9 of 10 *H. bilis*-infected *mdr1a*^{-/-} mice had developed significant IBD, while only 4 of 15 uninfected

Table 4. Pathology of Large Bowel Regions in *H. bilis*-Infected and Uninfected *mdr1a*^{-/-} and FVB Mice

Mouse	Age at necropsy (mo)	Infection	Time PI (wk)	Cecum	Proximal colon	Middle colon	Distal colon	Rectum	Total score
<i>mdr1a</i> ^{-/-}	2.5–3	<i>H. bilis</i>	6–8	11 (6–13)	11 (6–13)	11 (3–14)	11 (0–14)	10 (0–13)	59** (15–66)
<i>mdr1a</i> ^{-/-}	2.5–3	Broth	6–8	0	2 (0–5)	1 (0–4)	1 (0–3)	0	4 (0–12)
FVB+/+	2.5–3	<i>H. bilis</i>	6–8	0	0	0	0	0	0
FVB+/+	2.5–3	Broth	6–8	0	0	0	0	0	0

Scores are means and ranges derived from 3–5 mice; ** $P = 0.01$ (Student's *t* test). Data are from 1 of 3 experiments with similar results. PI, post-infection.

mdr1a^{-/-} mice had developed spontaneous IBD; disease pathology was similar to that seen in the previous study (data not shown).

H. hepaticus-Infected *mdr1a*^{-/-} Mice Have Less Severe IBD than Uninfected *mdr1a*^{-/-} Mice with Spontaneous IBD

Studies with *H. hepaticus*-infected *mdr1a*^{-/-} mice were longer term than *H. bilis* infection studies since no diarrhea or body weight changes were noted until 15 weeks PI in either the *H. hepaticus* or the uninfected groups. Notably, *H. hepaticus*-infected *mdr1a*^{-/-} mice showed a significantly elevated body weight gain at 15 weeks PI compared to uninfected *mdr1a*^{-/-} mice, which exhibited body weight loss as spontaneous IBD developed (Figure 2C). All mice were 5 months of age at the time of necropsy. Unexpectedly, *H. hepaticus*-infected mice had less severe disease in all portions of the large bowel as indicated by lower mean total pathology scores (22) compared with uninfected *mdr1a*^{-/-} mice with spontaneous disease (63) (Table 5). When disease developed, IBD in the colon of *H. hepaticus*-infected *mdr1a*^{-/-} mice was characterized by moderate epithelial hyperplasia and inflammatory cell infiltrate in the lamina propria which occasionally extended into the submucosa (Figure 3E). These lesions were less extensive than that seen in uninfected *mdr1a*^{-/-} mice with spontaneous IBD (Figure 3F) and ulcers were infrequently observed in *H. hepaticus*-infected *mdr1a*^{-/-} mice. These results suggest that *H. hepaticus* infection delayed or attenuated the development of spontaneous IBD in *mdr1a*^{-/-} mice. That *H. hepaticus* infection delays onset and severity of colitis in *mdr1a*^{-/-} mice was confirmed in two additional infection studies which compared the development of spontaneous IBD in uninfected *mdr1a*^{-/-} mice with disease development in *H. bilis* or *H. hepaticus* infection in *mdr1a*^{-/-} mice. In one of these studies, by 27 weeks PI, only 2 of 16 *H. hepaticus*-infected *mdr1a*^{-/-} mice had developed colitis, while 9 of 15 uninfected *mdr1a*^{-/-} mice had developed spontaneous colitis and all 10 *H. bilis*-infected *mdr1a*^{-/-} mice had developed colitis (Table 6). Histopathological analysis confirmed the presence or absence of disease (data not shown).

