THE EFFECTS OF P-GLYCOPROTEIN DEFICIENCY ON INTESTINAL INTEGRITY AND HOMEOSTASIS

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A DISSERTATION

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P-glycoprotein (P-gp) is an ATP-binding transmembrane pump associated with substrate extrusion in cells of epithelial and hematopoietic lineage. Polymorphisms in multidrug resistance genes (MDR), which encode this protein, are associated with development of inflammatory bowel disease in patient subsets. Furthermore, FVB/N animals deficient in P-gp expression develop spontaneous colitis. Disease in FVB.mdr1a-/- animals is reported to be the result of P-gp deficiencies in intestinal epithelial cells. We developed a neonatal model of bone marrow reconstitution to accurately evaluate the contribution of P-gp deficiencies in either epithelial or immune cell populations. Our data indicate that deficiency of P-gp in epithelial tissue alone is not sufficient to initiate spontaneous colitis.

It has been theorized that P-gp extrudes bacterial ligands from the intracellular environment. To address this we conferred P-gp deficiency onto C67BL/6 mice, in hopes of studying colitic rescue in animals jointly expressing mutations to innate pattern recognition sensors. B6.mdr1a-/- mice were protected from developing colitis. Further analysis demonstrated these animals were resistant to colitis induction by bacterial inoculation with Helicobacter bilis, and by inhibition of prostaglandin synthesis with piroxicam. B6.mdr1a-/- animals demonstrated an increased susceptibility to colitis induction following epithelial disruption using dextran sodium sulfate, indicating a role for P-gp in epithelial maintenance and injury repair.
To evaluate the role of P-gp deficiency in injury repair, B6.mdr1a\textsuperscript{−/−} and B6 mice were lethally irradiated with 12 Gy X-ray irradiation and crypt regeneration was assessed. B6.mdr1a\textsuperscript{−/−} and B6 animals demonstrated cyclooxygenase dependent crypt regeneration following exposure to irradiation. LPS pretreatment is known to induce radioprotection when administered prior to irradiation. B6 and B6.mdr1a\textsuperscript{−/−} animals demonstrated LPS induced radioprotection, however radioprotection was significantly more profound in B6 animals. Following LPS pretreatment and exposure to radiation, B6 animals expressed significantly more IL1\textalpha, and IL22 when compared to B6.mdr1a\textsuperscript{−/−} animals.

We demonstrate that a lack of P-gp expression on lymphocytic populations plays a contributory role in the development of spontaneous colitis. We show that P-gp deficiency renders cells unable to properly respond to systemically applied bacterial stimuli. Additionally, we demonstrate P-gp deficiency may affect rates of epithelial apoptosis, and intestinal injury repair.

Keywords: P-glycoprotein, neonatal, epithelium, injury repair, gastrointestinal, colitis
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INTRODUCTION

Inflammatory Bowel Disease

The term inflammatory bowel disease (IBD) refers to diseases of unknown etiology characterized by recurrent, spontaneous inflammation of the gastrointestinal tract (GI). There are two known IBD phenotypes, ulcerative colitis (UC) and Crohn’s disease (CD). Both diseases initiate mucosal inflammation, but are distinct from one another in disease pathogenesis and progression (66). UC affects colonic tissue; inflammation typically initiates at the rectum, and spreads proximally in an uninterrupted fashion potentially involving the entirety of the large bowel. Inflammation in this model tends to be limited to the mucosa, and takes the form of crypt abscesses. CD has a less homogenous phenotype; inflammation is present in the form of discontinuous inflammatory lesions which may occur at any point throughout the large bowel and distal small bowel. Inflammation in this model is more extensive, displaying transmural inflammation, and is characterized by the formation of granulomas (47). Inflammation in IBD is characterized by increased production of inflammatory cytokines, classically tumor necrosis factor alpha, and interferon gamma (TNFα and IFN-γ). Although IBD is not directly associated with increased mortality, patients suffer not only from diffuse abdominal pain, rectal bleeding and inflammatory lesions; but also crippling extra-
intestinal affects such as growth retardation, weight loss, anemia, joint symptoms, and in pediatric patients, delayed puberty (47).

**The Hygiene Hypothesis**

IBD has been shown to be on the rise specifically in western nations where it affects approximately 100-200 out of every 100,000 people (66). It has been theorized this may be a function of increased hygiene in industrialized nations, and a subsequent lack of exposure to some classes of microorganisms (75). The hygiene hypothesis states that childhood exposure to bacteria, viruses, parasites and/or natural environmental antigens such as pollen and dusts help to regulate or “educate” the immune response, potentially preventing allergic responses, and auto-immune dysregulation (75). A variety of studies have been conducted to investigate the protective effects of exposure to different stimuli. Protection has been demonstrated to result from exposure to natural environmental stimuli such as endotoxin, helminth infection, and lactobacilli (6, 8, 92).

Protection has historically been believed to be conferred as a result of polarization of T-cell subsets. Early childhood exposure to bacterial antigens directs the developing immune system towards a characteristically “pro-inflammatory” T-helper1 response inducing production of IFN-γ and IL12; there-by inhibiting production of the “allergic” T-helper 2 response characterized by production of IL4, 5 and 13. Failure to encounter adequate stimuli during childhood is proposed to lead to weak T-helper 1 biasing, and produce an unrestrained T-helper 2, response leading to allergic reactions and potentially a predisposition for autoimmune disorders (92).
The most recent theory involving mechanisms of hygiene induced protection, termed the “old friends” theory, focuses on production of regulatory cells. This theory is framed around the concept that the immune system, particularly in under-developed nations, is constantly exposed to bacteria; whose constitutive presence induces continuous activation of T-regulatory, and dendritic cells. It goes on to state that increased production of suppressive dendritic cells in intestinal tissue would lead to increased sampling of luminal contents, and a subsequent production of regulatory cells directed at the luminal contents, potential allergens, and self antigen (75).

**Genetics of IBD**

Evidence suggests IBD is the result of genetic predispositions for altered immune function, leading to aberrant immune responses directed at the intestinal microbiota (44). Studies conducted on European twin pairs demonstrate high concordance rates for development of both CD and UC in monozygotic twins (84, 86). In fact a positive family history of IBD is one of the greatest risk factors associated with disease development (15). However, evidence also suggests that while genetic predisposition maybe the largest known contributing factor in IBD development; actual disease initiation may be the result of environment stimuli (47). Twin data clearly suggests that environmental stimuli/exposure may be critical to inducing disease in genetically inclined populations (89). A variety of meta-analysis, and genome wide association studies have been conducted to identify genes associated with the development of IBD. One such gene is the multi drug resistance gene (MDR).
**MDR Polymorphisms and IBD**

The human *MDR* gene is expressed on chromosome 7 (7q21.1) more commonly known as IBD susceptibility loci 2 (76). Human studies have confirmed that *MDR* mutations are associated with decreased P-gp expression and function; however, studies to date have been inconsistent with regards to the significance of *MDR* polymorphisms in relation to the development of IBD (35, 91). *MDR* polymorphisms have been shown to be significantly expressed in patients with IBD in Scottish populations (34). It has been shown to be particularly associated with pediatric UC or refractory CD in European and Slavic populations (24, 68).

Conversely, it has been reported that MDR polymorphism is not associated with IBD in Greek and European patient populations (7, 28). Interestingly, FVB/N mice lacking the *mdr1a* gene have been shown to develop spontaneous unremitting colitis when housed under specific pathogen free conditions (65). FVB.*mdr1a*–/– animals treated either prophylactically (16 weeks), or therapeutically (for 10 weeks following clinical signs of colitis) with broad spectrum antibiotics were shown to be protected against the development of colitis, confirming the role of the intestinal microbiota in disease initiation and progression (65).

**P-gp Expression and Function**

The *MDR* genes were originally discovered in Chinese hamster ovary (CHO) cells, and were so named for their ability to prevent intracellular drug accumulation, thereby conferring cells (and tumors) with drug resistance (26). The functional product of the *MDR* gene is P-glycoprotein (P-gp). P-gp, also known as *abcb1a*, is a glycosylated
transmembrane protein, and a member of the ATP-binding cassette super family of transporters. P-gp functions as a drug efflux pump effectively removing hydrophobic and amphipathic substrates from the intracellular environment (26). There are 2 MDR genes in humans (MDR1 and MDR3) and 3 mdr genes in mice (mdr1a, mdr1b and mdr2) (19, 38). The MDR2 genes are expressed in the liver, and actively pump phosphatidylcholine into bile (79). The MDR1 genes, those linked to colitis, are expressed at multiple tissue locations. They are present not only on the apical surface of epithelial cells in the colon and small intestine, but also 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 (18, 43, 83). Murine mdr1a and mdr1b have been shown to sub-stratify, with mdr1a being present predominantly in the intestine, liver and brain; while mdr1b is expressed primarily in the adrenal glands, the placenta and the uterus.

P-gp Deficient Animal Models of IBD

Expression of mdr1a on both lymphocytic and epithelial cell populations prompted experiments to determine the relevant cell population for driving spontaneous IBD in FVB animals. Adult FVB.mdr1a^{−/−} mice and wild type control animals were lethally irradiated and reconstituted with either, FVB.mdr1a^{−/−} or wild type bone marrow. Animals were maintained for 38-52 weeks and monitored for clinical signs of colitis. At the time of sacrifice only animals FVB.mdr1a^{−/−} deficient in P-gp expression in epithelial tissue developed histologically relevant colitis. Data from these initial experiments were interpreted to mean that expression of mdr1a in epithelial and radiation resistant cell populations is critical in protecting from spontaneous colitis (65).
Subsequent work utilizing this animal model has focused on the role of P-gp deficiency in the intestinal epithelium and its interactions with the intestinal microbiota. Initial reports of disease incidence in FVB.\textit{mdr1a}^{-/-} animals indicated that spontaneous colitis occurred in 20-25% of P-gp deficient animals; however, incidence rates as high as 100% with disease occurring earlier, and with increased severity in male mice have been reported at other facilities (65, 73). Discrepancies in disease incidence are thought to be the result of endemic bacteria in specific animal facilities.

FVB.\textit{mdr1a}^{-/-} animals have been shown to demonstrate reduced functioning of the intestinal epithelial barrier prior to the onset of clinical symptoms of colitis. Transepithelial Resistance and barrier permeability to fluorescently tagged substrates was assessed in FVB.\textit{mdr1a}^{-/-} compared to wild type controls. FVB.\textit{mdr1a}^{-/-} demonstrated decreased tissue resistance and stimulated ion transport, and increased barrier permeability to fluorescent molecules as early as 4 weeks of age. Furthermore, culture of lymphoid organs indicated greater bacterial translocation in FVB.\textit{mdr1a}^{-/-} animals when compared to controls. Altered intestinal function was associated with decreased phosphorylation of proteins expressed at epithelial cell junctions, which serve to maintain intestinal integrity, and separate luminal contents from systemic exposure (73).

**Epithelial Barrier**

The mammalian gastrointestinal (GI) tract contains $10^{12}$ colony-forming units of bacteria (out numbering the bodies’ own cells 10:1), all of which express microbial products capable of eliciting an inflammatory response (5). The intestinal epithelium is a continuous cellular monolayer extending the length of the alimentary tract. It creates a
physical barrier separating luminal contents from systemic inflammatory response elements such as basolateral sensors on epithelial cells, and adaptive immune cells in the lamina propria and draining lymph nodes (32). The intestinal epithelium actively aids in host defense through the production of mucins, and antimicrobial proteins (67). These barrier characteristics allow the host to enjoy a symbiotic relationship with commensal bacteria; which aid in nutrient metabolism, tissue development, and resistance to pathogen colonization (5, 48, 49). Disruption of this barrier, as occurs in IBD, leads to inflammation, and enhanced immune reactivity to the intestinal microbiota (52).

Recent evidence suggests that intestinal epithelial cells (IECs) may play a more active role in mediating the mucosal inflammatory response. Epithelial cells secrete a variety of cytokines and chemokines in response to infection by pathogenic bacteria (41, 67). A mechanism for this response was demonstrated when it became evident that IECs express Toll-like receptors (TLRs).

**Innate Pathogen Recognition Receptors**

TLRs are a family of innate pathogen recognition sensors that serve to recognize repetitive, conserved molecular patterns expressed on bacteria, and in bacterial products (57). There are 13 known mammalian TLRs, all of which are expressed throughout the GI tract on various cell lineages (45). TLRs are expressed on multiple cell types, including monocytes, macrophages, and dendritic cells, as well as intestinal epithelial cells (11). Murine IECs have been shown to express TLR 2, 4, 5 and 9 at varying levels in the small intestine and colon (humans express RNA for TLRs 1-9) (1, 56). Upon ligand recognition, TLRs activate signaling cascades that ultimately activate MAPK, NF-
κB and interferon response factors (13). There is evidence that intestinal epithelial are
tolerized with respect to exposure to low levels of bacterial ligands (50). Epithelial cells
are exposed to bacterial products on a constant basis, and must be unresponsive to
stimulation from the luminal contents (42).

TLR signaling may be internally regulated by alterations in cellular localization
and expression, and alterations in the expression of secondary signaling molecules (1).
While the precise role of TLRs in the intestinal epithelium remains unclear, it has been
demonstrated that TLR expression is integral in maintaining intestinal homeostasis, and
protection from injury (72).

TLR2 is expressed at low levels in the intestinal epithelium. It recognizes
lipotiechoic acid (LTA), a component of gram-positive cell walls (42). Studies done
using epithelial cell lines indicate that TLR2 localizes to a subapical location until
stimulation induces basolateral expression (12). However, IEC derived cell lines have
been shown to be unresponsive to TLR2 ligands (58).

TLR4 is present in the colonic and intestinal epithelium. Evidence indicates it
recognizes lipopolysaccharide (LPS), a component of gram-negative cell walls (42).
TLR4 is thought to be intracellularly expressed, requiring ligand internalization for
 celluar activation (36). Stimulation of IECs with LPS can induce a dramatic
inflammatory response (13, 78). However, when repeatedly stimulated with LPS,
primary IECs can become tolerized (50, 51).

TLR5 is present at high levels in the colonic epithelium. It recognizes bacterial
flagellin, a component of both Gram negative and positive bacteria (1). Work with
epithelial cell lines has shown that TLR5 expression is limited to the basolateral surface
in IECs; however this has not been confirmed in vivo (29). Stimulation of epithelial cell lines with flagellin induces secretion of pro-inflammatory cytokines (1).

TLR9 is expressed in the colonic and intestinal epithelium. It recognizes unmethylated CpG dinucleotides that are uniquely present in bacterial DNA (1). The responsiveness of TLR9 signaling in cultured epithelial cells is in direct correlation with the CG content of a given bacterial DNA sample (21). TLR9 is thought to be expressed intracellularly, though recent work indicates it can differentially affect NF-κB signaling in response to basolateral versus apical stimulation (46).

NOD proteins are a family of intracellular pathogen receptor proteins homologous to TLRs. They are expressed in effector cell populations, as well as epithelial cells of the gastrointestinal tract. These cytoplasmic proteins have been shown to activate the NF-κB pathway inducing an inflammatory response upon stimulation with bacterial ligands (56). Both NOD1 and NOD2 recognize components of bacterial peptidoglycan (PGN) in the intracellular environment. NOD1 responds to γ-D-glutamyl-meso-diamino-pimelic acid (iE-DAP), a derivative of gram-negative bacterial cell walls, while NOD2 responds to muramyl dipeptide a component of PGN found in gram-negative or gram-positive bacteria (56).

P-gp deficiency induces drug accumulation in a variety of tissues, demonstrating its function as an intracellular efflux pump (40, 74). Additionally, P-gp expression correlates with bacterial load in the gastrointestinal tract indicating a potential involvement in regulation of the inflammatory response to commensal bacteria (59). This evidence in concert with the known development of colitis in the FVB.mdr1a/-
mouse has lead to the belief that P-gp may function to extrude xenobiotic stimuli from the intracellular environment.

**Junctional Proteins and Barrier Integrity**

The application of bacterial stimuli to epithelial cells has been shown not only to induce the production of pro-inflammatory cytokines, but also to affect barrier integrity through the expression of junctional proteins (33).

Barrier integrity at epithelial cell borders is maintained by the apical junction complex, which link the cells of epithelial barrier and selectively inhibit paracellular flux. The apical junction complex is formed by both tight and adherins junctions; complexes of multiple transmembrane proteins that bind cells together at their apical poles. Studies as to the exact structure of the junctional complex continue, however, the tight junction is believed to be composed of the transmembrane proteins occludin, claudin, and JAM; which are bound to the cytoskeletal elements through zonula occludin (ZO-1) (15).

The apical junction is found directly subapical to the tight junction; it consists of a single transmembrane protein (E-cadherin) and is bound to the cytoskeleton through α, β, and γ catenins (39). Regulation of these proteins is incompletely understood, but it has been suggested that protein localization is regulated by protein phosphorylation state (2).

Bacterial toxins affecting cytoskeletal elements have been shown to indirectly disrupt the junctional complex and induce increased barrier permeability (33). Additionally, studies using intestinal cell lines indicate that inflammatory cytokines also induce disruption of junctional complexes, demonstrably causing protein internalization.
and redistribution (14, 53, 60, 93). Studies in CD patients indicate that increased barrier permeability predates inflammatory episodes (20, 90).

**Induced Colitis Models**

Animal research has enabled scientists to identify a variety of factors proven to initiate IBD in genetically susceptible hosts; primary among them are inhibition of the suppressive immune response, and barrier disruption, either by infection with invasive bacteria or by physical manipulation of epithelial tissue.

Barrier disruption and initiation of IBD in genetically susceptible hosts has been proposed to be the result of bacterial colonization or infection with pathogenic bacteria. Disease initiation in this model is believed to be the result of both the uncontrolled immune response initiated following epithelial invasion by certain bacterial agents, or the resultant alterations in luminal bacterial populations following infection (10).

