Supplementary Materials and Methods

Animals

Mice were maintained in specific-pathogen–free facilities at Massachusetts General Hospital. Card9-null mice on the C57/BL6j background (back-crossed 10 times) were provided by Takashi Saito (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and have been described previously.[1] All mice were maintained on food and water ad libitum. Littermate and sex-matched mice were used at 7–9 weeks of age and co-housed for all experiments.

Histology

Colon tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections (5-μm) were stained with H&E. Tissues were scored blindly using established methods for both DSS and *C rodentium* colitis.[2,3] The results presented show the histologic evaluation of the mid-part of the colon.

qRT-PCR

Proximal, medial, and distal colon tissues were harvested at the end of each course of colitis and stored in RNA*later* (Ambion, Austin, TX). RNA was extracted from homogenized tissues using the RNeasy Kit (Qiagen, Valencia, CA). RNA samples were reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). By using the iQ SYBR Green Supermix (Bio-Rad) for quantitative PCR, messenger RNA levels were determined using the iCycler with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Reaction conditions consisted of 40 cycles of PCR with 58°C or 59°C annealing temperature. The following primers were used: TNF-α: forward: GACGTGGAACTGGCAGAAGAG; reverse: TTGGTGGTTTGTGAGTGTGAG; IFNγ: forward: ATGAACGCTACACACTGCATC; reverse: CCATCCTTTT GCCAGTTCCTC; IL-6: forward: GTAGCTATGGTACT CCAGAAGAC; reverse: ACGATGATGCACTTGCAGAA; IL-10: forward: AGAAGCATGGCCCAGAAATCA; reverse: GGCCTTGTAGACACCTTGGT; IL-17A: forward: TTTAA CTCCCTTGGCGCAAAA; reverse: CTTTCCCTCCGCAT TGACAC; IL-17F: forward: TGCTACTGTTGATGTTG GGAC; reverse: AATGCCCTGGTTTTGGTTGAA; IL-21: forward: CGCCTCCTGATTAGACTTCG; reverse: CAG GGTTTGATGGCTTGAGT; IL-22: forward: CATGCAG GAGGTGGTACCTT; reverse: CAGACGCAAGCATTTCT CAG; IL-23: forward: AGCGGGACATATGAATCTACTA AGAGA; reverse: GTCCTAGTAGGGAGGTGTGAAGTTG; IL-23R: forward: AGCAAAATCATCCCACGAAC; reverse: GCCACTTTGGGATCATCAGT CCL20; forward: GCCTC TCGTACATACAGACGC; reverse: CCAGTTCTGCTTTG GATCAGC; β-defensin 1: forward: AGGTGTTGGC ATTCTCACAAG; reverse: GCTTATCTGGTTTACAGGT TCCC MCP1; forward: CTGGATCGGAACCAAATGAG; reverse: AAGGCATCACAGTCCGAGTC; RegIIIγ: forward: TCCTGTCCTCCATGATCAAAA; reverse: CATCCACCTC TGTTGGGTTCA.

The threshold cycle for each sample was determined for each gene and normalized to the threshold cycle value of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Data were calculated using a comparative threshold cycle method.

Lymphocyte Isolation and Measurement of Cytokine Production

Lymphocyte suspensions were prepared from the MLN and spleen by pressing cells through a 70-μmol/L Falcon nylon cell strainer (BD Biosciences, San Jose, CA) in complete Dulbecco's modified Eagle medium (10% fetal calf serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U penicillin/mL, 100 μg streptomycin/mL, 50 μmol/L 2-mercaptoethanol, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate; Invitrogen Life Technologies, Carlsbad, CA). Cells (5×10^6 cells/mL) were cultured in 24-well plates in the presence or absence of plate-bound anti-CD3 monoclonal antibody (10 μg/mL) or *C rodentium* Antigen (50 μg/mL), and culture supernatants were collected 72 hours later and stored at -20°C. IFNγ, IL-17A, IL-6, and TNF-α cytokines were assayed with an enzyme-linked immunosorbent assay using the following reagents: purified anti-mouse IL-17A (eBioscience, San Diego, CA), biotin anti-mouse IL-17A (eBioscience), purified anti-mouse IFNγ (BD Biosciences, San Diego, CA), biotin anti-mouse IFNγ (BD Biosciences), purified anti-mouse IL-6 (BD Biosciences), biotin anti-mouse IL-6 (BD Biosciences), purified anti-mouse TNF-α (BD Biosciences), biotin anti-mouse TNF-α (BD Biosciences), streptavidin-HRP (BD Biosciences), and OptEIA TMB Substrate (BD Biosciences).

Femtosecond Laser Injury to the Colon Epithelium

We used a custom-built, 2-photon endomicroscopy system with a side-view probe.[4] After anesthetizing a mouse with an intraperitoneal injection of ketamine/xylazine solution (75 mg/kg ketamine and 15 mg/kg xylazine), we intravenously administered 2M-Da fluorescein isothiocyanate dextran (Sigma-Aldrich; 100 μL of 3% wt/vol). The mouse then was placed on a heated translation stage and fluorescent colonoscopy was performed as described previously.[5] After achieving stable positioning to the colonic epithelium, we increased the laser power to 200 milliwatts for 1 minute to induce photo-damage to the epithelium. Successful photo-damage was confirmed by observing the formation of intravascular clots and extravasation of the fluorescent dye.

