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Original Research

Effects of Azithromycin on Behavior, Pathologic Signs, and Changes in Cytokines, Chemokines, and Neutrophil Migration in C57BL/6 Mice Exposed to Dextran Sulfate Sodium

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Here we characterized the murine dextran sulfate sodium (DSS) model of acute colitis. Specifically, we evaluated azithromycin and metronidazole treatment regimens to assess their effects on animal wellbeing, pathologic changes, barrier function, cytokine and chemokine profiles, and neutrophil migration in colon tissue. Azithromycin treatment significantly reduced the severity of colitis, as assessed through body weight change, water consumption, macroscopic lesions, and animal behaviors (activity level, climbing, and grooming), but did not alter food consumption or feeding behavior. Mucosal barrier function (evaluated by using FITC-labeled dextran) was decreased after DSS exposure; azithromycin did not significantly alter barrier function in mice with colitis, whereas metronidazole exacerbated the colitis-related deficit in barrier function. In addition, metronidazole appeared to exacerbate disease as assessed through water consumption and animal behaviors (overall activity, climbing, grooming, and drinking) but had no effect on weight loss, macroscopic lesions, or eating behavior. Pathologic changes were typical for DSS treatment. Antibiotic treatment resulted in reduced levels of proinflammatory cytokines and chemokines and decreased neutrophil adhesion and emigration in DSS-exposed mice. The results highlight the importance of clinical and behavioral assessments in addition to laboratory evaluation as tools to evaluate animal welfare and therapeutic efficacy in disease models. Data from this study suggest that azithromycin may convey some benefits in the mouse DSS colitis model through modulation of the immune response, including neutrophil migration into tissues, whereas metronidazole may exacerbate colitis.

Abbreviations: DSS, dextran sodium sulfate; IBD, inflammatory bowel disease

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Inflammatory bowel disease (IBD), including Crohn disease and ulcerative colitis, encompasses a group of disorders characterized by lifelong relapsing intestinal inflammation and injury that affect an estimated 5 million people worldwide.[9,38] Although the pathogenesis is still unclear, epithelial barrier breakdown and immune dysregulation appear to play key roles in IBD development.[4,12] Clinical outcomes have been greatly improved by the use of antiTNF drugs, which inhibit the proinflammatory cytokine TNFα, but more than two thirds of patients either fail to respond or experience intolerance or loss of response to therapy.[22] Therefore, additional effective treatments as well as increased understanding of the etiology and pathophysiology of IBD are needed.

Macrolide antibiotics and their derivatives, the azalides, are broad-spectrum bacteriostatic drugs that are often prescribed to patients with inflammatory airway disease.[17,18,33] The drugs have antiinflammatory and immunomodulatory effects, which appear to be independent of their antibacterial properties.[2,8,36] A growing body of evidence highlights that macrolides directly affect the functions of innate immune cells and alter levels of several inflammatory cytokines, including TNFα.[8,28,48] Macrolides stimulate neutrophil apoptosis,[8,15,20,21,28] improve phagocytosis, and alter the degranulation and oxidative burst functions of these cells.[11,30] These activities are often cited as contributors to the clinical benefits that patients with inflammatory airway disease experience when treated with these drugs.[48] Although the literature has focused on the benefits of macrolide use in respiratory diseases, the role they play in the treatment of IBD is unclear.

Metronidazole is a member of the nitroimidazole group of antibiotics and is active primarily against obligate anaerobic bacteria and protozoa.[10] When antibiotics are indicated, metronidazole is commonly prescribed to IBD patients.[6] Although studies examining its antiinflammatory properties are sparse, some evidence suggests that metronidazole alters

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microcirculation[32] and may modify the actions of reactive oxygen species.[37]

The murine dextran sulfate sodium (DSS) model of colitis is a well-established inducible model of disease that has been used in IBD research since the 1980s.[39,45] Administration of DSS in the water of rodents induces patchy colonic inflammation characterized by mucosal erosion and ulceration and extensive leukocyte infiltration.[46] Clinical signs typically include weight loss, hematochezia, and diarrhea.[46] Disease severity can be manipulated by changing the concentration of DSS used, and chronicity can be modulated by altering the duration of exposure.[39] Although the exact mechanism by which DSS induces disease is not known, the chemical is thought to act primarily by disrupting the permeability of the mucosal barrier through direct epithelial cytotoxicity.[46] This disruption, in turn, enables the translocation of luminal bacteria into the tissue, inducing inflammation that is driven primarily by cells of the innate immune system.[46] Others have suggested that DSS induces colitis through the formation of nanolipocomplexes with medium-chain fatty acids that can fuse with colonic epithelial cell membranes, potentially activating inflammation.[31]

Although the rodent DSS model is frequently used to assess potential IBD therapies, few studies focus on animal behavior as an indicator of clinical efficacy. We investigated the clinical, behavioral, pathologic, and immunomodulatory changes induced by the azalide azithromycin and the nitroimidazole metronidazole in the mouse DSS model of colitis. Our study highlights the importance of assessing behavioral outcomes and offers preliminary evidence of potential mechanisms involved in macrolide-mediated modulation of inflammation in the murine DSS model of colitis.

Materials and Methods

Animals. Male SPF C57BL/6 mice (age, 46 to 63 d; weight [mean ± 1 SD], 25.5 ± 1.6 g; Charles River Laboratories, Montreal, Quebec, Canada) were used for all experiments. Animals were housed under standard conditions comprising IVC (IVC Blue Line, Tecniplast, Toronto, Ontario, Canada) containing autoclaved aspen chip bedding (NEPCO, Warrensburg, NY), an autoclaved paper house (Shepherd Shack, Shepherd Specialty Papers, Watertown, TN), and autoclaved shredded paper (Sizzle Pack, Instabox, Calgary, Alberta, Canada). Irradiated food (5062, LabDiet, St. Louis, MO) and tap water were provided without restriction. Routine colony health surveillance was performed through monthly sentinel serology (MFIA Mouse Tracking Panel, Charles River Laboratories), with expanded panels performed quarterly (MFIA Mouse Assessment Plus, Charles River Laboratories). The mice were purchased from a *Helicobacter*-free source, and the *Helicobacter* status of these mice was not specifically determined during the experiments. Animals were acclimated for 1 wk prior to initiation of experiments. Experiments were performed at the University of Calgary Health Sciences Campus in compliance with procedures outlined in approved Animal Care Committee Protocols (AC13-0003 and renewed as AC17-0032). Clinical assessments by animal care staff and veterinary staff were used to implement humane endpoints according to criteria established in conjunction with the Animal Care Committee and these protocols. Euthanasia was performed for any animal that met the endpoint. Data collected from these animals were excluded when the endpoint occurred prior to day 3 but were included when the endpoint occurred on or after day 3.

Experimental design. Mice were assigned to experimental groups by using a randomized block design that considered supplier cage origin. Colitis was induced by delivering 3.5% (w/v) DSS (MW 40,000 to 50,000 g/mol, MJS BioLynx, Brockville, Ontario, Canada) in the drinking water for 7 d.[16,27,35] Animals received either 100 µL of untreated water (vehicle), 50 mg/kg azithromycin (Sandoz, Boucherville, Quebec, Canada) in water, or 10 mg/kg metronidazole (AAPharma, Toronto, Ontario, Canada) in water once daily through oral gavage. Gavage treatments began on day 0, in conjunction with colitis induction, and continued until euthanasia. Experimental groups were control, DSS only, DSS plus azithromycin, and DSS plus metronidazole; groups for azithromycin only and metronidazole only were included when examining neutrophil adhesion and emigration. Food intake, water intake, and animal body weight were recorded daily. Behavior measurements (described later) and circulating granulocyte counts were assessed on day 7. After euthanasia, colon tissue was harvested for measurements of colon length and thickness, macroscopic lesion scoring, histopathology scoring, and cytokine and chemokine quantification.

