Supplemental Methods

Measurement of gastrointestinal transit times

Carmine red (Sigma-Aldrich, St. Louis MO) was prepared as a 6% (w/v) solution in filter sterilized 0.5% methyl cellulose (Sigma-Aldrich). During a 3-day acclimation period, mice were individually housed, placed on a steel rack for 1-3h/d, and sham gavaged. Following an overnight fast, mice were gavaged with 0.3 ml of the carmine solution between 09:00 and 09:30 local time and monitored every 15-30 min for the appearance of the first red fecal pellet. The time from gavage to passage of first red pellet was recorded as total intestinal transit time.

Luminex immunoassay.

Protein extract was obtained by lysing tissue in Ripa Buffer (Sigma-Aldrich) supplemented with 1 mM phenylmethylsulfonyl fluoride, Protease Inhibitor Cocktail (1:100, Sigma), and Phosphatase Inhibitor Cocktail 2 (1:100, Sigma-Aldrich). Protein extracts were normalized to a final concentration of 1.5 mg/ml using Pierce BCA Protein Assay Kit (Pierce, Rockford IL). Standardized 38-plex Luminex immunoassays containing comprehensive assortments of mouse inflammatory cytokines and chemokines (Affymetrix, Santa Clara, CA) was performed in the Human Immune Monitoring Center at Stanford University according to the manufacturer's recommendations with modifications as described below. Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 h followed by overnight incubation at 4 °C. Room temperature incubation steps were performed on an orbital shaker at 500–600 r.p.m. Plates were vacuum-filtered and washed twice, and then incubated with biotinylated detection antibody for 2 h at room temperature. Samples were then filtered and washed twice as above and resuspended in streptavidin-phycoerythrin (PE). After incubation for 40 min at room temperature, two additional vacuum washes were performed and the samples resuspended in Reading Buffer. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument.

Western blotting.

Protein extract (15 μg) was subjected to electrophoresis on 4-12% NuPAGE Bis Tris gels (Invitrogen) and transferred to Hybond-P PVDF blotting membranes (GE Healthcare, Pittsburgh PA). The membranes were incubated with primary antibodies and HRP-conjugated secondary antibodies (supplemental table 1) and visualized using chemiluminescence (ECL, Pierce). Semi-quantitative measurements of band intensity using pixel intensity were performed using ImageJ (NIH) and expressed in densitometric units normalized to the loading control (GAPDH).

Immunofluorescence.

LMMP tissue (from mid-small intestine) was maximally stretched and pinned on Sylgaard-lined plates before fixing in 4% PFA in PBS for 20 minutes at room temperature. Tissue was permeabilized in 50% glycerol in PBS overnight at room at 4^{o}C, blocked in PBS containing 10% normal goat serum (NGS) with 0.2% Triton for 1 hour at room temperature (RT) before incubating at 4^{o}C with primary antibodies diluted in PBS containing 1.5% NGS and 0.01% sodium azide for 2 days. For pSTAT3 staining, tissue was pre-incubated in 100% methanol for 10 minutes at -20^{o}C prior to blocking. After washing with PBS, tissue was incubated overnight at 4^{o}C with secondary antibodies or conjugated antibodies. Please see Table S1 for a list of antibodies and dilutions used. After washing samples with PBS, coverslips were mounted onto glass slides using mounting media with or without DAPI (Vector Labs, Burlingame CA). Antibody specificity was confirmed using specimens incubated without primary antibody. Samples were examined and imaged with a CCD cooled camera on a Nikon C1 confocal microscope using 60X objective. Neuronal density was determined as previously described[1 2]. Briefly, a minimum of 10 randomly selected, undamaged myenteric ganglia containing a minimum of 5 neurons was analyzed for each animal. Neuronal density calculations were based on number of HuC/D^{+} neurons per ganglia area (calculated using ImageJ software).

Ex-vivo organotypic cultures and EdU uptake.

LMMP was placed in stem cell medium (SCM) consisting of Neurobasal medium (Invitrogen) containing B27 (Invitrogen), 2 mM L-glutamine and 100 U/ml PS, plus 10 ng/ml fibroblast growth factor, 10 ng/ml epidermal growth factor and 10 ng/ml glial cell-derived neurotrophic factor (all Invitrogen) and cultured in the presence of EdU at a concentration of 25 μM for 40 hours. Following fixation with PFA and glycerol permeabilization (described above), EdU imaging was performed with Alexa Fluor 488 azide using Click-iT EdU Imaging Kits (Invitrogen) according to the manufacturer's instructions. Three sections of LMMP were assayed for experiment. A minimum of 10 randomly selected ganglia containing a minimum of 5 neurons was assessed per tissue section. Ganglia area was calculated based on boundaries of Hu+ neurons using ImageJ software.

