Gut sterilization in experimental colitis leukocyte mediated colon injury, and effects on angiogenesis/lymphangiogenesis*

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ABSTRACT

Inappropriate responses to normal commensal bacteria trigger immune activation in both inflammatory bowel disease and experimental colitis. How gut flora contribute to the pathogenesis of inflammatory bowel disease is unclear, but may involve entrapment of leukocytes and remodeling of the vascular system. Here we evaluated how the progression and tissue remodeling in experimental colitis differ in a germ-free model of mouse colitis. Four treatment groups were used: control, antibiotic-treated (ABX), dextran sulfate colitis (DSS) and DSS pre- and co-treated with antibiotics (DSS + ABX). In days 0-3 of the study, germ-free mice received antibiotics (vancomycin, neomycin, and metronidazole). During the next 11 days, antibiotics were continued and DSS (3%) added to "colitis" groups. Disease activity, weight, stool form and blood were monitored daily. Mice were sacrificed and tissue samples harvested. Histopathological scores in controls (0.00) and in ABX (1.0 +/− 0.81) were significantly (p < 0.001) lower than DSS (12 +/− 0). Extents of injury, inflammation and crypt damage were all improved in DSS + ABX. The Disease Activity Index score (day 11) was significantly worse in the DSS group compared to the DSS + ABX group. Stool blood and form scores were also significantly improved among these groups. Importantly, myeloperoxidase was significantly reduced in DSS + ABX, indicating that neutrophil infiltration was blocked. Colitis was associated with an increase in blood and lymphatic vessels; both of these events were also significantly reduced by gut sterilization. Our experiment shows that clinical and histopathological severity of colitis was significantly worse in the DSS colitis group compared to the DSS + ABX group, supporting the hypothesis that development of IBD is likely to be less severe with appropriate antibiotic treatment. In particular, gut sterilization effectively reduces leukocyte-dependent (PMN) injury to improve outcomes and may be an important target for therapy.

Keywords: Crohn's Disease; Antibiotics; Myeloperoxidase; Lymphatics; Angiogenesis

1. INTRODUCTION

Inflammatory Bowel Diseases (IBD) are a group of gastrointestinal diseases including Crohn's disease (CD) and ulcerative colitis (UC). CD is characterized by granulomas, skipped lesions, transmural inflammation, goblet cell hyperplasia, fistulae and luminal stenosis involving any part of the GI tract. UC is characterized by mucosal inflammation, pseudopolyps, reduced goblet cells, absence of fistulization, stenosis, and granulomas, and is restricted to the colon. Several factors which can increase the risk for IBD include genetic background, stress, diet, and infections (e.g. atypical Mycobacteria) [1]. Recent increases of IBD incidence in developing nations and non-classical populations [2] also suggest socioeconomic status and environmental factors (e.g. dietary "hygiene", and "cold chain") may contribute to IBD etiology [1].

The etiology of CD and UC appears to represent inappropriate immune responses to normal gut bacteria which occur in genetically susceptible individuals. IBD associated genetic polymorphisms like the Nod2/Card15 locus [3] hyperactivate immune system responsiveness and reduce tolerance to intestinal flora, with sustained gut inflammation, fibrotic changes, and destruction of the intestinal mucosal barrier. Based on these findings, one potential IBD therapy could be reduction of the enteric

flora. This could reduce immune responses and be less toxic and more focused than immunosuppressive drugs [4].

Gut flora contributes to the etiopathology of animal models of IBD as well as human CD and UC. Several experimental immune models (HLA-B27, Samp1/Yit, TGF-bRII and IL-10-/-, TNFDARE) and erosive models (iodoacetamide, picrylsulfonic acid, TNBS, dextran sulfate sodium-DSS) have been used to study IBD mechanisms in animals. Both human and animal models of IBD suggest that barrier dysfunction is evident before disease onset, and contributes to development of these diseases [5]. DSS, a negatively charged dextran, disturbs gut barrier, allowing bacteria to penetrate the gut lamina propria, and is used as an experimental model of colitis. Similarly, iodoacetamide and TNBS also disturb gut barrier leading to inflammation [6]. Additionally, IBD genetic models [7] exhibit dysregulated gut barrier, which precedes the onset of IBD activity, and importantly display reduced inflammation following antibiotic therapy. Similar prodromal changes in gut epithelial barrier are also seen in human IBD [8,9]. Results in animal experimental models and in human trials using broad spectrum antibiotics (e.g. metronidazole plus ciprofloxacin) have demonstrated therapeutic success [10]. Thus, despite disturbances in gut barrier, reduced enteric bacterial loads reduce or eliminate inflammation and play an important role in IBD and therapy.

However, commensal enteric bacteria also have a wide range of beneficial effects in the GI tract, including digestion of complex carbohydrates, control of the intestinal mucosal barrier, modulation of inflammation, and regulating the activity of the enteric nervous system [11]. The gut vascular angiogenesis, in particular, is also known for development of gut injury in IBD, but whether and how gut commensals contribute to vascular disturbances in IBD is not known. Further, while angiogenesis is enhanced in IBD, whether or how the immune system and intestinal flora may modulate lymphangiogenesis is unclear [12].

Here we studied how complete gut sterilization alters disease activity, weight changes, histopathology of experimental IBD, and various inflammatory parameters like leukocyte infiltration, angiogenesis, and lymphangiogenesis. We analyzed these disease features in four different groups of mice: control, antibiotic treated (ABX), dextran sulfate colitis (DSS), and DSS pre and co treated with antibiotics (DSS + ABX). Importantly, as previous studies have linked IBD with changes in angiogenesis and lymphangiogenesis, our study demonstrates how commensal bacteria and broad spectrum antibiotics affect both angiogenesis and lymphangiogenesis in the 3% DSS model, and further shows the protective role of lymphatic expansion.

2. MATERIALS AND METHODS

2.1. Experimental Groups

Mice used in this study were male or female KBLACZ mice. The mice used in these experiments were 5 - 9 weeks of age, and had an average initial weight of 20.84 +/− 1.1(SE) gms on day 0. A total of 4 groups were selected: control (n = 4), ABX (n = 4); DSS (n = 5); and DSS + ABX (n = 5). Mice in different groups were separated into different cages. All mice were fed autoclaved alfalfa mouse chow and sterile drinking water and data was recorded of consumption each day. Animal protocols were reviewed and approved by the LSUHSC and University of Arizona Institutional Animal Care and Use Committees (IACUC).