Necropsy evaluations. Colon and rectal tissue, extending from the cecum to the anus, were collected and assessed together in a subset of animals. Colons were opened longitudinally, placed on a flat surface, and lengths were measured to the nearest millimeter. The mucosal and serosal surfaces were assessed for the presence of any of the following macroscopic findings: erythema, hemorrhage, edema, fecal blood, fecal mucus, diarrhea, strictures, ulcerations, and adhesions. Each lesion was assigned a score of 1, and scores were summed to produce an overall macroscopic finding score. The thickness of the descending colon was measured by using a digital caliper at a location approximately halfway between the transverse colon and the anus. Wet cecum weights, both full and emptied, were assessed in a subset of animals by using an analytic balance.

Histopathology. Pathologic changes in the colon were evaluated by using Swiss roll preparations, as previously described.[45] Briefly, colon tissue extending from the cecum to the anus was opened longitudinally, and the feces were carefully removed. The tissue was immersed in PBS, gently agitated to remove feces, and then rolled around a pin before fixation in 10% neutral buffered formalin. Tissues were sequentially dehydrated, embedded in paraffin, and sectioned (5 µm) for standard hematoxylin and eosin staining. Pathologic changes were scored by a reader blinded to the experimental group. The scoring system involved estimation of crypt damage (0 to 5),[31] mucosal ulceration (0 to 3),[31] inflammation (0 to 5),[7] goblet cell depletion (0 to 3),[23] and colon edema (0 to 2; with 2 indicating colonic tissue involvement of more than 25%).[7] No special staining for goblet cells was performed.

Food and water consumption. Food and fluid (water) intakes were determined by recording the daily weights of the food hopper and water bottle, respectively, for each cage. Daily consumption was calculated by subtracting the current weight of the appropriate item from the weight determined on the previous day. The average spillage that occurred when mounting the water bottle to the cage was calculated and subtracted from the daily water intake for each cage.

Behavioral assessments. On the final day of the experiment, a subset of mice from each group was assessed behaviorally. The animals were placed individually in the Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS, Metris, Hoofddorp, The Netherlands) for a total of 23 h per mouse. Validation studies indicate a 90% to 95% correlation between the LABORAS system and human observer scores.[40] The system quantifies animal behaviors including times spent in locomotion, immobility, climbing, grooming, eating, and

drinking and the frequency of each behavior. To minimize the diluting effect of a normal sleep cycle, only data from the 10-h period during which the mice were most active were analyzed.

Barrier function. To assess intestinal barrier permeability, a subset of mice from each group received 10 mg of FITC-conjugated dextran (catalog no. 68059, MW 4000, Sigma Aldrich, St Louis, MO) in 100 µL PBS by gavage at 4 h prior to euthanasia on day 7. Mice were anesthetized by using isoflurane, and blood was collected through terminal cardiocentesis. Sera from these blood samples were analyzed for fluorescence by using a standard plate reader at 490 nm excitation and 530 nm emission wavelengths.

Circulating granulocyte counts. A subset of mice from each group was anesthetized by using isoflurane, and heparinized whole blood was collected through terminal cardiocentesis. As described in the manufacturer instructions, all whole-blood specimens were analyzed within 2 h of collection for granulocyte counts by using a HemaTrue Hematology Analyzer (Heska, Loveland, CO).

Colonic cytokines and chemokines. Colon tissue from a subset of mice from each group was weighed; standard cell lysis buffer containing protease inhibitors (catalog no. 05892791001, complete ULTRA Tablets Mini EDTA-free, Roche, Laval, Quebec, Canada) was added to each sample at a ratio of 10 µL buffer per 1 µg tissue. After homogenization and centrifugation (10,000 × *g* for 10 min at 4 °C), supernatants were collected for multiplex cytokine and chemokine capture ELISA quantification (catalog no. MD31, Eve Technologies, Calgary, Canada).

Neutrophil adhesion and emigration. To investigate the emigration behavior of neutrophils in situ, mice were injected intravascularly with 2 µg rat antimouse Ly6G-PE (clone 1A8, BioLegend, San Diego, CA) and 10 µg rat antimouse PECAM1 that was coupled (catalog no. AF660, protein labeling kit, ThermoFisher Scientific, Waltham, MA) to Alexa Fluor 660 (clone 390, eBioscience, San Diego, CA) at 10 min prior to the collection of distal colon collection. The colon tissue was removed, cut lengthwise, gently washed with PBS, mounted onto a slide by using fluorescence mounting medium (Dako, Santa Clara, CA), and coverslipped. Spinning disk confocal microscopy was performed by using an upright microscope (model BX51W1, Olympus, Center Valley, PA) equipped with a 20×/0.95 XLUM Plan Fl water-immersion objective and a confocal light path (WaveFx, Quorum, Guelph, Ontario, Canada) on a Yokogawa head (model CSU-10, Yokogawa Electric Corporation, Tokyo, Japan). Laser excitation at wavelengths of 561 nm and 640 nm (Cobalt, Stockholm, Sweden) was used, and fluorescence in the RFP and Cy5 channels was visualized by using the appropriate long pass filter (593 ± 40 nm and 692 ± 40 nm, respectively; Semrock, Rochester, NY). Exposure time was 300 ms for RFP and 400 ms for Cy5. A 512×512-pixel back-thinned EMCCD camera (model C9100-13, Hamamatsu, Bridgewater, NJ) was used for fluorescence detection. Volocity acquisition software (Improvision, Lexington, MA) was used to drive the confocal microscope, and the captured images were processed and analyzed in Volocity 4.20. Fluorescence-labeled cells were enumerated by selecting at least 5 random fields of view at 20× magnification.

Statistical analyses. Significance was defined at $P < 0.05$ for all analyses. All statistical analyses were performed by using R version 3.4.1,[41] unless stated otherwise. Body weight, food consumption, and water consumption were assessed by using linear mixed-effects regression through the lme4 package. Linear mixed-effects regression models behave similarly to the repeated-measured ANOVA test but can accommodate unbalanced designs, missing data points, and the variance of random factors, including individual mouse and time points. Homoscedasticity and linearity of the model were assessed by using diagnostic plots of the residuals. F tests and *P* values were adjusted for multiple testing by using the Benjamini–Hochberg procedure for estimating the false discovery rate. Behavioral data were analyzed by using multivariate generalized linear models. For cytokine and chemokine analysis, values for TNFα, IL1α, KC/CXCL1, MIP2/CXCL2, GCSF, IL17, and IL6 were analyzed separately to minimize type II errors, whereas eotaxin, GMCSF, IFNγ, IL1β , IL2, IL3, IL4, IL5, IL7, IL9, IL10, IL12(p40), IL12(p70), IL13, IL15, IP10, LIF, LIX, MCP1, MCSF, MIG, MIP1α , MIP1β, MIP2, RANTES, and VEGF were tested under a separate model. To account for intercorrelation between cytokines and chemokines, we used multivariate generalized linear models within the mvabund package and assumed a negative binomial distribution. Posthoc testing was then performed by using a custom function Kruskal–Wallis test followed by the Conover test to compare the group pairwise differences with *P* values that were corrected for false discovery rate.[3] For all stand-alone variables (histologic scoring, colon length and thickness, cecum weight, serum FITC, and neutrophil adhesion and emigration), we used one-way ANOVA or the Kruskal–Wallis nonparametric test when assumptions of the ANOVA were not met. Pairwise comparisons were made by using the Tukey honest significant difference test.

