Supplementary Information for

Ornithine decarboxylase supports ILC3 responses in bacterial infection and autoimmune colitis through positive regulation of IL-22 transcription

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This PDF file includes Materials and Methods, and Figures S1 and S2
Materials and Methods

Mice

All mice were bred and maintained in specific pathogen-free facilities at Washington University in Saint Louis. Rorc\textsuperscript{Cre} mice were provided by A. Tumanov. Odc1\textsuperscript{flox} mice were a generous gift from J. Cleveland. Rag1\textsuperscript{+/-} mice (#00216) were obtained from The Jackson Laboratory. Mice used for experiments were between 8-16 weeks of age and were on a C57BL/6 background unless otherwise indicated. Control mice were littermates unless otherwise indicated. For ornithine feed studies, mice were fed 5% ornithine (w/v) for two weeks by drinking water. All studies were conducted in accordance with the Washington University Animal Studies Committee.

Tissue preparation and single cell isolation

siLP and cLP were prepared as previously described \(^1\). Briefly, intestines were dissected out and flushed with cold 1X HBSS. Mesenteric fat and Peyers’ patches were removed and intestine was cut longitudinally. Intestines were incubated in HBSS-EDTA to remove intestinal epithelium. Intestines were washed and shaken in collagenase IV for tissue digestion (40 minutes of small intestine, 60 minutes for colon) and strained through a 100µm strainer. Lamina propria cells further purified through a 40/70 Percoll density gradient.

Antibodies and flow cytometry

The following antibodies and corresponding dilutions were used for FACS: NK1.1 (PK136, 108710, BioLegend) (1:200), CD11b (M1/70, 101228, BioLegend (1:200), CD11c (N41B, 45-0114-82, ThermoFisher) (1:200), CD3e (145-2C11, 100328,
BioLegend) (1:100), CD19 (6D5, 115534, BioLegend) (1:100), CD45 (30-F11, 103116, BioLegend) (1:200), CD5 (53-7.2, 100624, BioLegend) (1:100), KLRG1 (2F1, 138408, BioLegend) (1:200), RORgt (AFKJS-9, 12-6988-82, ThermoFisher) (1:100), IL-22 (1H8PWSR, 12-7221-82, ThermoFisher) (1:100), IL-17A (TC11-18H10, 560220, BD BioSciences) (1:100), Thy1.2 (53-2.1, 105312, BioLegend) (1:500), NKp46 (29A1.4, 137616, BioLegend) (1:100), CD4 (RM4-5, 100546, BioLegend) (1:200), CD8α (53-6.7, 100750, BioLegend) (1:200), FoxP3 (MF-14, 124608, BioLegend). For staining, single-cell suspensions were incubated with Fc block for 10 min and then stained with antibodies and Fc block for 20 min at RT or 4°C. Dead cells were excluded using either the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific), 7-aminoactinomycin D, or DAPI. Intracellular proteins were stained using either the BD Biosciences Fixation/Permeabilization Solution Kit or the eBioscience Transcription Factor Staining Kit. BD FACSymphony A3 was used for FACS analysis. BD FACS ARIA II was used for cell sorting. Analysis was performed using BD FACS Diva Software v 8.0.1 and FlowJo v10 (FlowJo LLC).

Reagents and Cell Stimulation

siLP lymphocytes were incubated with IL-23 (10ng/mL) and/or IL-1β (10ng/mL) for 4 hours with brefeldin A being added for the last 3.5 hours at 37°C. In anti-CD40-induced colitis experiments, cLP lymphocytes were stimulated with 50 ng/mL PMA, 500 ng/ml Ionomycin and brefeldin A for 4h at 37°C as previously described². MNK-3 cells were a gift from J. R. Carlyle. MNK-3 cells were maintained in complete RPMI at 37°C supplemented with in-house generated mouse 5% IL-7 and 5% IL-2. For polyamine studies, reagents were used at the following concentrations: Putrescine (10mM, Sigma),
Spermidine (100nM, Sigma), Spermine (100nM, Sigma). Final concentrations were determined by the maximum possible dose which did not reduce cell viability during the indicated treatment periods.