*Helicobacter bilis* is a microaerophilic bacterium initially characterized as colonizing the bile in the liver and the lower GI tract in murine hosts (25). *H. bilis* is a gram-negative, rod shaped, spiral bacteria. It is known to be endemic in many animal research facilities, but has been shown to induce colitis in genetically susceptible hosts. *H. bilis* has been shown to accelerate colitis in IL10 deficient animals unable to regulate their immune response (9). It has also been shown to induce colitis in immune deficient mice and rats unable to mount an immune response (9, 30, 77). Additionally, it has been shown to accelerate the development of IBD in FVB.*mdr1a,/* reportedly as a result of increased barrier permeability seen in these animals (54). Colitis induction following *H. bilis*
inoculation indicates disease susceptibility as a result of deficiencies in both innate and adaptive immune responses.

Inhibition of immune-regulatory elements has also been shown to play an integral role in colitis induction in animal models. Prostaglandins are lipid mediators known to play a critical role in multiple physiological processes. Prostaglandins are crucial for initiation of proper wound healing and blood clotting; and they have proven to be pivotal in maintenance of the intestinal epithelial tissue by regulating blood flow and bicarbonate secretion (23, 61, 62, 87). Synthesis of prostaglandins is regulated by expression of the cycloxygenase (COX) enzymes 1 and 2, which function to reduce arachadonic acid to prostaglandin precursors (88). COX-1 is constitutively expressed at multiple tissue locations and has been more closely associated with homeostatic maintenance. COX-2 expression is believed to be induced as a result of inflammation (3, 80). Prostaglandin production has been shown to regulate the immune induced inflammation in response to stimulation with dietary antigen (62). Furthermore, inhibition of prostaglandins in IL10 deficient animals has been shown to accelerate disease onset (4). Colitis induction following prostaglandin inhibition is an effective means of determining colitis susceptibility as a result of defects in immunosuppressive responses.

One of the most commonly utilized methods for disrupting intestinal tissue is administration of dextran sodium sulfate (DSS). DSS is a sulfated polysaccharide that has been shown to induce colitis following oral administration in mice and rats (63, 81). DSS can be utilized to mimic both acute and chronic colitis, and has been shown to induce disease histologically and symptomatically similar to human UC; however, its precise mechanism of disease induction remains unclear (17, 63). It has been shown that the adaptive immune
response plays little to no role in disease induction in this disease model, as mice lacking lymphoid cells are not protected from disease (22). DSS is toxic to the intestinal epithelium, effectively stripping epithelial tissue from large areas of the cecal and colonic tissue, and affecting barrier integrity in the bowel. Development of colitis in this animal model is known to be contingent upon host bacterial interactions; germ-free and antibiotic treated animals do not develop significant inflammation following treatment (85). Animals resistant to DSS induced colitis typically develop a resolvable inflammation. Genetic background has been shown to have a profound impact on the development of disease, with animals displaying differential susceptibility to colitis as a result of specific strain background (55).

**Cellular Homeostasis and Radiation Induced Injury Repair**

Susceptibility to IBD has also been linked to dysregulation of epithelial cell cycling, and maintenance of intestinal homeostasis. It has been shown that MDR may play a role in regulating cell cycle. Expression of P-gp is associated with cellular resistance to apoptosis. Kidney proximal tubules cells were shown to be protected from cadmium and reactive oxygen species induced apoptosis as a result of increased P-gp expression (82). *In vitro* studies using blasts from patients with acute myeloid leukemia have shown that over-expression of MDR correlates with increased cell survival or decreased apoptosis (64). P-gp expression increases distally along the GI tract, correlating strongly with bacterial load (59). However, the potential effect of mdr1a deficiency on apoptotic resistance and cellular homeostasis in intestinal tissue has yet to be examined. One of the most well defined methods for studying cellular maintenance
and injury repair in the small intestine is crypt regeneration following radiation induced injury (69-71).

The intestinal epithelium exists in a state of perpetual regeneration, with epithelial cells being derived in intestinal crypts even as their predecessors are being sloughed off into the intestinal lumen (27, 31). Homeostatic balance, and epithelial cell generation is maintained by gastrointestinal stem cells located at the base of intestinal crypts. Stem cells divide in crypt bases producing daughter cells which subdivide, mature and eventually differentiate as they migrate away from intestinal crypts and into the proliferative zone (71). Radiation injury targets rapidly dividing cells, eliminating cells in the proliferative zone; effectively testing the ability of crypt stem cells to effectively repopulate intestinal tissue (69-71).

Increased susceptibility to apoptosis in P-gp deficient cells may induce higher levels of baseline intestinal regeneration subsequently causing increased cycling of stem cells at the base of intestinal crypts. Increased stem cell cycling puts crypt stem cells at an increased risk for radiation induced cell death, leaving tissue unable to regenerate following radiation induced injury.

**Aims of Dissertation**

It was the aim of this dissertation to clarify the role of the epithelial cells and the adaptive immune systems in the development of spontaneous colitis in the FVB\(mdr1a^{-/-}\) animal; to determine the effects of P-gp deficiency on colitis susceptibility in the C57BL/6\(mdr1a^{-/-}\) animal model; and to elucidate the role P-gp deficiency homeostasis and injury repair in the small intestine.
NEONATAL MODEL OF BONE MARROW RECONSTITUTION-A NEW MODEL FOR STUDYING INFLAMMATORY BOWEL DISEASE IN FVB.mdr1a^{-/-} MICE

by

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ABSTRACT

Background:

P-glycoprotein (P-gp), the product of the multi-drug resistance gene (MDR), is an ATP-dependent transmembrane pump known to extrude substrates from the intracellular environment. P-gp is expressed on the apical surface of epithelial cells and cells of a hematopoietic lineage. FVB/N animals deficient in mdr1a expression develop spontaneous colitis. Studies evaluating adult bone marrow chimeras indicate colitis development is contingent on P-gp deficiency in epithelial and stromal cells as well as other radiation resistant cell lineages. However, these studies utilized FVB.mdr1a−/− animals that already expressed increased levels of inflammatory cytokines in their colonic tissue. These cytokines could be a reflection of sub-clinical disease that exists in FVB.mdr1a−/− animals prior to reconstitution.

Methods:

To more accurately assess the influence of epithelial versus adaptive cells in inducing colitis in FVB.mdr1a−/− animal; we developed a neonatal model of bone marrow reconstitution. 7 day old FVB.mdr1a−/− animals were lethally irradiated and reconstituted with bone marrow from FVB or FVB.mdr1a−/− donors. Animals were monitored up to 20 weeks for the development of colitis.
Results:

FVB.mdr1a<sup>−/−</sup> reconstituted with FVB.mdr1a<sup>−/−</sup> deficient bone marrow developed colitis more rapidly than wild type FVB.mdr1a<sup>−/−</sup> mice. 80% of FVB.mdr1a<sup>−/−</sup> mice reconstituted with FVB/N bone marrow failed to develop disease, as confirmed by weight, histology, colonic length, intestinal permeability to FITC-Dextran and reduced expression of inflammatory cytokines.

Conclusions:

Unlike previous reports neonatal reconstitution of FVB.mdr1a<sup>−/−</sup> animals with FVB/N bone marrow suggests that P-gp deficiency in epithelial tissue does not play a significant role in the initiation of spontaneous colitis in FVB.mdr1a<sup>−/−</sup> animals.
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, also known as abcb1a, is a member of the ATP-binding cassette super-family of transporters. It is known to be 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 hematopoetic 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 has been shown to be associated with decreased P-gp expression and function [6-8].

FVB/N animals deficient in expression of mdr1a, have been shown to develop a spontaneous, unremitting colitis, characterized by diarrhea, rectal prolapsed, and death [9,10]. Treatment of these animals with antibiotics has been shown to ameliorate colitis when administered either prophylactic or therapeutically, confirming the role intestinal microbiota in inducing intestinal inflammation [9].

To assess the role of the epithelial and other radiation resistant cell lineages versus bone marrow derived immune cells in mitigating the inflammatory response and resultant colitis, studies were conducted utilizing bone marrow chimeras. Adult FVB and FVB.mdr1a-/- animals were lethally irradiated and reconstituted with bone marrow from
FVB, or FVB.\textit{mdr1a}^{-/-} donors. Results indicated that only FVB.\textit{mdr1a}^{-/-} recipients developed colitis, highlighting the importance of epithelial cell populations in preventing disease development [9]. Furthermore, it was shown that FVB.\textit{mdr1a}^{-/-} animals demonstrate increased barrier permeability at an early age prior to the onset of colitis, as well as decreased phosphorylation of proteins of the tight junction [10]. This data suggest that P-gp may interfere with the formation of tight junctions in the intestinal epithelium, ostensibly causing a leaky barrier, and ultimately colitis.

It has been recently shown that FVB.\textit{mdr1a}^{-/-} animals express increased levels of inflammatory cytokines such as INF-\textgamma and IL17 early in development [11,12]. \textit{In vitro} experiments using colonic epithelial cell lines demonstrate that TNF\alpha, and IFN\gamma has 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]. Expression of inflammatory cytokines at such an early developmental time point may be inducing barrier deficiencies, and predisposing FVB.\textit{mdr1a}^{-/-} mice towards developing IBD as a result of aberrant responses to bacterial exposure.

We hypothesize that in order to more accurately assess the responsibility of the intestinal epithelial cells in promoting susceptibility to colitis in FVB.\textit{mdr1a}^{-/-} animals it is necessary to reconstitute animals with wild type bone marrow, 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-Abcbltm1BorN7 (FVB.mdr1a/-) were originally derived by Schinkel et al., and were purchased from Taconic Farms, Inc. (Hudson, NY) [18]. Animals were bred and maintained in specific pathogen free (SPF) conditions in Thoren Isolator racks (Hazleton, PA) under positive pressure and were given sterile drinking water and autoclaved NIH-31 rodent diet (Harlan Teklad, Madison, WI) ad libitum. Mice were acclimatized to our facility 2 weeks prior to mating. Mdr1a deficiency is confirmed by periodic genotyping by polymerase chain reaction (PCR). In brief, tail clippings are collected, and DNA is extracted using Sigma’s RED Extract-N-Amp tissue PCR kit (St Louis, MO). Mdr1a phenotype is confirmed as indicated by Taconic using the primers MDR1A S2 CTCCTCCAAGGTGCATAGACC, MDR1A W2 CCCAGCTCTCATCTAACTACCCCTG, and MDR1A K02 CTTCCAGCCTCTGAGCCCCAG 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 are then run on a 1.5% agarose gel, and presence or absence of mdr1a gene expression is confirmed by analyzing banding patterns produced by amplification products (Invitrogen, Carlsbad, CA). Animals are monitored for colitic symptoms, including extreme 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, murine norovirus, 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

FVB.\textit{mdr1a}\textsuperscript{+} and wild type recipient animals were exposed to a lethal dose (900 RADs) of gamma irradiation using the GC40 \(\gamma\)-iradiator containing a cesium 137 core. Animals were exposed to radiation at 7 days of age. Irradiated mice were reconstituted with donor bone marrow at a ratio of 1 donor providing for 2 recipients, or approximately 3\(\times\)10\(^6\) cells injected intraperitoneally (i.p.) in neonates, or retro-orbitally in adults. Dams were treated with Bactrim 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, or subsequent to losing more than 20\% of maximal achieved body weight.

Reconstitution

To generate bone marrow, FVB.\textit{mdr1a}\textsuperscript{+} and FVB/N donor animals were anesthetized with isoflourane 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 µM Nitex® membrane (Tetko, Elmsford, NY) to remove debris. Cells were washed twice in R10 (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, Onatario, 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 (approximately 3x10⁶ cells/animal) 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 5µm 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. Blood samples were coagulated at 4º 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 it’s 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. For a complete list of genes utilized and primer probes and sequences please see supplemental table I.
**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, while non-continuous data was evaluated by applying the Fisher’s Exact analysis. Comparisons having a P<0.05 were considered statistically significant.
RESULTS

Effects of Radiation Exposure on Barrier Integrity in Neonatal and Adult Tissue

It has previously been reported that 100% FVB.mdr1a<sup>-/-</sup> animal reconstituted with FVB.mdr1a<sup>-/-</sup> bone marrow developed colitis at a higher rate than disease incidence in conventionally house FVB.mdr1a<sup>-/-</sup> mice in that colony, suggesting potential effects of radiation induced damage to the intestinal epithelium [9]. 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, barrier permeability is inherently different in non-treated neonatal and adult tissue, with neonatal tissue being significantly more permeable (Fig. 1).

Weight Loss and Morality in Neonatal Bone Marrow Chimeras

It has previously been reported in adult animals that FVB.mdr1a<sup>-/-</sup> animals reconstituted with FVB/N bone marrow develop colitis within 12 weeks from reconstitution; furthermore, that by 52 weeks from reconstitution 25% of animals these animals had developed disease (a rate comparable with the incidence rates of spontaneous
FIGURE 1. Serum Levels of FITC-Dextran following Irradiation. Neonatal (day 7 from birth) and 6-8 week FVB/N mice were exposed to 900 RADS $\gamma$-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.
colitis in FVB.\textit{mdr1a}^{-/-} animals in that facility) [9]. To evaluate disease progression in our colony neonatal FVB.\textit{mdr1a}^{-/-} animals reconstituted with FVB/N or FVB.\textit{mdr1a}^{-/-} bone marrow, along with non-treated FVB/N, and FVB.\textit{mdr1a}^{-/-} controls were routinely weighed and monitored for physiological symptoms of colitis.

FVB.\textit{mdr1a}^{-/-} animals reconstituted with FVB.\textit{mdr1a}^{-/-} bone marrow demonstrated dramatically altered growth and development, indicating reduced weight gain as early as 8 weeks of age, and consistently demonstrated significantly reduced weight from all other experimental groups starting at 12 weeks of age. FVB.\textit{mdr1a}^{-/-} animals reconstituted with FVB/N bone marrow demonstrated growth and weight gain similar to non-treated FVB/N controls, displaying normal growth from weaning to 10 week, and increased weight gain at week 20 (Fig 2A).

Animals were maintained for 20 weeks following reconstitution (or birth), and were sacrificed upon evidence of weight loss greater than 20% maximal weight or incidence of fecal blood. FVB.\textit{mdr1a}^{-/-} animals neonatally reconstituted with FVB.\textit{mdr1a}^{-/-} bone marrow demonstrated 100% mortality at 18 weeks from reconstitution. However FVB.\textit{mdr1a}^{-/-} animals reconstituted with FVB/N demonstrated only a 20% mortality rate by 20 weeks of age, similar to non-treated FVB.\textit{mdr1a}^{-/-} controls, demonstrating that irradiation and reconstitution may not be altering disease progression in a neonatal model (Fig 2B).

**Clinical Indicators of Colitis in Neonatally Reconstituted animals**

Animals were evaluated for evidence of colitic symptoms throughout growth and development. Occurrence of diarrhea, as well as fecal blood and rectal prolapse were
Figure 2: Weight Changes and Mortality in Neonatally Reconstituted Bone Marrow Chimeras and FVB/N and FVB.mdr1a⁻/⁻ non-treated irradiated controls. 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 non-treated controls 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. # indicates statistical significance of P<0.05 when compared to non-treated FVB controls, * indicates significance of P<0.05 when compared to FVB.mdr1a⁻/⁻ controls, and § indicates significance of P<0.05 when compared to FVB.mdr1a⁻/⁻ animals reconstituted with FVB.mdr1a⁻/⁻ bone marrow. Statistical analysis was conducted using the Student’s T-Test.
recorded. Not surprisingly FVB\(^{mdr1a^{-/-}}\) animals reconstituted with FVB\(^{mdr1a^{-/-}}\) bone marrow showed an increased incidence of fecal blood, rectal prolapse and mortality when compared to non-treated FVB/N and FVB\(^{mdr1a^{-/-}}\) controls. FVB\(^{mdr1a^{-/-}}\) animals reconstituted with FVB bone marrow displayed disease indices more similar to non-treated FVB/N animals; demonstrating statistically similar incidence rates of prolapse, fecal blood, and mortality (Table 1).

Colitis induces macroscopic physiological changes in colonic tissue. As inflammation develops over the course of disease progression, colonic tissue has been shown to both shorten and become heavier, as a result of cellular infiltration, and edema. At sacrifice whole colons were removed and weight and length were evaluated to assess colitic progression. FVB\(^{mdr1a^{-/-}}\) animals reconstituted with FVB\(^{mdr1a^{-/-}}\) bone marrow demonstrated significantly reduced colonic length when compared to non-treated FVB/N animals and FVB\(^{mdr1a^{-/-}}\) animals reconstituted with FVB/N. FVB\(^{mdr1a^{-/-}}\) animals reconstituted with FVB bone marrow showed increased colonic ratios, statistically higher than non-treated FVB\(^{mdr1a^{-/-}}\) animals, and similar to those of FVB/N mice, indicating relative tissue health (Fig 3).

