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Critical role for P-glycoprotein expression in hematopoietic cells in the FVB.*mdr1a*^{-/-} model of colitis

Elizabeth M. Staley, PhD^{*,#}, Reed A. Dimmitt, MD, MPH^{*,†,#}, Trenton R. Schoeb, PhD, DVM[‡], Scott M. Tanner[§], and Robin G. Lorenz, MD, PhD^{*,§}

^{*}Department of Microbiology, University of Alabama at Birmingham

[†]Department of Pediatrics, University of Alabama at Birmingham

[‡]Department of Genetics, University of Alabama at Birmingham

[§]Department of Pathology, University of Alabama at Birmingham

Abstract

Objective—P-glycoprotein (P-gp), the functional product of the multi-drug resistance gene (*mdr*), is a transmembrane protein that extrudes substrates from the intracellular environment. P-gp is expressed on the apical surface of epithelial cells and on cells from the hematopoietic lineage. Human *MDR* polymorphisms have been associated with increased risk inflammatory bowel disease, and FVB/N animals deficient in *mdr1a* expression develop spontaneous colitis. Previous studies utilizing adult bone marrow chimeras indicated colitis development in this animal model was contingent on P-gp deficiency in radiation resistant epithelial cells. However, the use of adult animals may mask the role of hematopoietic immune cells in colitis initiation, due to pre-existing epithelial abnormalities.

Methods—To assess the importance of P-gp expression in intestinal epithelial and hematopoietic derived cells on colitis induction in FVB.*mdr1a*^{-/-} animal we developed a neonatal model of bone marrow reconstitution. FVB/N and FVB.*mdr1a*^{-/-} adult and neonatal animals were lethally irradiated and reconstituted with bone marrow from FVB/N or FVB.*mdr1a*^{-/-} donors. Animals were observed for 20 weeks.

Results—Adult FVB/N animals deficient in P-gp expression in hematopoietically derived immune cells developed colitis similar to adult animals deficient in P-gp expression in radiation resistant epithelial/stromal cells. However, neonatal animals deficient in P-gp expression in hematopoietically derived immune cells developed a more histologically significant colitis than those deficient in P-gp expression in epithelial tissue.

Conclusions—The use of a neonatal model of bone marrow reconstitution has revealed a critical role for P-gp expression in hematopoietically derived immune cells in colitis development in the FVB.*mdr1a*^{-/-} model.

Keywords

P-glycoprotein; bone marrow chimera; colitis; neonatal

Address correspondence and reprint requests to: Dr. Robin G. Lorenz, Department of Pathology, University of Alabama at Birmingham, 1825 University Blvd., SHEL 602, Birmingham, AL 35243-2182. Phone: 205-934-0676. Fax: 205-996-9113. rlorenz@uab.edu.

[#]Drs. Staley and Dimmitt share authorship equally.

INTRODUCTION

P-glycoprotein (P-gp), is a glycosylated transmembrane protein known to pump a variety of amphipathic and hydrophobic substrates from the intracellular environment (1, 2). P-gp, the functional product of the multidrug resistance genes (*MDR*, *ABCB1*), is a member of the ATP-binding cassette super-family of transporters. It is expressed at multiple locations including the apical surface of epithelial cells in the colon and small intestine, on epithelial cells of the pancreas, kidney, and adrenal glands, on endothelial cells at the blood brain barrier, and on cells of the hematopoietic lineage (3–5). Expression of *MDR* polymorphisms has been associated with the development of inflammatory bowel disease (IBD) in European and Slavic patient populations; and is associated with decreased P-gp expression and function (6–8).

FVB/N animals deficient in expression of *mdr1a* develop a spontaneous, unremitting colitis, characterized by diarrhea, rectal prolapsed, and death (9–11). Treatment of these animals with antibiotics ameliorates colitis when administered either prophylactic or therapeutically, confirming the role of the intestinal microbiota in inducing intestinal inflammation (9). Disease incidence rates have been shown to vary dramatically between animal colonies, ostensibly as a result of differences in bacterial composition within animal facilities (9–11).