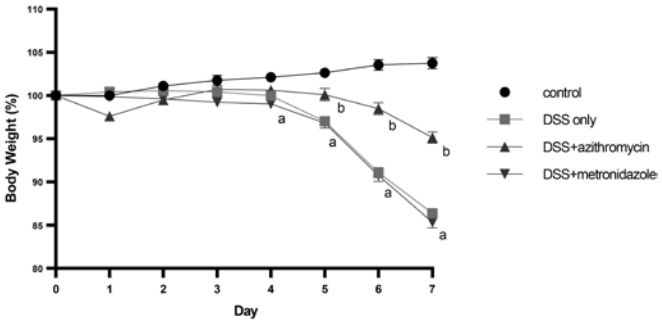

Figure 1. Percentage daily body weight change (% loss or gain from initial day 0 weight) for control (n = 32); DSS only (n = 79); DSS+azithromycin (n = 38); and DSS+metronidazole (n = 42) groups. Data are expressed as mean ± SEM [a], DSS only and DSS+metronidazole mice lost significantly ($P < 0.05$) more body weight than controls on days 4, 5, 6, and 7; [b], weight loss on days 5, 6, and 7 was significantly ($P < 0.05$) ameliorated in DSS+azithromycin mice compared with mice given DSS only or DSS+metronidazole.

Results

Body weight. Control mice gained body weight over time, whereas DSS-exposed animals lost a significant amount of body weight (maximum, 15%) over the course of the experiment (Figure 1). Clinical signs appeared after 3 to 4 d of DSS exposure and gradually increased in severity over time. On all days, animals given both DSS and metronidazole experienced similar weight loss to those given DSS only. In contrast, weight loss was ameliorated in the DSS-treated mice that received azithromycin. On days 5, 6, and 7, mice given DSS and azithromycin were significantly ($P < 0.05$) heavier than those given DSS only or DSS and metronidazole.

Necropsy findings. DSS exposure resulted in a significantly ($P < 0.05$) shorter colon than in control animals (Figure 2 A), and azithromycin treatment significantly ($P < 0.05$) attenuated this effect. Exposure to DSS significantly ($P < 0.05$) thickened colons (Figure 2 B) compared with the control animals. However, the colons of DSS-treated mice that received azithromycin

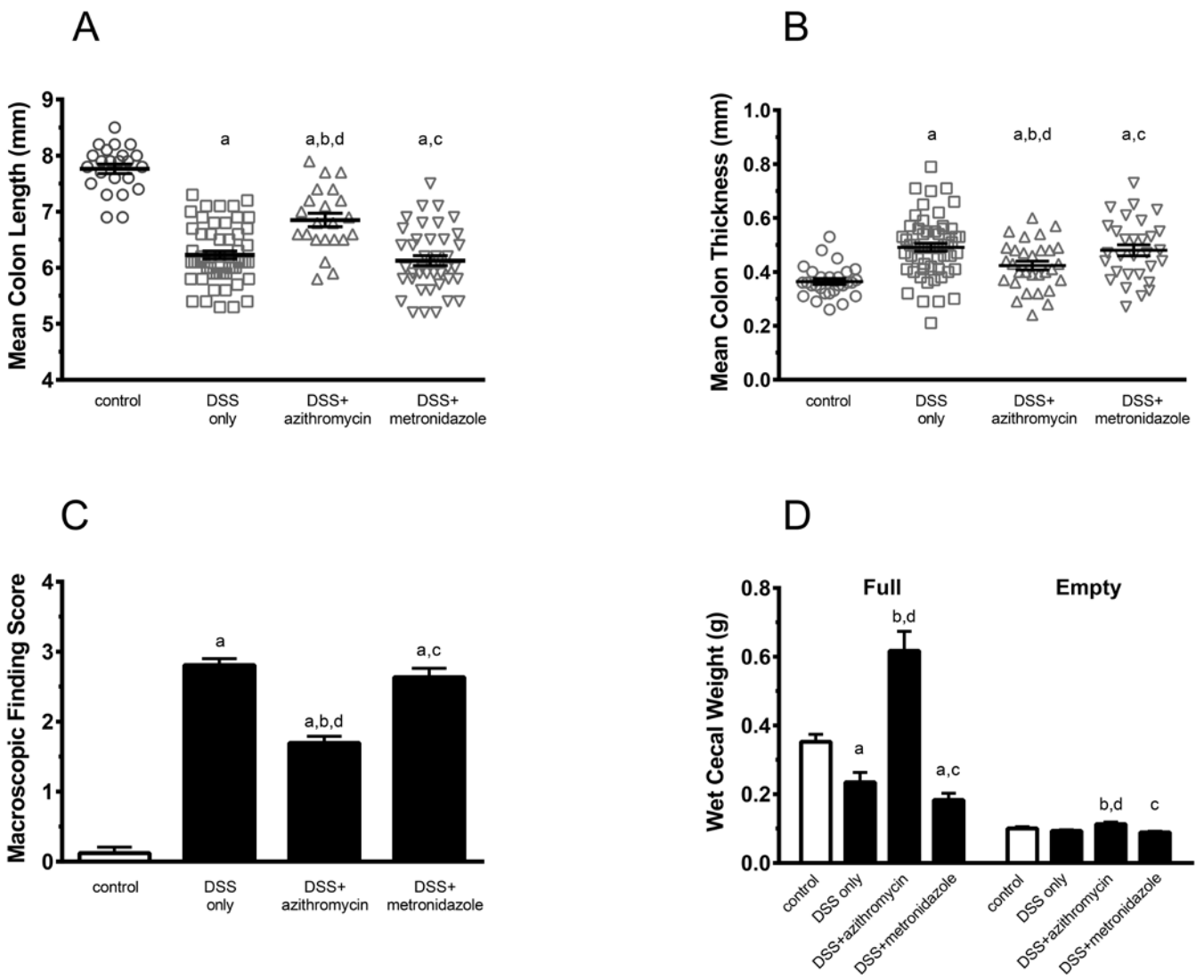

Figure 2. Necropsy findings of control, DSS only, DSS+azithromycin, and DSS+metronidazole groups. All data are expressed as mean ± SEM. (A) Colon length in control (n = 23), DSS only (n = 55), DSS+azithromycin (n = 22), and DSS+metronidazole (n = 41) groups. (B) Colon thickness in control (n = 23), DSS only (n = 55), DSS+azithromycin (n = 22), and DSS+metronidazole (n = 41) groups. (C) Macroscopic findings score in control (n = 33), DSS only (n = 73), DSS+azithromycin (n = 36), and DSS+metronidazole (n = 41) groups. (D) Weights of full and emptied ceca in control (n = 8), DSS only (n = 22), DSS+azithromycin (n = 11), and DSS+metronidazole (n = 19) groups. [a], Significantly ($P < 0.05$) different from control; [b], significantly ($P < 0.05$) different from DSS only; [c], significantly ($P < 0.05$) different from DSS+azithromycin; [d], significantly ($P < 0.05$) different from DSS+metronidazole.

were significantly ($P < 0.05$) thinner than those given DSS only or DSS with metronidazole. All DSS-exposed mice had more pathologic changes in the colon than control animals (Figure 2 C). However, the DSS-treated mice given azithromycin had significantly ($P < 0.05$) lower macroscopic finding scores, involving exclusively the presence of diarrhea and edema, than the other 2 DSS-exposed groups. DSS-treated mice given azithromycin had significantly ($P < 0.05$) heavier filled ceca than mice in the other DSS-exposed groups (Figure 2 D). This difference can mostly be attributed to cecal contents, because the differences in the weights of the emptied ceca were much smaller (Figure 2 E).