**Histological Evidence of Colitis in Neonatally Reconstituted Mice**

To evaluate colitis development at a microscopic level, tissue was fixed at sacrifice for histological evaluation. Tissue was evaluated and given disease scores 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. FVB\(^{mdr1a^{-/-}}\)
### Table 1: Clinical Symptoms of Colitis

<table>
<thead>
<tr>
<th>Donor into Recipient</th>
<th>Diarrhea</th>
<th>Fecal Blood</th>
<th>Prolapse</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB/N</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>FVB.\textit{mdr1a}^{-/-}</td>
<td>3/6</td>
<td>1/6</td>
<td>0/6</td>
<td>1/6</td>
</tr>
<tr>
<td>FVB.\textit{mdr1a}^{-/-} into FVB.\textit{mdr1a}^{-/-}</td>
<td>4/10</td>
<td>8/10*#</td>
<td>5/10#</td>
<td>10/10*#</td>
</tr>
<tr>
<td>FVB/N into FVB.\textit{mdr1a}^{-/-}</td>
<td>3/15</td>
<td>2/15</td>
<td>0/15§</td>
<td>3/15§</td>
</tr>
</tbody>
</table>

* Indicates significance of P<0.05 when compared to FVB.\textit{mdr1a}^{-/-} animals.
# Indicates significance of P<0.05 when compared to FVB/N.
§ Indicates significance of P<0.05 when compared to FVB.\textit{mdr1a}^{-/-} animals reconstituted with FVB.\textit{mdr1a}^{-/-} bone marrow.
Figure 3: Colonic Length to Weight Ratio in Neonatally Reconstituted Bone Marrow Chimeras, and FVB/N and FVB.mdr1a/- non-treated irradiated controls. Colonic tissue was harvested at sacrifice and assessed for weight and length measurements. # indicates statistical significance of P<0.05 when compared to non-treated FVB controls, * indicates significance of P<0.05 when compared to FVB.mdr1a/- controls, and § indicates significance of P>0.05 when compared to FVB.mdr1a/- animals reconstituted with FVB.mdr1a/- bone marrow. Statistical analysis was conducted using the Student’s T-Test.
animals reconstituted with FVB.mdr1a<sup>−/−</sup> bone marrow demonstrated significant disease pathology, in both cecal and colonic tissue. Inflammation in colonic tissue from FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB.mdr1a<sup>−/−</sup> bone marrow was significantly more severe at sacrifice than inflammation present in non-treated FVB.mdr1a<sup>−/−</sup> controls (Fig 4). However, FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB bone marrow displayed significantly lower histology scores when compared to non-treated FVB.mdr1a<sup>−/−</sup> animals in both colonic and cecal tissue. Colonic and cecal histologic scores from FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB bone marrow was not statistically different from tissue in non-treated FVB/N animals, indicating that the majority of FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB bone marrow are not developing colitis (Fig 4).

**Gene Expression of Inflammatory Cytokines in Neonatally Reconstituted Animals**

It has been previously shown that FVB.mdr1a<sup>−/−</sup> animal display increased levels of inflammatory cytokines prior to the onset of colitis. As our FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB bone marrow were shown to histologically free of colitis, we next wanted to evaluate levels of inflammatory cytokines, to assess potential for disease development. RNA was isolated from colonic tissue from FVB/N, FVB.mdr1a<sup>−/−</sup>, and reconstituted animals at sacrifice and RT-PCR analysis was conducted to evaluate gene expression. Gene expression was normalized to expression in FVB/N animals. FVB.mdr1a<sup>−/−</sup> non-treated controls, and FVB.mdr1a<sup>−/−</sup> animals reconstituted with bone marrow from FVB.mdr1a<sup>−/−</sup> animals displayed significantly increased levels of IL17, and IFNγ, when compared to FVB/N and FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB/N bone marrow. Expression of inflammatory cytokines in FVB.mdr1a<sup>−/−</sup> animals
Figure 4: Colonic and Cecal Histology from FVB.mdr1a<sup>−/−</sup> mice and Neonatally Reconstituted FVB.mdr1a<sup>−/−</sup>. Cecal and colonic tissue was from FVB mice (A), FVB.mdr1a<sup>−/−</sup> (B), FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB.mdr1a<sup>−/−</sup> bone marrow (C), and FVB.mdr1a<sup>−/−</sup> mice reconstituted with FVB.mdr1a<sup>−/−</sup> mice bone marrow (D). Tissue was harvested at sacrifice and fixed for histological analysis. Histological scoring was conducted under blinded conditions and was assessed by virtue of epithelial hyperplasia, goblet cell loss, superficial and crypt epithelial degeneration and loss, crypt exudate, and inflammatory cell accumulation in lamina propria and submucosa, as well as the presence or absence of mucosal ulceration. Histology was evaluated from colonic (E) and Cecal (F) tissue. Scores range from 0-7. # indicates statistical significance of P>0.05 when compared to non-treated FVB controls, * indicates significance of P>0.05 when compared to FVB.mdr1a<sup>−/−</sup> controls, and § indicates significance of P>0.05 when compared to FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB.mdr1a<sup>−/−</sup> bone marrow. Statistical analysis was conducted using the Student’s T-Test.
reconstituted with FVB/N bone marrow was not significantly different from FVB/N animals (Fig 5).
Figure 5: RNA expression of Inflammatory Cytokines in Neonatally Reconstituted 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 non-treated FVB animals. Data is shown on a log 2 scale. All values demonstrating a 2 fold alteration in gene expression from control values are considered physiologically altered from control values. Notably expression of IFN$_{\gamma}$, IL17 and TNF$\alpha$ is significantly increased in FVB.mdr1a$^{-/-}$ animals reconstituted with FVB.mdr1a$^{-/-}$ bone marrow when compared to both FVB.mdr1a$^{-/-}$, and FVB.mdr1a$^{-/-}$ animals reconstituted with FVB/N bone marrow. Furthermore, expression of inflammatory cytokines in FVB.mdr1a$^{-/-}$ mice reconstituted with FVB/N bone marrow is not significantly different from FVB/N animals.
DISSCUSSION

Schinkel et al. originally derived the FVB.129P2-Abcb1<sup>tm1BorN7</sup> (FVB.<sup>mdr1a</sup><sup>−/−</sup>) 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 in the intestinal epithelium was subsequently realized when it was demonstrated that FVB.<sup>mdr1a</sup><sup>−/−</sup> animals develop a spontaneous unremitting colitis when maintained in a specific pathogen free environment [9]. Efforts to elucidate the nature of this colitis indicated it was the result of aberrant reactions directed at the intestinal microbiota; when it was shown that both prophylactic and therapeutic antibiotic treatment were capable of ameliorating disease in this animal model [9].

Human studies confirmed the importance of P-gp gene function in protecting human populations from the development of colitis. The human <i>MDR</i> gene is found on chromosome 7(7q21.1), IBD susceptibility loci 2 [24]. Recent meta-analysis demonstrate that <i>MDR</i> polymorphisms are strongly associated with the development of IBD, and more specifically UC [25,26]. One recent study has implicated <i>MDR</i> polymorphisms in the development of early on-set UC [6]. Additionally, <i>MDR</i> polymorphisms have been specifically linked refractory UC and CD in Slovian populations [8]. Expression of <i>MDR</i> polymorphisms has been shown to be associated with decreased P-gp expression and function as assessed by cellular ability to flux fluorescent dye [27].

P-gp has been shown to be expressed a multiple tissue locations including the apical surface of epithelial cells in excretory organs, as well as cells of hematopoietic lineage [3-5]. Initial studies using this animal model were aimed at clarifying the role of
epithelial versus immune cells in disease induction. Panwala et al. lethally irradiated adult FVB/N and FVB.mdr1a\(^{-/-}\) animals prior to reconstitution with bone marrow from either FVB/N, or FVB.mdr1a\(^{-/-}\) animals [9]. Reconstituted animals were maintained for 38 to 52 weeks and observed for clinical indicators of colitis. At sacrifice FVB/N animals reconstituted with FVB.mdr1a\(^{-/-}\) bone marrow remained clinically and histopathologically free of colitis while, FVB.mdr1a\(^{-/-}\) animals reconstituted with FVB/N bone marrow developed colitis clinically observable at 12 weeks of age, at an incidence rate comparable to that typically observed in non-reconstituted FVB.mdr1a\(^{-/-}\) animals in that facility [9]. This data was interpreted such that the absence of P-gp expression on epithelial cells was necessary and sufficient to induce colitis in the FVB.mdr1a\(^{-/-}\) animal model, while P-gp expression on immune cells had little to no role in disease initiation.

As a result of these experiments, subsequent research in this animal model has been focused on examining the effects of P-pg deficiency on the integrity of the intestinal epithelium. FVB.mdr1a\(^{-/-}\) animals have been shown to develop altered permeability to molecularly tagged substrates at 12 weeks of age, prior to the development of colitis [10]. Permeability changes have been shown to correlate with altered phosphorylation of junctional proteins occludin and zonula occludin-1 (ZO-1) [10]. These data clearly indicate that histological and phenotypic evidence of colitis occurs secondarily to the development of altered intestinal barrier function.

However, it was also recently shown that FVB.mdr1a\(^{-/-}\) animals demonstrate an increased expression of inflammatory cytokines IFN\(\gamma\), TNF\(\alpha\), and IL17, and an increased responsiveness to LPS early in development, also prior to the development of intestinal inflammation [11,12]. In vitro studies utilizing human colonic T84 cells clearly indicate
that the presence of IFN-γ has a significant effect on localization and expression of proteins of the tight junction, dramatically reducing barrier permeability [13,14,16]. Furthermore, it has been shown that TNFα facilitates this effect, increasing the barrier remodeling in response to IFN-γ expression [15]. These data indicate that altered expression of inflammatory cytokines, potentially as a result of aberrant immune function in P-gp deficient immune cells; may affect expression of junctional proteins, early in development, potentially predisposing animals towards developing colitis.

It was our hypothesis that to more precisely assess the role of the intestinal epithelial cells in promoting susceptibility to colitis in FVB\textit{mdr1a}^{-/-} animal model it would be necessary to reconstitute animals at an early stage in development prior to any potentially harmful changes in the cytokine milieu.

FVB\textit{mdr1a}^{-/-} animals were lethally irradiated at day 7 from birth and reconstituted with bone marrow from FVB/N or FVB\textit{mdr1a}^{-/-} animals. Reconstituted animals, as well as non-treated FVB/N and FVB\textit{mdr1a}^{-/-} animals were maintained for 20 weeks following reconstitution and were monitored for clinical signs of colitis as well as changes in growth and development, and increased mortality.

FVB\textit{mdr1a}^{-/-} animals reconstituted with FVB/N bone marrow displayed a marginally increased mortality when compared to the FVB/N animals, with 20% of animals being sacrificed prior to 20 weeks of age. However, the surviving animals failed to develop disease to the same magnitude as that seen in even non-treated FVB\textit{mdr1a}^{-/-} controls. Surviving FVB\textit{mdr1a}^{-/-} reconstituted with FVB/N proved to be clinically and histologically free of colitis. Furthermore, these animals failed to demonstrate the up-regulation of inflammatory cytokines IFN-γ, TNFα, and IL17 seen in both FVB\textit{mdr1a}^{-/-}
animals reconstituted with FVB.\textit{mdr1a}^{-/-} bone marrow as well as FVB.\textit{mdr1a}^{-/-} controls, remaining statistically consistent with gene expression in FVB/N animals.

As previously indicated FVB.\textit{mdr1a}^{+/-} animals reconstituted with FVB.\textit{mdr1a}^{+/-} bone marrow spontaneously developed a severe unremitting colitis. Animals demonstrated reduced weight gain when compared to non-treated control FVB/N animals between 6-8 weeks from birth. FVB.\textit{mdr1a}^{+/-} animals reconstituted with FVB.\textit{mdr1a}^{+/-} bone marrow displayed an increased mortality of 100% by 18 weeks of age. Colitis in these animals was confirmed histologically and disease was shown to extend from the cecum throughout colonic tissue. Disease in these animals was significantly more severe than disease that occurs naturally in FVB.\textit{mdr1a}^{+/-}. It may be that exposure to radiation is more damaging to FVB.\textit{mdr1a}^{+/-} epithelial cells, and that damage is regulated by adaptive immune cells. Absence of P-gp expression in both epithelial cells, and adaptive immune cells in this reconstituted animal, may render them unable to recover from radiation induced insult.

Our neonatal model shows that the absence of P-gp expression in the intestinal epithelium may play a smaller role than previously suggested in the initiation of spontaneous colitis in the FVB.\textit{mdr1a}^{+/-} model. FVB.\textit{mdr1a}^{+/-} reconstituted with FVB.\textit{mdr1a}^{+/-} deficient bone marrow developed colitis phenotypically similar to that seen in wild type FVB.\textit{mdr1a}^{+/-} mice. However, 80% of FVB.\textit{mdr1a}^{+/-} reconstituted with FVB bone marrow remained disease free indicating a potential role for P-gp deficiency in immune cells as an instigator for spontaneous colitis in the FVB.\textit{mdr1a}^{+/-} animal model.
ACKNOWLEDGEMENTS

This work was supported by the NIH grants P01 DK071176, and T32AI07051 and University of Alabama at Birmingham Digestive Diseases Research Development Center Grant P30DK064400. We would like to thank Peggy R. McKie-Bell, Reed Dimmitt, and Jamie L. McNaught for their assistance and members of the Lorenz Lab for valuable advice. We thank Dr. Chuck O. Elson for use of the Synergy Microplate Reader and Wayne Duck for experimental input and comments. We thank Dr. Susanne Michaleck for use of the GC-40 irradiator and Gregg Harbor for technical assistance.
REFERENCES


colonic epithelium that precede increased gut permeability during colitis development in mdr1a(-/-) mice. Inflamm Bowel Dis 2008;14:620-631.


**Supplement Table 1.** Primer-probes pairs utilized for quantitative RT-PCR.

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DIFFERENTIAL SUSCEPTIBILITY OF P-GLYCOPROTEIN DEFICIENT MICE TO COLITIS INDUCTION BY ENVIRONMENTAL INSULTS

by

ELIZABETH M. STALEY, TRENTON R SCHOEB, AND ROBIN G. LORENZ

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ABSTRACT

Background

P-glycoprotein (P-gp), the product of the multi-drug resistance gene (MDR), is an ATP-dependent transmembrane pump, which is expressed in multiple cell lineages including epithelial and hematopoetic cells. The human MDR gene is located on chromosome 7 (7q21.1), a susceptibility loci for inflammatory bowel disease (IBD). A significant number of IBD patients carry mutations in this gene and P-gp deficient FVB/N mice develop a severe spontaneous colitis, characterized by impaired intestinal barrier function and immune reactivity to intestinal bacterial antigens.

Methods:

In this work we explored the role of mouse strain, as well as environmental insults on the development of colonic inflammation in the absence of P-gp. Among the induction methods utilized, dextran sodium sulfate (DSS) disrupts the intestinal epithelium, while Piroxicam is a non-steroidal anti-inflammatory (NSAID) drug which inhibits prostaglandin production, and initiates colitis in IL10 deficient animals. *Helicobacter bilis (H.bilis)* is a known mediator of bacterial induced colitis.

Results:

We demonstrate that crossing this mutation onto the C57BL/6 strain confers protection from spontaneous colitis. C57BL/6.mdr1a deficient animals demonstrated increased histological inflammation, colonic shortening, fecal blood, and reduced body
weight after 7 days of treatment with 2.25% DSS. C57BL/6.mdr1a deficient mice treated with piroxicam or infected with *H. bilis* showed no weight loss, or alterations in colonic histology.

**Conclusions:**

These data indicate that the effects of P-gp deficiency are significantly modulated by background strain influences, but that the epithelium continues to have increased susceptibility to chemical injury in the C57BL/6 model.

**Key Words:** colitis, p-glycoprotein, mouse models, helicobacter
INTRODUCTION

Inflammatory bowel diseases (IBD) are diseases of unknown etiology characterized by recurrent, spontaneous inflammation of the gastrointestinal tract (GI).(1) IBD consists of two disease phenotypes, ulcerative colitis (UC) and Crohn’s disease (CD). These diseases are proposed to be the result of genetic predispositions for either altered immune function leading to aberrant immune responses to natural stimuli, or overall autoimmune dysregulation.(2) Studies show that 20% of patients with IBD have a relative with either UC or CD, in fact a positive family history is thought to be the single greatest predictive factor associated with the disease.(1, 3)

Numerous genes are being examined as carriers of mutations that may result in a predisposition towards IBD, of particular interest is the human multi-drug resistance gene (MDR), an ATP-binding cassette subfamily B member 1A (Abcb1a). In humans the MDR gene is expressed on chromosome 7 (7q21.1) a susceptibility loci for IBD.(4) Studies to date have been inconsistent with regards to the significance of mutations in the MDR gene in relation to the development of IBD. Several recent meta-analysis studies have shown that a significant number of IBD patients carry mutations to this gene, and further studies have demonstrated that MDR mutations are associated with decreased P-gp expression and function.(5-8) Additionally, it was shown that mutations to this gene are strongly associated with early onset UC in German populations.(9) Conversely, it has been reported that subsets of IBD patients in Greek and European populations bear no correlation to MDR mutations.(10, 11)
The functional product of the *MDR* gene is P-glycoprotein (P-gp). P-gp is a glycosylated transmembrane protein, which functions as a drug efflux pump effectively removing hydrophobic and amphipathic substrates from the intracellular environment.(12) There are 2 *MDR* genes in humans (*MDR1* and *MDR3*) and 3 *mdr* genes in mice (*mdr1a* (*abcb1a*), *mdr1b* (*abcb1b*), and *mdr2*(*abcb4*).(13, 14) The *MDR3* (and *mdr2*) genes are expressed in the liver, and actively pump phosphatydcholine into bile.(15) The *MDR1* genes, those linked to colitis, are found in multiple tissue locations. They are present not only on the apical surface of epithelial cells in the colon and small intestine, but also on epithelial cells of the pancreas, kidney, and adrenal glands, on endothelial cells at the blood brain barrier, and on cells of the hematopoetic lineage.(16-18) Due to the expression of P-gp on the apical epithelium of organs with excretory function, and due to the known role of P-gp in drug extrusion; it has been postulated that P-gp’s physiologic function is to extrude xenobiotic substrates from the intracellular environment.(19)

A murine model of this genetic mutation has lent some insight into the potential physiological role of P-gp. FVB/N mice deficient in *mdr1a* gene expression develop a spontaneous colitis as early as 3-4 months of age.(20, 21) These FVB. *mdr1a*−/− mice have deficiencies in tight junction proteins in the intestinal epithelium, as well as an increased intestinal permeability prior to disease development.(21, 22) These mice have increased colonic IFNγ and IL8 and treatment of these animals with antibiotics was shown to be prophylactic and therapeutic in mitigating disease progression, confirming the role intestinal microbiota in inducing intestinal inflammation.(20, 23)
To expand our knowledge of the mechanisms of the spontaneous colitis in this disease model and to investigate the role of different genetic backgrounds in disease susceptibility, our lab endeavored to backcross the mdr1a genetic deletion on to the C57BL/6J (B6) mouse strain. B6.mdr1a<sup>−/−</sup> mice were established by 10-generations of backcross breeding to B6 control mice. We initially characterized disease development in these animals; however, the P-gp deficiency on the B6 genetic background did not result in any clinical or histological evidence of spontaneous colitis in animals up to one year of age.