To assess the role of the epithelium and other radiation resistant cell lineages versus bone marrow derived immune cells in mitigating the inflammatory response and resultant colitis, previous studies used adult bone marrow chimeras. Adult FVB and FVB.*mdr1a*^{-/-} animals were lethally irradiated and reconstituted with bone marrow from FVB, or FVB.*mdr1a*^{-/-} donors. Results indicated that only FVB.*mdr1a*^{-/-} recipients developed colitis over a period of 38–52 weeks, highlighting the importance of P-gp expression in epithelial cell populations in preventing disease development (9). Unfortunately, these experiments were conducted in a facility with limited disease penetrance (20–25%). We hypothesized these experiments might have dramatically different results when conducted in a facility with greater incidence of disease/different constituent colonic flora.

It has been shown that FVB.*mdr1a*^{-/-} animals demonstrate increased barrier permeability at an early stage of development, prior to the onset of colitis; in correlation with decreased phosphorylation of proteins involved in maintaining intestinal tight junctions (10). This data suggests that P-gp deficiency interferes with the formation of tight junctions in the intestinal epithelium, ostensibly causing a leaky barrier, and ultimately colitis.

However, it has also been shown that FVB.*mdr1a*^{-/-} animals express increased levels of inflammatory cytokines such as INF- γ and IL17 early in development (11, 12). *In vitro* experiments using colonic epithelial cell lines demonstrate that TNF α , and IFN γ have profound effects on expression and localization of proteins at the tight junctions, which serve to fuse intestinal epithelial cells, and regulate intestinal integrity (13–16). Increased colonic barrier permeability, fosters penetration of luminal bacteria, and antigens promoting colonic mucosal injury and inflammation (17). Elaboration of inflammatory cytokines at an early developmental time point may induce barrier deficiencies, potentially predisposing FVB.*mdr1a*^{-/-} mice to develop IBD as a result of aberrant responses leading to increased bacterial exposure. Previous experiments were conducted using only adult animals, and thus the impact of P-gp deficiency on cell function during early post-natal development remains unclear.

We hypothesize that in order to more accurately assess the responsibility of the intestinal epithelial cells in promoting susceptibility to colitis in FVB.*mdr1a*^{-/-} animals, it is necessary to reconstitute animals early in development, prior to any changes in the cytokine milieu. To

this end we have developed a neonatal model of bone marrow reconstitution for adaptation in models where early developmental changes may play a role in experimental out-come.

MATERIALS AND METHODS

Animals

FVB.129P2-*Abcb1^{tm1Bor}*N7 (FVB.*mdr1a*^{-/-}) were originally derived by Schinkel et al., and they, as well as the FVB/NTac wildtype controls) were purchased from Taconic Farms, Inc. (Hudson, NY) (18). Animals were bred and maintained in Thoren Isolator racks (Hazleton, PA) under positive pressure in specific pathogen free (SPF) conditions. Animals were provided sterile drinking water and autoclaved NIH-31 rodent diet (Harlan Teklad, Madison, WI) ad libitum. *Mdr1a* expression was confirmed as previously described by periodic genotyping utilizing polymerase chain reaction (PCR). In brief DNA was extracted using Sigma's RED Extract-N-Amp tissue PCR kit (St Louis, MO) from mouse tail clippings. *Mdr1a* phenotype expression was confirmed as indicated by Taconic using the primers MDR1A S2 CTCCTCCAAGGTGCATAGACC, MDR1A W2 CCCAGCTCTTCATCTAACTACCCTG, and MDR1A K02 CTTCCCAGCCTCTGAGCCCAG to amplify DNA using the PCR settings 1 cycle at 95° for 15 minutes, 35 consecutive cycles of 94° for 45 seconds, 60° for 1 minute, and 72° for 1 minute, followed by 1–5 minute cycle at 72°. PCR products were then run on a 1.5% agarose gel, and presence or absence of *mdr1a* gene expression is confirmed by analyzing expression of amplification products (Invitrogen, Carlsbad, CA).

Animals were monitored for presence of colitic symptoms such as weight loss, diarrhea, and/or fecal blood. All experiments were approved by the Institutional Care and Use Committee of the University of Alabama at Birmingham. SPF conditions at UAB include absence of the following organisms, as determined by serological screening: mouse parvoviruses, including MPV-1, MPV-2, and minute virus of mice; mouse hepatitis virus, Theiler's murine encephalomyelitis virus; mouse rotavirus (epizootic diarrhea of infant mice), Sendai virus; pneumonia virus of mice; reovirus; *Mycoplasma pulmonis*; lymphocytic choriomeningitis virus; mouse adenovirus; ectromelia (mousepox) virus; K polyoma virus; and mouse polyoma virus. Testing and other methods were as described at <http://main.uab.edu/Sites/ComparativePathology/surveillance/>.