Histopathology. Pathologic changes visible in hematoxylin-and-eosin–stained sections of colon included transmural inflammation, which is consistent with colitis induced by DSS.[14] Substantial edema, leukocyte infiltration, and mucosal erosion were evident, and some animals had crypt abscesses. Whereas the colons from mice in the control group (Figure 3 A) were largely normal, DSS exposure resulted in significant ($P < 0.05$) pathologic changes (Figure 3 B through D). Whereas metronidazole treatment of DSS-exposed animals did not significantly alter histopathology scores, treatment with azithromycin yielded a general trend ($P = 0.059$) toward modest reductions in scores.

Food and water consumptions. Appetite and food consumption are often considered to be revealing clinical signs in diseased animals. All groups that were exposed to DSS ate less food than controls (Figure 4 A). However, no significant differences were detected among the 3 DSS-exposed groups except for on day 6, when the mice treated with both DSS and azithromycin had significantly ($P < 0.05$) greater food consumption than those given DSS only or with metronidazole. In addition, eating frequency (Figure 4 B) and the time spent eating (Figure 4 C) showed no significant differences among groups.

Because the colitis-inducing agent was delivered in drinking water, it was imperative to assess water consumption. The control animals and those given both DSS and azithromycin drank significantly ($P < 0.05$) more water on days 5, 6, and 7 than mice

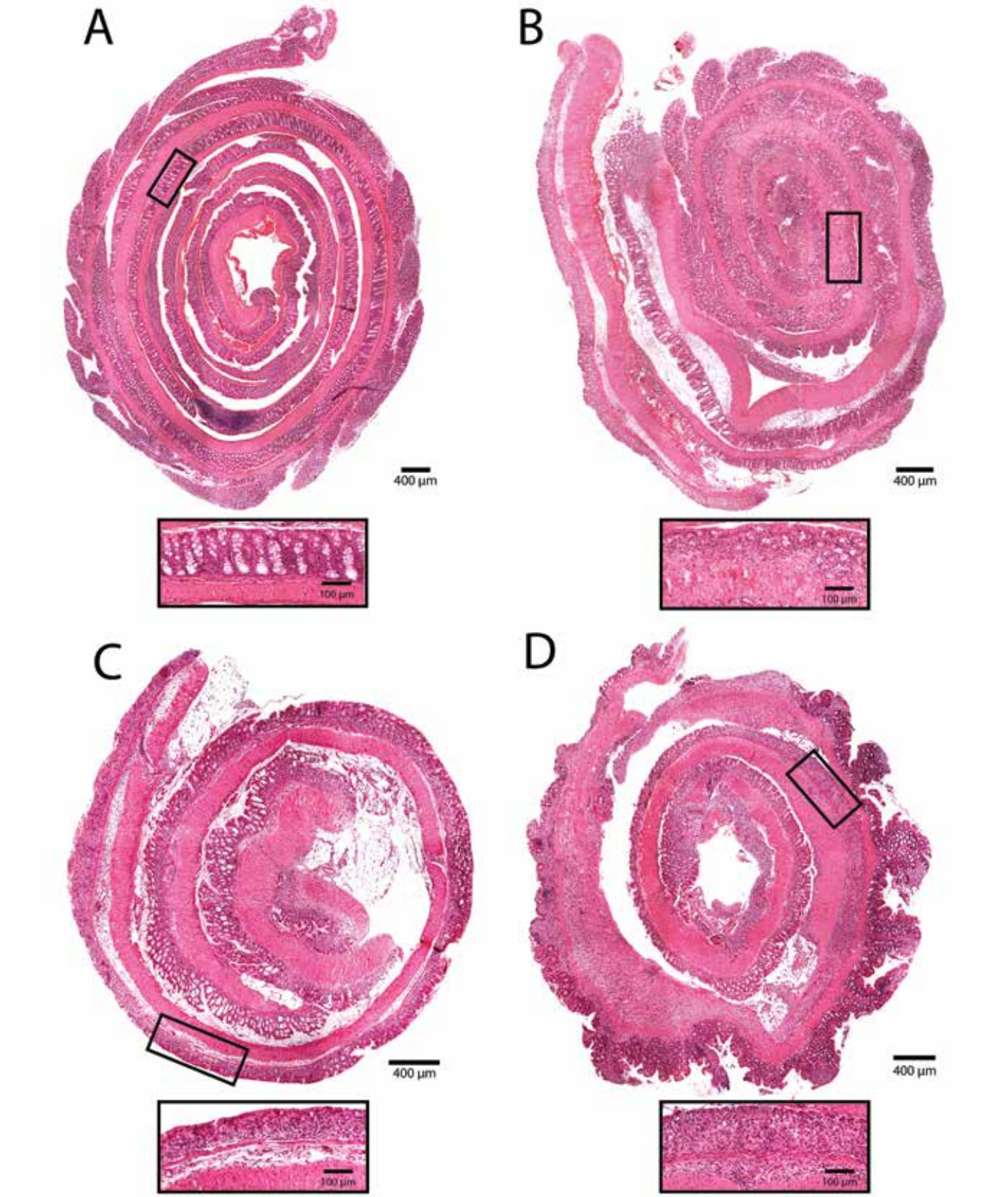

Figure 3. Photomicrographs of representative hematoxylin-and-eosin–stained sections of mouse colon from (A) control, (B) DSS only, (C) DSS+azithromycin, and (D) DSS+metronidazole groups. The animals that received DSS only exhibited areas of colonic transmural inflammation, including edema, leukocyte infiltration, crypt abscesses, and focal areas of mucosal erosion. Bar, 400 µm (entire colon); 100 µm (colon section).

given DSS only or with metronidazole (Figure 4 D). No significant differences were found between control animals and those given both DSS and azithromycin at any time, except on day 6, when DSS-treated mice given azithromycin drank more often ($P < 0.05$) than mice in the other DSS-treated groups, in amounts not significantly different from control (Figure 4 E and F).

