Materials and Methods

Mice

All studies were performed with C57BL/6J mice (WT) and *PHIL,*[1] C57BL/6J female mice. Eosinophil depletion studies were completed using a monoclonal antibody specific for Interleukin-5 (TRFK-5; Cayman Chemical, Ann Arbor, MI). Experimental animals were injected with seven intraperitoneal (*i.p.*) doses of TRFK-5 antibody (10mg/kg) or saline vehicle daily during Dextran Sodium Sulfate (DSS) colitis. Animals were maintained in micro-isolator cages housed in a specific pathogen-free (SPF) facility at the University of Colorado or Mayo Clinic Arizona. Rodent diet contained 0.3% C18:3ω3 Linolenic and 3.1% C18:2ω6 Linoleic polyunsaturated fatty acids, 1.3% monounsaturated and 0.9% saturated fatty acids. Age and sex matched littermate WT mice were used as controls. Studies involving animals were performed in accordance with National Institutes of Health, University of Colorado and Mayo Clinic Arizona IACUC guidelines.

Dextran Sodium Sulfate (DSS), 2,4,6-Trinitrobenzenesulfonic acid (TNBS) & Oxazolone-*induced colitis*

Colitis was induced by administering DSS (2.5-3.5%(w/v)) 36,000-50,000 kDa; (MP Biomedicals, Santa Ana, CA) to 10-week old mice in drinking water (*ad libitum*) for up to 8 days (i.e., protocol days 0-8). All studies were performed following 6 days of DSS treatment unless otherwise shown. DSS was replaced every 3 days with fresh DSS solution. No differences in volumes consumed were observed. TNBS and Oxazolone colitis was induced in 10-week old mice as follows. On day 0, a 2cm x 2cm area of abdominal skin of anesthetized mice was shaved and TNBS (100µl of a 1%(w/v) solution of TNBS in 80% ethanol vehicle) or Oxazolone (100ul of 3%(w/v) solution of Oxazolone in 100% ethanol vehicle) was applied to the skin surface to initiate the sensitization

phase of the protocol. Sensitized mice were administered a rectal gavage of 2.5%(w/v) TNBS in 40% ethanol vehicle or 1%(w/v) Oxazolone in 50% ethanol vehicle on protocol day 7 (challenge phase of the protocol). Vehicle control animals (either Wild Type or *PHIL* mice) were sensitized as noted above and challenged with 40% or 50% ethanol vehicle alone. Daily assessments of disease activity were measured following colitis induction (i.e., weight loss, stool consistency, and fecal blood) were performed blinded to experimental conditions as previously described.[2] Upon necropsy, colons were excised and colonic lengths were recorded.

In some studies, the commercially available DHA-derived protectin D1 isomer (10S, 17S-dihydroxydocosahexaenoic acid (10S,17S-diHDoHE))[3, 4] (hereafter referred to as PD1-isomer) (0.05mg/kg) (Cayman Chemical, Ann Arbor, MI) was administered to *PHIL* mice via six *i.p.* doses (100μl total volume) daily from days 1 to 6 of colitis. Mice were sacrificed 24 hours following final treatment (day 7) and endpoint measures of colonic inflammation were assessed as above.

Generation of bone marrow chimeric mice

Six week old marrow recipient mice (*CD45.1*$^+$) were irradiated with 11Gy, and 1×10^7 donor bone marrow cells (*CD45.2*$^+$, isolated from femurs and tibia) were introduced into peripheral circulation via intravenous (i.v.) injection (100μL) into the retro-orbital plexus.[5] Tetracycline (100mg/L) was administered *i.p.* every third day for a two week-period following bone marrow adoptive transfer. Cell count and differential assessments were performed on mice following a 60-day recovery period. Bone marrow and colonic samples were excised at necropsy and the extent of donor cell engraftment in recipient mice was determined by FACS, assessing for the presence of leukocytes with the donor-derived *CD45.2*$^+$ marker.[5]

Tissue processing and histologic analysis

Whole length colonic tissue was removed and fixed with 10% neutral buffered formalin, paraffin embedded and cut into 5µm sections.[5] Sections were stained with H&E (Sigma-Aldrich) according to the manufacturer's specifications, and a global inflammatory score based on a quantitative index was assigned by a pathologist blinded to the experimental conditions (P.J.) modified to account for area of tissue effected from that described previously.[2] Two histological parameters (i.e., inflammatory cell infiltration and tissue injury[2]) were assessed with indices of equal weight given to each parameter to account for the extent of tissues affected. Cell infiltration was scored as: 0, occasional inflammatory cells in the lamina propria (LP); 1, increased infiltrate in the LP predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; 3, transmural extension of infiltrate. Tissue damage was scored as: 0, no mucosal damage; 1, partial (up to 50%) loss of crypts in large areas; 2, partial to total 50–100% loss of crypts in large areas, epithelium intact; 3, total loss of crypts in large areas and epithelium lost. Each score is then adjusted to reflect the area of tissue effected by each parameter: 1, 0-25% effected; 2, 25-50% effected; 3, 50-75% effected and 4, 75-100% effected. Thus a maximum score of 24 could be applied to an individual specimen.