Irradiation and Reconstitution

Adult (age between 2 and 4 months) and neonatal (day 7) FVB.*mdr1a*^{-/-} and FVB/NTac wild type recipient animals were exposed to a lethal dose (900 RADs) of gamma irradiation using the GC40 γ -irradiator containing a cesium 137 core. Irradiated mice were reconstituted with donor bone marrow at a ratio of 1 donor providing for 2 recipients, or approximately 3×10^6 cells injected intraperitoneally (i.p.) in neonates, or retro-orbitally in adults. Irradiated animals were given with Bactrim (sulfamethoxazole/trimethoprim) in drinking water for 10 days following irradiation. Animals were monitored for signs of distress; weight loss, fecal blood, or diarrhea. Animals were sacrificed at 20 weeks of age (for neonatal reconstitution) or 20 weeks following reconstitution (for adult reconstitution), or subsequent to losing more than 20% of maximal achieved body weight (if prior to the 20 week timepoint).

To generate bone marrow, FVB.*mdr1a*^{-/-} and FVB/N donor animals were anesthetized with isoflurane and euthanized by cervical dislocation. Tibia and femurs were harvested and stripped of fat and muscle. Marrow was flushed from bone using a 26 gauge needle into HBSS+ (Hanks buffered saline solution containing 25mM HEPES, 1% Hyclone defined fetal bovine serum, and 1% penicillin/streptomycin (Fisher, Pittsburgh, PA)). The cell

suspension mechanically dispersed by passage through a 26 gauged needle, suspension was then filtered using a 70 μ M Nitex® membrane (Tetko, Elmsford, NY) to remove debris. Cells were washed twice in R1.5 (RPMI 1640, 1.5 gms bovine serum albumin (Fisher), 1% Glutamax, and 5mg Gentamicin (Invitrogen, Carlsbad, CA) and counted. Activated T-cells were depleted using an Thy 1.2 antibody and rabbit compliment (Cedarlane, Burlington, Ontario, Canada). Cells were again washed twice in R10, and counted. Cells were resuspended at in PBS at 50ul/recipient for neonates or 100ul/recipient for adults and were injected into lethally irradiated recipients.

Histology/Injury Scoring

At time of sacrifice mice were anesthetized with isoflourane and then euthanized by cervical dislocation. Distal small intestine, colon, and cecal segments were flushed with PBS and the colon was assessed for changes in weight and length. The tissue was then opened longitudinally, and oriented as strips, mucosa up in tissue cassettes. Tissue was fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue was cut into 5 μ m sections and stained with standard H&E for histologic examination. Experimental conditions were concealed until after the slides were examined. Cecum, proximal colon, and distal colon were evaluated separately. For each segment, crypt epithelial hyperplasia, goblet cell loss, superficial and crypt epithelial degeneration and loss, crypt exudate, inflammatory cell accumulation in lamina propria and submucosa, submucosal edema, mucosal ulceration, transmural inflammation, fibrosis, and dysplasia were evaluated. Severity of each change was scored 0, 1, 2, or 3 for absent (normal), mild, moderate, and severe, respectively. The distribution of each change present also was scored 1, 2, 3, or 4 for 25%, 25%–50%, 50%–75%, or 75%–100%, respectively, of the segment affected. Lesion scores for each segment were calculated as the sum of severity scores multiplied by distribution scores, with changes indicating severe inflammation or injury, including crypt epithelial degeneration, ulceration, transmural inflammation, and dysplasia, weighted by a factor of 1. An overall colonic lesion score was calculated as the average of the scores for the two colonic segments.