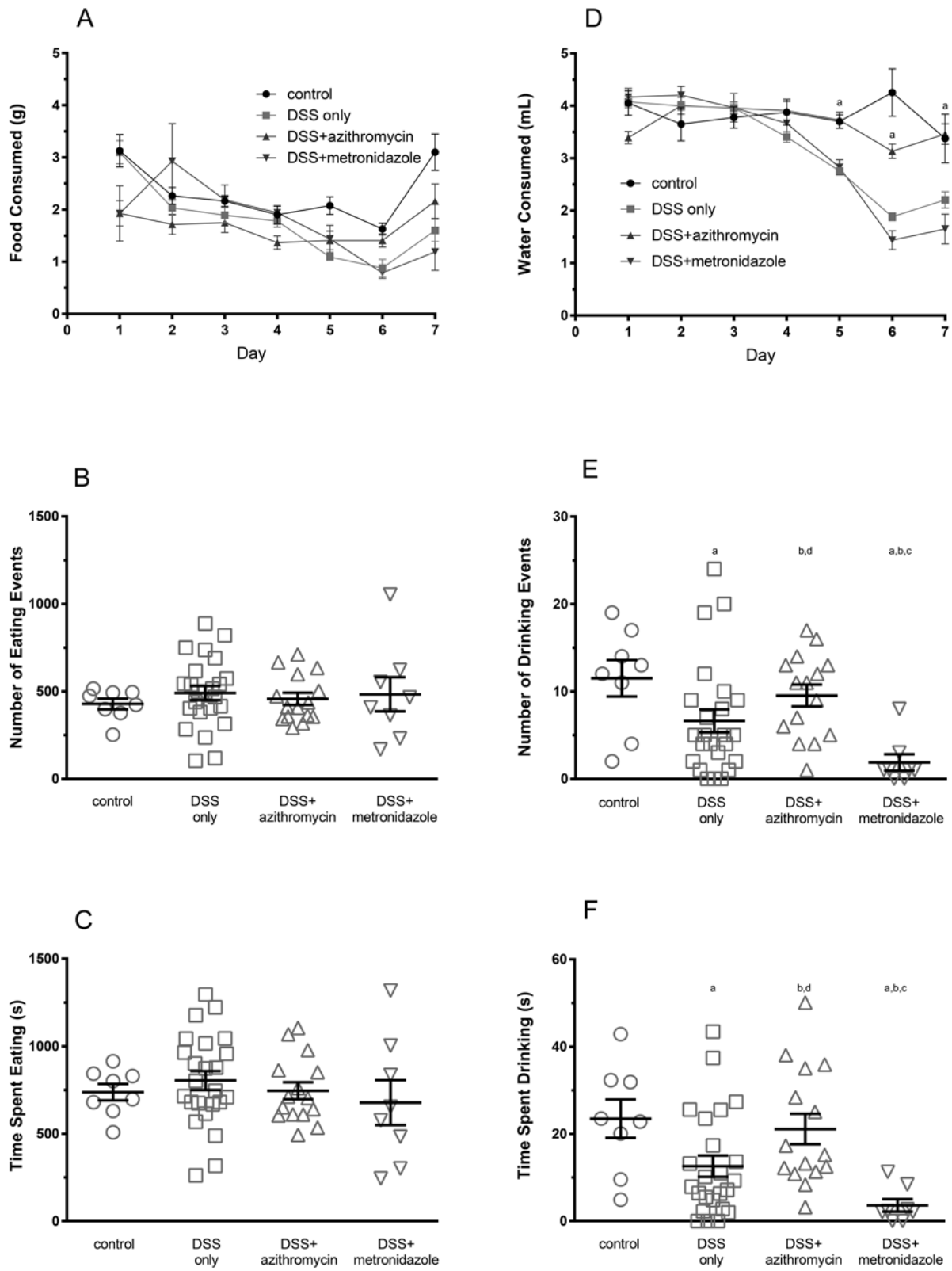

Figure 4. Daily food and water consumption and consumptive behaviors of control (n = 8), DSS only (n = 24), DSS+azithromycin (n = 15), and DSS+metronidazole (n = 8) groups. All data are expressed as mean ± SEM. (A) Daily food consumption. (B) Number of eating events. (C) Time spent eating. (D) Daily water consumption. [a], control and DSS+azithromycin mice drank significantly ($P < 0.05$) more water on days 5, 6, and 7 than DSS only and DSS+metronidazole animals. (E) Number of drinking events. [a], Significantly ($P < 0.05$) different from control; [b], significantly ($P < 0.05$) different from DSS only; [c], significantly ($P < 0.05$) different from DSS+azithromycin; [d], significantly ($P < 0.05$) different from DSS+metronidazole. (F) Time spent drinking. [a], Significantly ($P < 0.05$) different from control; [b], significantly ($P < 0.05$) different from DSS only; [c], significantly ($P < 0.05$) different from DSS+azithromycin; [d], significantly ($P < 0.05$) different from DSS+metronidazole.

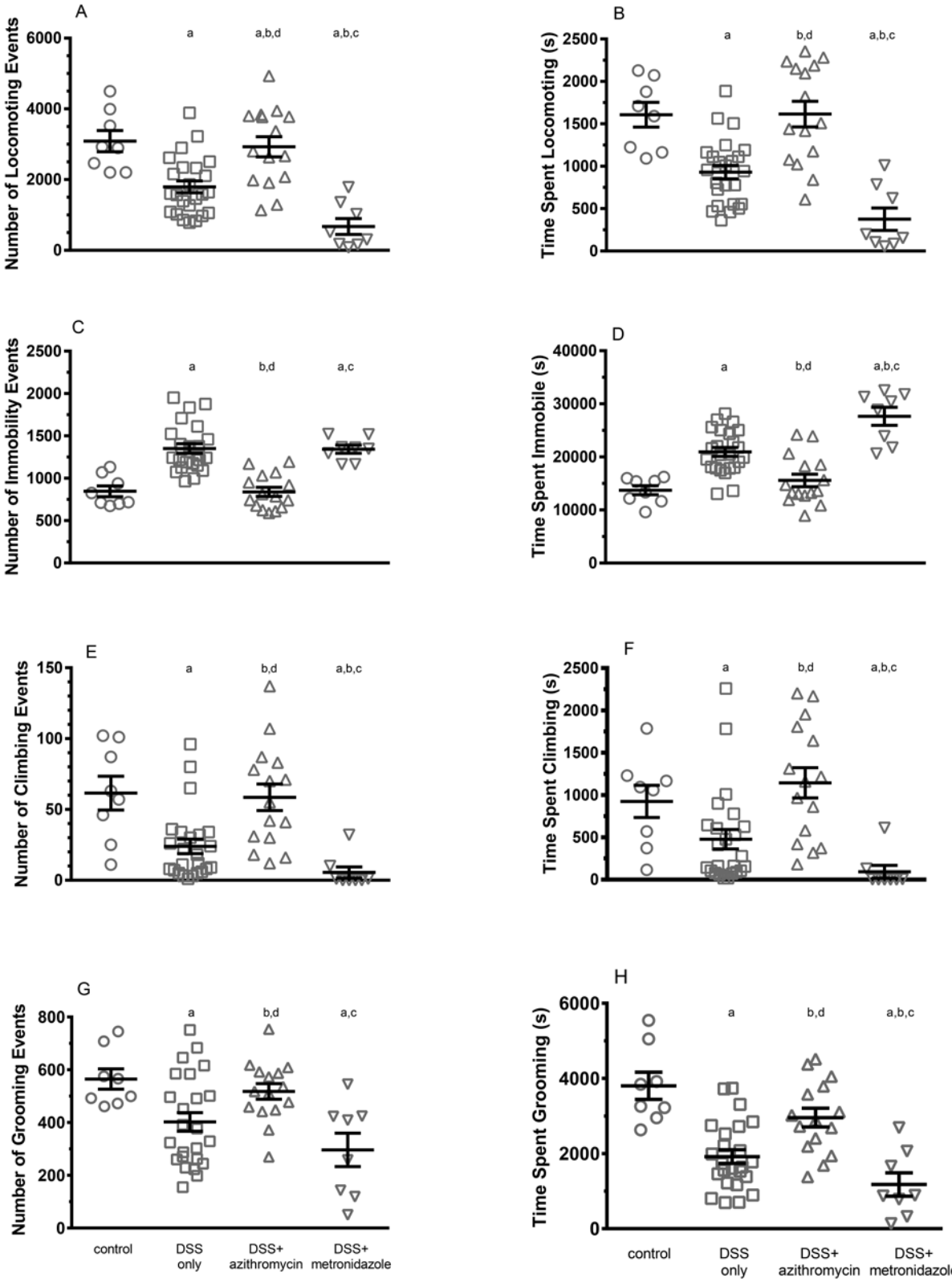

Figure 5. Number of behavior events and time spent performing the behavior over a continuous 10-h period on day 7 for control ($n = 8$); DSS only ($n = 24$); DSS+azithromycin ($n = 15$), and DSS+metronidazole ($n = 8$) groups. All data are expressed as mean ± SEM. (A) Number of locomotion events. (B) Time spent in locomotion. (C) Number of immobility events. (D) Time spent immobile. (E) Number of climbing events. (F) Time spent climbing. (G) Number of grooming events. (H) Time spent grooming. [a], Significantly ($P < 0.05$) different from control; [b], significantly ($P < 0.05$) different from DSS only; [c], significantly ($P < 0.05$) different from DSS+azithromycin; [d], significantly ($P < 0.05$) different from DSS+metronidazole.

