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Supplementary Materials for

Dendritic cell-derived hepcidin sequesters iron from the microbiota to promote mucosal healing

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Materials and Methods

Mice, antibiotics and fecal microbiota transplantation

C57BL/6 were purchased from Jackson Laboratories and used at 6-12 weeks of age. All conventional mice were maintained in specific pathogen-free facilities at Weill Cornell Medicine. *Hamp*$^{-/-}$ mice were generated by Dr. Vaulont. *Hamp*$^{F/F}$ mice were generated by Dr. Peyssonnaux. *Slc40a1*$^{C326Y\text{-floxed}}$ mice were generated by Dr. Lakhal-Littleton. *Zbtb46*^{Cre} mice were kindly provided by Dr. Nussenzweig (Rockefeller University). Albumin^{Cre} (stock number 003574), Villin^{Cre} (stock number 021504), and CD11c^{Cre} (stock number 008068) mice were purchased from Jackson Laboratories. For FMT studies, donor fecal samples were collected, weighed, and re-suspended at 150 ng/ml in anaerobic PBS supplemented with 10% glycerol in an anaerobic chamber. Fresh fecal suspension (200 µl per mouse) was then administered to recipient germ-free C57BL/6 mice by oral gavage. Germ-free C57BL/6 were maintained within sterile vinyl isolators at Weill Cornell Gnotobiotic Mouse Facility and monitored for germ-free status by weekly aerobic and anaerobic culturing. After FMT, recipient mice were housed in sterile cages. For antibiotic experiments, ampicillin (1 g/l) and gentamicin (1 g/l) were supplemented with 1 packet of artificial sweetener (Sweet'N Low) per 250 ml of drinking water. Antibiotics were administered beginning 2 weeks prior to DSS administration, and antibiotic treatment continued throughout the DSS experiment. For *Bifidobacteria* administration, a mixture of lyophilized *Bifidobacterium* species (*B. bifidum, B. longum, B. lactis and B. breve*, Seeking Health) was suspended in sterile aerobic PBS at 2.5×10^9 CFU/ml and 200 µl per mouse were given by oral gavage. For DSS experiments, *Bifidobacteria* was administered 3X/week for 2 weeks prior to the administration of DSS. For measurement of colon gene expression, *Bifidobacteria* was administered 3x/week for 2 weeks and before analysis of colon gene expression. Deferoxamine mesylate (Sigma) was suspended in sterile PBS at 20 mg/ml. One hundred microliters (2 mg) of DFO or PBS was administered daily via intraperitoneal injection starting at Day 0 of DSS. Modified iron diets were purchased from Envigo. Low-iron diet (TD.10210) contained 2-6 ppm iron, and control diet (TD.10211) contained 35 ppm iron as ferric citrate. Mice were maintained on modified-iron diets for 4 weeks and fecal *Bifidobacteria* levels were compared to baseline. All experiments were performed according to the guidelines of the Weill Cornell Medical College Institutional Animal Care and Use Committee or by the Ethical Committee for Animal Experimentation of Paris Descartes University (Agreement n° CEEA34.CP.003.13) and in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals (Council of Europe, ETS 123, 1991).

DSS administration

Colitis-grade dextran sulfate sodium salt with average MW of 36,000-50,000 Da (MP Biomedicals) was added to drinking water at day 0. Mice were weighed at the same time of day at indicated time points. All DSS experiments used sex-matched mice at age 7-10 weeks. DSS was administered ad libitum until substantial inflammation was induced as evidenced by significant weight loss. For experiments with *Slc40a1*$^{C326Y\text{-floxed}}$ mice and *Hamp*$^{\Delta CD11c}$ mice, littermate mice were used as controls. For experiments with *Hamp*$^{-/-}$ and *Hamp*$^{\Delta Zbtb46}$ mice,

wild-type C57BL/6 mice were used as controls after co-housing for at least 3 weeks. After DSS was removed, mice were euthanized when significant weight recovery had occurred in at least one group. To compare recovery between experimental groups, weights at sacrifice (as a fraction of the starting mouse weight) were compared by an unpaired two-tailed Student's *t*-test for the last day (*:p<0.05; **:p<0.01; ***:p<0.001; ****:p<0.0001). Additionally, colon length was measured (and compared by an unpaired two-tailed Student's *t*-test), and distal colon tissue samples were collected for histology. DSS was used at a concentration of either 2.5% or 3.5%, depending on the manufacturer lot used (titration experiments were performed to determine the DSS concentration required to induce similar inflammation and weight loss across manufacturer lots). Mice remained on DSS until 10% body weight was lost in at least one group, and then switched to water.