Barrier Permeability

Barrier permeability was assessed by analyzing serum concentrations of FITC-dextran following oral gavage as described (19). Briefly, mice were orally gavaged 4 hours prior to sacrifice using an 80mg/ml stock of FITC-dextran, for a total dose of 60mg/100g of body weight (MW 4,000; Sigma). Total blood volume was collected at sacrifice via cardiac puncture. Neonatal animals were pooled with 2 animals providing one sample. Blood samples were coagulated at 4° in the dark for one hour, prior to centrifugation and serum harvest. Samples were analyzed using the Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek Technologies, Winooski, Vermont) and standardized to FITC loaded control serum, FITC was detectable between 25-0.195ng/ml.

RNA Isolation

RNA was isolated from colonic tissue using Trizol® as previously described by Chomczynski et al (Invitrogen®) (20). Contaminating genomic DNA was removed from tissue samples using the Turbo DNA-free kit® available from Applied Biosystems® (Foster City, CA). The Transcriptor First Strand cDNA Synthesis Kit® was utilized to synthesize cDNA from purified RNA samples (Roche®, Pensburg, Germany). Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using Applied Biosystems® gene specific primer probe sets in combination with TaqMan Universal PCR Mix® (Invitrogen®). RNA expression was quantified by calculating the threshold of detectable fluorescence as provided by the RT cycler MX3000P® (Stratagene®, La Jolla, CA). Fluorescence thresholds were averaged generating a gene specific numeral which then could be normalized to the average expression of the 18S housekeeping gene and further

stratified by particular strain and experimental condition. We utilized the 18S housekeeping gene as the gene of choice for this study as a result of recent publications indicating its relative stability or expression under inflammatory conditions (21–23). Gene expression was calculated as an average fold change when compared to control strain values, and shown on a log 2 scale as fold changes from the control baseline (=1). The protocol for this data analysis format is provided in the Applied Biosystems manufacturer's instructions (4371095 Rev A, PE Applied Biosystems). Data has been considered physiologically relevant if alterations in expression levels exceed a 2 fold change from control strain values.

Statistical Analysis

Statistical analysis was performed using the Quick Calcs® program available from Graph Pad® (La Jolla, CA). Statistical analysis for continuous data was performed using an unpaired Student's T-Test, or the Dunn's Multiple Comparisons Test. Statistical Analysis for weight comparisons and survival was performed using the Mantel-Cox Test. Comparisons having a $P < 0.05$ were considered statistically significant.

RESULTS

An Adult Model of Bone Marrow Reconstitution

The incidence of spontaneous colitis in the facility where initial bone marrow reconstitution experiments were conducted was 20–25% in FVB.*mdr1a*^{-/-} (9). We have previously shown that in our facility colitis penetrance is 100%, causing a 50% mortality rate as early as 8 months of age (11). Given the discrepancies in reported disease incidence rates between animal facilities, we initially chose to recapitulate the original adult bone marrow reconstitution studies conducted by Panwala et. al.(9).

Four groups were used in the course of this study. All donor mice were males 6–8 weeks of age, and all recipient mice were 2–4 month old lethally irradiated females. Group 1A consisted of FVB/N mice reconstituted with bone marrow from FVB/N mice (Wild Type into Wild Type, WT $\Rightarrow$ WT). Group 2A contained FVB/N recipients and FVB.*mdr1a*^{-/-} bone marrow donors (Knock Out into Wild Type (KO $\Rightarrow$ WT). Group 3A consisted of FVB.*mdr1a*^{-/-} mice reconstituted with FVB/N bone marrow (WT $\Rightarrow$ KO). Finally, Group 4A contained FVB.*mdr1a*^{-/-} mice reconstituted with bone marrow from FVB.*mdr1a*^{-/-} (KO $\Rightarrow$ KO).

As expected Group 1A (WT $\Rightarrow$ WT) animals maintained stable growth curve following exposure/radiation. Also, unsurprisingly Group 4A (KO $\Rightarrow$ KO) demonstrated significantly reduced weight shortly following reconstitution, and an increased mortality over the course of the experiment (Fig. 1A & B). Interestingly, both Group 3A (WT $\Rightarrow$ KO, those expressing P-gp only in hematopoietically derived cells), as well as Group 2A (KO $\Rightarrow$ WT, those expressing P-gp only radiation resistant epithelial/stromal cells) demonstrated stable growth curves following reconstitution (Fig. 1A).