Figure 3. *H. bilis*-infected *mdr1a*^{-/-} mice develop rapid onset of severe IBD, while *mdr1a*^{-/-} mice infected with *H. hepaticus* have delayed development, and a less severe form, of IBD. Mice infected with *H. bilis* are 6–8 weeks PI and *H. hepaticus*-infected *mdr1a*^{-/-} mice are 15 weeks PI. *H. bilis*-infected *mdr1a*^{-/-} mice showed epithelial cell hyperplasia, crypt loss, extensive inflammatory cell infiltrates into the mucosa sometimes extending into the submucosa, and crypt abscesses (arrow) in the distal colon (A), while no lesions were seen in *H. bilis*-infected FVB mice (B). The cecum was similarly affected (C), although crypt abscesses were rarely seen. Uninfected *mdr1a*^{-/-} mice showed no evidence of disease in the cecum at this time point (D). *H. hepaticus*-infected *mdr1a*^{-/-} mice inconsistently developed intestinal inflammation in the colon within the 15-week time period of a separate study. When present, there was moderate mucosal hyperplasia and inflammatory cell infiltrate into the mucosa, which occasionally extended into the submucosa. A representative lesion from the proximal colon is shown here (E). In contrast, uninfected *mdr1a*^{-/-} mice developed severe diffuse IBD, at this same time point. A representative section from the middle colon is shown here (F). Hematoxylin & eosin; original magnification, $\times 10$ (A–F).

Ulcer Formation Characterizes Spontaneous IBD but Not Helicobacter-Induced IBD in *mdr1a*^{-/-} Mice

The spontaneous IBD that develops in *mdr1a*^{-/-} mice was characterized by ulcers in cecum, colon, and rectum, while *Helicobacter*-induced IBD rarely included ulcer formation. Combining several groups of uninfected spontaneously colitic *mdr1a*^{-/-} mice with total pathology scores of 35 to 72, 24 of 26 animals (92%) had ulcers in multiple sites; their mean age ($\pm$ SD) was 14 ($\pm$ 9.8) weeks at necropsy. However, in a combined group of *H. bilis*-infected colitic *mdr1a*^{-/-} mice with total pathology scores of 44 to 68, only 1 of 20 mice (5%) had ulcer

Table 5. Pathology of Large Bowel Regions in *H. hepaticus*-Infected and Uninfected *mdr1a*^{-/-} and FVB Mice

Mouse	Age at necropsy (mo)	Infection	Time PI (wk)	Cecum	Proximal colon	Middle colon	Distal colon	Rectum	Total score
<i>mdr1a</i> ^{-/-}	5	<i>H. hepaticus</i>	15	8 (6–13)	5 (0–11)	2 (0–5)	2 (0–4)	5 (0–11)	22** (6–43)
<i>mdr1a</i> ^{-/-}	5	Broth	15	10 (6–13)	11 (9–14)	14 (13–14)	14 (14–15)	14 (12–15)	63 (58–71)
FVB+/+	5	<i>H. hepaticus</i>	15	0	0	0	0	0	0
FVB+/+	5	Broth	15	0	0	0	0	0.5 (0–2)	0.5 (0–2)

Scores are means and ranges derived from 3–4 mice; ***P* = 0.008 (Student's *t* test). Data are representative of 2 experiments with similar results. PI, post-infection.

formation limited to the proximal colon; their mean age was 13.6 ($\pm$ 3.6) weeks at necropsy. A large ulcer in the middle colon and rectum of a *mdr1a*^{-/-} mouse with spontaneous colitis is shown in Figure 1, G and H.

mrp^{-/-} Mice Do Not Develop IBD When Infected with *H. bilis*

mrp^{-/-} mice lack the multidrug resistance-associated protein, *Mrp1*, gene which encodes for another ATP-transport protein.³² *Mrp1* is expressed ubiquitously including the intestinal tract.³³ In contrast to *mdr1a*^{-/-} mice, *H. bilis* did not trigger IBD in *mrp*^{-/-} mice. More than 5 months PI, 10 of 10 *H. bilis*-infected *mrp*^{-/-} mice showed no diarrhea or clinical signs suggestive of IBD. This was confirmed histologically in all 10 mice necropsied (data not shown).

