Supplementary Methods

Repeat restraint stress (RS)

Experiments were performed on adult male Wistar rats (200–225 g), six per group, as described previously.[1, 2] The rats were housed in plastic cages, three per cage, and maintained on a 12h light, 12 hr dark cycle. Each rat was submitted to a 120-min restraint period in plastic cylinder (diameter 6.5 cm and length 15 cm). This restraint procedure minimized the space around the animal, prevented turning, and provided a rather strong stressful stimulus without causing lasting harm. After restraint, the rat was returned to its cage. The rats in the control group remained in their cages, undisturbed, throughout the experiment.

Microbial DNA isolation

The microbial genomic DNA was isolated from the contents of the distal 8 cm of each rat ileum. During necropsy, the ileal contents, weighing an average 0.5 g, were harvested immediately, frozen in liquid nitrogen and stored at -80°C until processed. Genomic DNA was extracted by using a modified protocol of the Qiagen DNeasy Blood & Tissue kit. These modifications included (i) a bead-beating step using UltraClean fecal DNA bead tubes (Mo Bio Laboratories, Solana Beech, CA) that were shaken for 1.5 min using a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK), (ii) an increased amount of buffer ATL in the initial steps of the protocol (from 180 μL to 360 μL), (iii) an increase in the volume of proteinase K (from 20 μL to 40 μL), and (iv) a decreased in the amount of buffer AE to elute the DNA at the end of the protocol (from 200 μL to 85 μL).

Quantitative PCR of 16S rRNA Gene

Quantitative PCR (qPCR) was used to quantify the total number of 16S rRNA gene copies/gram in our samples. Reactions were performed on a Lightcycler 480 (Roche) using the following protocol: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, and hold at 37°C. To detect bacterial signal, 2 pmol of each of the forward and reverse primers and 1 pmol of the fluorogenic probe were included in the reaction mixtures. Primer and probe sequences were as follows: forward primer (5′-TCCTACGGGAGGCAGCAGT-3′), reverse primer (5′-GGACTACCAGGGTATCTAATCTT-3′), and 16S probe (5′-[6-carboxyfluorescein]-CGTATTACCGCGGCTGCTGGCAC-[6-carboxytetramethylrhodamine]-3′). A standard curve was constructed using 10 2-fold dilutions, run in triplicate, of *Helicobacter pylori* DNA (a bacterium known to have two copies of the 16S rRNA gene in its genome), starting at 1000 ng.

Operational taxonomic unit, phylotype assignment, and diversity measurements

The open-source, platform-independent, community-supported software program, mothur (http://www.mothur.org) was used to bin 16S rRNA gene sequences into operational taxonomic units (OTUs) and phylotypes following the Schloss standard operating procedure (SOP) (http://www.mothur.org/wiki/Schloss_SOP) referenced in mothur.[3] OTUs were assigned at a cutoff of 0.03 and were classified using a Bayesian approach with an 80% confidence threshold using RDP training set version 7. Classified sequences were then binned into phylotypes that had the same taxonomy. Phylotypes were assigned at the level of phylum and family. During curation of the 454 data any rare phylotypes

that did not comprise at least 1% of the total number of sequences were removed before any analyses. Within-community diversity (α-diversity) was calculated using the Shannon diversity index (H′) applied to the normalized phylotype data. Between-community diversity (β-diversity) was determined using the Yue and Clayton (θ_{YC}) distance metric on the normalized phylotype abundance data. Nonmetric multidimensional scaling (NMDS), in 3 dimensions, was used to ordinate the β-diversity data. An analysis of molecular variation (AMOVA) was used to test for significant differences among the centroids of the different treatment groups and controls on the NMDS plot. Phylotype data was converted to relative abundance ± standard error of the mean (mean ± SEM). Statistical analyses (ANOVA with Tukey post hoc test and Kruskal–Wallis with Conover–Inman post hoc test) were performed using Systat 13; AMOVA was performed using mothur; a statistic was considered significant at $P < .05$.