Immunohistochemical assessment of eosinophil, myeloperoxidase and F4/80 positive cellular inflammation

Eosinophils were localized in mouse colon tissue sections by immunohistochemistry using a rat anti-mouse eosinophil major basic protein-1 (MBP-1) monoclonal antibody (Clone MT-14.7) as previously described.[6] MBP-1 immunohistochemically positive cells were identified through visualization of a Permanent Red chromogen (Dako,

Carpinteria, CA) and slides counterstained with Methyl Green (Sigma, St Louis, MO). Neutrophils were immunolocalized in mouse colons using polycolonal goat anti-mouse myeloperoxidase (MPO) antibody (R&D Systems, Minneapolis, MN). F4/80 was used as a surrogate marker for macrophage infiltrating cells, using a rat anti-mouse F4/80 monoclonal antibody (Clone BM8) (BMA Biomedicals, Augst, Switzerland). MPO or F4/80 positive cells were identified through visualization of a DAB chromogen (Vector Labs, Burlingame, CA) and slides counterstained with Gills Hematoxylin (Sigma, St. Louise, MO). MPO or F4/80 positive cells were quantified in 4-6 random high-powered fields per animal. Immunolocalization of eosinophils in human histological specimens was completed with a monoclonal mouse anti-eosinophil peroxidase (EPX) antibody (MM25–82.2.1) as previously described.[7, 8] EPX immunopositive cells were identified through visualization of a Permanent Red chromogen (Dako, Carpinteria, CA) and slides counterstained with Methyl Green (Sigma, St Louis, MO). Negative control sections were performed by replacing the primary antibody in each case with either no antibody or rat IgG2a isotype control antibody (Vector Labs, Burlingame, CA). This study received IRB approval, 07-0888.

In situ hybridization assessment of CXCL1 production

Formalin fixed paraffin embedded sections were subjected to *in situ* hybridization with probes targeted to CXCL1 mRNA as per manufacturers recommendations (Advanced Cell Diagnostics, Hayward, CA). Slight modifications in tissue pre-treatment were as follows. Briefly, tissues were subjected in heat-induced pre-treatment #2 by boiling in a pressurized dekloaking chamber for 20 minutes. Probes directed towards UBC and DapB were used as positive and negative controls respectively.

Colonic lamina propria and bone marrow leukocyte isolation for flow cytometry

Leukocytes were isolated from the colonic mucosa as previously described.[5, 6, 9] Colonic leukocytes were recovered from resected colons cut longitudinally, washed briefly in 1X PBS/1mM EDTA/15mM HEPES (Invitrogen, Grand Island, NY) and digested with 100-200 U/ml collagenase (Sigma, St Louis, MO) as previously described.[5, 6] Clumps of colonic cells were disrupted by pipetting and disassociated cells were recovered following transfer to a 15 ml conical tube and centrifugation at 4ºC for 10 minutes (1200rpm). Red blood cell contamination was removed by hypotonic shock using red blood cell lysis buffer (Sigma, St Louis, MO). Colonic leukocytes were passed through a 40µm cell strainer (BD Biosciences, San Jose, CA) and cell counts and viability determined with a hemocytometer and trypan blue exclusion.

Bone marrow cells were isolated from femurs and tibias using sterile PBS. Briefly, femur and tibia were recovered, cleaned of attached musculature, the epiphyses were removed and marrow was recovered from each bone by fitting a 25G needle attached to a 3ml syringe to one end and flushing the marrow with physiological saline.[10] Aspirates were disrupted to single cells by pipetting. Marrow cells were recovered following transfer to a 15 ml conical tube and centrifugation at 4ºC for 10 minutes (1200rpm). Red blood cell contamination was removed by hypotonic shock using red blood cell lysis buffer (Sigma, St Louis, MO). Marrow leukocytes were passed through a 40µm cell strainer (BD Biosciences, San Jose, CA) and cell counts and viability determined with a hemocytometer and trypan blue exclusion.