Lymphocyte Changes in *mdr1a*^{-/-} mice Infected with *H. bilis* or *H. hepaticus*

MLN from mice with *H. bilis*-induced IBD had increased cellularity (3.8 $\times$ uninfected) which was due to expansion of CD4⁺ and CD8⁺ T cells, B cells, macrophages, and NK cells (Figure 4, A and C). Interestingly, FVB mice infected with *H. bilis* but not exhibiting IBD also showed increased MLN cellularity (2.4 $\times$) with similar changes in lymphocyte subsets. Mice shown in Figure 4, A and C are approximately 3 months old and uninfected *mdr1a*^{-/-} mice are not yet showing spontaneous IBD. MLN from *mdr1a*^{-/-} mice infected with *H. hepaticus* similarly showed increased cellularity compared with uninfected *mdr1a*^{-/-} mice (1.9 $\times$) which was also due to an expansion of CD4⁺ and CD8⁺ T cells, B cells, macrophages, and NK cells (Figure 4, B and D). However, lymphocyte expansion did not correlate with the presence or severity of IBD since uninfected *mdr1a*^{-/-} mice exhibited severe spontaneous IBD while *H. hepaticus*-infected *mdr1a*^{-/-}

mice showed less severe IBD. Mice shown in Figure 4, B and D are approximately 5 months old. Similar to *H. bilis*, FVB mice infected with *H. hepaticus* and not exhibiting IBD had increased MLN cellularity (2 $\times$).

Lymphocyte Cultures from Infected FVB and *mdr1a*^{-/-} Mice

Mdr1a^{-/-} mice with *H. bilis*-induced colitis had increased lymphocyte proliferative responses to *H. bilis* antigens compared to the lower proliferative responses seen in cells from non-colitic *H. bilis*-infected FVB mice (Figure 5, A and C). *H. hepaticus*-infected *mdr1a*^{-/-} mice with mild colitis exhibited lymphocyte proliferative responses that were similar to non-colitic *H. hepaticus*-infected FVB mice (Figure 5, B and D). Supernatants derived from lymphocyte cultures from *H. bilis*-infected *mdr1a*^{-/-} mice showed elevated levels (ng/ml) of IFN- γ and detectable levels (pg/ml) of TNF- α and IL-10 with a dose response to *H. bilis* antigens (Figure 6, A–C).

Infected Mice Mount Helicobacter-Specific Antibody Responses

Mdr1a^{-/-} mice showed increased serum immunoglobulin levels in response to *Helicobacter* infection but there were some differences in responses between *H. bilis*-infected colitic mice and *H. hepaticus*-infected mice with mild to no colitis. Figure 7 shows serum immunoglobulin levels from *H. bilis*-infected mice taken at 58 days post-infection (left panels) compared with *H. hepaticus* infected mice taken at 17 weeks post-infection (right panels). *H. bilis* infected *mdr1a*^{-/-} mice showed elevated serum IgA, IgG1 and particularly IgG2a (left panels). Despite the absence or development of less severe colitis, *H. hepaticus*-infected *mdr1a*^{-/-} mice also mounted an *H. hepaticus*-specific immunoglobulin response which was primarily of the IgG2a isotype (lower right panel). It is noteworthy that *H.*

Table 6. *mdr1a*^{-/-} Mice Developing Colitis after Infection with *H. bilis*, *H. hepaticus*, or Broth

Mouse	<i>n</i>	12 wk PI, colitic/ non-colitic	16 wk PI, colitic/ non-colitic	27 wk PI, colitic/ non-colitic
<i>mdr1a</i> ^{-/-} + broth	15	2/13	4/11	9/6*
<i>mdr1a</i> ^{-/-} + <i>H. bilis</i>	10	8/2	9/1	10/0
<i>mdr1a</i> ^{-/-} + <i>H. hepaticus</i>	16	2/16	2/16	2/16

*4 of 6 non-colitic *mdr1a*^{-/-} + broth mice were euthanized early in the study to obtain samples for cytokine analysis. PI, post-infection; *n*, number of mice in each group.