Histological Evidence of Colitis in Adult Reconstituted Mice

To evaluate colitis development at a microscopic level, tissue was harvested at sacrifice for histological evaluation. Tissue was evaluated and histologically scored in a blinded fashion based on evidence of crypt epithelial hyperplasia, goblet cell loss, superficial and crypt epithelial degeneration and loss, crypt exudate, as well as inflammatory cell accumulation in lamina propria and submucosa, and incidence of mucosal ulceration. As indicated by weight data, Group 1A (WT $\Rightarrow$ WT) were macroscopically and histologically free of disease; while Group 4A (KO $\Rightarrow$ KO) demonstrated significant disease (Fig. 2A & D–F). As indicated by Panwala et al., FVB.*mdr1a*^{-/-} animals reconstituted with FVB/N bone marrow (Group 3A)

demonstrated colonic disease (Fig. 2C, E–F) (9). However, in contrast to that report, FVB/N animals reconstituted with bone marrow from P-gp deficient animals (Group 2A) also developed significant colonic disease; indicating that hematopoietic derived cells also play a role in the development of spontaneous disease in FVB.*mdr1a*^{-/-} mice (Fig. 2B, E–F).

A Neonatal Model of Bone Marrow Reconstitution

Our adult data demonstrates that animals deficient in P-gp expression both in radiation resistant epithelial cells or hematopoietically derived immune cells go on to develop spontaneous colitis. However, these experiments fail to take into account potential pre-existing epithelial damage already present in adult animals, or developmental changes that occur in animals during the neonatal period; thus, we chose to evaluate chimeras generated during the neonatal period. It has been proposed that exposure to radiation in the course of generating chimeras, may damage epithelial tissue and predispose animals towards spontaneous colitis; and that these effects may be more profound in neonatal animals whose developing tissue may be more susceptible to the effects of radiation. To evaluate possible mitigating effects of radiation damage on neonatal as opposed to adult tissue, we quantified the effects of exposure to irradiation on barrier integrity in both groups. FVB/N mice at 6–8 weeks and 7 days post-birth were exposed to 900 RADS radiation and were sacrificed approximately 72 hours later. Barrier integrity was evaluated by measuring serum content of FITC-Dextran, four hours following oral gavage. Exposure to radiation had no significant effect on barrier permeability in FVB/N adult or neonatal animals. However, baseline barrier permeability is inherently different between neonatal and adult tissue, with neonatal tissue being significantly more permeable (Fig. 3).

As these experiments demonstrated no differential effects of irradiation on barrier function during the neonatal period, we lethally irradiated and reconstituted with donor bone marrow mouse pups at 7 days of age. Animals were routinely weighed and monitored for physiological symptoms of colitis. Group 4N (KO $\Rightarrow$ KO) animals demonstrated dramatically altered growth and development, indicating reduced weight gain as early as 8 weeks of age, and consistently demonstrated reduced weight from all other experimental groups starting at 12 weeks of age (Fig. 4A). Animals deficient in P-gp expression in hematopoietic or epithelial tissue alone (Groups 2N and 3N, respectively) showed growth and weight gain similar to controls, displaying normal growth from weaning to 10 weeks, and increased weight gain at week 20 (Fig. 4A).

Animals were sacrificed either at 20 weeks after reconstitution or upon evidence of weight loss greater than 20% maximal weight or incidence of fecal blood. Group 4N (KO $\Rightarrow$ KO) demonstrated 100% mortality by 18 weeks after reconstitution (Fig. 4B). Group 3N (WT $\Rightarrow$ KO, animals deficient in P-gp expression in radiation resistant epithelial cells) demonstrated a 20% mortality rate by 20 weeks of age, but this was not statistically different from Groups 1N or 2N (Fig. 4B).

Histological Evidence of Colitis in Neonatally Reconstituted Mice

To evaluate colitis development at a microscopic level, tissue was harvested at sacrifice for histological evaluation. Tissue was evaluated and scored as described above. Group 4N (KO $\Rightarrow$ KO) animals demonstrated significant disease pathology, in both cecal and colonic tissue (Fig. 5D–F), while Group 1N (WT $\Rightarrow$ WT) remained disease free (Fig. 5A, E–F). As suspected by our adult data, 50% of the Group 2N (KO $\Rightarrow$ WT, animals deficient in P-gp expression in hematopoietic cells) also displayed a significant disease pathology developing inflammation in both cecal and colonic tissue (Fig. 5B, E–F). Group 3N (WT $\Rightarrow$ KO, animals deficient in P-gp expression in radiation resistant epithelial cells) displayed lower

histology scores, demonstrating scores not significantly different from control Group 1N (WT $\Rightarrow$ WT) (Fig. 5C, E–F).