Expression of genetic mutations may leave individuals predisposed towards colitis development in response to mitigating factors, such as a bacterial colonization or infection.(1) We therefore hypothesized that the B6.mdr1a<sup>−/−</sup> mice might be more susceptible to colitis induction after a secondary environmental insult. Environmental factors tested included Helicobacter bilis, and dextran sodium sulfate (DSS), which have both been previously reported to accelerate the development of colitis in the FVB.mdr1a<sup>−/−</sup> model.(24, 25) DSS has also been shown to alter mdr1a expression prior to clinical colitis symptoms.(26) Additionally, results from our B6.mdr1a<sup>−/−</sup> animals indicated that prostaglandin (PG) regulation may play a protective role in preventing colitis in this seemingly resistant strain. Therefore, we tested the affects of altering PG synthesis by blocking the key regulatory enzyme of the PG biosynthesis pathway, cyclooxygenase (COX) in our B6.mdr1a<sup>−/−</sup> animal model. We administered the non-steroidal anti-inflammatory drug (NSAID), Piroxicam, which inhibits both COX isoforms and rapidly induces colitis in IL10 deficient mice.(27)
MATERIALS AND METHODS

Animals

FVB.129P2-Abcb1\textsuperscript{tm1Bor}N7 (FVB.mdr1\textsuperscript{a-/-}) as originally described by Schinkel et al. mice were purchased from Taconic Farms, Inc. (Hudson, NY). Animals were bred and maintained under specific pathogen free (SPF) conditions in Thoren Isolator racks (Hazleton, PA) under positive pressure and were fed autoclaved NIH-31 rodent diet (Harlan Teklad, Madison, WI), and sterile drinking water ad libitum. Animals were acclimatized to our facility 2 weeks prior to mating. Mice were backcrossed onto the C57BL/6J background (Jackson Laboratories, Bar Harbor, ME), for >10 generations under a Research Crossbreeding Agreement with Taconic Farms, Inc., and genotype is confirmed by periodic genotyping by polymerase chain reaction (PCR). In brief, tail clippings are routinely collected, and DNA is extracted using Sigma’s RED Extract-N-Amp tissue PCR kit (St Louis, MO). Mdr1\textsuperscript{a} phenotype is confirmed as indicated by Taconic using the primers MDR1A S2 CT CCTCCAAGGTGCATAGACC, MDR1A W2 CCCAGCTCTTCATCTA ACTACCCTG, and MDR1A K02 CTTCCCAGCCTCTGAGCCCA G 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 are then run on a 1.5% agarose gel, and presence or absence of mdr1\textsuperscript{a} gene expression is confirmed by analyzing banding patterns produced by amplification products (Invitrogen, Carlsbad, CA). Animals were routinely monitored for presentation of a colitic phenotype,
including 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, murine norovirus, 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/.

**Helicobacter Infection**

*H. bilis* (ATCC# 51630) was obtained from the American Type Tissue Collection (Manassas, VA). Organisms were streaked onto Brucella blood agar plates and grown at 37° under microaerophilic conditions (90% N₂, 5% H₂ and 5%CO₂) for 24-28 hours. Bacteria were then inoculated into Brain Heart Infusion broth (supplemented with 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific), 2.5 μg/ml amphopeteracin B, 3μg/ml vancomycin, and 10 μg/ml Trimethoprim) and grown under micoraerophilic conditions at 37° for 24 hours in a continuous shaker. Viability was confirmed via phase microscopy. Organisms were pelleted and quantified at an optical density of 450nm (one OD₄₅₀=10⁹ bacteria.). *Mdr1a⁻/⁻* and control strain mice of 6-10 weeks of age were orally inoculated with 2x10⁷ cfu bacteria three times over the course of one week. Mice were
monitored weekly for presentation of a colitic phenotype, including weight loss, diarrhea, and/or fecal blood. Animals were sacrificed at 4 or 12 weeks post infection.

**DSS Treatment**

B6 or B6. *mdrla*−/− animals from 6-10 weeks of age were given 2.25% w/v DSS (m.w. 36,000-50,000, MP Biomedicals, Solon Ohio) in drinking water ad libitum for 1 week prior to sacrifice (acute). Alternatively animals were treated with 2% w/v DSS in drinking water for 4 alternating cycles of: 1 week of treatment, followed by 2 weeks of recovery during which DSS is replaced with sterile drinking water (chronic). Mice were monitored for presentation of a colitic phenotype, including weight loss, diarrhea, and/or fecal blood.

**Piroxicam Diet**

B6 or B6. *mdrla*−/− animals from 6-10 weeks of age were given 280 ppm piroxicam, (Sigma, Aldrich, St Louis, MO) in pelleted rodent diet NIH-31 from Harlan Tecklad (Madison, WI) as described by Lynch *et al.*(27) Animals were given access to chow ad libitum, and were either treated for 1 week prior to sacrifice (acute), or treated for 2 weeks and sacrificed 4 weeks post treatment (chronic). Mice were monitored for presentation of a colitic phenotype including weight loss, diarrhea, and/or fecal blood.

**Histological Analysis**

At time of sacrifice mice were anesthetized with isoflurane 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 5μm sections and stained with standard H&E for histologic examination. Experimental conditions were concealed until after the slides were examined. Cecum, proximal colon, middle 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 2. An overall lesion score was calculated as the average of the scores for the four segments.

**Barrier Permeability**

Barrier permeability was assessed by analyzing serum concentrations of FITC-dextran following oral gavage as described.(29) Briefly, B6 and B6.mdr1a−/− 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-Aldrich, St. Louis, MO).
Total blood volume was collected at sacrifice via cardiac puncture. Blood samples were coagulated at 4º 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

Colon segments were snap frozen in liquid nitrogen and RNA was isolated from tissue using Trizol® as previously described (Invitrogen®, Carlsbad, CA).(30) Genomic DNA contamination was cleared from samples using the Turbo DNA-free kit from Applied Biosystems®. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit® (Roche, Pensburg, Germany). Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using TaqMan Universal PCR Mix® (Invitrogen®, Carlsbad, CA) in combination with Applied Biosystems® gene specific primer probe sets. RNA expression level was calculated using the crossing threshold of detectable fluorescence level as determined by the RT cycler MX3000P® (Stratagene®, La Jolla, CA). Crossing thresholds were then averaged to get a gene specific value which was then normalized to the average expression of the 18S housekeeping gene for each strain and experimental condition studied. The 18S housekeeping gene was utilized as the housekeeping gene in this study as published results indicate it is expressed with relatively stability under inflammatory conditions.(31-33) Changes in gene expression in the experimental groups were then calculated as an average fold change when compared to control strain values, and
expressed on a log 2 scale as fold changes from the control baseline (=1). This data analysis is as described in the Applied Biosystems manufacturer’s instructions (4371095 Rev A, PE Applied Biosystems, Foster City, CA). Data is considered physiologically relevant if changes in expression exceed a 2 fold change from control strain values. For a complete list of genes utilized and primer probes and sequences please see supplemental table III.

Elisa for H. bilus-Specific Serum IgG and Fecal IgA

ELISAs were performed to determine the specific IgG response to *H. bilus* in serum samples. In brief, a 96-well Immunlon Assay Plate (Fisher Scientific, Pittsburgh, PA) was coated with *H. bilis* sonicate (10 μg/mL in PBS) overnight at 4°C. Plates were washed 5 times with PBS with 0.05% Tween and nonspecific binding sites were blocked with 5% bovine serum albumin (BSA, Fisher Scientific, Pittsburgh, PA) in PBS for one hour at room temperature (RT). The plate was washed as before and then samples diluted in 1% BSA in PBS were incubated for two hours at room temperature. After a wash of five cycles, alkaline phosphatase-linked goat anti-mouse IgG (diluted 1:2000 in 1% BSA in PBS) was added to the wells and incubated for 2 hours at RT (IgG, Cat. #1030-04, 1ml stock solution, Southern Biotech; Birmingham, AL). The plate was washed another five times, and then the bound secondary antibody was detected using pNPP substrate solution (N-2770, Sigma, St. Louis, MO). The plates were read on a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. To determine the concentration of IgG anti-*H. bilis*, a standard curve of the corresponding IgG (5300-01; Southern Biotech) was run on each plate as described above with the exception of
coating the plate with 10μg/mL goat anti-mouse Ig (#1010-01, Southern Biotech). To
determine *H. bilis* specific fecal IgA, a similar protocol was followed with the exception
that alkaline phosphatase-linked goat anti-mouse IgA (diluted 1:2000 in 1% BSA in PBS)
was added to the wells and incubated for 2 hours at RT (IgA, Cat. #1040-04, 1ml stock
solution, Southern Biotech), and the standard curve utilized IgA (5300-01; Southern
Biotech).

**Statistical Analysis**

Statistics on continuous data was performed using the unpaired t-test in GraphPad
InStat 3® (San Diego, CA). Statistics on continuous data was performed using Student’s
T-test, and non-continuous data was analyzed using the Mann Whitney U test.
Comparisons having a p<0.05 were considered significant and are indicated by asterisks.
RESULTS

Absence of Spontaneous Colitis in B6.mdr1a⁻/⁻ mice

B6.mdr1a⁻/⁻ mice were sacrificed at 2, 5, 8, and 12 month of age, and examined for histological and phenotypic evidence of colitis. These time points were selected based on previous data from our lab indicating the significance of these times in the development of colitis in FVB.mdr1a⁻/⁻ animals. FVB.mdr1a⁻/⁻ in our facility demonstrate no clinical symptoms and are histologically free of colitis at 2 months (Fig. 1 B/C). By 5 months of age FVB.mdr1a⁻/⁻ animals begin to demonstrate weight loss and diarrhea and upon sacrifice 4 out of 10 animals showed histological evidence of colitis (Fig. 1 E/F). At 8 months of age, FVB.mdr1a⁻/⁻ demonstrate a 66% mortality rate (Fig. 2C) and 6 out of the 7 animals analyzed demonstrated histologically significant colitis (Fig 1 H/I)). Significant numbers of FVB.mdr1a⁻/⁻ mice at 12 month of age proved difficult to attain, as few of the animals survived to this time point; however, the three mice that were analyzed all had significant colitis (Fig. 1 K/L).

Over the lifespan of B6.mdr1a⁻/⁻ mice, clinical and gross observation and histologic examination showed that they were free of colitis at all selected time points (Fig. 2). This was confirmed by histological analysis (Fig 1). RNA was isolated from colonic tissue at the 2, 5, and 8 month time points to foster a better understanding of inflammatory conditions in the FVB.mdr1a⁻/⁻ large intestine as colitis progressed, and to enable us to better understand potential mitigating factors preventing colitis development in the B6.mdr1a⁻/⁻ colons (for a complete list of genes selected and fold change see
FIGURE 1. B6.mdr1a<sup>-/-</sup> and FVB.mdr1a<sup>-/-</sup> colonic histology. Representative examples of histology and histological scores are given for B6 and FVB P-gp deficient mice at 2 (A-C), 5 (D-F), 8 (G-I), and 12 (K-L) months of age. Images captured at 100X magnification. Bar=100µm. * Indicates statistical significance of P<0.05 as determined by Mann Whitney analysis. Values were significantly different when compared both between P-gp deficient strains, and when compared to control strains.
FIGURE 2. Weight changes and survival curves of B6 and FVB animals deficient in P-gp expression. Changes in body weight (A, B) are expressed from weaning at 4 weeks of age. Survival (C, D) was determined up to 8 months of age. Weights and survival were derived from an N of 8 for the B6, B6.mdr1a−/− and FVB animals, and 7 for the FVB.mdr1a−/−. § Indicates the incidence of diarrhea in FVB.mdr1a−/− animals. * Indicates statistically significant weight loss of P<0.05 in FVB.mdr1a−/− animals when compared to wild type controls, as determined by a Student’s T-test.
Supplemental Tables III & IV). The 12 month time point was not included due to the severely reduced survival of the FVB.mdr1a<sup>−/−</sup> animals.

It has been previously been shown that colitis in the FVB.mdr1a<sup>−/−</sup> model is characterized by an increase in colonic expression of inflammatory cytokines and chemokines such as IFN<sub>γ</sub>, TNFα, and IL6, which correlates to changes seen in colitic human tissue.(22, 23, 25) Our data demonstrates that colonic tissue from FVB.mdr1a<sup>−/−</sup> animals not only display dramatically increased production of the inflammatory cytokine IFN<sub>γ</sub>, IL6, and TNFα, but also IL17, MIP-2, and IL1β (Fig. 3A/B and Supplemental Table II). Moreover, it further demonstrates that in the FVB.mdr1a<sup>−/−</sup> animals production of these cytokines increases with age and/or development of colitis. Interestingly, the expression of a key enzyme (COX-2) required for production of lipid mediators of inflammation, prostaglandins, remain unchanged in the FVB.mdr1a<sup>−/−</sup> colons (Fig 3D). Conversely, data from the B6.mdr1a<sup>−/−</sup> colons indicates that these animals, despite a moderate increase in the gene expression of the inflammatory cytokines and chemokines IFN<sub>γ</sub>, MIP-2, TNFα, and IL1β at 2 months of age, fail to demonstrate the increased production of inflammatory cytokines in later months as seen in the corresponding FVB.mdr1a<sup>−/−</sup> animals (Fig. 3 and Supplemental Table IV). Furthermore, these animals display a moderate increase in Cox-2 production at early time points, indicating this may be a mitigating factor in strain linked pathology (Fig 3D). Given our observation that the B6.mdr1a<sup>−/−</sup> are resistant to the development of spontaneous colitis, we evaluated their resistance to the development of chemical- or bacterial-induced colitis.
FIGURE 3. Colonic gene expression in B6 and FVB animals deficient in P-gp expression. Gene expression was derived from RNA isolated from whole colonic tissue. Target gene expression was normalized to expression of the 18S housekeeping gene, and the average fold change in gene expression from control strain values. Range is calculated from the standard deviation of the \( \Delta \Delta CT \) value and the average gene expression is plotted with upper and lower limits of the range shown. Data is shown on a log 2 scale. Dotted lines demonstrate the range of physiological relevance, all values demonstrating a 2 fold alteration in gene expression from control values are considered physiologically different from control values. Gene expression of IFN\( \gamma \) (A), IL17 (B), IL10 (C), and COX-2 (D) are represented graphically. Expression of other genes of interest can be found in Supplemental Table II. A minimum N of 2, 4, and 6 animals from control and mutant strains were analyzed for the 2, 5, and 8 month time points respectively. * Indicates statistical significance of P<.05 as determined by the Student's T-test when the B6.mdr1a\(^{-/-}\) is compared to the FVB.mdr1a\(^{-/-}\). § Indicates statistical significance of p<.05 between P-gp deficient and control animals as determined by Student’s T-Test
**H. bilis infection does not induce colitis in B6.mdr1a^{-/-} mice**

It has been previously reported that inoculation of FVB.mdr1a^{-/-} animals with *H. bilis* accelerates the development of colitis, corresponding with an increased inflammatory response in the mesenteric lymph node cells. FVB.mdr1a^{-/-} animals were shown to develop diarrhea and hunching within 3-4 weeks of inoculation (9-10 weeks of age), and severe colitis by 16 weeks post-inoculation (22 weeks of age). Similar to previous reports we found that in our colony, FVB.mdr1a^{-/-} but not FVB control strain animals, developed a significant colitis at 12 weeks post inoculation (18 weeks of age, data not shown). B6.mdr1a^{-/-} and B6 controls were sacrificed at both 4 and 12 weeks post inoculation with *H. bilis* and examined for evidence of colitis. At 4 weeks post-inoculation B6 and B6.mdr1a^{-/-} demonstrated no clinical or macroscopic indicators of colitis. Given the inherent resistance of the B6.mdr1a^{-/-} strain, we went on to examine animals at 12 weeks post-inoculation. B6.mdr1a^{-/-} animals demonstrated reduced weight gain over the course of the experiment when compared to inoculated wild type controls, however, at sacrifice they were shown to be free of diarrhea, fecal blood and alterations to intestinal permeability (Fig 4 and Table I). Serum and feces harvested at the time of sacrifice were analyzed for the presence of antibodies specific for *H. bilis*. B6 and B6.mdr1a^{-/-} inoculated animals showed expression of serum IgG specific for *H. bilis*, while levels of *H. bilis* specific fecal IgA remained below quantifiable limit. There was no significant difference in the levels of anti-*H. bilis* IgG between *H. bilis* infected B6 and B6.mdr1a^{-/-}, although control strain B6 animals had a tendency towards higher levels of antibody expression (Fig. 5). Additionally, antibody levels were not comparable to those seen in inoculated FVB.mdr1a^{-/-} animals, which were shown to be in excess of...
FIGURE 4. Evaluation of colitis in B6 and B6.mdr1a<sup>−/−</sup> mice following inoculation with <i>H. bilis</i>. Alterations in body weight in B6 and B6.mdr1a<sup>−/−</sup> following inoculation with <i>H. bilis</i>. (A) Colonic histology of B6 (B) and B6.mdr1a<sup>−/−</sup> (C) animals at sacrifice. Colonic length (D) and histological scoring of inoculated animals (E). Images captured at 100X magnification. Bar=100µm. * Indicates statistical significance of P<0.05 when comparing P-gp deficient and control animals inoculated with <i>H.bilis</i> as determined by a Student’s T-test.
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<tr>
<td>DSS</td>
<td>1/9</td>
<td>0/9</td>
<td>0/9</td>
<td>4.72 ± 1.90</td>
</tr>
<tr>
<td>Mock</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>1.00 ± 1.31</td>
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<tr>
<td>Piroxicam</td>
<td>0/10</td>
<td>3/10</td>
<td>3/10</td>
<td>0.49 ± 0.38</td>
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</tbody>
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*Indicates statistical significance of $P < 0.05$ as determined by Student’s t-test when B6.mdr1a/−/− is compared with FVB.mdr1a/−/−.