2.2. Induction of DSS Colitis and Antibiotic Treatment

The experiment was divided in 2 phases: In the first phase from day −3 to 0, mice in the ABX and DSS + ABX group received antibiotics preemptively in their drinking water to sterilize the gut. The control and DSS groups received water only. The antibiotic solution used in the first 3 days consisted of 500 mg/L vancomycin, 350 mg/L neomycin and 600 mg/L metronidazole (to eliminate Enterococci, gram-negatives, and anaerobes (Bacteroides/Clostridium respectively) [13,14]. In the second phase (Day 0 to 11): On the 0 day, the antibiotic concentration in water was reduced by 50%, and 3% DSS [as described, Soriano *et al.*], ad libitum (DSS, MW 1/4 36 - 50 kDa; ICN Biomedicals, Costa Mesa, CA) was added to the drinking water of the DSS and DSS + ABX group for the duration of the study [9]. At 3 - 4 days, progressive weight loss, diarrhea, occult blood, leukocyte infiltration, colon shortening, loss of intestinal epithelial barrier, and histopathological changes in colon structure were observed. Mouse weight, stool form, occult blood, food (grams), and liquid (mL) consumption were recorded daily.

2.3. Drug Consumption

Table 1 shows the drug consumption in ABX and DSS + ABX group over 14 days. The first phase includes from day −3 to day 0, and second phase includes from day 1 to day 11. During the first phase, antibiotics were given with drinking water, and in the second phase antibiotic concentration was halved in the DSS + ABX group after initiation of 3% DSS administration. The antibiotic concentration used in this experiment was 500 mg/L of vancomycin, 350 mg/L of neomycin and 600 mg/L of metronidazole. The main issue with the combination of antibiotics is the toxicity of the regimen, but the concentration of

Table 1. Antibiotic loading from days −3 to day 0 and days 1 to 11. First value is mg/kg/day in pretreatment and second value is mg/kg/day during colitis induction phase. Values are based on medicated water consumption per day/cage of mice.

Treatment groups	Antibiotics consumption (doses in mg/kg/day) (day −3 to 0/day 1 to 11)		
	Neomycin	*Vancomycin*	*Metronidazole*
ABX (n = 4)	29.75/27.91	42.50/39.88	51.00/47.85
DSS + ABX (n = 5)	31.02/23.82	44.32/34.02	53.18/40.84

antibiotics was lower than LD 50. The dosages are calculated with average daily consumption of antibiotics per average initial weight of mice.

2.4. Necropsy

At the end of the study, mice were anesthetized with a Ketamine (50 mg/ml) and Xylazine (2.85 mg/ml) mixture; mice were sacrificed and blood and tissue specimens were collected. The ceca were removed and colon length and weight were measured. Spleen and cecum weights were also recorded. Mice were then sacrificed by cardiac puncture (under ketamine/xylazine anesthesia). Histological samples were fixed in cold 3.7% phosphate-buffered formalin, or frozen at 20 C for myeloperoxidase (MPO) and Western blotting analysis.

2.5. Gut Sterilization

To determine that this level of combined antibiotics was sufficient to eliminate gut flora, fecal samples (100 μl of 1 gms mouse feces dispersed in 1 ml water) were plated onto 1.5% selective agar plates. These plates contained the combined antibiotic regimen at concentrations equivalent to the levels of antibiotics consumed by the mice or nutrient agar without antibiotics. Plates were incubated at 37°C overnight and resulting colonies were counted.

2.6. Evaluation of Clinical Colitis

Body weight, stool form, and occult blood were scored daily as described [15] and disease activity index (DAI) was determined as the average of these scores: 1) weight change (calculated as: percent difference between original body weight and weight on any given day with a score between 0 and 4) (0: <1%, 1: 1% - 5%, 2: 5% - 10%, 3: 10% - 15%, 4: >15%); 2) stool consistency score based on qualitative examination (0—very firm, dry, non-adherent; 1—firm, moist, adherent; 2—soft, very adherent; 3—very soft, pliable; 4—formless, liquid); 3) occult blood score based on results using "Colo-screen" kits (Helena Labs, Beaumont, TX): (0—no color development, 1—greenish blue reaction, 2—consistent blue color, 3—rust color stools + blue reaction, 4—wet blood + dark blue reaction) [16-19].

2.7. Histopathological Analysis

Formalin-fixed colon sections were paraffin embedded and 10 μm sections stained with hematoxylin/eosin. Slides were analyzed for evidence of histopathological injury using criteria established by Cooper *et al.* [20]. This system includes edema, extent of injury, leukocyte infiltration, crypt abscesses and loss of goblet cells. These were scored on inflammation severity (0—none, 1—slight, 2—moderate, 3—severe), extent of injury (0—none, 1—mucosal, 2—mucosal + submucosal, 3—transmural), and crypt damage (0—none, 1—basal 1/3 damaged, 2—basal 2/3 damaged, 3—only surface epithelium intact, 4—loss of entire crypt and epithelium). Each value was multiplied by an extent index which reflects the amount of involvement for each section (x1: 0% - 25%, x2: 26% - 50%, x3: 51% - 75%, x4: 76% - 100%). The final score, based on at least three different colon samples, were analyzed and equaled the sum of the individual extent-adjusted scores [20]. A maximum possible histopathological score for this assay is 40.

2.8. Measurement of Tissue MPO Content

MPO activity was measured as described by Grisham *et al.* [21], and as modified by Hausmann *et al.* [22]. 25 mg samples of colon tissue were frozen under N_2 [23], crushed and freeze-thawed 3X in 0.5% HETAB buffer, sonicated for 10 seconds at 50% max power [24], and cleared by centrifugation (10,000 × g, 5 min) before MPO activity of supernatants was measured (using 0.1% o-dianisidine substrate) at an absorbance of 450 nm/min/mg tissue.

2.9. Immunohistochemistry

Colon sections fixed >12 h in 3.7% phosphate buffered formalin were embedded in paraffin. Sections (10 μm) were collected onto Superfrost-Excell slides, and deparaffinized prior to antigen retrieval. Slides were incubated in primary antibody (1:125, 1 hr), washed in 0.1% Bovine serum albumin (BSA), reacted in HRP-conjugated secondary antibody (1:2500, 1 h), and reacted with DAB/peroxide (5 mins). The 3.7% phosphate-buffered formaldehyde fixed tissue sections (5 μm) were immunostained using DAB/ peroxidase for vascular endothelial growth factor receptor-3 (VEGFR-3) (lymphatic vessels) and MECA-32 (mouse endothelial cell antigen-32) monoclonal antibody [25,26], a blood vascular specific marker [26] obtained from Developmental Hybridoma Studies Bank (Iowa City, IA) and prepared on-site. Slides were hematoxylin counterstained (30 s) and sealed in Permount.

2.10. Trans Endothelial Electrical Resistance (TEER)

24 well PETP (polyethylene terephthalate) transwell in-

serts (BD Biosciences; San Jose, CA) were coated with 2% gelatin and seeded with young adult mouse colon cells (YAMCs) in RPMI medium with 5% FCS, 1% insulin/transferrin/selenite and 5 U/ml INF-*γ* and allowed to reach confluence. At confluence, medium was removed and replaced with experimental RPMI media containing 3%, 1% or 0.5% DSS or control medium in both upper and lower chambers. Resistance readings were taken at 0, 1, 2, 18 and 24 hours using an epithelial volt-ohmmeter (World Precision Instruments; Sarasota, FL) and data were converted to percent initial resistance. Data were analyzed by repeated measures ANOVA with Dunnett's post-hoc test to determine significant (p < 0.05) departure from basal resistance.