Gene Expression of Inflammatory Cytokines in Neonatally Reconstituted Animals

It has been previously shown that FVB.*mdr1a*^{−/−} animals display increased levels of inflammatory cytokines prior to the onset of colitis. As our data clearly indicates animals deficient in P-gp expression in hematopoietically derived tissue can develop colitis, we wanted to assess whether increased expression of inflammatory cytokines elaborated by these aberrant immune cells may be contributing to the development of disease. RNA was isolated from colonic tissue from neonatal reconstituted animals at sacrifice and RT-PCR analysis was conducted to evaluate gene expression. Gene expression was normalized to expression in Group 1N animals (WT $\Rightarrow$ WT). Not surprisingly, Group 4N (KO $\Rightarrow$ KO) demonstrated increased expression of a panel of inflammatory cytokines (IL17, IFN γ , IL6, and MIP2) (Fig. 6). Interestingly animals deficient in P-gp expression in hematopoietically derived tissues (Group 2N, KO $\Rightarrow$ WT) expressed levels of all inflammatory cytokines similar to those elaborated by Group 4N (KO $\Rightarrow$ KO), despite demonstrating consistently lower histological scoring. Furthermore, animals deficient in P-gp expression in radiation resistant epithelial/stromal tissue alone (Group 3N, WT $\Rightarrow$ KO) expressed significantly lower levels of inflammatory cytokines when compared to either of the other two groups (Fig. 6). This data supports our hypothesis that P-gp deficiency in hematopoietically derived tissue contributes to the development of spontaneous colitis in the FVB.*mdr1a*^{−/−} animal model, and indeed may lead to disease progression more rapidly than deficiency of P-gp expression in epithelial/stromal tissue alone.

DISCUSSION

Schinkel et al. originally derived the FVB.129P2-*Abcb1*^{tm1Bor}N7 (FVB.*mdr1a*^{−/−}) for the purposes of studying drug extrusion and barrier integrity in epithelial cells of the blood brain barrier (18). The importance of P-gp expression to intestinal function was subsequently realized when it was discovered that FVB.*mdr1a*^{−/−} animals develop a spontaneous unremitting colitis when maintained in a specific pathogen free environment. Efforts to elucidate the nature of this colitis induced by non-pathogenic commensal microbiota indicated it was the result of aberrant reactions directed at the intestinal microbiota; as it was shown that both prophylactic and therapeutic antibiotic treatment were capable of ameliorating disease in this animal model(9).

Initial studies were aimed at characterizing colitis in the FVB.*mdr1a*^{−/−} animals. To clarify the role of cell populations in disease induction Panwala et al. derived adult bone marrow chimeras (9). Reconstituted animals maintained for 38 to 52 weeks indicated that FVB/N animals reconstituted with FVB.*mdr1a*^{−/−} bone marrow remained clinically free of colitis, while FVB.*mdr1a*^{−/−} animals reconstituted with FVB/N bone marrow developed colitis at an incidence rate comparable to that typically observed in FVB.*mdr1a*^{−/−} animals their facility (9). Unfortunately complete histological data from these experiments was never shown, and these experiments took place in a facility where disease incidence was very low, presumably as a result of different constituent microbiota in their animal facility.

Further experiments utilizing this animal model have identified the effects of P-gp deficiency on the intestinal epithelium. FVB.*mdr1a*^{−/−} animals develop altered permeability to molecularly tagged substrates, as early as 4–12 weeks of age, prior to the development of colitis (10). Permeability changes correlate with altered phosphorylation of junctional proteins (10). These data indicates that histological and phenotypic evidence of colitis occurs secondarily to the development of altered intestinal barrier function.