Figure 4. Lymphocyte cellularity and phenotype in MLN from *mdr1a*^{-/-} and FVB mice. Cellularity is increased in the MLN of colitic *mdr1a*^{-/-} mice infected with *H. bilis* compared with non-colitic uninfected *mdr1a*^{-/-} and infected and uninfected FVB mice (A). Colitis in *H. bilis*-infected *mdr1a*^{-/-} mice is associated with expansion of T and B cells, macrophages, and NK cells (C). Cellularity is also increased in mildly colitic *H. hepaticus*-infected *mdr1a*^{-/-} mice relative to uninfected *mdr1a*^{-/-} mice with severe colitis (B). *H. hepaticus*-infected mice with mild colitis have marked expansions of T and B cells, macrophages, and NK cells as compared with uninfected *mdr1a*^{-/-} mice with severe colitis (D). Due to kinetics of clinical signs, uninfected (broth) mice were non-colitic at the time *H. bilis* animals became sick, yet had developed spontaneous colitis by the time *H. hepaticus* recipients were harvested. *H. bilis* infection study (A, C) was done at 41 days post-infection and is representative of three experiments. *H. hepaticus* infection study (B, D) was done once at 17 weeks post-infection. Numbers reflect means derived from pooled MLN of 2–4 mice in each group except FVB *H. hepaticus* (*n* = 1).

bilis infected FVB mice had minimal to no detectable *H. bilis* specific immunoglobulin responses while *H. hepaticus*-infected FVB mice had substantial *H. hepaticus*-specific IgG1 and IgG2a elevations (right panels). The lack of serum immunoglobulin responses in *H. bilis* infected FVB mice may be related to the duration post-infection which was 58 days with *H. bilis* infections and 17 weeks with *H. hepaticus* infections. *H. hepaticus*-specific IgG responses in A/JCr mice are not detectable until 16 weeks post-infection.¹⁶

Figure 5. Lymphocyte proliferation in *H. bilis*-infected and *H. hepaticus*-infected *mdr1a*^{-/-} and FVB mice. MLN cells from *mdr1a*^{-/-} mice with *H. bilis*-induced colitis have increased proliferative responses to *H. bilis* antigens (A). These cells are also responsive to polyclonal stimulation via anti-CD3 (C). *H. hepaticus*-infected *mdr1a*^{-/-} mice with mild colitis have lymphocyte proliferative responses that are similar to non-colitic *H. hepaticus*-infected FVB mice (B and D). Numbers reflect means and standard deviations comprising triplicate wells of pooled MLN from 2 to 4 mice in each group except FVB *H. hepaticus* (*n* = 1).

Figure 6. Levels of IFN-γ (A), TNF-α (B) and IL-10 (C) in supernatants derived from *H. bilis* stimulated lymphocytes from *H. bilis*-infected *mdr1a*^{-/-} colitic and FVB non-colitic mice. MLN cells were cultured for 72 hours, then supernatants were harvested and surveyed via ELISA. Following exposure to *H. bilis* antigens, cytokines were produced in a dose-dependent manner. Immunoregulatory cytokines were only detected in cells from *H. bilis*-infected *mdr1a*^{-/-} animals. Numbers reflect means and standard deviations comprising triplicate wells of pooled MLN from 2 to 3 mice in each group.

Cytokine mRNA Levels Are Elevated in Colonic Tissue of *H. bilis* or *H. hepaticus*-Infected *mdr1a*^{-/-} Mice with IBD

Since lymphocyte cultures from lymph nodes draining the bowel of infected *mdr1a*^{-/-} mice indicated that cytokines previously associated with IBD were elaborated, we analyzed colonic tissue for alterations in pro- and anti-inflammatory cytokine mRNA levels. *H. bilis*-infected *mdr1a*^{-/-} mice with colitis showed increased transcripts for IFN-γ, a Th1 cytokine, when compared with uninfected and infected FVB and uninfected *mdr1a*^{-/-} mice not exhibiting colitis. Despite the presence of IBD, *H. bilis*-infected *mdr1a*^{-/-} mice also showed increased transcripts for the down-regulatory cytokine IL-10 by RT-PCR (Figure 8A). This was confirmed quantitatively by