2.11. Effect of DSS on Colonic Epithelial Metabolism

YAMCs were plated in 96 well plates coated with 2% porcine gelatin in RPMI and allowed to grow to confluence. When cells reached confluence, the culture medium was removed and replaced with experimental RPMI media containing 3%, 1% or 0.5% DSS or control medium. To determine the effect of DSS on metabolism, each of the groups was divided in two (n = 10) and either treated for eight hours with DSS or treated for eight hours with DSS followed by a 24 hour recovery period in RPMI culture medium. Upon the end of treatment, media was changed to RPMI without phenol red containing 0.5 mg/mL (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) for two hours. Next, medium was aspirated and converted MTT was released by addition of acidified isopropanol. Absorbance was measured at 570 nm with background subtraction at 650 nm. Results were analyzed by one-way ANOVA with Dunnett's post-hoc test.

2.12. Statistical Analysis

Disease activity studies were evaluated using repeated-measures ANOVA with Dunnett's post testing, Immuno-histochemistry, macroscopic, and microscopic data were analyzed with one way ANOVA with Tukey Kramer multiple comparison test. MPO data were analyzed by 1 way ANOVA with Dunnett's post testing (Graph Pad In stat 3 software, San Diego, CA).

2.13. Microphotography

Images of immunostained tissue sections were photographed using Nikon bright field microscopy station (Olympus CK2) and analyzed by a double-blinded expert for the numbers of blood and lymphatic vessels and vessel dimensions using Image-J (NIH, Bethesda, MD).

3. RESULTS

3.1. Gut Sterilization

We found that the antibiotic treatment used in this study was sufficient to prevent the growth of any colonies on nutrient agar. This appeared to be both bactericidal and bacteriostatic since feces from mice undergoing antibiotic water treatment were found to contain sufficient bacteria to yield viable 24 nutrient broth cultures (data not shown).

3.2. Disease Activity

Antibiotics reduce the disease activity: Disease activity represents the development of cumulative symptoms (weight change, stool consistency, and occult blood). The DSS group showed onset of disease activity on day 3 (p < 0.01[**]) and it continually increased toward day 11, while the antibiotic-treated DSS group (DSS + ABX) showed a similar onset initially, but after day 5, the disease activity substantially slowed (**Figure 1(a)**). The ABX group showed mild disturbances, but did not develop colitis.

Weight of the mice: Body weight remained constant in the control group and increased gradually in the ABX group (p < 0.01[**]). The DSS group was characterized by a consistent weight loss (p < 0.01[**]), as expected in the colitis model (**Figure 1(b)**). Conversely, the weight of the mice in the DSS + ABX group remained fairly constant compared to the control group (p > 0.05).

Stool consistency: (**Figure 1(c)**) In the DSS group, diarrhea started on Day 1, immediately after the DSS administration and it worsened throughout the course of the study, whereas the DSS + ABX group demonstrated initial diarrheal symptoms on Day 2, but remained less severe than the DSS group (p < 0.01). The ABX and control group had no severe diarrheal symptoms.

3.3. Macroscopic Findings

Antibiotics prevents inflammatory shortening of colon & weight change: Due to severe inflammation in DSS-treated mice (**Figure 2(a)**), the colon length was much shorter (7.0 +/− 0.7 cm) than that of the control group (5.5 +/− 0.52 cm) at p < 0.01. Antibiotics preserved the colon length of DSS + ABX group (6.2 +/− 0.5 cm; p > 0.05). Inflammation induced swelling of the colon (**Figure 2(b)**), which caused an increase in the DSS mice colon weight (0.27 +/− 0.05 g) compared to the control (0.19 +/− 0.03 g) at p < 0.01. Whereas, antibiotics prevented the swelling of the DSS + ABX group colons (p > 0.05).

Spleen weights: The spleen weight may be an indirect index of immune response induced by inflammation. The DSS mice group exhibited an increase in spleen weight

(a)

(b)

(c)
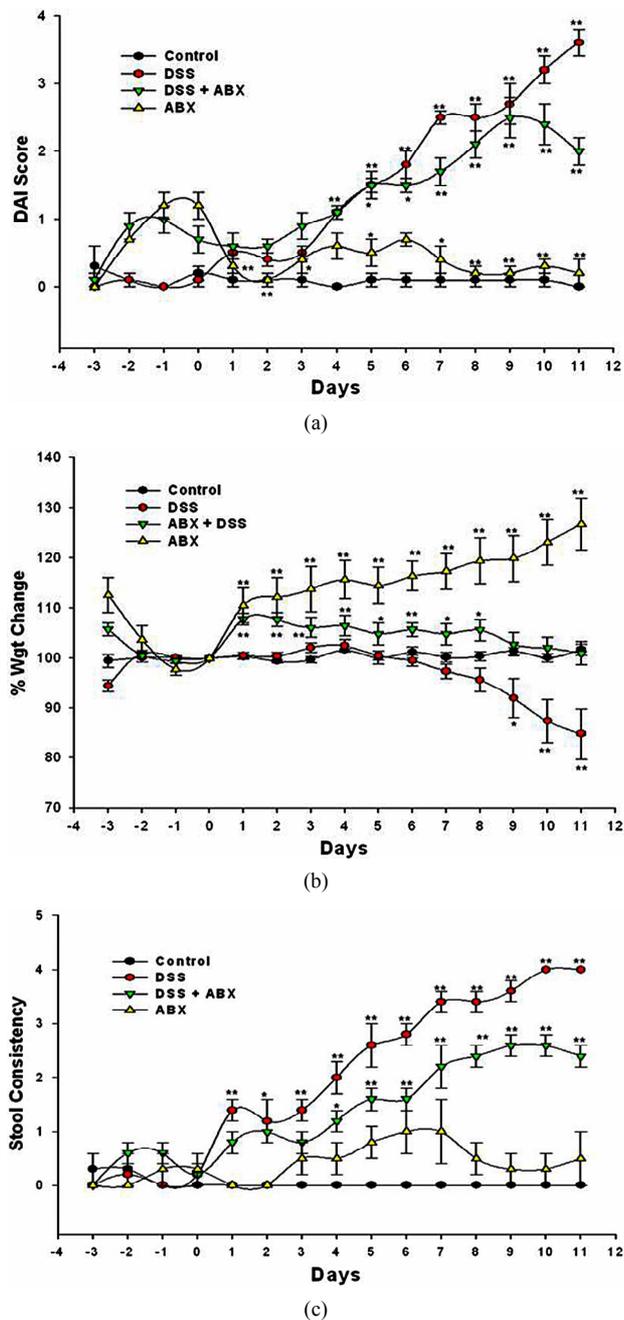
Figure 1. (a) Disease activity index was calculated with a cumulative score of weight change, stool form, and occult blood. The DSS group displayed significant elevation of DAI from baseline ($p < 0.01$) at Day 5 throughout Day 11; though DSS + ABX DAI scores differed from baseline, they were substantially lower than DSS; (b) Over 14 days, weight loss in DSS mice was maximum compared to the control ($p < 0.01$), while DSS + ABX group and control group had similar weight changes at Day 11; (c) Stool consistency (diarrhea) was significantly improved in the DSS+ABX group compared to DSS over 11 days ($p < 0.01$).