It has recently been shown that FVB.*mdr1a*^{-/-} animals demonstrate an increased expression of inflammatory cytokines IFN γ , and IL17, and an increased responsiveness to LPS, as early as 8 weeks of age, also prior to the development of inflammation (11, 12). *In vitro* studies utilizing human colonic cells clearly indicate that the presence of IFN- γ has a significant effect on localization and expression of tight junctional proteins, dramatically reducing barrier permeability (13, 14, 16). Altered elaboration of inflammatory cytokines, potentially as a result of aberrant immune function in P-gp deficient immune cells could predispose FVB.*mdr1a*^{-/-} animals to develop an inflammatory response to luminal microbiota and subsequent colitis.

Due to this knowledge of altered barrier function prior to colitis development, it was our hypothesis that the previous experiments were influenced by these pre-existing barrier changes in adult animals and may not accurately reflect the individual influences of P-gp deficiency in epithelial vs. hematopoietic-derived cells. As our data clearly suggests that neonatal animals have inherently leakier epithelial barriers when compared to adult animals, it is our supposition that during the neonatal period systemic immune cells would be exposed to more of the intestinal microbiota. Animals lacking P-gp expression in their immune cell populations may respond in an aberrant fashion, leading the development of unregulated inflammatory response. Thus performing the experiments during the neonatal period may provide more insight as to the role of P-gp expression in epithelial and immune cells in disease initiation and progression.

To serve as an appropriate basis for comparison we assessed bone marrow chimeras derived from adult animals in our experimental facility. As previously reported, 100% of Group 4A animals (KO $\Rightarrow$ KO) developed fatal disease; while Group 1A (WT $\Rightarrow$ WT) consistently maintained weight and remained disease free. We went on to show that as expected, animals deficient in P-gp expression in radiation resistant epithelial/stromal cells (Group 3A, WT $\Rightarrow$ KO) display evidence of disease at 20 weeks following reconstitution. However, surprisingly animals deficient in P-gp expression in hematopoietic tissue with normal epithelium (Group 2A, KO $\Rightarrow$ WT) also demonstrated increased mortality and consistently demonstrated histologic evident disease in both cecal and colonic tissue. We believe the difference in our results from those previously published is related to the increased incidence of disease found in our colony, which may be driving disease progression in a more distinct and accelerated fashion than seen in other facilities.

We then compared these results to disease development in animals reconstituted as neonates. As expected animals deficient in P-gp expression in both epithelial and hematopoietic tissue (Group 4N, KO $\Rightarrow$ KO) developed rapidly fatal spontaneous colitis, with animals dying more rapidly than was observed in adults. Additionally FVB/N animals reconstituted with FVB/N bone marrow (Group 1N, WT $\Rightarrow$ WT) gained weight, and were disease free at sacrifice. Animals deficient in P-gp expression in radiation resistant epithelial/stromal cells (Group 3N, WT $\Rightarrow$ KO) demonstrated a non-significant increase in mortality rate, and all animals examined 20 weeks following transplantation showed no histologic evidence of disease. Animals deficient in P-gp expression in hematopoietically derived tissue (Group 2N, KO $\Rightarrow$ WT) showed no increased mortality 20 weeks after transfer, however 50% of the animals demonstrated evidence of histological disease in both the colon and cecum at sacrifice.

One potential mechanism for the increased mortality of animals deficient in P-gp in radiation resistant epithelial cells, and the increased intensity/speed of disease progression in animals lacking P-gp expression in all tissues, is that P-gp may play a role in epithelial cell repair. Studies of *MDR* gene expression in humans has indicated that *MDR* plays a role in regulating cellular apoptosis. P-gp expression and function has been strongly associated with

increased resistance to spontaneous apoptosis(24). Lacking P-gp expression during the sensitive neonatal period may impair intestinal regeneration following exposure to radiation. Furthermore, if cellular ability to undergo apoptosis is impaired, this may participate in the propagation of an unchecked immune response to both non-pathogenic colonic bacterial microbiota.

Our neonatal model clearly demonstrates that the absence of P-gp expression in the intestinal epithelium is not the sole initiator of spontaneous colitis in the FVB.*mdr1a*^{-/-} model. The presence of histologically significant colitis in animals deficient in P-gp expression in hematopoietically derived cells alone clearly indicates a role for immune cells in the initiation and progression of colitis in the FVB.*mdr1a*^{-/-} disease model. Although P-gp functional activity has been shown to be expressed in multiple bone-marrow derived cell lineages, including the majority of NK cells and CD8⁺ cytotoxic T-cells very little is known about its role in the development and function of lymphocytes. What has been reported is that human P-gp can to pump cytokines such as IL2 and IL12, however the functional outcome of this activity is unclear and a second report has questioned this result(25, 26). It has also been demonstrated that P-gp is important in the development and function of both human DCs and monocytes(27). Specifically, P-gp antagonists alter DC and monocyte differentiation and reduce migration out of skin explants. The role of any of these potential mechanisms in the susceptibility to colitis remains under investigation.