Figure 7. Serum immunoglobulin levels from *Helicobacter*-infected *mdr1a*^{-/-} and FVB mice. Immunoglobulin isotypes (IgA, total IgG, IgG1, IgG2a) for *H. bilis*-infected mice (58 days post-infection) are shown in the left four panels and *H. hepaticus*-infected mice (17 weeks post-infection) are shown in the right four panels. *H. bilis* exposed *mdr1a*^{-/-} animals produce a broad immunoglobulin response, while *H. hepaticus*-exposed *mdr1a*^{-/-} animals express a more restricted repertoire. Numbers reflect means and standard deviations comprising triplicate wells of pooled sera from 2 to 3 mice in each group.

Taqman analysis (Figure 8B). Since *H. bilis* and *H. hepaticus* induced different pathology in *mdr1a*^{-/-} mice, we compared colonic cytokines in *H. bilis*-infected colitic mice, *H. hepaticus*-infected *mdr1a*^{-/-} mice with mild to moderate colitis, and *mdr1a*^{-/-} mice with spontaneous colitis by RTPCR (Figure 8C). Both spontaneous and *H. bilis*-infected colitic *mdr1a*^{-/-} mice showed increases in IFN- γ mRNA; in contrast, *H. hepaticus*-infected *mdr1a*^{-/-} mice had low levels of IFN- γ mRNA. Taqman analysis further verified and quantitated low levels of IFN- γ mRNA and lower levels of IL-10 mRNA in *H. hepaticus*-infected mice with mild to moderate colitis compared with uninfected *mdr1a*^{-/-} mice with severe spontaneous colitis (Figure 8D).

Discussion

The *mdr1a*^{-/-} mouse model of IBD differs from most other mouse models. The majority of other spontaneous colitis models occur in animals bearing a cytokine imbalance^{5,6,34} or T cell defect^{35,36} and hence are very susceptible to immunological dysregulation. *mdr1a* codes for P-glycoprotein, an ATP-membrane efflux transporter that is expressed in the intestinal epithelium and in lymphocytes and is absent in *mdr1a*^{-/-} mice.¹² We have

previously shown that it is a defect in epithelial *mdr1a*^{-/-} expression that leads to IBD susceptibility.¹³ It is not known why *mdr1a*^{-/-} mice develop spontaneous colitis as they age. Sequential fecal microbial analyses over time would need to be done to see if disease development could be related to the gradual acquisition of commensal luminal organisms. Rather than examine the development of spontaneous IBD that occurs in response to the acquisition of enteric microflora, we wished to determine whether *Helicobacter* infection, known to induce IBD in other mouse models, would accelerate and synchronize development of IBD in *mdr1a*^{-/-} mice.

The mechanism by which *H. bilis* accelerates development of IBD in *mdr1a*^{-/-} mice is unclear. P-glycoprotein is a membrane transporter important in drug efflux and its endogenous substrates are unknown. P-glycoprotein is expressed on the apical surface of intestinal epithelial cells and could play a role in protection against bacterial toxins. One possibility is that *Helicobacter*-induced IBD may be mediated via a family of bacterial toxins termed cytolethal distending toxins (CDT) that have been considered virulence factors in certain *Helicobacter* infections.³⁷ Genes encoding for CDT have been identified in *H. bilis*³⁸ and sonicates of *H. hepaticus* have been shown to cause progressive cell distension and cell cycle arrest *in vitro*³⁹ presumably related to their CDT activity. Further compromise of intestinal epithelial barrier function by elaboration of IFN- γ ,⁴⁰ which we noted in *mdr1a*^{-/-} mice with *H. bilis*-induced colitis, may allow more access of intestinal flora to macrophages, dendritic cells, and lymphocytes in the lamina propria to incite an *H. bilis* specific inflammatory response. Studies by Elson et al. have shown that the spontaneous colitis that develops in C3H/HeJBir mice is mediated by CD4⁺ T cells responding to enteric bacterial antigens.⁴¹ The ability to deal with one or more organisms capable of triggering IBD may reside in the intestinal expression of certain critical genes and *mdr1a*^{-/-} mice could be lacking in such critical genes. Recent evidence shows that the introduction of one commensal bacterial organism into gnotobiotic mice can modulate expression of multiple genes, including *mdr1a*.⁴² Although *mdr1a*^{-/-} mice develop IBD independent of *Helicobacter* infection, *H. bilis* infection induces IBD at a much earlier age, and the phenotypic appearance of spontaneous disease is similar, but not identical to *Helicobacter*-induced IBD. In a group of uninfected *mdr1a*^{-/-} mice with spontaneous colitis, 92% showed ulcers in all areas of the colon and rectum while only 5% of *H. bilis*-induced colitic mice, of comparable age and disease severity, exhibited ulcers in one location (proximal colon). This difference in IBD phenotype may be a useful model in sorting out factors associated with ulcer formation in colitis.