Quantitative PCR

cDNA was synthesized from RNA with Superscript III first-strand synthesis system for RT-PCR (Invitrogen). RNA expression was analyzed by quantitative PCR using Universal SYBR Green PCR Master Mix (Bio-Rad Laboratories) and an ABI7000 (Applied Biosystems). The following oligonucleotides were used: Il17f Fwd 5′-CTGGAGGATAACACTGTGAGAGT-3′, Il17f Rev 5′-TGCTGAATGGCGACGGAGTTCC-3′, Il22 Fwd 5′-GCTCAGCTCCTGTCACATCA-3′, Il22 Rev 5′-AGCTTCTTCTCGCTCAGACG-3′, Gapdh Fwd 5′-ACGGCAAATTCAACGGCAGAGTCA-3′, Gapdh Rev 5′-TGGGGGCACTCGGCAGAAGG-3′, Reg3g Fwd 5′-AACAGAGGTGGATGGGAGTG-3′, Reg3g Rev 5′-GGCCTTGAATTTGCAGACAT-3′.

scRNA-seq analysis

Processed count tables were download from GSE85157. Data from antibiotic-treated, germ-free, and non-Rorc-GFP mice were excluded from analysis. Data were normalized, reduced, and clustered using Seurat (v4.1.1) with the following functions: SCTransform, RunPCA, RunUMAP, FindNeighbors, and FindClusters. Analysis was performed using the top 30 PCs and a resolution of 1.4. Lymphocyte clusters were isolated and re-clustered using the same strategy. ILC subsets were identified using cluster-specific marker genes and expression of RORγT, KLRG1, and NKp46 recorded from index sorting.
Metabolomics

For untargeted metabolomics, small intestinal ILCs were sort-purified. ILC2s were purified by sorting live, lymphocyte-sized cells that were negative for CD3ε, CD5, CD19, CD11b, CD11c, and NK1.1; positive for KLRG1 and CD45. ILC3s were purified by sorting live, lymphocyte-sized cells that were negative for CD3ε, CD5, CD19, CD11b, CD11c, NK1.1, and KLRG1; positive for CD90.2; and intermediate for CD45. Cells were directly sorted into 80% methanol (for amines and cations) or isopropanol (for lipids) and snap frozen on dry ice. Two liquid chromatography tandem mass spectrometry (LC-MS) methods were employed to measure metabolites in cell extracts. For identification of lipid species, 230 known chemical standards were run in parallel. For identification of amines and cationic species, 106 known standards were used. Targeted validation was performed using LC/MS and labeled chemical standards. For analysis of untargeted metabolomics, only species reliably annotated by standard run in parallel were considered for analysis. Relative intensity values were divided by input cell number for each sample. Species which were not detected in more than half of all samples were removed from downstream analysis. Zero values were transformed into 0.5×minimum intensity value for each metabolic species. Values were then log10 transformed and multiplied by a scalar of 10^6. Log10 values were fit to a linear model with the following function \( \text{lm(log10} \sim \text{celtype}) \) for each metabolite. Multiple test correction was then performed using the Benjamini-Hochberg method.

RNA-seq

MNK-3 cells were plated in 12-well plates and allowed to rest overnight. Putrescine pre-treatment was performed for 4 hours and cells were stimulated with IL-23 (10ng/mL) for
4 hours. CCR6+ ILC3 cells were sort purified from SIs of ODC1 cKO and WT controls using a FACSARia II (BD Biosciences) as live, lymphocyte-sized cells that were negative for CD3ε, CD5, CD19, CD11b, CD11c, NK1.1, and KLRG1; positive for CD90.2 and CCR6; and intermediate for CD45. Total RNA was extracted from samples using an RNeasy Micro Plus Kit (Qiagen). Total RNA integrity was determined using Agilent Bioanalyzer or 4200 TapeStation. Library preparation was performed with 10 ng total RNA with a Bioanalyzer RNA integrity number score greater than 8. Double standard complementary DNA (cDNA) was prepared using the SMARTer Ultra Low RNA kit for Illumina Sequencing (Takara-Clontech) per manufacturer's protocol. cDNA was fragmented using a Covaris E220 sonicator using peak incident power 18, duty factor 20%, and cycles per burst 50 for 120 s. cDNA was blunt ended, had an A base added to the 3′ ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 12 to 15 cycles using primers, incorporating unique dual index tags. Fragments were sequenced on an Illumina HiSeq. Basecalls and demultiplexing were performed with Illumina’s bcl2fastq software and a custom Python demultiplexing program with a maximum of one mismatch in the indexing read. RNA-seq reads were then aligned to the Ensembl release 76 primary assembly with Spliced Transcripts Alignment to a Reference version 2.5.1a. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.6-p5. Aligned gene counts were processed using the DESeq2 package with R (version 4.1) ⁵. Genes with fewer than 10 counts among all of the samples were excluded. DEGs were defined as protein-coding genes with an average expression > 100 counts and false discovery rate < 0.05. Gene ontology analysis was performed
using Metascape with default settings\textsuperscript{6}.