Interestingly, data from our neonatal model demonstrates that colitis does not appear to be as severe in animals deficient in P-gp expression in either hematopoietically derived immune cells, or radiation resistant epithelial/stromal cells alone, as it appears to be in animals globally deficient in P-gp expression. Taken together results suggest that there exists a collaborative effect of impaired barrier function initiating and exacerbating an aberrantly excessive immune response driving an ultimately fatal response to the intestinal microbiota.

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Figure 1.

Weight Changes and Mortality in Adult Bone Marrow Chimeras. Mice were 2–4 months of age at reconstitution. Reconstituted animals were monitored for weight loss (A) or increased mortality (animals were sacrificed following loss of approximately 20% of highest body weight) (B). Animals were sacrificed 20 weeks following reconstitution. † indicates an animal died or was sacrificed at this time point. Group 4A was significantly different ($P < 0.05$) from the other groups at or after week 7 (weight loss) and week 15 (mortality).

Figure 2.

Histologic Analysis of Adult Reconstituted Bone Marrow Chimeras. Mice were 2–4 months of age at reconstitution. Representative photomicrographs of H&E stained distal colonic tissue from Group 1A, WT ⇒ WT (A), Group 2A, KO ⇒ WT (B), Group 3A, WT ⇒ KO (C), and Group 4A, KO ⇒ KO (D). Histological scoring data from cecal and colonic tissue from reconstituted animals (E & F). * indicates significance of $P < 0.05$. Reference bar = $1\mu\text{m}$.

Figure 3.

Serum Levels of FITC-Dextran following Irradiation in Neonatal and Adult Animals. Neonatal (day 7 from birth) and 6–8 week adult FVB/N mice were exposed to 900 RADS γ -radiation. 3 days post irradiation animal were orally gavaged with 60mg/100gm of body weight with FITC-Dextran. Total blood volume was collected at sacrifice and serum was evaluated for FITC-Dextran content. N.D. indicates not detected. * indicates significance of $P < 0.05$ vs. the FVB Adult.

Figure 4.

Weight Changes and Mortality in Neonatally Reconstituted Bone Marrow Chimeras. Mice were 7 days of age at reconstitution. FVB.*mdr1a*^{-/-} mice were lethally irradiated and reconstituted with bone marrow from FVB, or FVB.*mdr1a*^{-/-} donors, and monitored for weight loss (A) or increased mortality (B). Reconstituted FVB.*mdr1a*^{-/-} and FVB/N animals were sacrificed upon reaching 5 month of age, following the loss of 20% maximum weight gain, or demonstration of fecal blood. † indicates an animal died at this time point. Weight loss and mortality in Group 4N was significantly different ($P < 0.05$) from the other groups at or after week 14.

Figure 5. Histologic Analysis of Neonatally Reconstituted Bone Marrow Chimeras. Mice were 7 days of age at reconstitution. Representative photomicrographs of H&E stained distal colonic tissue from Group 1N, WT ⇒ WT (A), Group 2N, KO ⇒ WT (B), Group 3N, WT ⇒ KO (C), and Group 4N, KO ⇒ KO (D). Histological scoring data from cecal and colonic tissue from reconstituted animals (E & F). * indicates significance of $P < 0.05$. Reference bar = 1 μm

Figure 6.

RNA expression of Inflammatory Cytokines in Neonatally Reconstituted FVB/N and FVB.*mdr1a*^{-/-} animals. Colonic tissue for RNA isolation was harvested from animals at sacrifice. Target gene expression was normalized to expression of the 18S housekeeping gene, and to the average fold change in gene expression in Group 1N, WT $\Rightarrow$ WT. Data is shown on a log₂ scale. All values demonstrating a 2 fold alteration in gene expression from control values are considered physiologically altered from control values. N=4 in each data set. * indicates significance of P<0.05 vs. Group 1N.