The initial characterization of *mdr1a*^{-/-} mice using bone marrow chimeras suggested that these animals are immunologically normal.¹³ Recent studies have shown that *mdr1a*^{-/-} mice have altered intestinal intraepithelial lymphocyte populations associated with altered proliferation and cytokine responses⁴³ which may be contributing to their susceptibility to colitis; however, it may be hard to distinguish if these alterations in cell number and

Figure 8. Cytokine analysis of RNA derived from colonic tissue from uninfected and infected colitic and non-colitic *mdr1a*^{-/-} and FVB mice. RT-PCR shows increased transcripts for IFN-γ and IL-10 from *H. bilis*-infected colitic *mdr1a*^{-/-} mice compared with uninfected non-colitic *mdr1a*^{-/-} and FVB mice (A). Quantitative Taqman analysis of the same samples confirms IFN-γ and IL-10 elevations in *H. bilis*-infected colitic *mdr1a*^{-/-} mice (B). RT-PCR shows cytokine transcripts comparing *mdr1a*^{-/-} mice with colitis (*H. bilis*), spontaneous colitis (uninfected), *mdr1a*^{-/-} mice with moderate colitis (*H. hepaticus*) and *mdr1a*^{-/-} mice with mild to no colitis (*H. hepaticus*) (C). *Mdr1a*^{-/-} mice with *H. bilis*-induced or spontaneous colitis had increased transcripts for IFN-γ and IL-10, while decreased IFN-γ transcripts correlated with the lesser degree of colitis in *mdr1a*^{-/-} mice infected with *H. hepaticus*. One *mdr1a*^{-/-} mouse infected with *H. hepaticus* and exhibiting moderate colitis showed some elevation in IFN-γ transcript levels, while IFN-γ levels were minimal in *mdr1a*^{-/-} mice infected with *H. hepaticus* showing mild or no colitis. Quantitative Taqman analysis of the same samples confirmed IFN-γ and IL-10 elevations in *mdr1a*^{-/-} mice (D); *mdr1a*^{-/-} mouse with *H. bilis*-induced colitis is not shown in D. Representative histopathology samples were taken from *mdr1a*^{-/-} mice infected with *H. hepaticus* at time of RNA harvest. C, severe colitis; MoC, moderate colitis; M, mild colitis; NC, no colitis.

phenotype are indicative of the early IBD process. Characterization of colitis studies in germ-free *mdr1a*^{-/-} mice would help clarify the role of luminal bacteria in mucosal lymphocyte changes with or without colitis but we are unaware of any colitis studies done in germ free *mdr1a*^{-/-} mice. We noted an expected expansion of lymphocyte numbers in draining lymph nodes of *mdr1a*^{-/-} mice but this did not necessarily correlate with colitis. Both uninfected and infected *mdr1a*^{-/-} mice developed expansion of all major lymphocyte subsets in lymph nodes draining the bowel. However, *H. hepaticus*-infected *mdr1a*^{-/-} mice had the greatest expansion of lymphocytes accompanied by the mildest colitis (Figure 4).