*Indicates statistical significance of $P < 0.05$ between Pgp deficient and control animals as determined by Student’s t-test.
FIGURE 5. Levels of serum *H. bilis* antibodies in inoculated B6.*mdr1a*-/ and control B6 mice. Serum levels of anti-*H. bilis* IgG were determined using an *H. bilis* specific ELISA, and quantified using total IgG controls. N=5-12 animals per strain/treatment type.
9000ng/ml. Colonic tissue appeared healthy at time of sacrifice and displayed no alterations in either length or weight (Fig 4D and data not shown). Histological examination confirmed these results (Fig 4B, C & E)).

**B6.mdr1a<sup>−/−</sup> mice have increased susceptibility to DSS-induced colitis**

P-gp deficiency has been shown to affect epithelial permeability, and is associated with altered phosphorylation of proteins involved in the formation of tight junctions, resulting in impaired intestinal integrity.(21) It has been previously reported that FVB.mdr1a<sup>−/−</sup> animals show an accelerated colitis development following exposure to DSS.(25) DSS serves to disrupt the intestinal epithelium, and has been shown to induce colitis in susceptible models. B6.mdr1a<sup>−/−</sup> and wild type B6 animals were given 2.25% DSS in drinking water for 7 days and sacrificed on day 11. B6.mdr1a<sup>−/−</sup> animals developed a more severe colitis in response to DSS treatment, as evidenced by a significant reduction in weight and an increased incidence of fecal blood and diarrhea (Fig 6A, and Table I). Colonic length was significantly reduced in B6.mdr1a<sup>−/−</sup> mice and they demonstrated macroscopic evidence of colitis at time of sacrifice (Fig 6C,D). Histological examination confirmed these findings and demonstrated that DSS-treated P-gp deficient animals had significantly higher colonic histology scores than did DSS-treated B6 control mice (Fig 6E). The DSS-treated B6.mdr1a<sup>−/−</sup> additionally demonstrated an increased colonic permeability to FITC-dextran as measured by serum levels following oral gavage (Table I). Results were mirrored in the long term DSS colitis models (Supplemental Fig. 1). Interestingly, it has been previously observed in the FVB.mdr1a<sup>−/−</sup> model that males demonstrate an increased incidence of colitis when
**FIGURE 6.** Evaluation of colitis in B6 and B6.mdr1a^-/- mice following treatment with DSS. Reductions in body weight in B6 and B6.mdr1a^-/- undergoing treatment with DSS (A). The black bar represents time period of DSS treatment. Colonic histology of B6 (B), and B6.mdr1a^-/- (C) animals at sacrifice. Average colonic length (D) and histological scoring (E) of treated animals and controls. Closed circles represent female animals, open circles represent male animals. Images captured at 100X magnification. Bar=100µm.* Indicates statistical significance of P<0.05 as determined by Mann Whitney (A & C), or P<0.05 as determined by Student’s T-test (B) when comparing P-gp deficient and control animals treated with DSS.
compared to female animals. Our experiments contained mixed gender populations, which seems to be the source of the variance displayed in our histology scoring. B6.mdr1a−/− male animals treated with DSS typically had higher histological scores when compared to female animals (Fig. 6E and Supplemental Fig. 1).

**Piroxicam-diet does not induce colitis in B6.mdr1a−/− mice**

Our initial colonic gene expression data suggested a potential role for elevated prostaglandin (PG) synthesis in modulating disease development in B6.mdr1a−/− mice, as gene expression was elevated in the colons isolated from the 2 month B6.mdr1a−/− mice. PGs are lipid mediators that have been shown to play a role in mediating inflammation in arthritis and inflammatory bowel disease.(34-36) PG synthesis is tightly regulated through expression of the COX enzymes. To better examine the potential role of COX in preventing or dampening intestinal inflammation in these animals, we treated B6.mdr1a−/− and wild type B6 mice with piroxicam, a NSAID that inhibits COX function.(27) B6.mdr1a−/− and B6 controls were initially treated for 7 days with piroxicam supplemented chow. Despite early clinical signs of disease including weight loss, and increased mortality, none of the animals treated showed any histological evidence of colitis (Supplemental Fig 2). We next looked at the effects of a long term piroxicam diet on P-gp deficient animals. Animals were treated with piroxicam supplemented chow for 2 weeks followed by a 4 week recovery period during which they returned to normal rodent diet. Animals were monitored on a weekly basis for weight loss and other physiologic evidence of colitis. B6.mdr1a−/− mice demonstrated increased weight loss during the period of piroxicam treatment, and additionally one animal had had transient
fecal blood (Table I, Fig 7A). However, at sacrifice animals had an unaltered colonic phenotype including unperturbed colonic length, barrier permeability, and colonic histology (Table I, Fig 7). B6.mdr1a\(^{+/-}\) animals displayed a slightly increased mortality in response to piroxicam treatment that was not seen in control strain animals, however, upon sacrifice this appeared to be associated with the development of liver inflammation (Table I, Fig 8). Liver inflammation was notably absent in both B6 controls, B6.mdr1a\(^{+/-}\) controls, and B6 piroxicam treated animals (Fig 8).
FIGURE 7. Evaluation of colitis in B6 and B6.mdr1a<sup>-/-</sup> mice following treatment with piroxicam. Body weight in B6 and B6.mdr1a<sup>-/-</sup> undergoing treatment with Piroxicam (A). Colonic histology of B6 (B), and B6.mdr1a<sup>-/-</sup> (C) animals at sacrifice. Colonic length (D) and histological scores of treated animals (E). Images captured at 100X magnification. Bar=100µm. * Indicates statistical significance of P<0.05 as determined by Mann Whitney analysis when comparing piroxicam treated P-gp deficient and control strain animals.
FIGURE 8. Liver histology from B6 control and P-gp deficient piroxicam treated animals. B6 (A), B6.mdr1a-/-(B) and piroxicam treated B6 wild type animals demonstrated no significant liver histological changes or inflammation, while B6.mdr1a-- piroxicam treated animals showed severe liver inflammation. Images captured at 400X magnification. Bar=50µm
DISCUSSION

The FVB \textit{mdr1a} deficient animal was originally derived for the purposes of drug extrusion studies at the blood brain barrier.\(^{(28)}\) It was subsequently discovered that P-gp’s pumping capabilities were integral to the function of the intestinal epithelium, as indicated by the development of spontaneous colitis in these animals.\(^{(20)}\) P-gp function is of particular importance with respect to interactions involving the intestinal microbiota, as it has been demonstrated that prophylactic treatment with antibiotics prevented colitis development for up to 16 weeks in P-gp deficient animals. Additionally, it was shown that 10 weeks of antibiotic treatment ameliorated colitis in FVB \textit{mdr1a}^-^-^- mices.\(^{(20)}\)

Chimeric animals were generated to examine the respective roles P-gp deficiency in the intestinal epithelium, as compared to in immune effector cells with regards to colitis development. It was shown that P-gp deficiency in the intestinal epithelium was necessary and sufficient for the development of colitis FVB \textit{mdr1a}^-^-^- animals.\(^{(20)}\)

Subsequent work has focused on examining the effects of P-pg deficiency on the integrity of the intestinal epithelium. Evidence indicates that FVB \textit{mdr1a}^-^-^- animals develop an altered permeability to molecularly tagged substrates at approximately 12 weeks of age, and prior to the development of any clinical or histological evidence of colitis.\(^{(21)}\) These permeability changes correlate with altered phosphorylation of junctional proteins occludin and zonula occludin-1 (ZO-1).\(^{(21)}\) These data clearly indicate that histological and phenotypic evidence of colitis occurs secondarily to the development of altered intestinal barrier function. Although the precise mechanism remains unclear it has also recently been demonstrated that FVB \textit{mdr1a}^-^-^- demonstrate an increased expression of inflammatory cytokines, and an increased responsiveness to LPS
prior to the development of intestinal inflammation. It was our initial hypothesis that alterations in P-gp expression would affect the animal’s ability to respond to bacterial stimuli. In hopes of better understanding the affects of P-gp expression to individual bacterial ligands we re-derived the mdr1a mutation on the C57BL/6 background and began our efforts to characterize the development of colitis on this background.

P-gp deficient mice on the B6 and FVB backgrounds were first analyzed at 2 months of age. Although neither strain demonstrated histological colitis, both the B6.mdr1a−/− and the FVB.mdr1a−/− colons had increased expression of the inflammatory cytokines and chemokines IFNγ, MIP2, TNFα and IL1β. The strains differed in their expression of colonic IL17 (high in FVB.mdr1a−/−) and COX-2 (high in B6.mdr1a−/−), therefore providing the first clue that genetic strain background was modifying the intestinal effects of P-gp deficiency. This genetic background effect was even stronger at the later times examined, as the FVB.mdr1a−/− continued to have increased inflammatory cytokine and chemokine expression and significant histological colitis, while the B6.mdr1a−/− was similar to its B6 wildtype control in both colonic cytokine levels and histology. This modulation of colitis incidence by genetic background may help to explain the conflicting data on the association of P-gp mutations and colitis incidence in human populations. Due to this genetic resistance to colitis in the absence of P-gp, we investigated other environmental insults known to induce colitis in murine models.

*H. bilis* is a microaerophilic gram-negative organism which colonizes the liver and lower GI tract of susceptible hosts. It has been shown to induce colitis in a number of immune-dysregulated animal models including immunodeficient rats, SCID mice, and B6 mice deficient in IL10 gene expression. It has previously been
shown that bacterial colonization of the FVB.\textit{mdr1a}^{-/-} mice with \textit{Helicobacter bilis} induces a more rapid, and phenotypically distinct colitis.(24) FVB.\textit{mdr1a}^{-/-} animals display a significant alterations intestinal permeability as early as 4-6 weeks of age, prior to the development of colitic phenotype.(21, 22) It may be that the accelerated colitic phenotype seen in this model system is a result of the increased immune access resulting from the impaired intestinal integrity. To determine whether \textit{H. bilis} would serve as an adequate environmental factor to initiate inflammatory bowel disease we inoculated our newly derived B6.\textit{mdr1a}^{-/-} mice. Our results demonstrated that contrary to the colitis induced in FVB.\textit{mdr1a}^{-/-} mice, inoculation with \textit{H. bilis} was not a sufficient to induce colitis in B6.\textit{mdr1a}^{-/-}. It should be noted that the B6 and B6.\textit{mdr1a}^{-/-} mice did recognize the \textit{H. bilis} innoculum, as demonstrated by the serum IgG response; however, this immune response was not associated with colitis and did not extend to a mucosal fecal IgA response.

As the FVB.\textit{mdr1a}^{-/-} disease model has aberrant functioning of the intestinal epithelium, we hypothesized that chemical disruption of the intestinal epithelium may increase colitis susceptibility in B6.\textit{mdr1a}^{-/-} deficient animals. Additional support for this theory comes from the recent report that FVB.\textit{mdr1a}^{-/-} showed accelerated colitis development following DSS treatment.(25) Furthermore, mice deficient in expression of the multi-drug resistant protein-1, a transport protein of similar function and substrate specificity to \textit{mdr1a}, although resistant to spontaneous colitis show an increased susceptibility to both DSS and TNBS induced colitis.(41) Interestingly, the \textit{mrp-1} deficient mouse was also derived on the FVB background, which has subsequently been shown to be innately more resistant to DSS colitis, requiring far higher doses DSS than
those reported those previously reported for the C57BL/6 animal. (25, 42) This indicates both an increasingly important role for P-gp in mitigating DSS colitis, and implied that colitis induction in our strain would take place at much lower doses than those described for FVB. mdr1a deficient animals.

DSS is a sulfated polysaccharide, and although the exact mechanism of colitis induction is unknown; it has been shown to have toxic affects on the intestinal epithelium and cause stripping of epithelial cells. It was our hypothesis that animals deficient in P-gp would be more susceptible to damage induced at the epithelial level. To investigate the effects of epithelial insult as a mitigating factor in disease development, B6. mdr1a−/− and control strain mice were treated with DSS. B6. mdr1a−/− animals were shown to develop a significant colitis in response to treatment with DSS in both long term and short term treatment models. It has been suggested that P-gp expression plays a role in cellular apoptosis, and regulation of cell cycle. (43) It may be that the increased susceptibility to DSS-induced colitis in the P-gp deficient animals is directly linked to impaired cell cycling, thus affecting wound repair. Further studies will need to be conducted to determine whether this plays a factor.

Our initial studies done to characterize strain specific disease development revealed that B6. mdr1a−/− mice demonstrated altered colonic gene expression at 2 months of age, when compared to B6 controls, even though they never developed spontaneous colitis. B6. mdr1a−/− animals expressed elevated levels of IFNγ, MIP2, TNFα, IL1β, and COX-2 at 2-months of age. This increased gene expression returned to normal by 5-months of age. The COX enzymes tightly regulate the synthesis of the lipid inflammatory mediators prostaglandins (PG), thromboxanes and prostacyclins. (44) PGs
have been shown to have strong immunomodulatory effects and can inhibit LPS-induced inflammation. (45, 46) COX-1 expression is considered constitutive and has been associated with intestinal maintenance and homeostatic mechanisms, while COX-2 expression is induced in response to inflammatory stimuli and cytokines. (47-49) It has been previously shown that intestinal inflammation can be initiated through inhibition of COX-2, and is thought to be a function of disregulation of its biosynthetic bi-products. (50) COX-2 has been previously shown to be integral in regulating the immune response in IL10 deficient animals on the resistant C57BL/6 background, perhaps serving as a potential immune modulator in the absence of IL10. (27) In keeping with this hypothesis, treatment of B6.IL10 deficient animals with the COX-inhibitor, piroxicam, has been shown to induce an acceleration of colitis onset. (27) We hypothesized that in our model system early upregulation of COX-2 gene expression might be acting to modulate the immune response therefore preventing the development of colitis. To clarify the potential role of COX-2 gene expression in mitigating colitis development, B6.mdr1a<sup>−/−</sup> animals were treated with piroxicam. We show that COX inhibition has no affect on the colitis resistance seen in B6.mdr1a<sup>−/−</sup> mice. This is perhaps not surprising as it has also been previously shown that increased COX-2 expression reduces expression of P-gp, and that P-gp expression is decreased in IL10 deficient animals. (51) It may be that the diminished or even absent P-gp expression enhances the affect of COX-2 products, such as prostaglandins, on the intestinal epithelium, and that the initiation of inflammation after inhibition of COX function requires additional inhibition of IL10 expression.
One possible explanation for the lack of colitis development in our B6.mdr1a\textsuperscript{+/-} animals could be linked to unaltered barrier permeability. Initial studies utilizing this animal model indicate that these animals show no evidence of alteration in intestinal barrier function, as quantified by permeability to FITC-Dextran. This theory also is supported by the fact that we see no evidence of colitis in our \textit{H. bilis} inoculated animals. It may be that the lack of invasive properties of this bacterium, in conjunction with unaltered barrier function in our animals, prevented the bacterium from initiating a significant mucosal immune response. This would the complete lack of fecal antibodies seen in our inoculated animals. It may be that an organism more capable of penetrating the intestinal epithelium would be better suited to initiate colitis in the B6.mdr1a\textsuperscript{+/-} model. Furthermore we have shown that an insult to the intestinal epithelium serves as an initiating factor for colitis development in our animals, arguing that upon epithelial insult these animals develop colitis similar to that seen in the FVB.mdr1a\textsuperscript{+/-} animal model.
ACKNOWLEDGEMENTS

This work was supported in part by the NIH grants P01 DK071176, T32A107051 and the University of Alabama at Birmingham Digestive Diseases Research Development Center Grant #P30 DK064400. We would like to thank Peggy R. McKie-Bell and Jamie L. McNaught for their technical assistance and members of the Lorenz for valuable advice. We thank Dr. Chuck O. Elson for use of the Synergy Microplate Reader and Wayne Duck for experimental input and comments. We would also like to thank the Gnotobiotic and Genetically Engineered Mouse Core Facility for technical assistance.
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44. van der Heijde DM. The continuing challenge of predictive factors in rheumatoid arthritis: prediction or association? J Rheumatol. 1997;24:6-8


Supplement 1. Colitis in B6 and B6.mdr1a<sup>−/−</sup> mice following long term treatment with DSS. Average colonic length (A) and histological scoring (B) of B6.mdr1a<sup>−/−</sup> and wild type B6 controls. Clinical measures of colitis are assessed in panel C, data is given for diarrhea, presence of fecal blood, increased mortality following treatment, and increased intestinal permeability to FITC-Dextran.* Indicates statistical significance of P<.05 as determined by Mann Whitney (A, and C) when comparing P-gp deficient and control animals treated with alternating cycles of DSS infused drinking water.
Supplement 2. Evaluation of colitis in B6 and B6.mdr1a\textsuperscript{−/−} mice following short term treatment with piroxicam. Average colonic length (A) and histological scoring (B) of B6.mdr1a\textsuperscript{−/−} and wild type B6 controls. Clinical measures of colitis are assessed in panel C. Data is given for diarrhea, presence of fecal blood, mortality following treatment, and intestinal permeability to FITC-Dextran.