Quantitative or semiquantitative RT-PCR for inflammatory cytokines and tight junction protein

Total RNA was extracted from tissue samples of the distal 8 cm of ileum using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Promega, Madison, WI). Quantitative PCR on inflammatory cytokines and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with an iCycler IQ real-time detection system (Bio-Rad Laboratories, Hercules, CA) using SYBR Green detection. Primer sequences used for PCR were as follows: forward tumor necrosis factor-α (TNF-α), 5′-AGATGTGGAACTGGCAGAGG-3′; reverse TNF-α, 5′-

CCCATTTGGGAACTTCTCCT-3′; forward interleukin (IL)-1β, 5′-CTGTGACTCGTGGGATGATG-3′; reverse IL-1β, 5′-GGGATTTTGTCGTTGCTTGT-3′; forward IL-17, 5′-ACAGTGAAGGCAGCGGTACT-3′; reverse IL-17, 5′-GCTCAGAGTCCAGGGTGAAG-3′; forward IL-6, 5′-CCGGAGAGGAGACTTCACAG-3′; reverse IL-6, 5′-ACAGTGCATCATCGCTGTTC-3′; forward IL-10, 5′-CCTGCTCTTACTGGCTGGAG-3′; reverse IL-10, 5′-TGTCCAGCTGGTCCTTCTTT-3′; forward interferon-γ (INF-γ), 5′-CTTCTTCAGCAACAGCAAGGCGAAAA-3′; reverse INF-γ, 5′-CCCCCAGATACAACCCCGCAATCA-3′; forward GAPDH 5′-TGGCCATACTTCTCCTCACC-3′; reverse GAPDH, 5′-TCTGTTCCGTCTGGGTTTTC-3′. The PCR conditions were as follows: one cycle of 94°C for 10 min, followed by 35 two-temperature cycles of 94°C for 1 min and 55°C for 1 min. PCR amplifications were performed in a total volume of 25 μL, containing 10× PCR buffer with $MgCl_2$, 10 nmol/L dNTPs, 200 nmol/L primers, 5 μL cDNA, 100 nmol/L Taq polymerase GOLD, and 2.5 μL SYBR Green (Invitrogen, Carlsbad, CA). Cytokine transcript levels were normalized with that of GADPH, and relative gene expression was expressed as the fold change ($2^{-\Delta\Delta Ct}$) relative to expression in the control samples.

Semiquantitative PCR on tight junction protein occludin (NM_031329), and GADPH was performed. Primer sequences used for PCR were as follows: forward occludin, 5′- GTGTACATAATGGGAGTCAAC-3′; reverse occludin, 5′-GGTGGAGGCATGTCTTGTGTG-3′. PCR was performed with Taq DNA polymerase (Promega, Madison, WI) using the following conditions: 30 cycles of denaturation (30 s

at 94°C), annealing (30 s at 50°C), and extension (30 s at 72°C), followed by final extension (10 min at 72°C). The PCR products were loaded in a 1.2% Tris-borate-EDTA–buffered agarose gel, and the resulting bands were visualized after gel electrophoresis by ethidium bromide staining and ultraviolet light illumination. The bands were scanned and analyzed using ImageJ software (National Institutes of Health (NIH), Bethesda, MD).

Western blot analysis

The distal ileal tissues were homogenized in RIPA buffer (1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS in Tris-buffered saline solution 1×; pH 7.4), supplemented with 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 1× phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 14,000 *g* for 10 min. Protein samples were run on Ready Gel 10% Tris-HCl (Bio-Rad, Hercules, CA) for 1.5 h at 80 V. Proteins were transferred to polyvinylidene difluoride membranes for 1 h at 80 V. The membranes were blocked with StartBlock buffer T20 (Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature, probed with rabbit anti-occludin antibody (Zymed Laboratories, San Francisco, CA) at 1:500 dilution at 4°C overnight, and then washed in Tris-buffered saline for 1 h. The membranes were then probed with peroxidase–conjugated goat anti-rabbit IgG at 1:1000 dilution for 1 h at room temperature, and the bands were visualized by electrochemiluminescence (PerkinElmer, Waltham, MA). Signals were quantified using ImageJ (NIH, Bethesda, MD) and normalized to controls.

Histology and immunohistochemistry

Segments of the distal 8 cm of the ileum were fixed in 10% formalin. For morphologic analysis, dissected ileal segments were embedded in paraffin, sectioned at 4-μm thickness, and subsequently stained with H&E. Neutrophils, mononuclear cells, and eosinophils were counted at a 400× magnification in 12 different areas above the muscularis mucosae of each section using a micrometer grid and expressed as the number of cells/mm^2, as described previously.[4] For immunohistochemical staining, dissected segments were snap frozen and cut with a cryostat (10 μm: Leica CM1800, Leica Microsystems, Wetzlar, Germany) and permeabilized in PBS with 0.3% Triton X-100 for 10 min. After blocking with 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS with 0.3% Triton X-100, sections were incubated with polyclonal rabbit anti-human lysozyme (1:500; Dako, Carpinteria, CA). Cy3-conjugated donkey anti-rabbit antibody (1:500; Jackson ImmunoResearch, West Grove, PA) were used as a secondary antibody to detect rabbit anti-lysozyme. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and visualized with an Olympus BX51 fluorescence microscope (Olympus, Melville, NY).