Inflammatory bowel disease TissuScan Arrays

cDNAs in OriGene TissueScan Arrays (CCRT101) were used to assess gene expression in characterized biopsies of ulcerative colitis (n=21), Crohn's disease (n=21) and control (n=5) tissue biopsies. Clinical information, histology slides of each biopsy, and the quality control data for RNA isolation and cDNA preparations are available (https://www.origene.com/catalog/tissues/tissuescan/ccrt501/tissuescan-crohn-s-colitis-cdna-array-i). The original tissues were collected from accredited medical institutions in the United States using IRB-approved protocols, selected by board-certified pathologists and then deposited into the OriGene tissue biorepository along with all of the available clinical data supporting the pathology diagnoses. The specific array plates used contained cDNA prepared from RNA extracted from these pathologist-verified tissues.

RNA extraction, reverse transcription and quantitative PCR were performed as previously described (*24*). All samples were normalized to the threshold cycle value for cyclophilin-A. The primers used are presented in the table below. Spearman correlation analysis was used to test the correlation between *HAMP* expression and *ITGAX* (CD11c), *CCR7, DEC205, C5AR1,* or *CD68*.

Name	Forward primer 5′-3′	Reverse primer 5′-3′
CYCLOA	GTCAACCCCACCGTGTTCTT	CTGCTGTCTTTGGGACCTTGT
HAMP	CTCTGTTTTCCCACAACAGACG	CGTCTTGCAGCACATCCCACAC
ITGAX	ACGTTCGACACATCCGTGTA	TTTGCCTCCTCCATCATTTC
CD68	GCAACTCGAGCATCATTCTTTC ACC	GATGCAGGAGGTGGTGCCTT
C5AR1	GTGGTCCGGGAGGAGTACTTT	GCCGTTTGTCGTGGCTGTA
CCR7	TGCATCAGCATTGACCGCTA	TATCCAGATGCCCACACAGGA
DEC205	TGGCTTCATGGGTCATGTTA	CCAGCCAAAAACTTCTCATTT

Histologic analyses

For histological analysis, distal colon tissues were fixed in 4% paraformaldehyde and sent to IDEXX Laboratories (USA) for paraffin embedding, cutting, slide preparation and staining. Images were acquired on a Nikon Eclipse Ti microscope (Nikon) using a Nikon DS-Fi2 camera and a 4X objective lens (40X total magnification).

Iron measurements

Serum iron was determined using a ferrozine-based colorimetric assay (Pointe Scientific). For iron measurements, each condition was compared to the control group by an unpaired two-tailed Student's *t*-test (*:p<0.05; **:p<0.01; ***:p<0.001; ****:p<0.0001).

To measure iron in colon lumen contents, either 2-3 fresh pellets were collected from live mice (for the naïve condition), or complete colon contents were collected at sacrifice (for the DSS-treated condition). Samples were dried for 72 hours in iron-free tubes at 60°C. For non-heme iron measurements, oven-dried samples (8-84 mg) were digested with 300 µl of NHI Acid (10% trichloroacetic acid in 3 M HCl) overnight at 65°C. Samples were cooled, vortexed and centrifuged at 2,000 rpm (376 × g) for 15 s. Equal volumes of sample or iron standard (25 µg/ml-0.39 µg/ml using NHI Acid) were incubated in a 1-ml cuvette for 5-10 min at RT with 800 µl of BAT Buffer (0.2% thioglycolic acid, 0.02% bathophenanthroline sulfonate in 1/2 saturated NaAc solution). Samples were read at 535 nm with unknowns calculated from a standard curve. For heme iron measurements, the remaining pellet after NHI Acid digestion was digested in a 1:3 v/v dilution of ultra-pure HNO_3 for 2 hours at 95°C, cooled, and then further diluted 1:1 with 30% H_2O_2 and digested for another 1 hour at 95°C. Equal volumes of sample or iron standard were then measure by graphite furnace atomic absorption spectroscopy, with sample iron concentrations calculated from a standard curve. Total iron was calculated as [heme iron] + [non-heme iron], and non-heme iron fraction was calculated as [non-heme iron] / [total iron].

Flow cytometry and cell sorting

Mouse mesenteric lymph nodes were harvested at necropsy and single-cell suspensions were prepared by mechanical disruption through a 70-µm nylon mesh. For mouse colon lamina propria preparations, attached fat was removed and tissues were cut open longitudinally. All luminal contents were removed by gentle scraping and subsequent washes with cold PBS. Epithelial cells and intraepithelial lymphocytes were collected by two sequential incubations with shaking in strip buffer (1 mM EDTA, 1 mM DTT, and 5% FCS in PBS) at 37°C for 10 min. To isolate lamina propria lymphocytes, the remaining tissue was digested with 0.5 mg/ml collagenase/dispase (Roche), and 20 µg/ml DNaseI (Sigma) for 30 min at 37°C. Unlabeled anti-CD16/32 (clone 2.4G2, BD biosciences) was used to block Fc receptors when analyzing myeloid cells. Dead cells were excluded from analysis using the Live/Dead Aqua viability kit (Invitrogen). For staining cytokines, cells were stimulated with 50 ng/ml PMA, 750 ng/ml ionomycin, 10 µg/ml Brefeldin A (all from Sigma-Aldrich) for 4 hours at 37°C ex vivo. For staining of transcription factors and cytokines, cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD biosciences).