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<th>Death</th>
<th>Serum FITC-Dextran (ng/ml)</th>
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<td>B6</td>
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<td>0/4</td>
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<td>0.60 ±0.54</td>
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<td>Piro</td>
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<td>0/6</td>
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<td>0/8</td>
<td>0/8</td>
<td>0.49 ±0.83</td>
</tr>
<tr>
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<td>Piro</td>
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<td>1.47 ±1.52</td>
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**Supplement 3.** Primer Probe Pairs Utilized for quantitative RT-PCR.

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**Supplement 4.** Colonic RNA Production in B6 and FVB P-gp Deficient Animals.

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<td>FVB. mdr1a&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>61.32 (9.3-406.7)</td>
<td>$*40.90$ (4.7-353.6)</td>
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<td>IL17</td>
<td>B6. mdr1a&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.35 (0.3-0.5)</td>
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<td>43.9 (21.9-88.2)</td>
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<td>*2.03 (1.1-3.7)</td>
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<td>68.59 (50.1-93.9)</td>
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<td>4.56 (1.2-17.2)</td>
<td>2.01 (29.5-0.1)</td>
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<td>IL1β</td>
<td>B6. mdr1a&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>11.24 (9.3-13.5)</td>
<td>$*0.02$ (0.01-0.05)</td>
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<td>FVB. mdr1a&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2.52 (1.0-6.1)</td>
<td>* 3.50 (0.9-13.6)</td>
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* Indicates statistical significance of $P<0.05$ as determined by the Students T-test when the B6. mdr1a<sup>−/−</sup> is compared to the FVB. mdr1a<sup>−/−</sup>
§ Indicates statistical significance of $p<0.05$ between P-gp deficient and control animals as determined by Student’s T-Test
P-GLYCOPROTEIN DEFICIENCY ALTERS RESPONSIVENESS TO BACTERIAL LIGANDS AND CONFERS RADIOPROTECTION

by

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Format adapted for dissertation
ABSTRACT

P-glycoprotein (P-gp) is the functional product of the multidrug resistance genes (MDR). P-gp is an ATP dependent transmembrane pump expressed at multiple tissue locations, and is known to extrude amphipathic and hydrophobic substrates from the intracellular environment. It was recently shown that P-gp regulates cellular apoptosis, indicating a role for P-gp in mediating intestinal injury and repair. Radiation injury is the best characterized model for studying intestinal injury repair. Injury repair following irradiation injury is mediated via cyclooxygenase 1 (COX1) and subsequent production of prostaglandins (PGE\textsuperscript{2}). LPS has been shown to confer radioprotection by inducing production of TNF\textgreek{a} and subsequently COX2 and PGE\textsubscript{2}. We utilized a clonogenic assay to study the role of P-gp expression in repair of radiation induced injury.

C57BL6.mdr1a\textsuperscript{-/-} mice and wild type controls were subjected to 12Gy total body X-ray irradiation; surviving crypts in the proximal jejunum and distal colon were evaluated 3.5 days following irradiation. B6.mdr1a\textsuperscript{-/-} exhibit normal COX dependent crypt regeneration following irradiation, and demonstrate minor LPS induced radioprotection. However, LPS induced radioprotection is significantly higher in wild type animals. LPS treatment in wild type animals induces increased gene expression of radioprotective cytokines IL22, and IL1\textgreek{a} not seen in B6.mdr1a\textsuperscript{-/-}, both of which may play a role in increasing crypt regeneration following exposure to irradiation.

P-glycoprotein, X-ray radiation, prostaglandin (PGE\textsubscript{2}), epithelial cell.
INTRODUCTION

P-glycoprotein (P-gp) is the functional product of the multidrug resistance genes (MDR). It is a glycosylated transmembrane protein 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 (8, 19, 39). P-gp, also known as abcb1a, is a member of the ATP-binding cassette super-family of transporters and is known to pump a variety of amphipathic and hydrophobic substrates from the intracellular environment (12, 20). Expression of MDR polymorphisms has been associated with the development of inflammatory bowel disease (IBD) in specific patient populations, particularly most recently with pediatric ulcerative colitis (UC) (10). Deficiency in expression of the murine homologue, mdr1a, has been shown to induce a bacterial mediated spontaneous colitis in conventionally housed animals on the FVB/N background (10, 15, 24, 26). Colitis in P-gp deficient animals is associated with impaired mucosal integrity prior to the development of clinical symptoms of colitis (31). Introduction of the P-gp deficiency onto an animal model known to be resistant to colitis, has been shown to render them more susceptible to colitis resulting from epithelial disruption, indicating a role for mdr gene expression in maintaining intestinal homeostasis and injury repair (37).

The intestinal epithelium exists in a state of continuous regeneration maintained by multipotent stem cells found at the base of intestinal crypts. Stem cells divide producing daughter stem cells, or highly proliferative transit cells (2, 4). Transit cells
migrate to the proliferative zone found in the lower portion of each crypt and further
divide and differentiate to produce mature epithelial cells (29). Mature epithelial cells
then migrate onto the intestinal villi where they remain, unless damaged, for anywhere
from 3 to 5 days prior to being sloughed off into the intestinal lumen (13, 14).

One of the best characterized models for studying injury repair and homeostasis in
the intestinal epithelium is the radiation-induced injury model (27-29). Radiation injury
targets rapidly dividing cells, by inducing DNA damage, causing cell cycle arrest and
apoptosis. High doses of radiation effectively eliminate rapidly dividing cells in the crypt
proliferative zone, and denude the intestinal epithelium. Regeneration of the intestinal
barrier then becomes the sole responsibility of crypt stem cells (27-29).

Crypt regeneration following radiation injury has been shown to be dependent on
induction of cyclooxygenase 1 (COX1) and subsequent synthesis of prostaglandins
(PGE₂) (17). PGs are lipid mediators known to play an integral role in multiple
biological processes such as wound healing and blood clotting, and are crucial in
maintenance of the intestinal mucosa (9, 22, 23, 40). Prostaglandin synthesis is controlled
via cyclooxygenase (COX) enzymes, and serves to mediate inflammation by controlling
mucosal blood flow, and bicarbonate secretion (41).

Lipopolysaccharide (LPS) mitigates radiation induced injury in the murine small
intestine by stimulating further PGE₂ synthesis (32). LPS acts to stimulate production of
TNFα from immune cells. TNFα has been shown to signal through TNFR1 on
subepithelial fibroblasts to stimulate production of COX2, ultimately driving epithelial
synthesis of PGE₂ and reducing the effects of radiation injury and radiation induced
epithelial cell apoptosis (32).
Expression of P-gp has also been associated with apoptotic resistance. Kidney proximal tubules cells were shown to be protected from cadmium and reactive oxygen species induced apoptosis as a result of increased P-gp expression (38). *In vitro* studies using blasts from patients with acute myeloid leukemia have shown that over-expression of *MDR* correlates with increased cell survival or decreased apoptosis (25).

We have recently shown that the spontaneous colitis seen in the FVB/N.*mdr1a*\(^{+/-}\) animal model is not observed if the *mdrla* mutation is introduced onto the C57BL/6 background (37). The C57BL/6 background strain not only conferred resistance to spontaneous colitis, but RNA analysis of colonic tissue showed altered patterns of inflammatory cytokines. Analysis also showed increased expression of COX2 throughout development, potentially mitigating damage to mucosal integrity, and playing a role in wound healing and repair (37). We chose to utilize B6*mdrla*\(^{-/-}\) animals for the purposes of these studies to rule out potential artifact induced by spontaneous disease seen in FVB animals lacking P-gp expression.

The purpose of this study was to investigate the role of *mdr* gene expression on epithelial maintenance and injury repair in the murine small intestine. Furthermore, it was our goal to evaluate the potential radioprotective affects of both LPS and COX gene expression in this model.
MATERIALS AND METHODS

Animals

C57BL/6J.129P2-Abcb1tam1BorN7 (B6.mdr1a−/−) were derived as previously described (37). The recently derived B6.mdr1a−/− were subsequently mated with C57BL6.tlr4−/− which were a kind gift from Drs. Shizou Akira, and Doug Golenbock. Animals are bred and maintained under specific pathogen free (SPF) conditions in Thoren Isolator racks (Hazleton, PA) under positive pressure and are fed autoclaved NIH-31 rodent diet (Harlan Teklad, Madison, WI), and sterile drinking water ad libitum. Animals were acclimatized to our facility 2 weeks prior to mating. Genotype is confirmed by periodic genotyping by polymerase chain reaction (PCR). In brief, tail clippings are collected, and DNA is extracted using Sigma’s RED Extract-N-Amp tissue PCR kit (St Louis, MO). Phenotype (or lack of mdr or tlr expression) is confirmed as indicated by using the primers MDR1A S2 CTCCCTCCAAGGTGCATAGACC, MDR1A W2 CCCAGCTCTTCATCTAACTACCCTG, and MDR1A K02 CTTCCCAGCCTCTTGAGCCCAG or TLR4A CGTGTAAACCAGCCAGGTTTTGAAGGC, TLR4B TGTTGCCCTTGCATCTTTGACTACCTG, and TLR4C ATCGCCTTATCAGCTTCTTTTGGAGC 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 are then run on a 1.5% agarose gel, and presence or absence of mdr1a or tlr4 gene expression is confirmed by analyzing banding patterns produced by amplification products (Invitrogen,
Carlsbad, CA). 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, murine norovirus, 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**

Animals were subjected to either sub-lethal (two doses of 3Gy), or lethal (12Gy) doses of X-ray irradiation at 8-12 weeks of age in the X-RAD 320 irradiator (Precision X-Ray Inc. N. Branford, CT). Animals were sacrificed at either 6 hours (for apoptotic studies) or 3.5 days (for crypt regeneration studies) following lethal irradiation. Colon and proximal jejunum were harvested and fixed in bouins solution (Fisher). Tissue was then cut into 5mm segments and embedded longitudinally in paraffin to allow for cross-sectional analysis. The distal ileum was harvested and placed in RNA later at 4° for 24 hours, and then removed and stored at -20° prior to RNA isolation (Ambion®, Austin, TX).
LPS Injection

B6.mdr1a−/− and wild type control animals were given intraperitoneal (i.p.) injections of with 0.5 mg/kg LPS Escherichia coli (0111:B4, Sigma) dissolved in sterile pyrogen free phosphate buffered saline (PBS, Mediatech, Manassas, VA), or PBS alone 24 hours prior to irradiation.

Indomethacin Treatment

Indomethacin (Sigma) was dissolved at 12.5mg/ml into 70% ethanol and further diluted in 5% sterile phosphate buffered saline prior to use. B6.mdr1a−/− animals were treated with 1.5 mg/kg indomethacin i.p. every 8 hours beginning 14 hours prior to irradiation.

Crypt Micro Colony Assay

Regeneration cells were labeled with BrdUrd as described by Lorenz et al (34). Crypt regeneration was quantified in animals 3.5 days following irradiation as described previously by (32). Animals were injected i.p. with bromodeoxyuridine (BrdUrd) to label cell synthesis (Sigma). Tissue was fixed at sacrifice using cold bouins (Fisher, Pittsburg, PA). Paraffin embedded tissue sections were sliced in 5 um sections and deparaffinized using Citrisolv (Fisher). Cellular DNA was denatured by exposing the slides to 2N hydrochloric acid for 30 minutes (Fisher). Non-specific protein binding sites were blocked through incubation blocking buffer containing 1% bovine serum albumin (Fisher), 0.2% non-fat powdered dry milk (Nestle, Vevey, Switzerland), and 0.3% Triton-X 100 (Fisher) dissolved in PBS. BrdUrd incorporation was detected using a polyclonal
goat anti-BrdUrd antibody, and kind gift of Dr. S. M. Cohn of the University of Virginia
diluted in blocking buffer, and incubated at 4° overnight (6). BrdUrd antibodies were
labeled with Cy3, and nuclei were stained using Hoechst bisbenzamide 33258
(Sigma). Cellular regeneration was assessed via fluorescence microscopy using the Zeiss
Axioskop 2 (Carl Zeiss Inc, Thornwood, NY). A regenerative crypt was defined as
containing at least 5 cells demonstrating BrdUrd incorporation-and average of 6 intestinal
cross-sections were analyzed per mouse.

**Apoptosis**

Cellular apoptosis was confirmed visually using microscopy by the presence of
morphological characteristics-cell shrinkage, chromatin condensation, margination, and
cellular fragmentation. However, for more accurate quantization of apoptotic activity
tissue sections were subjected to terminal deoxynucleotidyl transferase mediated dUTP
nick end labeling (TUNEL) using the ApopTag apoptosis kit available from Millipore
(Billerica, MA). Nuclei were again stained using Hoechst bisbenzamide. To quantify
apoptotic cells 1 image was captured per cross-section at 200X magnification. The villi
and muscularis were removed from the image, and alternate channels were extracted
separately, and inverted to achieve a black staining pattern on a white background.
Results are expressed as the ratio of the positive area extracted from TUNEL channel
over the positive area extract from the Hoescht channel.
RNA Isolation and RT-PCR

RNA was isolated from distal ileal tissue using Trizol® as described in Sacchi et al (Invitrogen®, Carlsbad, CA) (5). 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®, Carlsbad, CA). 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 it’s relative stability or expression under inflammatory conditions (1, 33, 35). 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. For a complete list of genes utilized and primer probes and sequences please see supplemental table I.
PGE₂ ELISA

PGE₂ production following irradiation was quantified using the Prostaglandin E₂ EIA Kit available from Caymen (Ann Arbor, MI). Tissue from the proximal ileum was harvested at sacrifice and snap frozen in liquid nitrogen until use. Tissue was weighed prior to mechanical disruption in 5mls homogenization buffer (0.1M phosphate buffer containing 1mM EDTA, 10µM indomethacin). Protein was diluted 1:400 and applied to ELISA plate as directed. Sample was detected at range of 1-0.078 ng/ml. Protein is expressed as ng/mg of tissue homogenized.

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.
RESULTS

Effects of Sub-lethal Irradiation

It has been previously shown that B6.mdr1a\textsuperscript{\textminus/}\textsuperscript{\textminus} animals are more susceptible to colitis induction following chemical disruption of the intestinal epithelium (37). To assess potential effects of P-gp deficiency on injury repair mechanisms in the murine intestine; we administered sub-lethal doses of irradiation to B6 and B6.mdr1a\textsuperscript{\textminus/}\textsuperscript{\textminus} animals. Animals were exposed to 6Gy X-ray irradiation in two doses of 3Gy administered 4 hours apart. Animals were monitored for weight loss and increased mortality. B6.mdr1a\textsuperscript{\textminus/}\textsuperscript{\textminus} animals demonstrated increased weight loss, and increased mortality following exposure to low doses of irradiation (Fig 1).

Baseline and Radiation Induced Intestinal Apoptosis

Radiation targets rapidly dividing cells by damaging DNA during cell synthesis (27-29). It has been previously shown that over-expression of P-gp increases cellular resistance to apoptosis (25, 38). B6.mdr1a\textsuperscript{\textminus/}\textsuperscript{\textminus} animals may demonstrate an increased susceptibility to radiation damage as a result of increased rates of apoptosis, conversely increasing cell cycling. Clonogenic assays have been previously developed to assess apoptosis, and epithelial cell cycle in the proximal jejunum (17). As P-gp is expressed in the jejunum we sought to initially evaluate the effects of mdr1a gene deficiency in small intestinal tissue.

To examine levels of apoptosis in the intestinal epithelium following radiation injury B6.mdr1a\textsuperscript{\textminus/}\textsuperscript{\textminus} and B6 control animals were lethally irradiated and sacrificed 6 hours
**Figure 1.** Weight chart and survival curve for Sublethally Irradiated B6.mdr1a⁻/⁻ and B6 Control Mice. 8-12 week old Male B6 (●), and B6.mdr1a⁻/⁻ (■) were exposed to a total of 6Gy of X-ray radiation, administered as 2 doses of 3Gy approximately 4 hours apart. Animals were monitored for weight loss (A), and mortality (B). Animals were sacrificed upon losing 20-30% of initial body weight. N=7 for irradiated and non-irradiated B6.mdr1a⁻/⁻ animals, N=3 for irradiated B6 animals and N=2 for non-irradiated controls. * indicates statistical significance of P<0.05 between irradiated and non-irradiated animals of the same strain, and § indicates statistical significance of P<0.05 between B6 and B6.mdr1a⁻/⁻ irradiated animals.
following irradiation. Apoptosis was detected by TUNEL, and quantified as a ratio of apoptotic area to the area of nuclear staining. B6.mdr1a−/− animals demonstrated a non-significant increase in apoptotic area at baseline and following radiation injury when compared to wild type controls; indicating increased apoptosis may play a role in increased mortality of B6.mdr1a−/− animals following multiple exposures to low dose irradiation (Figure 2A-C). One pathway involved in regulating cellular apoptosis is controlled via signaling through caspase enzymes. To confirm the involvement of caspase signaling pathway in this radiation model irradiation, tissue was labeled with antibody targeting expression of capase-3. Caspase immune-labeling confirmed TUNEL results; B6.mdr1a−/− animals demonstrated increased labeling of caspase-3 in intestinal tissue following exposure to radiation (Fig. 2D &E).