Epithelial Barrier Function Assay

At day 4 after femtosecond laser injury, we examined epithelial barrier function. The mouse was anesthetized by an intraperitoneal injection of ketamine/xylazine solution, the descending colon was washed by injecting 0.5 mL of warm PBS with a rubber-tipped syringe. Then, 0.5 mL of Evans Blue solution (4% wt/vol in

PBS) was instilled in the colon for 5 minutes and flushed with PBS to eliminate residual dye. The mouse was killed and the descending colon was collected for microscopic evaluation. The laser-injured region was found by residual green fluorescence from the leaked vascular dye. Bright-field images were taken in the control and injured regions to measure Evans Blue permeability.

SFB and Fungi Quantification in Fecal Microbiota

Fecal DNA was extracted from 150 mg of stool using a DNA Stool Mini Kit (Qiagen). By using the iQ SYBR Green Supermix (Bio-Rad) for quantitative PCR, 16S or 18S ribosomal RNA gene levels were determined using the iCycler with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Reaction conditions consisted of 40 cycles of PCR with a 59°C annealing temperature. The following primers were used: all bacteria: forward[6]: CGGTGAATACGTTCCCGG; reverse: TACGGCTACCTT GTTACGACTT; SFB: forward[7]: GACGCTGAGGCATGA GAGCAT; reverse: GACGGCACGGATTGTTATTCA 18S; forward[8]: ATTGGAGGGCAAGTCTGGTG; reverse: CCGA TCCCTAGTCGGCATAG.

The threshold cycle for each sample was determined for each gene and normalized to the C_T value of the all-bacteria 16S ribosomal RNA gene. Data were calculated using the $2^{-\Delta\Delta C(T)}$ method.

C rodentium *Antigen Preparation*

C rodentium antigen was prepared from an overnight culture of *C rodentium* in Luria broth. The bacterial culture was washed 3 times with PBS and sonicated on ice. The homogenate then was centrifuged (14,000 rpm) at 4°C for 30 minutes. Supernatants were collected and aliquots were stored at -20°C.

Lamina Propria Isolation

Colonic and small intestine LP cells were isolated as previously described with slight modification. Briefly, colons and small intestine were harvested and placed in ice-cold Hank's balanced salt solution. After removal of residual mesenteric fat tissue, Peyer's patches were excised from small intestine, and the colon and small intestine were opened longitudinally. Tissues then were washed in ice-cold Hank's balanced salt solution and cut into 1-cm pieces. After 3 more washes in Hank's balanced salt solution, tissues were incubated twice in 20 mL of serum-free media with 5 mmol/L EDTA and 0.145 mg/mL dithiothreitol at 37°C at 250 rpm for 20 minutes. The pieces were washed 3 times with 10 mL of serum-free media with 2 mmol/L EDTA. After each incubation and wash, the epithelial cell layer containing the intraepithelial lymphocytes was removed by intensive vortexing and passing through a 40-μm cell strainer, washed, and kept on ice until Percoll separation. Colon and small intestine pieces were next digested in serum-free media containing liberase (Roche Applied Science, Indianapolis, IN) and DNase I at 37°C at 250 rpm for 30 minutes. Cells were washed and passed through a 70-μm cell strainer. Cells were resuspended in 4.5 mL of 44% Percoll and placed on 2.3 mL of 67% Percoll. Percoll gradient separation was performed by centrifugation for 20 minutes at 600 $\times$ *g* at room temperature. Lymphoid fractions were collected at the interphase of the Percoll gradient, washed once, and resuspended in fluorescence-activated cell sorter buffer or culture medium. Cells were used immediately.

Flow Cytometry

Cells were washed in PBS supplemented with 3% fetal bovine serum. For surface staining, cells were incubated with 2.4G2 Mouse Fc block in PBS supplemented with 3% fetal bovine serum (BD Pharmingen, San Diego, CA) for 20 minutes at 4°C. Cells were washed and stained with fluorescent-conjugated antibodies for 20 minutes at 4°C. The following antibodies were used for our analysis (BD Pharmingen): CD3–fluorescein isothiocyanate, CD4-PercP, IL-17A–allophycocyanin, and IFNγ-phycoerythrin. Fluorescently labeled LP cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo Analysis Software (Tree Star, Inc, Ashland, OR). For intracellular cytokine staining, immediately after isolation the cells were incubated for 5 hours at 37°C with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich), 1 μmol/L ionomycin (Sigma-Aldrich), and 1 μL/mL GolgiPlug (BD Biosciences). Surface staining was performed followed by intracellular staining using the BD Cytofix/Cytoperm Kit (BD Biosciences).

Ethical Approval

All animal studies were conducted under protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, which serves as the Institutional Animal Care and Use Committee as required by Public Health Service policies on the Humane Care and Use of Laboratory Animals.

Supplementary References

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