(0.14 +/− 0.005 g) compared to control (0.05 +/− 0.005 g; $p < 0.01$), and although the DSS + ABX group also ap-

peared to be increased, this change was not statistically significant (0.10 +/− 0.03 g; $p > 0.05$). This justifies the ablation in inflammatory immune response. The weights of the ABX group were similar to the control (0.045 +/− 0.002 g; $p > 0.05$). This eliminates the possibility of antibiotics playing a role in spleen weight change in control conditions (**Figure 2(c)**).

Colon cross-sectional area: The cross-sectional area indicates the morphological changes induced by inflammation to destroy the integrity of colon. The DSS group demonstrated mean cross-sectional area (**Figure 2(d)**) of 30.8 +/− 6.7 mm^2, which was higher than any of the other groups at $p < 0.001$. Antibiotics preserved cross-sectional areas of the ABX/DSS group (20.1 +/− 5.98 mm^2) because this group did not differ from the control or ABX groups (mean cross-sectional area of 21.03 +/− 4.83 and 18.20 +/− 3.77, respectively; $p > 0.05$).

3.4. Histopathological Colonic Injuury

Histopathological analysis was based on H&E stained colon sections under light microscopy scored by an expert with double blind experimental conditions. The total histopathology score (**Figure 3(a)**) includes 3 parameters: severity of inflammation (**Figure 3(b)**), extent of injury (**Figure 3(c)**), and crypt damage (**Figure 3(d)**). The control group demonstrated no notable epithelial lesions on light microscopy (mean total score 0 +/− 0), whereas the DSS group displayed substantial histological damage (38 +/− 1.581, $p < 0.001$), including focal erosions of the epithelium, crypt dilation, and acute inflammatory infiltrates of lymphocytes and granulocytes in the sub-epithelium and lamina propria (**Figure 3(e)**). Vascular congestion and goblet cell loss, in conjunction with moderate to severe edema, was also observed in this group. All 3 parameters of histopathological score increased significantly in DSS at $p < 0.001$, compared to controls. Meanwhile, antibiotics were able to reduce inflammatory colon damage in the DSS + ABX group (15.2 +/− 3.27; $p < 0.001$). In all 3 parameters, however, it did differ considerably from the control group ($p < 0.001$).

3.5. Colonic Angiogenesis

Blood vascular density (**Figure 4(a)**) indicates the neo-vascularization secondary to acute inflammation. The DSS group showed growth of new vessel formation with a mean count of 84.37 +/− 7.20 vessels per section, which was the only group with any change from control at $p < 0.001$. Antibiotics suppressed vessel counts 2.02 times lower for the ABX/DSS group (41.66 +/− 5.34 vessels per section) and it is approximately identical to the control group (40.90 +/− 10.93 vessels per section; $p > 0.05$) and the ABX group (mean 30.16 +/− 7.81; $p > 0.05$).

(a)

(b)

(c)

(d)
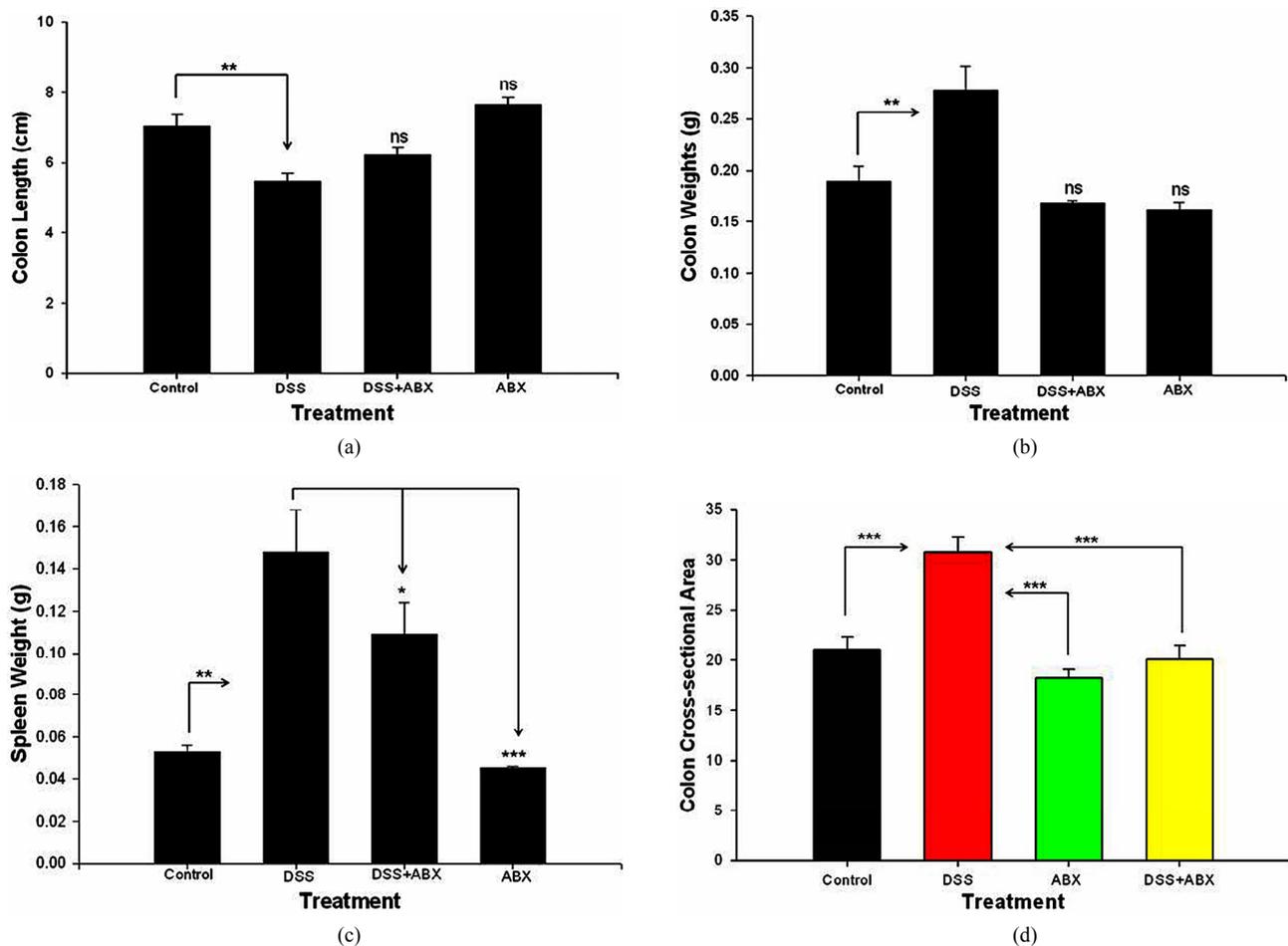
Figure 2. (a) Antibiotics prevent colon shortening: DSS induced inflammatory colon shortening was exacerbated in DSS (5.48 +/− 0.52 cm) compared to controls (7 +/− 0.7 cm; p < 0.01). Antibiotics preserved the colon length in DSS + ABX (6.22 +/− 0.5 cm); (b) Colon weight: Edematous swelling of the colon caused the weight gain in DSS (0.27 +/− 0.05 gm); this significantly differs from control (0.18 +/− 0.02 gm; p < 0.01), while DSS + ABX (0.16 gm) was identical to control; (c) Effects of antibiotics on spleen weight: DSS + ABX (0.108 +/− 0.03 gm) and DSS (0.14 +/− 0.04) showed splenic enlargement, which is elevated from baseline controls (0.005 +/− 0.005 gm); (d) Colon cross-sectional area: DSS with colonic inflammation had higher cross-sectional area than control (p < 0.001). Antibiotics prevent disruption of the integrity of colon architecture in DSS + ABX (20.1 +/− 5.98 mm^2), which did not differ from the control or ABX (mean cross-sectional area of 21.03 +/− 4.83 and 18.20 +/− 3.77, respectively; p > 0.05).