**Crypt Regeneration in the Small Intestine**

Increased rates of apoptosis in intestinal epithelial tissue may correspond with increased proliferation of crypt epithelial cells, rendering them more susceptible to radiation induced cell death (3). Death of crypt epithelial cells renders intestinal villi unable to repopulate following radiation damage, and impairs the ability of the intestinal epithelium to repair itself following injury. B6.mdr1a−/− and wild type control animals were exposed to lethal doses of irradiation (12G), and sacrificed 3.5 days later to evaluate crypt regeneration. Animals were injected with BrdUrd 90 minutes prior to sacrifice; BrdUrd is incorporated into cells during DNA synthesis. Regenerative crypts were quantified as those containing at least 5 positively labeled cells (Fig. 3A& B).
Figure 2: Caspase and Cellular Apoptosis in Small Intestinal Crypts from B6.mdr1a<sup>−/−</sup> and B6 Animals Following Exposure to Radiation. B6 (A and D), and B6.mdr1a<sup>−/−</sup> (B and E) mice were exposed 12Gy X-ray radiation and sacrificed 6 hours later. Apoptotic cells were labeled by TUNEL (FITC-green) or antibodies directed at caspase-3 (Cy3-red), and nuclei were labeled using Hoescht dye (DAPI-blue). Apoptosis is quantified as the ratio of apoptotic area to area of nuclear stain in irradiated and non-irradiated B6, and B6.mdr1a<sup>−/−</sup> animals (C) Images were captured at a 200X magnification and the area of positively labeled nuclear, and apoptotic areas were calculated. 1 image was capture from each of 3 cross-sections per animal, with an of N of 5 animals per group. Bar=50µm.
B6.\textit{mdr1a}^{-/-} animals demonstrated a non-significant reduction in crypt regeneration following radiation injury when compared to B6 control animals (Fig. 3C). Animals demonstrated a non-significant reduction in crypt regeneration following radiation injury when compared to B6 control animals (Fig. 3C).

**LPS Induced Radioprotection in the Small Intestine**

Treatment with LPS has been shown to be protective in radiation induced injury; functioning to increase the number of regenerating crypts following irradiation (17). However, it has been suggested that P-gp may actively extrude bacterial ligands, potentially interfering with intestinal responses to stimulation through intracellular receptors (26). Toll-like receptor 4, a receptor known to recognize LPS, has been shown to be expressed intracellularly in IECs (16). To investigate the effects of LPS treatment on crypt regeneration in B6.\textit{mdr1a}^{-/-} we injected P-gp deficient and wild type animals with LPS 24 hours prior to irradiation. Animals were irradiated and sacrificed as described above, and crypt regeneration was quantified. B6.\textit{mdr1a}^{-/-} and B6 controls both displayed significantly increased levels crypt regeneration following treatment with LPS, however there was a significant difference in the magnitude of radioprotection (Fig 4). B6.\textit{mdr1a}^{-/-} animals demonstrated significantly diminished crypt regeneration following LPS treatment when compared to B6 control animals.

**Crypt Regeneration and Radioprotection in the Distal Colon**

P-gp expression increases in GI tract similarly to bacterial load, with expression increasing distally towards the rectum (21, 39). In order to assess the effects of P-gp
Figure 3: Small intestinal Crypt Regeneration in Lethally Irradiated B6.mdr1a^-/- and B6 Mice. B6 (A), and B6.mdr1a^-/- (B) mice were exposed to lethal doses of X-ray radiation. Animals were sacrificed 3.5 days later. Mice were injected with BrdUrd 90 minutes prior to sacrifice, and proximal jejunal tissue was harvested for immunostaining. Images were captured at a 200X magnification, and BrdUrd positive cells were counted per crypt. Crypt regeneration was quantified as a crypt containing a minimum of 5 BrdUrd positive cells, 6 cross-sections were counted/animal. Data was presented as the average number of regenerative crypts/cross-section (C). All groups contained N=5 animals. Bar=50µm.
Figure 4: Small Intestinal Crypt Regeneration in Lethally Irradiated, B6.mdr1a<sup>−/−</sup> and B6 Mice in the Following LPS Stimulation. B6 (A), and B6.mdr1a<sup>−/−</sup> (B) mice were treated with LPS 12 hours prior to exposure to lethal doses of X-ray radiation. Mice were injected with BrdUrd 90 minutes prior to sacrifice, and tissue was subjected to immunostaining. Nuclei are stained blue (DAPI) with Hoescht dye, and regenerating (cycling cells) are labeled red with Cy3. Statistical analysis was conducted on crypt regeneration data (C). Images were captured at a 200X magnification. All groups contained N=5 animals. * Indicates statistically significant difference of P<0.05 between treatment groups as determined by a Student’s T-test. Bar=50µm.
deficiency where its absence may initiate more profound disturbances to cellular homeostasis, we next chose to examine crypt regeneration in the distal colon.

B6.mdr1a\(^{-/-}\) and B6 control animals demonstrated no differences in rates of colonic crypt regeneration following radiation injury. Interestingly crypt regeneration in both animal strains was much lower than values seen in small intestinal tissue (Fig. 5). Bacterial load increases distally throughout the GI tract; indicating that luminal levels of LPS will also be the highest in distal colonic tissue. Radioprotective effects of LPS treatment may be substantially altered in tissue continuously exposed to LPS stimulation. To examine the effects of LPS treatment on crypt regeneration in colonic tissue animals were injected with LPS prior to irradiation, and sacrificed as described. LPS induced colonic crypt regeneration was significantly different between animal strains. B6 control animals demonstrated higher levels of crypt regeneration following LPS treatment, than did B6.mdr1a\(^{-/-}\) animals (Fig. 5).

**Effects of TLR4 Expression on Crypt Regeneration in the Absence of P-gp Expression**

The reduced magnitude LPS induced crypt regeneration in B6.mdr1a\(^{-/-}\) colonic tissue indicates P-gp deficiency may be directly interfering with responsiveness to LPS stimulation. To assess the importance of P-gp expression in the absence of any LPS stimulation, B6.mdr1a\(^{-/-}\) tlr4\(^{-/-}\) animals and B6.tlr4\(^{-/-}\) animals were exposed to lethal irradiation and crypt regeneration was assessed as described above. Crypt regeneration was marginally higher in B6.tlr4\(^{-/-}\) following irradiation, however the difference was not
Figure 5: Distal Colonic Crypt Regeneration in Lethally Irradiated, B6.mdr1a<sup>−/−</sup> and B6 Mice in the Presence and Absence of LPS Stimulation. B6, and B6.mdr1a<sup>−/−</sup> mice were treated with vehicle or LPS 12 hours prior to exposure to lethal doses of X-ray radiation. Crypt regeneration was assessed. All groups contained N=5 animals. * Indicates statistically significant difference of P<0.05 between treatment groups as determined by a Student’s T-test.
significant indicating altered bacterial sensing as result of P-gp deficiency is not driving baseline radioprotection in B6.mdr1a<sup>−/−</sup> animals (Fig 6).

**LPS Induced Expression of TNFα and COX Enzymes**

LPS has been shown to induce crypt regeneration by increasing production of TNFα and subsequently inducing increased expression of COX enzymes, the upstream regulators of PGE<sub>2</sub> (32). We next wanted to evaluate the role of LPS induced expression of TNFα, and COX 1 and 2 in crypt regeneration following irradiation and LPS treatment in colonic tissue. RNA was harvested from ileal tissue and RT-PCR was performed to evaluate levels of TNFα and COX enzymes following treatment. B6 and B6.mdr1a<sup>−/−</sup> animals express similar baseline (non-irradiated/non-treated) amounts of both TNFα and PGE<sub>2</sub>, interestingly both animal strains produce significantly increased expression of TNFα following irradiation, but only the B6.mdr1a<sup>−/−</sup> animals demonstrate increased production of PGE<sub>2</sub>(Fig 7A and data not shown). Both animals strains similarly show no changes in TNFα expression when LPS is administered prior to irradiation (Fig.7A).

Similar results are seen with regards to expression of COX enzymes. Expression of both COX enzymes in non-treated, non-irradiated B6.mdr1a<sup>−/−</sup> animals is significantly higher when compared to B6 animals. Irradiation significantly increases expression of COX2 (inducible) in both strains of animals, (PGE<sub>2</sub> and COX1 are only significantly increased in B6.mdr1a<sup>−/−</sup> animals). However, increased COX in response to irradiation is demonstrably reduced in B6.mdr1a<sup>−/−</sup> animals if LPS is administered prior to irradiation (Fig 7B & C). This suggests that in our animal model LPS induced radioprotection is not solely dependent upon TNFα, COX1, COX2 or PGE<sub>2</sub>, as B6.mdr1a<sup>−/−</sup> animals
Figure 6: Distal Colonic Crypt Regeneration in Lethally Irradiated, B6.\textit{mdr1}\textsuperscript{−/−}\textit{tlr4}\textsuperscript{−/−} and B6.\textit{tlr4}\textsuperscript{−/−}. Crypt regeneration was assessed. All groups contained N=4 animals.
Figure 7: Gene Expression of TNFα, COX1 and COX2 in B6 and B6.mdr1a−/− animals. Gene expression was data derived from RNA isolated from whole ileal tissue. Target gene expression was normalized to expression of the 18S housekeeping gene, and the average fold change in gene expression in B6 baseline (non-irradiated, non-treated) animals. Range is calculated from the standard deviation of the ΔΔCT value and the average gene expression is plotted with upper and lower limits of the range shown. Data is shown on a log 2 scale. Dotted lines demonstrate the range of physiological relevance, all values demonstrating a 2 fold alteration in gene expression from control values are considered physiologically different from control values. Gene expression of TNFα (A), COX1 (B), and COX2 (C), are represented graphically. N=5 animals for all groups except B6 irradiated LPS where N=3. * Indicates statistical significance of P<0.05 as determined by the Students T-test when values are compared to baseline B6 (non-treated/non-irradiated) controls. § Indicates statistical significance of P<0.05 between treated B6.mdr1a−/− and treated control animals as determined by Student’s T-Test.
display increased expression of COX1 and COX2 and yet demonstrated lower crypt regeneration following LPS treatment prior to irradiation.

**Indomethacin Induced Blockade of PGE2 Production**

While COX expression cannot explain the differences in LPS induced radioprotection between P-gp deficient mice, and wild type controls; it may be involved in baseline crypt regeneration following radioprotection in B6.*mdr1a*<sup>−/−</sup> animals. To investigate the role of PGE<sub>2</sub> production in crypt regeneration; B6.*mdr1a*<sup>−/−</sup> animals were treated with the non-specific COX inhibitor indomethacin, prior to exposure to irradiation. Animals were given 1.5mg/kg indomethacin every 8 hours until sacrifice, starting 14 hours prior to irradiation. PGE<sub>2</sub> inhibition was confirmed via PGE<sub>2</sub> ELISA, and crypt regeneration was quantified as described. As indicated above, irradiation significantly increased PGE<sub>2</sub> production in B6.*mdr1a*<sup>−/−</sup> mice; and as predicted administration of indomethacin completely ameliorated production of PGE<sub>2</sub> in B6.*mdr1a*<sup>−/−</sup> animals (Figure 8A). Elimination of PGE<sub>2</sub> production in B6.*mdr1a*<sup>−/−</sup> animals completely abolished crypt regeneration in distal colonic tissue, indicating PGE<sub>2</sub> production is critical for baseline crypt regeneration following irradiation in P-gp deficient animals (Fig. 8B).

**Role of IL1α and IL22 in Radioprotection Following LPS Exposure**

LPS treatment prior to exposure to irradiation initiates a more profound radioprotection in wild type animals when compared to animals deficient in P-gp expression. To investigate alternative mechanism of LPS induced protection we chose to
Figure 8: PGE2 Production and Crypt Regeneration in Indomethacin Treated B6.mdr1a<sup>-/-</sup> Animals. B6.mdr1a<sup>-/-</sup> mice were injected with 1.5mg/kg indomethacin every 8 hours, beginning 14 hours prior to irradiation. Protein was isolated from proximal ileal tissue and PGE2 content was assessed by ELISA (A). Distal colonic tissue was harvested and immunolabeled for Crypt Regeneration analysis (B). * Indicates statistical significance of P<0.05 as determined by the Students T-test.
examine RNA expression of cytokines known to effect injury repair in the GI tract. RNA was isolated from proximal illeal tissue as described; and gene expression in each strain was normalized to its’ own baseline (non-treated) control. Normalization was done in this manner to account for potential strain related differences in gene expression necessary for homeostatic maintenance; for a complete list of cytokines examined, and gene expression results see Table 1.

Increased expression of IL22 has been shown to down regulate expression of apoptotic proteins (30). Non-irradiated B6 animals express more IL22 in response to LPS treatment when compared B6.mdr1a<sup>−/−</sup> animals, although values did not achieve significance. Administration of LPS prior to exposure to radiation seems to amplify these affects as B6 animals are expressing significantly more IL22 when compared to B6.mdr1a<sup>−/−</sup> animals under these conditions (Fig 9A).

Treatment of animals with IL1α has been shown to be radioprotective in the murine small intestine (42). Non-irradiated B6 animals expressed increased levels of IL1α, following LPS treatment when compared to P-gp deficient animals, however differences were not significant. However, B6 animals express significantly more IL1α when LPS is administered prior to irradiation when compared B6.mdr1a<sup>−/−</sup> animals (Fig. 9B).
Figure 9: Gene Expression of IL22 and IL1a in B6 and B6.mdr1a−/− animals. RNA isolated from whole ileal tissue. Target gene expression was normalized to expression of the 18S housekeeping gene, and the average fold change in gene expression to each strains’ own baseline values. N=5 animals for all groups. * Indicates statistical significance of P<0.05 as determined by the Student’s T-test when values are compared to their own baseline. § Indicates statistical significance of P<0.05 between treated B6.mdr1a−/− and treated B6 control animals as determined by Student’s T-test.
Table 1. Small Intestinal RNA Production in B6 and B6.mdr1a−/− animals.

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<th>LPS/Irradiated</th>
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<tr>
<td>IL22</td>
<td>B6</td>
<td>$4.94 (1.20-20.39)$ * $§44.86 (27.95-72.01)$</td>
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<td>B6.mdr1a−/−</td>
<td>0.25 (0.04-1.43) 2.10 (0.4-11.07)</td>
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<tr>
<td>IL1α</td>
<td>B6</td>
<td>1.68 (0.41-6.87) *§15.55 (6.45-37.46)</td>
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<td>0.45 (0.11-1.82) 0.65 (0.18-2.41)</td>
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<tr>
<td>IL10</td>
<td>B6</td>
<td>1.14 (0.2-6.46) *0.1 (0.06-0.17)</td>
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<td>1.02 (0.26-4.02) §0.61 (0.3-1.23)</td>
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<td>0.48 (0.09-2.49) *0.12 (0.04-0.35)</td>
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</table>

*Indicates statistical significance of P<0.05 as determined by the Students T-test when compared to non-treated animals of the same strain
§ Indicates statistical significance of P<0.05 between P-gp deficient and control animals as determined by Student’s T-Test
DISCUSSION

FVB.129P2-AbcblmBorN7 (FVB. mdr1a−/−) were originally derived for the purposes of studying barrier function and drug extrusion at the blood brain barrier (36). It was subsequently discovered that these animals developed a spontaneous unremitting colitis when maintained in a specific pathogen free environment; and that polymorphisms in the human mdr1a homolog are significantly associated with the development of inflammatory bowel disease in human populations. Efforts to elucidate the nature of this colitis indicated it was the result of altered interactions between the intestinal epithelium and the intestinal microbiota. Disease was proven to be ameliorated by both prophylactic, and therapeutic antibiotic treatment; confirming the role of luminal bacterial contents in initiating disease (26).

Altered epithelial function as a colitic instigator was confirmed by production of adult bone marrow chimeras. Adult P-gp deficient animals, and wild type controls were lethally irradiated and reconstituted with bone marrow from the same or opposing strain, results demonstrated colitis only developed in those animals lacking mdr1a expression in their innate cell populations (26). Impaired intestinal integrity was again cited as the root of colitic development when it was shown that FVB. mdr1a−/− demonstrate altered phosphorylation patterns of junctional proteins. Altered protein phosphorylation was associated with decreased intestinal tissue resistance at early in development, prior to the development of clinical symptoms of colitis; highlighting the importance of P-gp expression in the intestinal epithelial cells (31).
Recently we demonstrated that introducing the mdr1a gene deficiency onto an animal strain known to be more resistant to colitis ameliorated the development of spontaneous disease. It was also demonstrated that these animals demonstrated increased production of both IFN-γ and COX2 early in development. B6.mdr1a−/− animals proved to be more susceptible to colitis development than control strain animals when exposed to chemically induced epithelial disruption; indicating a role for P-gp protein expression in regulating epithelial homeostasis, and injury repair (37).

To elucidate the role of P-gp deficiency in epithelial injury models we chose to examine the effects of low dose radiation injury in B6.mdr1a−/− mice. It was our hypothesis that animals deficient in P-gp expression would succumb more readily to radiation induced tissue damage. Male B6.mdr1a−/− and wild type control mice were exposed to a sublethal dose of 6Gy X-ray radiation, administered as 2 doses of 3Gy irradiation. Wild type animals responded as expected, showing a brief initial weight loss following irradiation, but subsequently recovering; regaining weight and health. However B6.mdr1a−/− animals never recovered from radiation induced injury, and had to be scarified at 3 weeks following treatment, having lost approximately 30% of their initial body weight. These data validate the role of P-gp in maintaining stem cell viability and injury repair.

Further studies of MDR gene expression in humans have indicated that MDR may play a role in regulating cellular apoptosis. P-gp expression and function has been strongly associated with increased resistance to spontaneous apoptosis in cultured blasts from patients with acute myeloid leukemia (25). Additionally, it was shown that in vivo cadmium treatment of rats induced increased expression of P-gp, in kidney proximal
tubule cells (38). Follow-up *in vitro* studies indicated that increased P-gp expression was a function of NF-κb stimulation, and furthermore correlated significantly with decreased cell apoptosis (38).