Flow cytometric analysis

Single-cell bone marrow and colon suspensions were stained for 25 minutes on ice with cell type-specific antibodies after Fc receptor blockade using 1µg/µl of Fc block

(CD16/32; BD Biosciences, San Jose, CA). Antibodies used for staining of specific cell surface markers include CCR3 (557974), SiglecF (E50-2440) (all BD-Biosciences, San Jose, CA); MHC-II (M5/114.15.2; Biolegend, San Diego, CA) and CD11b (M1/70), CD11c (N418), CD45 (30-f11), F4/80 (BM8), Ly6G (1A8), (all eBiosciences, San Diego, CA). Viable cells were identified for analysis with Live/Dead AquaVi staining (Invitrogen, Grand Island, NY). Fluorescence analysis was performed using a BD FACSCanto™ II (BD Biosciences, San Jose, CA). Data files were further analyzed using FLOWJo software (Tree Star Inc, Ashland, OR).

RNA isolation and real-time RT-PCR

Total RNA was prepared from whole colon biopsies with RNeasy Mini Kits (Qiagen, Valencia, CA) and hand-held laboratory homogeniser (PRO Scientific, Oxford, CT) as previously described.[6] First strand cDNA synthesis was performed from 500ng of total RNA using the High Capacity cDNA Archive kit (Applied BioSystems, Foster City, CA). Transcript expression was assessed using Taqman Gene Expression Assay probes (Applied Biosystems, Foster City, CA). RT-PCR reactions were performed with ABsolute^{TM} Blue QPCR ROX Mix (Thermo Scientific, Surrey, UK), cDNA and Taqman assay mixtures. Thermocycling and subsequent analysis was performed with ABI-7300 System and software. Data was normalized to 18S expression and calculated as RQ (Relative Quantity; $2^{-\Delta\Delta Ct}$, where Ct is cycle threshold for each sample).

Colon Lipidomic analysis

Colon tissues were harvested and immediately frozen in liquid nitrogen. Frozen tissue samples were homogenized in ice-cold methanol, and the supernatants were diluted with ice-cold water and adjusted to 10% (v/v) methanol. Deuterium-labeled internal standards were added to each sample, and clear supernatants acidified to pH 4.0 were

immediately applied to preconditioned solid phase extraction cartridges (Sep-Pak C18, Waters) to extract the lipid mediators. LC-MS/MS-based lipidomics analyses were performed using an HPLC system (Waters UPLC) with a linear ion trap quadrupole mass spectrometer (QTRAP5500; AB SCIEX) equipped with an Acquity UPLC BEH C_{18} column (Waters) as previously described.[11] MS/MS analyses were conducted in negative ion mode, and fatty acid metabolites were identified and quantified by multiple reaction monitoring (MRM).

Cell culture and in vitro transmigration assay

Caco-2 human intestinal epithelial cells were cultured as previously described.[12, 13] For physiologically relevant basolateral-to-apical polymorphonuclear (PMN) cell transmigration, Caco-2 cells were plated as inverted monolayers on collagen-coated permeable 0.33 cm^2 polyester ring supports, 3µm pore size (Costar Corp., Cambridge, MA, USA) and monolayers were used 10 days after plating.[12, 13] Human poly-morphonuclear (PMN) leukocytes were isolated from normal volunteers by a Dextran Sulphate sedimentation and Percol density gradient centrifugation technique and the recovered cells were resuspended in Hanks Balanced Salt Solution (minus Ca^{2+} and Mg with 10mM Hepes, HBSS, pH7.4) (Sigma, St Louis, MO) prior to use. Caco-2 monolayers were washed free of serum containing media with HBSS and 1 x 10^6 PMNs were added to the basolateral chamber in the presence of 0.2nM or 20nM PD1-isomer or vehicle alone. PMN transmigration through the epithelium in response to a gradient of 100nM Formyl-Methinyl-Leucyl-Phenylalanine (FMLP; Sigma, St Louis MO) was measured over a two-hour period at 37^{o}C. PMN migration was quantitated with a myeloperoxidase (MPO) assay of the apical chamber supernatant as previously described.[12, 13]

Statistical analysis

Statistical analyses of data outcomes were performed by Student's *t*-test, one-way ANOVA or Log-rank (Mantel-Cox) test (conservative) survival curve where appropriate. Data are expressed as means ± SEM. A *P*-value of ≤0.05 was considered as statistical significance although in some cases higher levels of significance are noted and described in the figure legends where applicable. **P*≤0.05, ***P*≤0.01, ****P*≤0.001. +*P*≤0.05, ++*P*≤0.01, +++*P*≤0.001.

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