3.6. Colonic Lymphangiogenesis

Lymphatic density (**Figure 4(b)**) indicates immune reactivity to inflammation by neo-lymphangiogenesis. The DSS group showed prominent lymphatic vessel proliferation in the mucosa, lamina propria, and sub-mucosa of the colon (113.25 +/− 14.05 vps). Conversely, with the effect of protective antibiotics, the ABX/DSS group is 1.74 times lower than the DSS group (64.86 +/− 4.95, p < 0.001). The control group (24.45 +/− 6.85) and the antibiotic group (32.38 +/− 10.28) were essentially identical.

3.7. Comparison of Colonic Angiogenesis Lymphaniogenesis

Blood vascular density and lymphatic proliferation are parallel parameters of inflammation. This experiment

shows how antibiotics suppress the native immune response by hindering the development of angiogenesis and lymphangiogenesis (**Figure 4(c)**). The DSS colitis group showed the normal immune response to chemical (DSS) induced inflammation by having the highest number on vessel counts compared to the control group at p < 0.001. Interestingly, angiogenesis in the ABX + DSS is identical to the controls, while lymphangiogenesis is 2.1 fold higher than baseline. This indicates that the antibiotics play different roles in suppression of inflammatory angiogenesis & lymphangiogenesis.

3.8. MPO Activity

Myeloperoxidase is a peroxidase enzyme present in neutrophil granulocytes, which indicates neutrophilia during

(a)

(b)

(c)

(d)

(e)
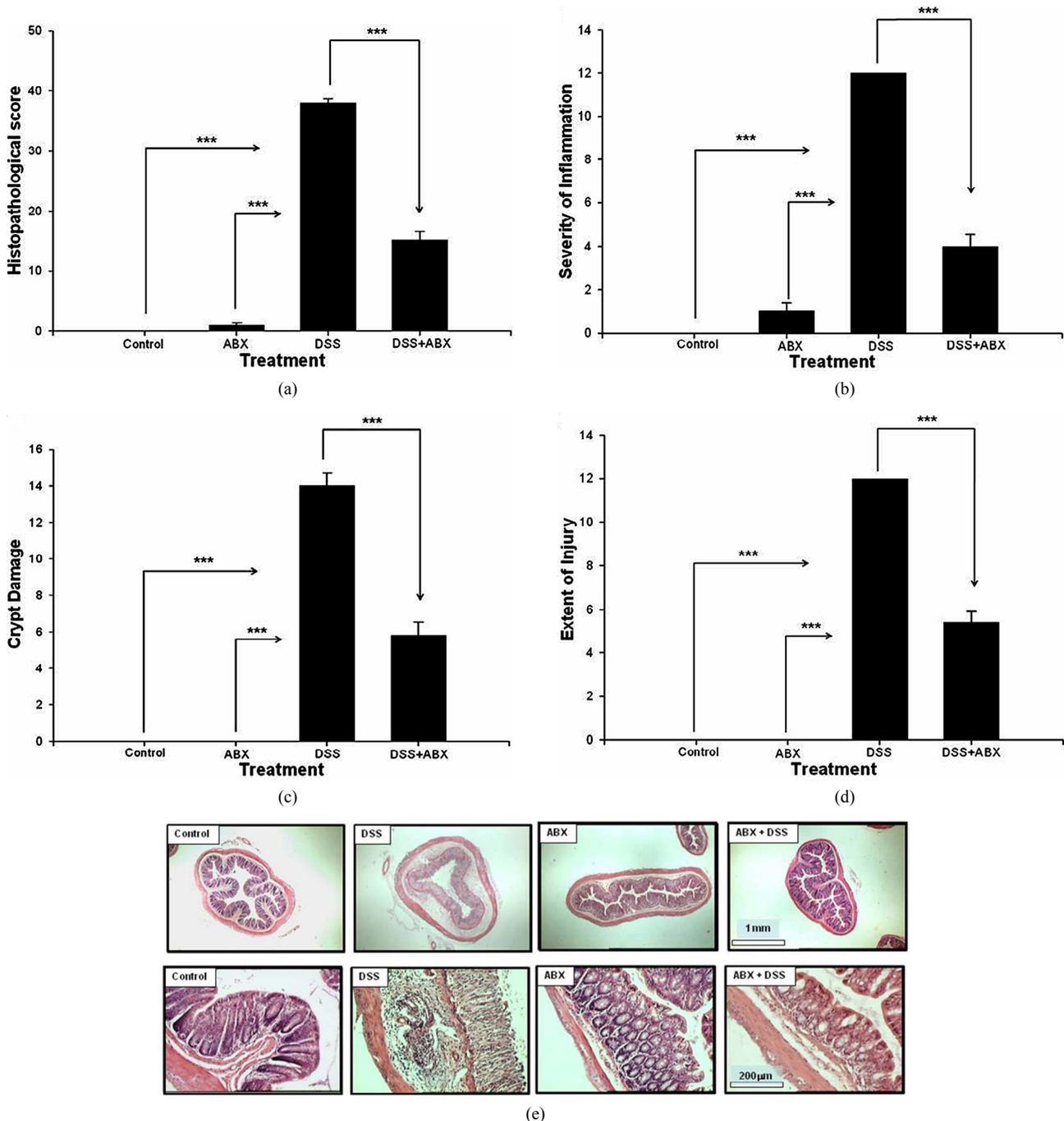
Figure 3. (a) Histopathological Scores: The DSS group had a cumulative histopathological score of 38 +/− 1.5 (normal score = 0, max injury = 40), which was significantly elevated compared to the controls. The DSS + ABX group was also elevated but less so than the DSS group; (b) Histopathological Scores: The DSS group had a significantly higher severity of inflammation score than the control, ABX, and DSS + ABX groups; (c) Histopathological Scores: The DSS group had a significantly higher crypt damage score than the control, ABX, or DSS + ABX groups; (d) Histopathological Scores: The DSS group had a significantly higher extent of injury score than the control, ABX, or DSS + ABX groups; (e) Pictures of various slide photographs of H&E staining of control, DSS, ABX and DSS + ABX groups.

acute inflammation. The DSS group exhibited the highest amount of neutrophil infiltration, as measured by myeloperoxidase (MPO) activity (**Figure 5**), with a mean absorbance change of 0.19 +/− 0.15 and a significant de-

viation from control (0.02 +/− 0.006; $p < 0.05$). The ABX/ DSS group (0.045 +/− 0.02) and the ABX group (0.01 +/− 0.006) didn't produce any significant change from control ($p > 0.05$).