\textit{Citrobacter rodentium infection}

6-8-week-old age- and sex-matched mice were orally administered \(1 \times 10^{10}\) CFU \textit{C. rodentium} (DBS100, ATCC). Body weight was measured until day 10. Feces were collected on day 7 for CFU counting. Briefly, feces were weighed, homogenized, and serially diluted up to \(10^{-8}\) in sterile PBS. Three consecutive dilutions per sampled were plated on MacConkey agar plates and incubated at 37 degrees C. Colonies were then counted and counts for three dilutions were averaged and normalized to feces weight.

\textit{Anti-CD40-induced colitis}

Anti-CD40-induced colitis was performed using 8-12 week-old, age- and gender-matched mice as previously described\textsuperscript{7}. Briefly, mice were injected i.p. with 0.75uL/gram body weight of ascites containing anti-CD40 antibody (prepared in the lab). The dose of anti-CD40 antibody was titrated to achieve a maximum body weight loss of approximately 20\% in \textit{Rag1}\textsuperscript{-/-} mice. Body weight was measured daily, and mice were euthanized on day 4 (for flow cytometry) or day 7 (for histology) after injection. Colons were removed, cleaned, and processed as previously described\textsuperscript{7}.

\textit{Histology}

Murine colons were removed and washed with ice-cold HEPES/HBSS, fixed in 10\% formalin overnight and then transferred to 70\% ethanol. Fixed tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). The severity of colitis was graded blindly based on published criteria\textsuperscript{7,8}. Each colon was given a score 0-4 in each of the 4 features: 1) epithelial cell depletion; 2) lamina propria cell infiltration; 3) percentage of mucosa affected; 4) severe inflammation (submucosal involvement,
crypt abscess). Scores for each feature were added to generate a total score for each sample of 0-16.

References for SI Appendix Materials and Methods

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SI Appendix, Fig. S1. Representative Flow Cytometry Gating Strategies.

Representative gating strategies for ILC2s, ILC3s, and T cell subsets examined in the study. For live cell sorting, DAPI was used for viability instead of LIVE/DEAD.
SI Appendix, Fig. S2. Characterization of intestinal ILC3 subsets and T cell subsets in control and Odc1ΔILC3,T mice at steady-state.

(A-D) Frequency of total ILC3 and individual ILC3 subsets in siLP of control and Odc1ΔILC3,T mice (n=4). (E-H) Frequency CD8+, CD4+, FOXP3+ CD4+ (Treg), and RORγT+ CD4+ (T_{H17}) cells in siLP of control (Cre-) and Odc1ΔILC3,T (Cre+) mice (n=3).

(I-L) Frequency of IL-22+ and IL-17A+ CCR6+ ILC3 from siLP lymphocytes unstimulated
or *ex vivo* stimulated with IL-23 (10ng/mL) (n=6-7). Data from **A-H** are representative of three independent experiments. Data from **I-L** are pooled from two independent experiments. P values were calculated using two-tailed Student’s t test.
SI Appendix, Fig. S3. Characterization of intestinal ILC3 and T cell subsets in control and Odc1ΔILC3,T mice during C. rodentium infection.

(A-D) Frequency of total ILC3 and individual ILC3 subsets in siLP of control (Cre-) and Odc1ΔILC3,T (Cre+) mice on day 10 of infection with C. rodentium (n=10-11). (E-H) Frequency CD8⁺, CD4⁺, FOXP3⁺ CD4⁺ (Treg), and RORγT⁺ CD4⁺ (TH17) cells in siLP of control and Odc1ΔILC3,T mice on day 10 of infection with C. rodentium (n=6). Data from A-D are pooled from two independent experiments. Data from E-H are representative of two independent experiments.