Behavioral assessments. All groups receiving DSS showed reductions in locomotion compared with control animals. However, among the DSS-exposed groups, azithromycin-treated mice were significantly ($P < 0.05$) more mobile than mice treated with metronidazole or vehicle alone (Figure 5 A and B). Similarly, the animals given DSS only or with metronidazole groups

Table 1. Amounts (pg/mL; mean + SEM) of cytokines and chemokines extracted from mouse colon tissue

	Control	DSS only	DSS+azithromycin	DSS+metronidazole
TNFα	4.1 ± 2.4	22.5 ± 4.8[a]	11.8 ± 1.9	14.3 ± 2.1[a]
IL1α	40.4 ± 2.3	233.7 ± 33.3[a]	151.4 ± 31.0[a]	162.8 ± 24.4[a]
KC	58.2 ± 7.5	1057.0 ± 117.5[a]	676.3 ± 111.7[a,b]	716.4 ± 66.2[a,b]
MIP2	79.3 ± 10.6	1370.7 ± 269.7[a]	390.0 ± 69.6[a,b,d]	1035.6 ± 199.8[a,c]
GCSF	28.8 ± 5.6	1346.2 ± 330.7[a]	487.9 ± 159.2[a,b,d]	1150.3 ± 118.2[a,c]
IL17	2.9 ± 0.5	58.5 ± 9.5[a]	38.0 ± 10.4[a]	43.4 ± 8.8[a]
IL6	46.1 ± 11.7	1475.0 ± 261.2[a]	1139.0 ± 243.7[a]	782.0 ± 57.7[a,b]
MCP1	81.9 ± 20.6	1097.1 ± 143.5[a]	689.1 ± 97.9[a,b]	660.1 ± 56.7[a,b]
IP10	34.9 ± 2.3	266.8 ± 44.4[a]	134.9 ± 18.2[a,b,d]	290.1 ± 57.7[a,c]
LIF	7.2 ± 0.3	70.4 ± 6.1[a]	57.0 ± 7.4[a]	60.7 ± 7.6[a]
Eotaxin	341.3 ± 26.3	887.5 ± 50.9[a]	697.4 ± 57.9[a,b]	794.1 ± 29.5[a]

Cytokines and chemokines that did not differ significantly between groups are not shown.
[a]Significantly ($P < 0.05$) different from control group.
[b]Significantly ($P < 0.05$) different from DSS only group.
[c]Significantly ($P < 0.05$) different from DSS+azithromycin group.
[d]Significantly ($P < 0.05$) different from DSS+metronidazole group.

were stationary significantly ($P < 0.05$) more often than mice that received DSS and azithromycin (Figure 5 C and D). No significant difference was found in immobility behavior between the DSS-treated mice given azithromycin and the control group; in addition, the DSS+azithromycin group climbed and groomed more frequently (Figure 5 E and G, respectively) and for longer periods of time (Figure 5 F and H, respectively) than the other DSS-treated groups, in amounts similar to the controls. Interestingly, DSS+metronidazole animals were less mobile (Figure 5 A and B), climbed less (Figure 5 E and F), spent more time immobile (Figure 5 D), and spent less time grooming (Figure 5 H) than animals given DSS only ($P < 0.05$ for all comparisons).

Colonic barrier function. DSS exposure resulted in a 3-fold increase ($P = 9.1 \times 10^{-5}$) in the leakage of FITC-labeled dextran from the gut lumen to serum, suggesting reduced barrier function in DSS-exposed animals. No difference was found between mice given DSS only or with azithromycin. However, leakage was increased ($P = 0.03$) 3-fold in the DSS+metronidazole group in comparison to the DSS only group.

Circulating granulocyte counts. Compared with controls, DSS exposure increased the number of circulating granulocytes in whole blood by 3-fold (data not shown). All DSS-exposed groups had similar numbers of circulating granulocytes (data not shown).

Colonic cytokines and chemokines. Compared with controls, mice exposed to DSS had significantly ($P < 0.05$) increased levels of colonic proinflammatory cytokines and chemokines, including TNFα, IL1α, GCSF, IL17, KC/CXCL1, MIP2/CXCL2, MCP1/CCL2, and IP10/CXCL10 (Table 1). All DSS-exposed groups had similar amounts of IL17 and of IL1α. However, azithromycin treatment significantly ($P < 0.05$) attenuated the increase in colonic TNFα to levels that did not differ from controls. In addition, the neutrophil chemoattractants KC and MIP2 were significantly ($P < 0.05$) decreased in the azithromycin-treated group compared with mice given DSS only, and levels of GCSF were significantly ($P < 0.05$) lower in the DSS+azithromycin group than in all other DSS-exposed animals. Both antibiotics significantly ($P < 0.05$) attenuated the increase in MCP1. Metronidazole treatment resulted in significantly ($P < 0.05$) reduced colonic IL6 and KC concentrations compared with treatment with DSS only.

Neutrophil adhesion and emigration. Neither neutrophil adhesion nor emigration differed between any DSS-naïve groups (Figure 6 A and B). Exposure to DSS significantly ($P < 0.05$) increased neutrophil adhesion and emigration within colon tissue. Treatment with either azithromycin or metronidazole significantly ($P < 0.05$) attenuated neutrophil adhesion and emigration in DSS-exposed animals. Colonic neutrophil emigration was significantly ($P < 0.05$) lower in the DSS+azithromycin group than the DSS+metronidazole group (Figure 6 B); neutrophil adhesion did not differ between these groups (Figure 6 A). Representative photomicrographs of fluorescence-stained cells and colonic vessels in the control, DSS only, DSS+azithromycin, and DSS+metronidazole groups are shown in Figure 7 A through D.

Discussion

The murine DSS model of colitis is widely used to study IBD, especially ulcerative colitis.[39] In our current study, DSS exposure led to significant body weight loss and reduced water consumption. Treatment with azithromycin improved the clinical health of the animals, as indicated by reduced weight loss and improved animal behavior (Figures 1 and 5). Importantly, the clinical picture of the DSS+azithromycin group was substantially and significantly more positive than the other DSS-exposed groups, even though the azithromycin-treated animals consumed more DSS (Figure 4). This finding highlights an obvious methodologic issue that can be overlooked when using this model, given that the severity of colitis is intuitively dependent on the amount of DSS consumed. However, the severity of challenge according to DSS-treated water intake is not uniformly accepted in the literature.[16] Note that our model, as part of the standard protocol, included antibiotic treatment beginning on day 0; thus, colitis was not fully established in any of the animals. Recognizing these limitations is important in any DSS model study.