(a)

(b)

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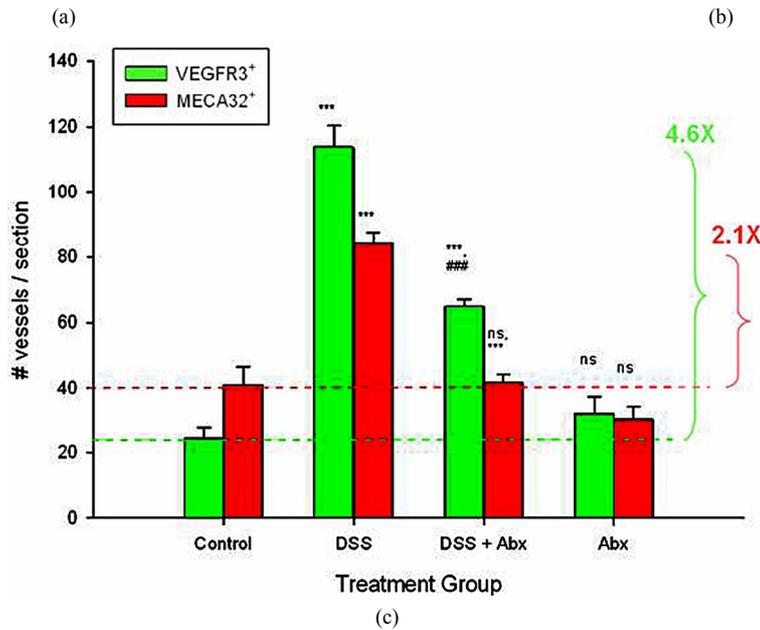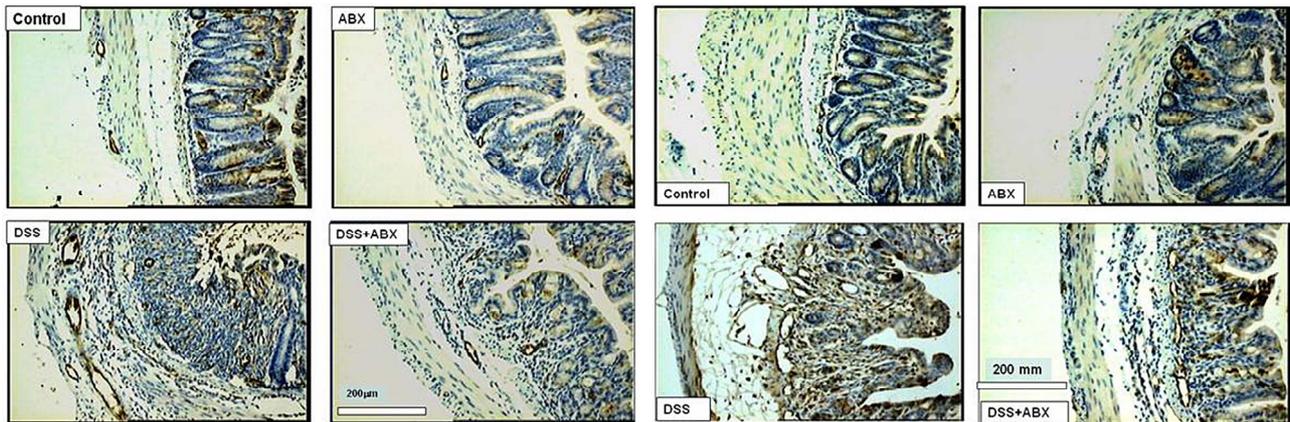
Figure 4. (a) MECA-32 stained blood vessels were significantly elevated in the colon of the DSS group (2.1-fold over control), while the ABX count was similar to the control group; (b) Lymphatic vessels (VEGFR-3) showed a 4.6-fold increase in DSS versus control, whereas the ABX group exhibited a 2.6 fold increase versus control; (c) Histogram of number of vessel/section (1-way ANOVA w/Bon-ferroni post testing).

3.9. Effect of DSS on Trans-Epithelial Electrical Resistance (TEER)

Depression in epithelial barrier function (**Figure 6(a)**) was found to be dependent on the dose of DSS. Controls showed only minor changes in barrier function, which increased slightly over 24 h. 0.5% DSS showed an initial drop in barrier, which recovered by 4 h. At 4 h, 1% DSS showed a drop in barrier, but this also recovered by 24 h. 3% DSS showed the largest drop in barrier, which progressively decreased to 84.1% of baseline by 24 h ($^{**}p < 0.01$).

3.10. Effect of DSS on Colonic Epithelial Cell Metabolism

DSS also produced a dose-dependent and reversible de-crease in epithelial cell metabolism (**Figure 6(b)**). All DSS groups showed a significant decrease in metabolism at 4 h, which was reversed during the recovery period, suggesting that DSS does not produce an irreversible injury to epithelial cells.

4. DISCUSSION

In recent years, animal experiments have demonstrated that gut commensal flora induced immunoinflammatory dysregulation, which contributes in the etiopathogenesis of colitis. While some forms of gut inflammation represent appropriate responses to abnormal or pathogenic gut flora, inflammation in IBD appears to result from an inappropriate response to normal gut flora. While many different antibiotics have been shown to decrease gut inflammation and colonic injury in human and animal
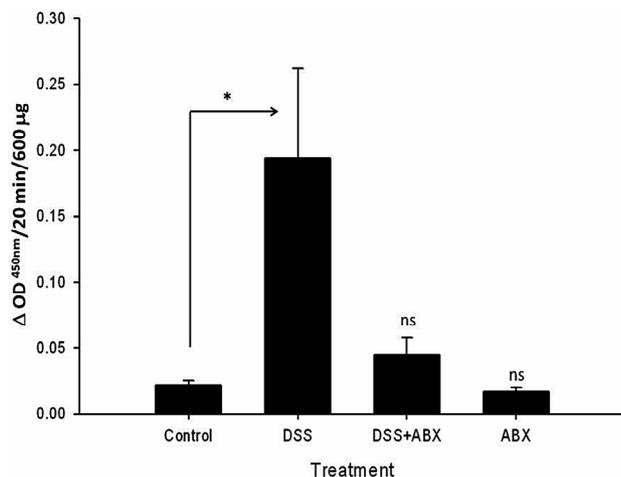
Figure 5. MPO content: Antibiotics suppress gut neutrophil infiltration in colitis. The level of necrophilia measured by MPO activity of the colonic tissue in the DSS group exhibited the highest myeloperoxidase activity versus that of the control group (p < 0.05), Antibiotics reduced MPO activity in the DSS + ABX group compared to the DSS group.

models of colitis, the use of antibiotics in IBD treatment still remains controversial.