Visceromotor response to colorectal distention

Measurement of visceral sensitivity in animals is mainly based on brainstem reflexes, which have been described as “pseudoaffective” responses. Details of the protocol for measuring visceromotor response (VMR) to colorectal distention (CRD) has been previously described.[5,6] Measurement of VMR to visceral stimulus was conducted in rats on days 0 and day 11 of the chronic WAS procedure or after sham WAS treatment. A 32-

gauge stainless steel wire was implanted in the external oblique pelvic muscle 4–6 days before beginning the experimental procedure. Animals were habituated in the testing room and placed in a Plexiglas cylinder for 30 min before the CRD procedure was initiated. A series of CRDs were conducted at constant pressures of 20, 40, and 60 mm Hg. Each distention consisted of 3 segments: a 20-s predistention baseline period, a 20-s distention period, and a 20-s period after termination of CRD with a 4-min interstimulus period. Electromyographic (EMG) activity was amplified and digitized using a SPIKE2/CED 1401 data acquisition interface (Cambridge Electronic Design, Cambridge, England). The responses were considered stable if there was less than 20% variability between 2 consecutive trials of each CRD. EMG activity was rectified, and the increase in the area under the curve of EMG amplitude during CRD over the baseline period before CRD was recorded as the response.

References

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Supplementary Figure 1. Effect of chronic treatment with rifaximin (150 mg/kg, twice daily, oral gavage) on bacterial community composition in the ileal contents (replicate experiment). (*A*) Relative abundance of selected phylotypes, identified at the level of family. (*B*) NMDS plots using a θ_{YC} distance matrix of the OTU-based data. n = 3 in each group (replicate experiment). For all studies, N.D. = not detected. *significantly different than sham WA; #significantly different than WA (Kruskal–Wallis with Conover–Inman post hoc test for multiple comparisons, $P < .05$).

Supplementary Table 1

Chronic WAS-induced inflammatory cell infiltration in ileal mucosa

	Neutrophils (cells/mm^2)	Mononuclear cells (cells/mm^2)	Eosinophils (cells/mm^2)
Sham WAS	37 ± 8	27 ± 8	35 ± 6
WAS	85 ± 12^a	62 ± 12^a	45 ± 9

NOTE. Rats were subjected to sham WAS or WAS 1 h each day for 10 consecutive days. Cell populations were counted at 400× magnification in 12 different areas above the muscularis mucosae of each section, using a micrometer grid. Values are means ± SE, n = 8 per group. $^aP < .05$ compared with sham WAS controls (Student *t* test).

Supplementary Table 2

Effect of chronic treatment with rifaximin on WAS-induced inflammatory cell infiltration in ileal mucosa

	Neutrophils (cells/mm^2)	Mononuclear cells (cells/mm^2)	Eosinophils (cells/mm^2)
Sham WAS + water	33 ± 6	23 ± 6	29 ± 4
WAS + water	69 ± 8[a]	60 ± 8[a]	34 ± 4
WAS + rifaximin	34 ± 8[b]	18 ± 4[b]	25 ± 7

Note: Rats were gavaged with rifaximin (150 mg/kg) or water twice daily, 6 h apart (AM and PM administrations), for 10 consecutive days. The rats were also submitted to a daily session of WAS or sham WAS 3 h after each AM administration of rifaximin. Cell populations were counted at 400× magnification in 12 different areas above the muscularis mucosae of each section, using a micrometer grid. Values are means ± SE, n = 8 per group (1-way ANOVA).

[a]$P < .05$ compared with sham WAS + water.

[b]$P < .05$ compared with WAS + water.

Supplementary Table 3. Effect of chronic treatment with neomycin on WAS-induced inflammatory cell infiltration in ileal mucosa

	Neutrophils (cells/mm^2)	Mononuclear cells (cells/mm^2)	Eosinophils (cells/mm^2)
Sham WAS + water	37 ± 7	27 ± 10	31 ± 7
WAS + water	77 ± 6[a]	64 ± 11[a]	38 ± 8
WAS + Neomycin	73 ± 10[a]	68 ± 13[a]	32 ± 4

Note: Rats were gavaged with neomycin (150 mg/kg) or water twice daily, 6 h apart (AM and PM administrations), for 10 consecutive days. The rats were also submitted to a daily session of WAS or sham WAS 3 h after each AM administration of neomycin. Cell populations were counted at 400× magnification in 12 different areas above the muscularis mucosae of each section, using a micrometer grid. Values are means ± SE, n = 6 per group (1-way ANOVA).

[a]$P < .05$ compared with sham WAS + water.

Supplemental Figure 1