Although the majority of studies using animal models of experimental colitis measure body weight loss as an indicator of clinical health, clinical assessments of behaviors, including grooming and activity level, are less commonly incorporated. Because one of the main goals of IBD treatment is to improve a patient's quality of life, evaluating animal behavior during preclinical research seems crucial. Unlike physical parameters such as weight loss and pathologic changes, animal behavior provides an indication of the severity of illness as experienced by the animal. Behavioral assessments can be sensitive indicators of

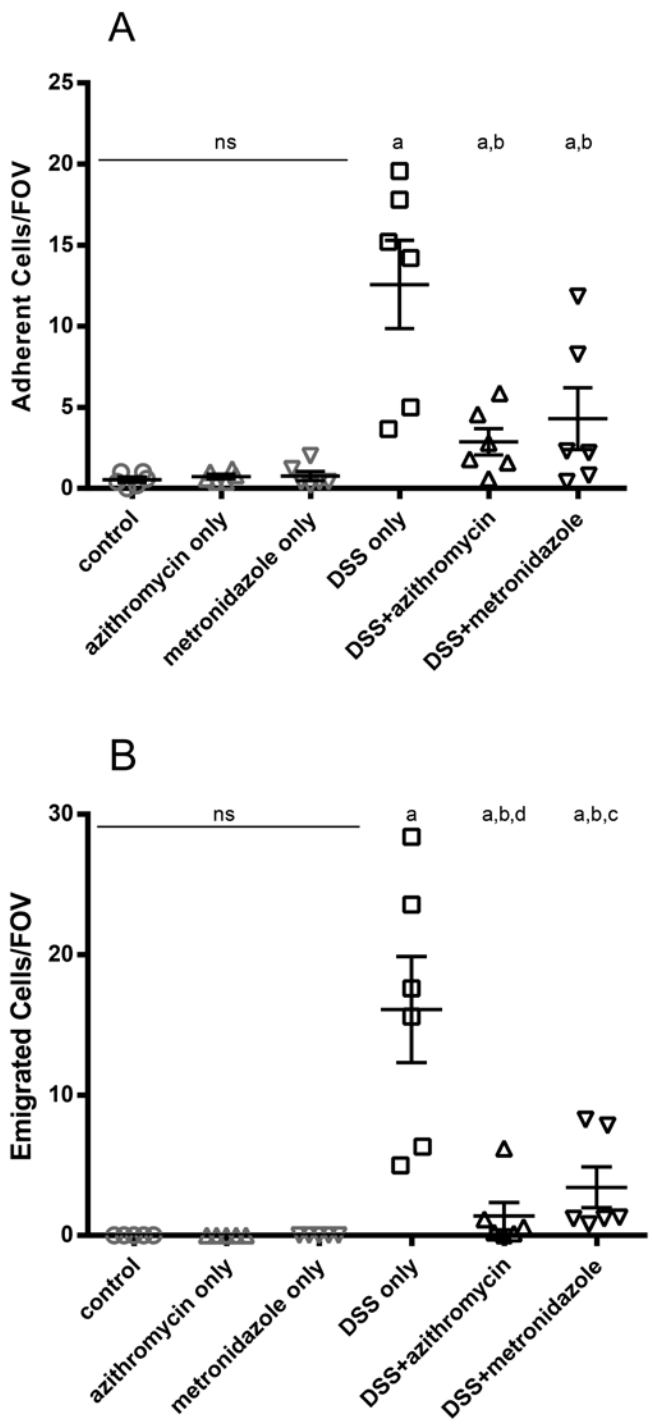

Figure 6. Neutrophil adhesion to venule walls and emigration from venules within colonic tissue from control ($n = 6$), azithromycin only ($n = 6$), metronidazole only ($n = 6$), DSS only ($n = 6$), DSS+azithromycin ($n = 6$), and DSS+metronidazole ($n = 6$) groups. Data are expressed as mean ± SEM. (A) Neutrophil adhesion to blood vessel walls. (B) Neutrophil emigration from blood vessels. FOV, field of view; [a], significantly ($P < 0.05$) different from control; [b], significantly ($P < 0.05$) different from DSS only; [c], significantly ($P < 0.05$) different from DSS+azithromycin; [d], significantly ($P < 0.05$) different from DSS+metronidazole.

therapeutic response and are reasonable parameters to measure in animal studies of experimental colitis. As our study demonstrates, behavioral assessments can be performed easily and are time-efficient, depending on the methodology used. One limitation of our study is that the mice could not be group-housed during the behavioral assessment, given that the technology relied on cage vibration to determine animal activity. Because mice are inherently social animals, single-housing may have affected these outcomes.[25,29] Video recording is an alternative, but video interpretation requires a much greater time commitment on the part of the researcher and can pose challenges when views are obscured by enrichment items or nesting material.[42] These limitations highlight the need for digital caging systems with the capacity to monitor and consolidate individual animal activity within the home cage of group-housed animals.

In our study, DSS exposure markedly reduced animal movement, climbing, and grooming behaviors. Similarly, immobility was significantly increased in DSS-exposed mice and served as a legitimate parameter to monitor. Azithromycin treatment improved animal behavior to levels similar to control values, suggesting that these animals experienced a less severe disease course than other DSS-treated mice (Figure 5). Conversely, treatment with metronidazole appeared to exacerbate the experience of DSS-induced illness, given that metronidazole-treated mice spent significantly less time performing normal behaviors and more time in a state of immobility than other DSS-treated mice.

Antibiotics have been used to treat both Crohn disease and ulcerative colitis with variable effects.[13] A meta-analysis suggests there are benefits from including antibiotics in IBD treatment regimes.[26] Epidemiologic research suggest cautions, however, because some studies have revealed early-life exposure to antibiotics as a risk factor for later development of IBD.[5] A recent review suggests that caution should be exercised when attempting to translate the effects of antimicrobial therapy across species,[19] given that we do not fully understand the effects of antibiotic treatment on microbiota and dysbiosis. Other authors described marked reduction in colonic myeloperoxidase activity in association with metronidazole in the mouse DSS model, suggesting reduced granulocyte infiltration[24] and immunomodulation. In the current study, we incorporated antibiotic treatments in light of the antibacterial and potential immunomodulatory effects of both azithromycin and metronidazole. Treatment with either antibiotic resulted in altered immune signaling and neutrophil migration within DSS-exposed groups (Table 1 and Figure 6). However, only azithromycin treatment resulted in clinical improvement and decreased pathology on necropsy examination (Figures 1, 2, and 5).

Mucosal barrier function, as assessed through FITC-dextran absorption, was significantly decreased in DSS-exposed mice. Barrier function was not improved with azithromycin treatment but was significantly impaired with metronidazole treatment. We do not fully understand the mechanisms of this deterioration of barrier function. It could be related to the reduction in anaerobic bacteria, which are the primary targets of this antibiotic.[10] These results suggest that the primary clinical benefits of these antibiotics are not mediated through actions on mucosal integrity. Our preclinical study suggests that when antibiotic treatment is required, azithromycin may be a better therapeutic choice than metronidazole, given that the negative effects on mucosal barrier function appeared much less prominent with azithromycin than with metronidazole. Careful clinical evaluation in human patients would be necessary to establish a similar recommendation.