4.1. Pro-Inflammatory Bacterial Trigger in Pathogenesis of IBD and Colitis

It is interesting to note that normal commensal gut flora distribution in the intestine exhibits increased density towards the distal end of colon, containing both beneficial and potentially detrimental species. Lactobacillus and Bifidobacteria species have been found to exert anti-inflammatory effects on the gut; administration of Lactobacillus-containing probiotics may even be beneficial in the treatment of UC and pouchitis [27-30]. Conversely, Bacteroides vulgates is a normal gut commensal which can initiate inflammation, while a high bacterial load of *E. coli* or Enterococcus can increase the intensity of gut inflammation [31]. Generally, many aerobes have been associated with local inflammation, while some anaerobes can create diffuse and severe fibrogenic transmural inflammation of the intestine [29]. Rakoff-Nahoum *et al.*, however, have shown that commensal microflora are required for normal homeostasis and maintenance of intestinal epithelial integrity, yet it is unclear how the presence of normal microflora influences tissue injury during disturbances of normal intestinal epithelial barrier [32]. Our studies here show that gut sterilization (using Vancomycin, Metronidazole, and Neomycin) prevents the colonic injury and histopathological damages in the DSS + ABX group indicating that following injury, normal microflora can provoke localized injury responses, some of which may support resolution of this injury.

(a)

(b)
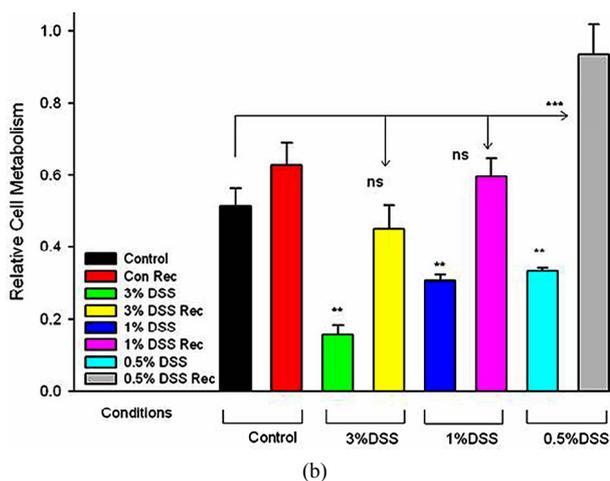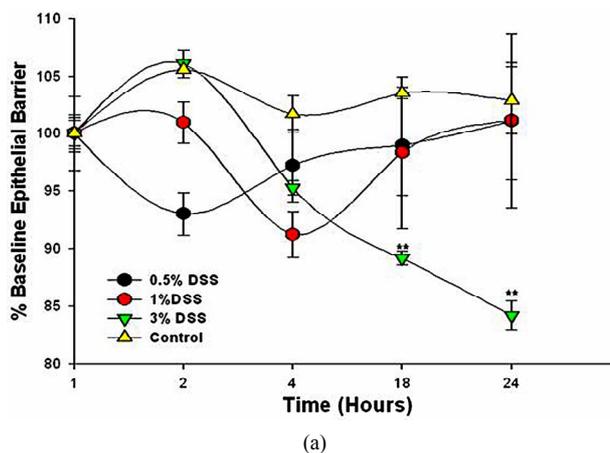
Figure 6. (a) DSS dysregulates epithelial barrier integrity: Epithelial electrical barrier changes in response to control, 0.5% DSS-treated, 1% DSS-treated, and 3% DSS-treated groups are shown at 1, 2, 4, 18 and 24 hours after treatment; (b) DSS transiently dysregulates epithelial metabolism: Relative epithelial cell metabolism changes during different DSS concentration exposures are shown. Exposure phases display inhibition of cell metabolism in all DSS groups (p < 0.01**) and during recovery period the 3% DSS and 1% DSS recovers similar to control.

4.2. Antibiotic Treatment and Gut Barrier Dysfunction

Changing the microbial mass or composition using antibiotics (particularly Metronidazole and Ciprofloxacin) has been effective for treating some Crohn's and UC patients, as well as IBD patients with septic complications [10,30]. Ciprofloxacin, Vancomycin-imipenem, and Amoxicillin/clavulanic acid have been shown to decrease inflammation in TNBS-induced colitis [33,34], while a combination of Metronidazole and Gentamicin is effective in reducing the number of translocated bacteria in acetic-acid induced colitis [35]. In mouse and rat studies, antibiotics have been successfully used to prevent the colitis that spontaneously develops in IL-10 deficient mice [13], to decrease the inflammation in DSS-induced

colitis [36], to treat transgenic HLA-B27-related colitis [31], and to attenuate acetic acid colitis in mice [37]. These mouse and rat studies used combinations involving Ciprofloxacin, Metronidazole, Neomycin, Vancomycin, and Imipenem, but Rifaximin and Tobramycin have also had short-term success in UC [30]. When metronidazole and ciprofloxacin are used in combination, their efficacy is comparable to steroids (methylprednisolone) [30]. Neomycin and Metronidazole both established protection by an increase in level of Lactobacillus along with elimination of detrimental bacterial species [31], and vancomycin exhibits a role in prevention and reversed the established colitis in IL-10 mice.

Because gut barrier dysfunction can usually be shown to precede the development of gut inflammation [23], antibiotic clearance of non-beneficial commensals is particularly useful during instances of a diminished gut barrier in IBD. Although IL-10 gene deficient mice spontaneously develop colitis when colonized by normal gut commensals, these mice do not develop colitis when raised in a germ free environment; this suggests that foreign pathogens may also be involved in the process [38]. One well-known role of IL-10 is to regulate responses to the inflammatory Th1 cytokines IFN-γ and TNF-α, which can dysregulate the integrity of the gut epithelial cell layer. This leads to a compromised gut barrier in IL-10-/- mice, and although etiology may be slightly different, similar pathology is also found in other animal models of IBD and in many cases of human IBD [10,26]. The barrier defect allows bacteria to gain access to the inner layers of the gut wall, as well as increase in numbers of adhesive bacteria, consequently leading to sustained inflammation. In fact, IL-10 deficient mice demonstrate a pre-existing increase in gut permeability to luminal contents even before colitis induction. Humans with IBD also exhibit decreased gut solute barrier function prior to development of inflammation [39]. In our study, even after DSS was used to disturb gut epithelial function, mice administered antibiotics failed to display significant changes in inflammation by a variety of measures; meanwhile, mice administered DSS without antibiotics did demonstrate clear signs of heavy inflammation. This finding furthers the notion that even when the epithelial barrier is disturbed, the progression of IBD requires the presence of bacteria.