ENSC culturing and TUNEL assay.

ENSCs were isolated by enriching for $CD49b^+$ cells using CD49b-magnetic beads (Miltenyi Biotec, San Diego, CA) according to manufacturer's instructions. Conditioned media was prepared by incubating LMMP from either 24 month old (Old-CM) or 3 month old (Young-CM) mice for 12 hours in SCM. ENSCs cultured in SCM on fibronectin-coated slides (BD Bioscience) were treated with CM +/- anti-IL-6 neutralizing antibody (1:400, Abcam) or rabbit IgG control (1:400, Sigma); or with recombinant murine IL-6 (Biolegend) +/- recombinant murine IL-6 Rα (200ng/ml, R&D Systems) for 12 hours. TUNEL assay was performed using Click-iT TUNEL Imaging Assay according to the manufacturer's instructions (Invitrogen). Cells were counted under fluorescence microscopy.

Bone marrow derived macrophages (BMDMs).

Mice were anesthetized, euthanized, and BM was harvested from femur and tibia by flushing with a syringe filled with complete RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (cRPMI) to extrude BM cells into a sterile petri dish. BM cells were treated with Red Blood Cell Lysing buffer (Sigma-Aldrich) for 5 minutes. BMDMs were prepared as previously described[3]. Briefly, BM cells were cultured in cRPMI supplemented with 50 ng/ml M-CSF (Peprotech, Rocky Hill NJ). On day 4, unattached cells were discarded, and medium was replaced with cRPMI with M-CSF. On day 6, BMDMs were cultured for 2 additional days with either cRPMI alone for M0 or containing either 10 ng/ml IFNγ for M1, or 20 ng/ml IL-4 and 20 ng/ml IL-13 (all from Peprotech) for M2. Cells were used for experimentation on day 8 or RNA extraction with Trizol was performed. For LPS stimulation, 100 ng/ml LPS was added to BMDMs prior to RNA extraction. For expression analysis, M1 and M2 states were normalized relative to M0 from young or WT.

Lymphocyte suppression.

To isolate $CD4^+$ T cells, spleen from 1 month-old C57/BL6 mouse was mechanically dissociated through a 100 µm nylon mesh to yield single-cell suspensions in HBSS containing 2% BCS. Cells were incubated in Red Blood Cell Lysing buffer (Sigma-Aldrich) and washed by centrifugation for 10 min at 300g. $CD4^+$ T cells were enriched by negative selection using $CD4^+$ T cell Isolation kit (Miltenyi Biotec) according to manufacturer's instructions. $CD4^+$ T cells were incubated with 2 µM CSFE (Invitrogen) in PBS at 37^{o}C for 15 minutes then activated with mouse anti-CD3 and anti-CD28 (1:1000, Biolegend) in cRPMI. T cells were added to 24-well plate containing BMDMs previously induced to M2 activation state (described above) at a ratio of 100 x 10^3 lymphocytes to 250 x 10^3 macrophages and cultured for 72 hours. Cells were blocked with 5% rat serum and mouse anti-CD16/CD32 (eBioscience) in HBSS containing 2% BCS then incubated for 30 minutes at 4^{o}C with APC-conjugated mouse anti-CD4, and isotype

controls (Biolegend). Following washing and fixation in 4% PFA, data was acquired on a LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc).

Generation of bone marrow chimeras.

Congenic BM chimeric mice were generated by lethally irradiating young (2 months) or old (19 months) C57BL/6 mice with 9.5-Gy y radiation in two doses ~3h apart, followed by transfer of 5 x 10^6 BM cells from young (2 months) or old (24 months) mice via retro-orbital injection. Chimeric mice were provided with antibiotics for the first two weeks and left to engraft for at least 8 weeks before experimentation.

References

1. Gulbransen BD, Bashashati M, Hirota SA, et al. Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis. Nature medicine 2012;**18**(4):600-4 doi: 10.1038/nm.2679[published Online First: Epub Date]|.
2. Phillips RJ, Kieffer EJ, Powley TL. Loss of glia and neurons in the myenteric plexus of the aged Fischer 344 rat. Anatomy and embryology 2004;**209**(1):19-30 doi: 10.1007/s00429-004-0426-x[published Online First: Epub Date]|.
3. Xue J, Habtezion A. Carbon monoxide-based therapy ameliorates acute pancreatitis via TLR4 inhibition. The Journal of clinical investigation 2013 doi: 10.1172/JCI71362[published Online First: Epub Date]|.