Mounting an immunological response to the inciting enteric bacterial antigen correlated with the development of disease in *mdr1a*^{-/-} mice as has been noted in other bacterial-related IBD mouse models^{21,44} as well as in human patients with IBD.⁴⁵ Lymphocytes from *H. bilis*-infected *mdr1a*^{-/-} mice (showing colitis) proliferated in response to *H. bilis* antigens while lymphocytes from *H. hepaticus*-infected *mdr1a*^{-/-} mice (showing mild colitis) did not (Figure 5). *Mdr1a*^{-/-} mice with *H. bilis*-induced

colitis produced elevated serum levels of *H. bilis*-specific IgA, IgG1, and IgG2A (relative to infected FVB mice) at 58 days post-infection while serum levels of *H. hepaticus*-specific IgA, IgG1, and IgG2A of *mdr1a*^{-/-} mice infected with *H. hepaticus* (mild colitis) were similar to or lower than *H. hepaticus*-infected FVB mice at 17 weeks post-infection (Figure 7).

Up-regulation of Th1-mediated cytokines like IFN-γ and TNF-α have been noted in other bacterial models of IBD⁴⁶ and have been reported to characterize *Helicobacter*-induced disease.^{21,26,47} Hence, it is not surprising that IFN-γ is similarly up-regulated in *H. bilis*-induced colitis in *mdr1a*^{-/-} mice. The significance of decreased IFN-γ transcripts from colonic tissue of *H. hepaticus*-infected *mdr1a*^{-/-} mice is unclear. IFN-γ, generally associated with inflammatory processes, may be absent simply because there is minimal disease in *H. hepaticus*-infected *mdr1a*^{-/-} mice. Alternatively, *H. hepaticus* infection may act to down-regulate this inflammatory cytokine and its role in delaying or preventing colitis in this susceptible mouse strain may be important. Determining which of these hypotheses may be true is the focus of ongoing studies. IL-10 is an important cytokine in modu-

lating inflammatory responses in IBD presumably through regulatory T cells as suggested by animal studies.^{48–50} However, human therapeutic trials have had variable success^{51,52} possibly because IL-10 may play different roles depending on the phase of disease development.⁵³ We found that *mdr1a*^{−/−} mice exhibited severe IBD, with or without *H. bilis* infection, that was accompanied by increased colonic IL-10. On the other hand, the apparent delay in IBD development in *H. hepaticus*-infected *mdr1a*^{−/−} mice was not accompanied by similar increases in IL-10, suggesting that this cytokine was not critical in this particular phase of disease development or protection. Future studies are aimed at evaluating the kinetics of IL-10 levels particularly early in *H. hepaticus* infection of *mdr1a*^{−/−} mice.

It is well known that bacterial flora play a role in IBD pathogenesis.^{1,54} Mouse models raised under germ-free conditions or treated with antibiotics confirm the necessity for microbial flora for development of colitis.^{13,55,56} T cell lines activated with enteric bacterial antigens induce colitis⁵⁷ and the presence of a particular bacterial species can prevent disease.⁵⁸ In our studies, the ability of *H. hepaticus* infection to delay the development of IBD in *mdr1a*^{−/−} mice was an unexpected finding and raises questions about the nature of the commensal populations capable of inciting an inflammatory and proliferative response in a genetically susceptible animal. We consider it unlikely that the *H. hepaticus* isolate used was not as pathogenic as the *H. bilis* isolate since we have repeatedly induced severe IBD in IL-10^{−/−} mice using the same *H. hepaticus* and *H. bilis* isolates.²⁶ Studies are underway to determine a) whether *H. hepaticus* infection postpones or prevents the development of spontaneous or *H. bilis*-induced IBD in *mdr1a*^{−/−} mice and b) the nature of the microbial populations that are associated with severe *versus* mild *versus* no IBD in *mdr1a*^{−/−} mice. The use of a mouse model, with a presumed intestinal “barrier” defect due to the absence of intestinal P-glycoprotein, and whose colitis can be modulated with a particular bacterium may have relevance to studying the human disorder^{59,60} and offers a unique opportunity to further dissect IBD pathogenesis.

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