Beneficial (e.g. pro-biotic) bacteria may also positively influence barrier through induction of Th2 cytokine responses and NF-kB inhibition. However, in the IL-10 KO model [23,39], the inability to mobilize IL-10 may lead to a shift towards barrier failure as seen in human IBD [11]. We found that DSS produced a rapid and reversible depression in gut epithelial metabolism, which was correlated with barrier changes. Therefore, DSS ap-

pears to produce a transient barrier disturbance that allows the penetration of bacterial antigens into the lamina propria to provoke inflammation. However, because epithelia appear to be relatively intact in colons of DSS + ABX treated mice on day 11, (**Figure 2(a)**) our findings suggest that commensal antigens trigger gut inflammation that leads to epithelial injury mediated by infiltrated leukocytes, rather than simple chemical erosion of the epithelia. This is supported by histologic maintenance of colonic epithelia in DSS + ABX treated mice.

4.3. Histopathological Analysis, MPO Content, and Attenuation of Inflammation

The most direct measure of intestinal inflammation and damage is gross and microscopic examination of the colons and spleens. Control and ABX colons showed the normal colonic epithelial architecture with no evidence of inflammation and edema of intestine. However, DSS mice had significantly shorter, heavier, and larger colons, as well as heavier spleens. The addition of antibiotics substantially lowered these measures of inflammation, as the sizes of the colons and spleens in DSS + ABX mice, which suggests a reduction of disease activity. The microscopic examination resulted in similar findings; antibiotic administration was associated with decreased inflammation and histological damage.

Light microscopy demonstrated prominent involvement of the distal colon with respect to the mid- and proximal colon, which becomes more extensively involved in the DSS group. Presence of atrophy, crypt abscesses, and distortion indicate severe inflammation in the distal colon. Human UC is also characterized by inflammatory changes mainly limited to the rectum and to the left colon and confined within the mucosa. The lamina propria appears edematous, with vascular congestion and the presence of mixed inflammatory infiltrates (granulocytes, lymphocytes, and plasma cells) as well as cryptitis and crypt abscesses with depletion of goblet cells, crypt architecture distortion, and lymphoid aggregates. Severe human IBD also shows inflammatory changes, with shortening of the colon as a result of muscular contraction. Together, these factors confirm the similarities between human ulcerative colitis and mouse DSS colitis.

Neutrophil infiltration is an index of acute inflammation. DSS-induced chemical inflammation triggers the infiltration of neutrophils and subsequently chemokines, cytokines, growth factors and proteolytic enzymes, which contributes to colonic injury and new blood and lymphatic vessel formation. Decrement effect of antibiotics in MPO content prevents significant colonic inflammation in the DSS + ABX group, explaining the preserved histologic integrity of the colon.

4.4. Modulation of Angiogenesis and Lymphangiogenesis with Gut Sterilization

We found that DSS-induced inflammation leads to increased gut angiogenesis and lymphangiogenesis, likely as a result of elevated levels of angiogenic factors like VEGF-C and VEGF-A in colitis [40,41]. The rise in vessel density in the DSS-treated colon was correlated with notable pathological changes in the colon, while the antibiotic-treated DSS group demonstrated a complete blockade of inflammatory angiogenesis, as well as a substantial decrease in lymphangiogenesis, possibly by preventing the remodeling of vascular tissue by ang1/ang2 regulation. The ratio of lymphatic to blood vessel density was twice as high in the DSS group (L/B = 1.3) than in the control group (L/B = 0.61), although this ratio was increased further (L/B = 1.55) in the DSS + ABX treated group. This is likely a consequence of the fact that blood vessel expansion mediates injury, while lymphatic vessel injury does not; conversely, increased lymphatic density appears to adaptively balance interstitial fluid accumulation, especially when epithelial barrier is altered (e.g. by DSS, **Figure 6(a)**).

Interestingly, at least part of the benefit of UC therapy using mesalamine (5-ASA) may be due to the normalization of the balance between angiogenic/ anti-angiogenic factors. In iodoacetamide induced ulcerative colitis, 5-ASA increased angiostatin/endostatin levels and decreased TNF-α, which down-regulates MMP-2 and MMP-9 [42]. Angiopoietin-2 is an important ligand of the Tie-2 receptor, which dysregulates blood and lymphatic vascular networks in both humans and mice. We reported in our previous study [43] that Ang-2-/- mice treated with DSS exhibited no remodeling of blood or lymphatic vessels, with significantly fewer infiltrated leukocytes but still exhibited increased gut fragility (occult blood), disease activity, weight loss, and diarrhea. While angiogenesis knockout may limit events in gut inflammation, simultaneous interference with lymphatics mediated by Ang-2-/- appears to eliminate some or all of the benefit of anti-hemangiogenesis (blood vessel remodeling). Several studies on angiogenesis blockade in IBD shows a decrease in DAI, histopathology and angiogenesis in IL-10-/- mice when treated with the hemangiogenesis blocker ATN-161 [44]. It is important to note that in the current study we find that antibiotic protection against injury reflects complete block in blood vessel growth, but persistence of lymphatic vascular growth. This appears to be most consistent with new blood vessel growth rather than lymphatic growth as mediating injury in experimental colitis.

The present study reveals certain characteristic effects of antibiotics (by removing commensals) on the complex acute inflammatory response on new blood and lymphatic vessel formation. The findings suggest that elimination of gut flora by antibiotics reduces injury and prevents leukocyte infiltration into the gut. Injury reduction is correlated with a complete blockade of neo-angiogenesis. These findings were further correlated with a reduction, but not elimination, of lymphangiogenesis. These findings support lymphatic expansion in colitis as a protective feature of gut injury which interacts with angiogenesis. Therapies to enhance lymphangiogenesis or normalize lymphatic function may improve clinical responses.

Antibiotics have been shown to ameliorate some features of IBD, and reduce the amount of inflammatory damage to the mouse intestine; however, they have not previously been directly related to inflammatory angiogenesis, an important new mechanism in IBD therapy. Further our data supports the protective ability of lymphangiogenesis against development of injury in this model. Therefore, in the future, antibiotics may be combined with anti-angiogenesis agents, as well as pro-lymphangiogenic agents, to prevent development or exacerbation of human IBD.

5. CONCLUSION

Diverse IBDs share immune dysregulation toward normal gut bacteria and altered gut barrier function. A reduction in gut bacteria, despite barrier disturbances, might lessen deleterious effects of the disease. Broad spectrum antibiotics decreased colon neutrophil infiltration, disease symptom severity and histopathological evidence of colitis. Antibiotics completely prevented inflammatory angiogenesis and partially reduced inflammation associated lymphangiogenesis, which may be important events both in the development and regulation of inflammation. Our data suggests that infiltration of bacterial antigens into the gut lamina propria is necessary for the initiation of angiogenesis. Lymphangiogenesis was induced despite prevention of colon injury and suggests that unlike angiogenesis, lymphangiogenesis may represent an adaptive response in colon inflammation.

6. ACKNOWLEDGEMENTS

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