Regulation of cellular apoptosis could have dramatic affects on cell cycle and tissue homeostasis; particularly in intestinal tissue where cell turn-over is known to be rapid, and P-pg is highly expressed. One of the most highly characterized methods of studying cellular turn-over in the intestine is the radiation induced injury model examining proximal jejunal tissue. It has been previously shown that small intestinal crypts with increased cycling epithelial cells are more susceptible to radiation induced injury, and therefore less likely to repopulate intestinal crypts following exposure to radiation (3). To investigate the role of P-gp expression in cellular turn-over we initially chose to utilize this well characterized model system and examine the effects of P-gp deficiency in murine small intestinal tissue.

To verify the role of P-gp expression in regulating intestinal apoptosis we exposed male B6.*mdr1a*<sup>−/−</sup> and B6 animals to lethal doses of X-ray radiation (12Gy) and quantified cellular apoptosis 6 hours later. We also looked at rates of apoptosis in non-irradiated wild type and P-gp deficient mice to determine there was a difference in baseline cellular apoptosis. In both cases the average amount of apoptosis in intestinal crypts was two-fold greater in B6.*mdr1a*<sup>−/−</sup> when compared to B6 controls. Values never achieved statistical significance as a result of biological variability, but the trends indicated that animals deficient in expression of *mdr1a* were more susceptible to apoptosis, both at baseline, and in response to injury.
As increased apoptosis can be associated with increased cell turn-over and therefore higher number of cycling cells. To evaluate number of cycling cells we initial injected non-irradiated control mice from each strain with BrdUrd and counted the number of positive cells. Cycling cell numbers in small intestinal crypts from B6 and B6.mdr1a^-/- animals was approximately the same. We next wanted to examine crypt repopulation, or crypt regeneration in P-gp deficient animals following radiation induced injury (3). To that end crypt regeneration was assessed in wild type and P-gp deficient animals following exposure to lethal doses of X-ray irradiation. We observed a slight, but not significant reduction in small intestinal crypt regeneration in B6.mdr1a^-/- animals.

It has been shown previously that LPS confers radioprotection to radiation induced injury via stimulation of TNFR1 and subsequent induction of cyclooxygenase (COX) enzymes and prostaglandin (PGE2) synthesis (32). However, it has been suggested that mdr1a may function to alter responsiveness to bacterial ligands (26). In fact, development of colitis in FVB.mdr1a^-/- animals has been shown to be ameliorated by inhibition of TLR4 signaling, indicating that altered response to bacterial ligands is a driving force in initiation of spontaneous disease (11). To investigate the effects of P-gp deficiency on LPS induced radioprotection, small intestinal crypt regeneration was quantified in B6.mdr1a^-/- and wild type control animals injected with LPS prior to exposure to lethal doses of X-ray radiation. Crypt regeneration data indicated that while both wild type, and B6.mdr1a^-/- animals demonstrated increased crypt regeneration following pre-exposure to LPS, the magnitude of the response was significantly less in P-gp mice.
Expression of P-gp has been shown to increase distally throughout the GI tract, correlating strongly with bacterial load (21). Given the previously described effects of P-gp deficiency on LPS induce radioprotection we therefore wanted to assess the effects of P-gp deficiency in tissue where it is more heavily expressed. Crypt regeneration following radiation induced injury was next evaluated in distal colonic tissue, in the presence and absence of LPS treatment. We demonstrated once again, that baseline levels of crypt regeneration were similar between the two animal strains, however LPS induced radioprotection was markedly different. LPS induced significant crypt regeneration in wild type animals, while no significant changes were observable in B6.<i>mdr1a</i><sup>−/−</sup> animals.

Crypt regeneration data indicate that P-gp deficient animals are less responsive to radioprotection induced by LPS stimulation. To clarify the effects of normal levels of LPS stimulation on crypt regeneration following exposure to radiation we evaluated in B6.<i>mdr1a</i><sup>−/−</sup> <i>tlr4</i><sup>−/−</sup> animals and B6.<i>tlr4</i><sup>−/−</sup> animals. Absence of TLR4 signaling has no significant effects on crypt regeneration in the distal colon, indicating that luminal LPS has no discernable effect on crypt regeneration. This data is consistent with previous results indicating that LPS is interacting with sub-epithelial fibroblasts to induce radioprotection.

LPS induced crypt regeneration has been proven to be contingent upon increased TNFα signaling through the TNFR1 inducing increased expression of COX and subsequently PGE2 (32). As P-gp deficient animals are not responding properly to LPS stimulation, we next wanted to evaluate levels of key cytokines in the crypt regeneration pathway, to investigate aberrant signaling. Interestingly in B6 animals irradiation did
induce increased expression of the COX enzymes, as well as TNFα, however LPS treatment prior to irradiation reduced expression of all cytokines back to baseline values. This data indicates is different from that previously published, however, this maybe the result of experimental differences. Previous data was derived from female, FVB mice; it may be that LPS is inducing radioprotection by a different mechanism in male C57BL/6 animals. B6.mdr1a−/− animals demonstrated dramatically increased expression of COX1, COX2, and TNFα following irradiation, indicating a potential role for COX and PGE2 in baseline response to irradiation injury in P-gp deficient animals.

Crypt regeneration has been shown to be regulated by COX1 induce PGE2 production (7). Given the dramatic radiation induced increase in COX1 expression evidenced only in B6.mdr1a−/− animals, we chose to investigate the effects of COX inhibition in crypt regeneration in this animal model. Indomethacin treatment of B6.mdr1a−/− animal completely abrogated distal colonic crypt regeneration, demonstrating the importance of PGE2 expression in response to irradiation injury.

To further investigate alternative mechanism for LPS induced radioprotection utilized by wild type control animals we next chose to investigate levels of IL1α and IL22, regulatory cytokines known to play role in injury repair. IL22 has recently been shown to be critical in protecting animals from the development of inflammatory bowel disease (43). Furthermore it has been shown to reduce expression of apoptotic proteins in human hepatocytes, indicating it may play a role in reducing cell cycling and protecting from radiation induced injury. Both B6.mdr1a−/− and wild type LPS treated animals exposed to radiation express increased IL22 RNA when compared to strain baselines.
While wild type animals do express IL22 to a greater magnitude in response to LPS stimulation, differences failed to achieve significance.

Treatment of mice with recombinant IL1α has been shown to induce greater crypt regeneration following irradiation by decreasing radiation induced apoptosis (18, 42). Wild type animals express significantly more IL1α than B6.mdr1a<sup>−/−</sup> animals following LPS treatment and exposure to radiation. Furthermore, they express more IL22 in response to LPS treatment alone.

Our results indicate that there an increase in cellular apoptosis at baseline and following exposure to radiation in B6.mdr1a<sup>−/−</sup> animals when compared to B6 controls. We have shown that exposure to lethal doses of irradiation effects crypt regeneration and stem cell survival similarly in small intestinal tissue from B6 and B6.mdr1a<sup>−/−</sup> animals. We have confirmed that similar to what has previously been reported, crypt regeneration following irradiation is contingent upon induction of PGE2 via COX1 and 2 enzymes in our animal model. We have demonstrated that while LPS does induce radioprotection in both small intestinal and colonic tissue it does not appear to by mediated by TNFα or COX enzymes in the B6 or B6.mdr1a<sup>−/−</sup> male animals. Furthermore, we have shown that LPS induced radioprotection is significantly reduced in B6.mdr1a<sup>−/−</sup> animals when compared to B6 controls. We have further demonstrated that pre-exposure to LPS induces significantly increased expression of the radioprotective cytokines IL22 and IL1α in B6 animals when compared to B6.mdr1a<sup>−/−</sup> animals. It is our belief that LPS induced radioprotection in B6 animals is mediated via expression of IL1α and IL22, and that failure of B6.mdr1a<sup>−/−</sup> animals to respond appropriately to LPS stimulation is a function of altered bacterial sensing, interfering with adaptive immune responses to
bacterial ligands. Furthermore, increased expression of P-gp on intestinal epithelial cells, increases rates of apoptosis, and may render P-gp deficient animals more susceptible to radiation injury.
ACKNOWLEDGMENTS

This work was supported in part by the NIH grants R01 DK059911, P0 DK071176, and the Molecular Pathology and Imaging Core of the University of Alabama at Birmingham Digestive Diseases Research Development Center (P30DK064400). Elizabeth Staley received partial salary support from T32A107051. Vanisha Yarborough was supported by a NIDDK StepUP Fellowship. Aspects of this project were conducted in biomedical research space that was constructed with funds and supported in part by the NIH grant C06RR020136. We would like to thank Peggy R. McKie-Bell and Jamie L. McNaught for their assistance and members of the Lorenz Lab for valuable advice.
REFERENCES


**Supplement Table 1.** Primer Probe Pairs Utilized for Quantitative RT-PCR.

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CONCLUSIONS

The use of animal models is unquestionably invaluable to the progress of medical research pertaining the treatment and understanding of human disease. The FVB.mdr1a⁻/⁻ deficient animal model physiologically and histologically resembles human IBD. Manipulation of *mdr1a* expression on specific cell types within this animal model will provide insight as to the importance of epithelial cells and immune responders in the initiation of spontaneous disease. Such information may serve to shape and guide research in the field, and subsequently aid drug companies to target the inflammatory response at the cellular level, before uncontrolled inflammation initiates.

Furthermore, the derivation of the B6.mdr1a⁻/⁻ animal model is uniquely fascinating because it provides confirmation of the importance of genetic background and environmental factors to the initiation and progression of IBD. The study of induced colitis and injury repair in the context of this animal model will highlight the importance of natural stimuli in disease induction in genetically susceptible hosts.

FVB.mdr1a⁻/⁻ animals when maintained under conventional conditions develop a spontaneous inflammation of the large bowel, initiating early in development with increased severity in male mice. Research in this animal model utilizing adult bone marrow chimeras indicates disease initiation is contingent upon expression of P-gp in intestinal epithelial cells (65). Decreased colonic tissue resistance is demonstrated in FVB.mdr1a⁺/⁺ animals prior to the on-set of colitis and is associated with decreased
phosphorylation of proteins expressed at epithelial cell junctions (73). It is also reported that very early in development, prior to demonstration of decreased barrier integrity, colonic tissue from FVB.mdr1a<sup>−/−</sup> animals displays increased expression of inflammatory cytokines, indicating a potential role for the adaptive immune system in disease initiation (16).

We developed a neonatal model of bone marrow reconstitution to assess the role P-gp expression in epithelial versus adaptive immune cells in the induction of spontaneous colitis. FVB.mdr1a<sup>−/−</sup> animals were lethally irradiated with 900 RADS γ-radiation and reconstituted with bone marrow from FVB.mdr1a<sup>−/−</sup> or FVB/N animals; animals were maintained for up to 20 weeks following reconstitution, and were observed for clinical signs of colitis. Animals were sacrificed upon evidence of fecal blood, rectal prolapse or loss of 20% maximal body weight.

As expected, FVB.mdr1a<sup>−/−</sup> animals reconstituted with bone marrow from FVB.mdr1a<sup>−/−</sup> animals develop a severe colitis characterized by decreased growth/weight gain, diarrhea, rectal bleeding and prolapse, and an increased mortality when compared to FVB/N, and FVB.mdr1a<sup>−/−</sup> animals. Interestingly, FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB.mdr1a<sup>−/−</sup> bone marrow developed disease more rapidly, and demonstrated an increased mortality when compared to non-treated FVB.mdr1a<sup>−/−</sup>, or FVB.mdr1a<sup>−/+</sup> animals reconstituted with FVB/N bone marrow; indicating radiation may induce damage in these animals, which is ameliorated by expression of P-gp on immune cells. FVB.mdr1a<sup>−/−</sup> animals reconstituted with FVB/N bone marrow demonstrated a slightly altered pattern of growth, demonstrating a significant reduction in weight gain when compared to FVB/N at 12 and 18 weeks of age. Animals reconstituted with FVB/N
marrow also displayed a non-significant increase in mortality, and evidence of fecal blood when compared to FVB/N animals. However, at sacrifice colonic and cecal tissue from FVB.\textit{mdr1a}\textsuperscript{−/−} animals reconstituted with FVB/N bone marrow was macroscopically and histologically normal and was not significantly different from untreated FVB/N tissue from animals of the same age. These data indicate that the lack of P-gp expression in adaptive immune cells may play a role in the development of colitis in FVB.\textit{mdr1a}\textsuperscript{−/−} animals.

It has previously been suggested that P-gp extrudes bacterial stimuli from the intracellular environment, preventing contact between bacterial ligands and intracellular receptors (65). In an effort to derive animals doubly deficient in both intracellular pattern recognition receptors and P-gp, we derived the B6.\textit{mdr1a}\textsuperscript{−/−} animal. We demonstrated that unlike the FVB.\textit{mdr1a}\textsuperscript{−/−} animal model, B6.\textit{mdr1a}\textsuperscript{−/−} animals do not develop colitis for up to 12 months of age, highlighting the importance of genetic background in the development of disease. We show that B6.\textit{mdr1a}\textsuperscript{−/−} demonstrated an increased expression of inflammatory cytokines and inflammatory mediators early in development, but failed to initiate colitis.

To evaluate the effects of P-gp deficiency on susceptibility to environmentally induced colitis in this animal model, B6.\textit{mdr1a}\textsuperscript{−/−} were exposed to colitis induction via infection, suppression of immune modulators, and epithelial disruption. B6.\textit{mdr1a}\textsuperscript{−/−} animals were inoculated with \textit{H. bilis}, a gram-negative bacterium shown to instigate development of colitis only in immune deficient animals, and known to accelerate colitis in FVB.\textit{mdr1a}\textsuperscript{−/−} (9, 30, 54). We demonstrate that B6.\textit{mdr1a}\textsuperscript{−/−} animals are clinically and histologically free of colitis following 12 weeks of \textit{H. bilis} infection.
B6.mdr1a<sup>−/−</sup> animals were treated with piroxicam to suppress expression of COX enzymes and subsequent production of PGE<sub>2</sub>. PGE<sub>2</sub> has been shown to have an effect suppression of inflammation in the GI tract, and inhibition of COX has been shown to initiate disease only in IL10 deficient mice (4, 62). Inhibition of COX expression did not initiate disease in B6.mdr1a<sup>−/−</sup> animals, indicating that regulatory cells are not impaired in this animal model.

B6.mdr1a<sup>−/−</sup> animals were treated with DSS to initiate disruption of the intestinal epithelium. DSS has been shown to initiate colitis in susceptible animals, including immune-compromised animals, indicating that the adaptive immune response plays little to no role in disease initiation in this model (22). We show that B6.mdr1a<sup>−/−</sup> animals develop a more severe disease following DSS treatment when compared to B6 control animals. This data indicates that epithelial insult is a colitic instigator in our animal model; which suggests that P-gp deficiency plays a role in epithelial injury repair.

It has been shown that increased expression of P-gp is associated with increased resistance to cellular apoptosis (64, 82). To directly investigate the role of P-gp expression in intestinal apoptosis and injury repair, we exposed B6.mdr1a<sup>−/−</sup> animals to lethal doses of irradiation and quantified small intestinal cellular apoptosis. We show that B6.mdr1a<sup>−/−</sup> animals displayed a non-significant trend towards increased apoptosis at baseline, and following exposure to radiation. To investigate the effects of increased apoptosis following radiation induced injury on susceptibility to radiation induced damage in B6.mdr1a<sup>−/−</sup> animals, we quantified small intestinal and colonic crypt regeneration following exposure to lethal doses of radiation. We demonstrate that
B6.\textit{mdr1a}^{-/-} animals regenerate colonic and small intestinal crypts following exposure to radiation in a COX dependent fashion similarly to B6 control animals.

Administration of LPS prior to exposure to radiation increases crypt regeneration in small intestinal crypts by increasing the production of TNF$\alpha$, and subsequently inducing increased expression of COX enzymes (37). P-gp has been suggested to extrude bacterial ligands from the intracellular environment, potentially interfering with LPS recognition by its intracellular receptor (65). We show that LPS induced radioprotection is significantly diminished in B6.\textit{mdr1a}^{-/-} animals. We go on to demonstrate that LPS induced radioprotection is not mediated via increased expression of TNF$\alpha$ and COX enzymes in male B6 animals, but may be a function of increased expression of the radioprotective cytokines IL22 and IL1$\alpha$.

My dissertation set out to examine the effects of P-gp deficiency in the epithelial cells of the small intestine and colon, and determine it’s relevance in the spontaneous development of colitis in the FVB.\textit{mdr1a}^{-/-} disease model. Studies were also designed to determine the effects of P-gp deficiency on an alternate genetic background and to evaluate the importance of environmental stimuli on the development of disease. We have shown that P-gp deficiency on both epithelial and adaptive immune cells contributes to the development of colitis. We have also demonstrated that genetic background is immensely important in disease progression and pathogenesis, and further that environmental stimuli which alter barrier integrity play a dramatic role in disease development in this animal model. We have shown that P-gp expression may affects cellular apoptosis in epithelial tissue. We have further shown that P-gp expression affects systemic responsiveness to bacterial stimuli. Our data suggests that P-gp deficiency
induces a low level inflammation, serving to promote barrier permeability, exposing the immune system to bacterial ligands. P-gp deficiency on immune cells further disrupts the immune response to bacterial ligands, leading to the development of colitis. My dissertation clarifies the role of P-gp expression in epithelial and immune cell populations, and highlights the importance of genetic background and environmental stimuli in the development of disease.


75. Rook GA. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 126: 3-11, 2009.


NOTICE OF APPROVAL

DATE: June 3, 2009

TO: Lorenz, Robin G
SHEL 402
934-0676

FROM: Judith A. Kopp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: PPG. Innate and Adaptive Microbial Immunity in IBD (Elson). Project # 2:
Epithelial and Innate Sensors of the Microbiota in IBD
Sponsor: NIH
Animal Project Number: 090507521

On June 3, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

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Animal use is scheduled for review one year from May 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090507521 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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FAX 205.934.1188

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