When we examined the cytokine profile in the colon of DSS-exposed mice, we found significant elevations in proinflammatory cytokines and chemokines, as expected with an acute inflammatory insult to the colon. Azithromycin treatment markedly reduced levels of GCSF and the neutrophil chemoattractants KC and MIP2 within colonic tissue (Table

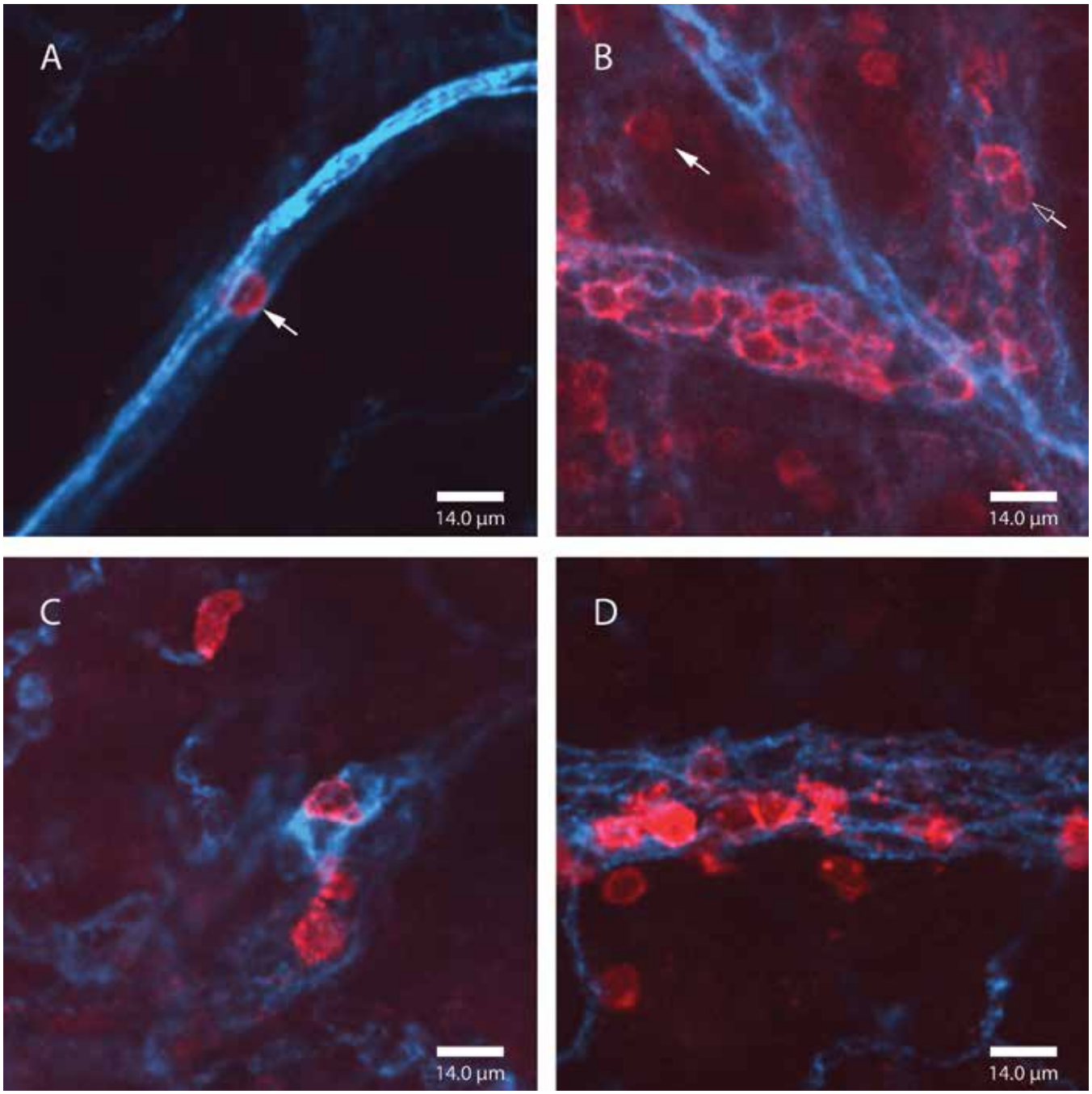

Figure 7. Photomicrographs of representative stained cells and vessels within colonic tissue from (A) control, (B) DSS only, (C) DSS+azithromycin, and (D) DSS+metronidazole mice. Neutrophils were visualized by using antiLy6G$^+$ (red), and blood vessels were labeled with antiPECAM1 (blue). Closed arrows, circulating (A) and emigrated (B) neutrophils; open arrow, adhered neutrophil (B). Bar, 14 µm.

1). These findings are consistent with the improved clinical health observed in this study, suggesting that clinical benefits may be mediated through alterations of acute inflammatory signals. Metronidazole treatment led to KC reduction but also reduced IL6. Because IL6 has both proinflammatory and antiinflammatory effects, decreased levels of this cytokine may be responsible for the exacerbation of behavioral abnormalities seen in the DSS+metronidazole group.[44] IL6 depletion in DSS+metronidazole-treated animals may contribute to the less pronounced resolution of inflammation in this group; however, this conclusion requires more detailed examination.

Of particular interest to us are the mechanisms of acute inflammation and the potential immunomodulatory effects of macrolide and azalide antibiotics in inflammation. The reductions in TNFα, KC, MIP2, and GCSF that occurred in the DSS+azithromycin group can be expected to modify the inflammatory response through neutrophil recruitment. Indeed, we observed this effect when we investigated neutrophil trafficking within colon blood vessels (Figures 6 and 7). Treatment with either antibiotic significantly decreased neutrophil adhesion and emigration (extravasation) without affecting total circulating granulocyte counts. Given that both drugs have antibacterial effects, they likely altered the gut microbiotas of these groups. We certainly observed marked alterations in cecum weight and size—parameters often linked to microbiologic alterations of the mouse gut (Figure 2).[1,34,43] Antibiotic-induced alterations in

microbial populations may have resulted in the immune signaling changes that we observed in our study. Direct effects of antibiotics on host cell metabolism have recently been proposed as mechanisms of antibiotic-associated immune modulation.[47] Such metabolite-related changes can be host-mediated or microbiota-related, although studies on peritoneal infection with *Pseudomonas aeruginosa* suggest that metabolite inhibition is host-mediated and independent of the gut microbiota.[47] Although both antibiotics significantly decreased neutrophil trafficking in our study, azithromycin treatment resulted in significantly fewer extravasated neutrophils than metronidazole, suggesting a role for both host-mediated and microbiota-related effects. We have previously demonstrated the immunomodulatory effects of macrolide and azalide antibiotics by preprogramming neutrophils to apoptotic cell death, as compared with the less controlled process of necrosis.[15,20,21,28] Future studies need to evaluate neutrophil function as well as alterations to the microbiota in DSS models using these antibiotics.

Overall, our study demonstrated that different classes of antibiotics can have markedly different effects on colitis severity. We showed that the azalide azithromycin is more clinically protective against DSS-induced murine colitis than metronidazole, even though both antibiotics resulted in immunologic changes. In addition, our study demonstrated the utility of behavioral assessments and clinical assessments as tools to better understand the pathophysiology of colitis and to evaluate therapeutic efficacy. Finally, our study highlights the differences in immune response between treatment with azithromycin and metronidazole within this experimental model of colitis. Future research should focus on elucidating the precise mechanism by which this difference occurs, because whether these immunologic changes are host-mediated or microbiota-related (or both) is unknown.

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