Ileum samples from pediatric Crohn's patients were obtained through Institutional Review Board approved protocols. Single-cell suspensions from intestinal tissues were obtained by incubating adjacent non-malignant tissue for 30 min at 37°C with shaking in stripping buffer (1 mM EDTA, 1 mM DTT, and 5% FCS) to remove the epithelial layer. Supernatants were then discarded. Remaining tissues were then mechanically dissociated with a sterile scalpel. The lamina propria fraction was obtained by incubating the dissociated tissues for 1 hour at 37°C with shaking in 2 mg/ml collagenase D (Roche), 0.1 mg/ml DNase I (Sigma), and 1 mg/ml of Trypsin Inhibitor

(Gibco) digestion solution. Remaining tissues were then filtered through a 70-µm cell strainer. All cells were then viably cryopreserved in 90% FBS and 10% DMSO for side-by-side analysis at a later time point. Following thawing and filtration through a 70-µm cell strainer, cells were stained with antibodies for flow cytometry acquisition. Human FcR blocking reagent (Miltenyi) was used at a dilution of 1:20. The anti-hepcidin antibody was labeled with an Alexa Fluor-647 protein labeling kit (Invitrogen). For hepcidin staining, cells were fixed and permeabilized using the eBioscience Foxp3/Transcription factor staining buffer set (eBioscience).

Stained cells were analyzed on a custom-configured 5-laser, 18-color BD LSRFortessa, or sorted on an identically configured FACSAria III (BD) with a 100 µm flow cell. Mouse and human DCs were isolated by gating on live, $CD45^+Lin^-MHCII^+CD11c^+CD64^-$ cells, where "Lin" comprised CD3, CD19 and CD20 in human samples, or CD3, CD19, NK1.1, and CD138 in mice. Macrophages were gated as live, $CD45^+CD64^+F4/80^+$ in mouse samples. In human tissue samples, macrophages were gated as live, $CD45^+Lin^-CD14^+$ or live, $CD45+Lin^-CD16^+$, where "Lin" comprised CD3, CD19 and CD20. Mouse cDC1 were gated as live, $CD45^+CD64^-F4/80^-Lin^-MHCII^+CD11c^+XCR1^{hi}CD172a^{lo}$ and mouse cDC2 as live, $CD45^+CD64^-F4/80^-Lin^-MHCII^+CD11c^+XCR1^{lo}CD172a^{hi}$, where "Lin" comprised CD3, CD19, NK1.1, and CD138. Neutrophils were gated as live, $CD45^+CD11b^+Ly6G^+$. T cells were gated as live, $CD45^+CD3^+$, and B cells were gated as live, $CD45^+CD19^+$. The following antibodies were used for flow cytometry and sorting:

Antigen	Fluorochrome	Source	Clone	Species	Staining concentration (in µg/ml)
CD3	PerCP-Cy5.5	eBioscience	145-2C11	Mouse	1
CD4	BV605	Biolegend	GK1.5	Mouse	1
CD5	PerCP-Cy5.5	Biolegend	53-7.3	Mouse	1
CD8	PerCP-Cy5.5	Biolegend	53-6.7	Mouse	1
B220	APC-e780	eBioscience	RA3-6B2	Mouse	1
CD11b	APC-e780 or PE-TexasRed	eBioscience	M1/70	Mouse	1
CD11c	APC-e780 or PE-Cy7	Biolegend	N418	Mouse	1
CD103	FITC	Biolegend	2E7	Mouse	2.5
MHCII	APC	eBioscience	M5/114.15.2	Mouse	1
CD64	BV421	Biolegend	X54-5/7.1	Mouse	1
Ly6G	BUV395	BD	1A8	Mouse	1
FoxP3	PE	eBioscience	FJK-16S	Mouse	1
Tbet	BV421	Biolegend	4B10	Mouse	1
IFNγ	PE-Cy7	eBioscience	XMG1.2	Mouse	0.5
RORγt	PE-e610	eBioscience	B2D	Mouse	1
IL22	APC	eBioscience	IL22JOP	Mouse	1
CD90.2	AF700	Biolegend	30-H12	Mouse	2.5
CD127	PE-Cy7	Biolegend	A7R34	Mouse	1
F4/80	AF700	Biolegend	BM8	Mouse	2.5
XCR1	FITC	Biolegend	ZET	Mouse	5

CD172a	PE-Dazzle594	Biolegend	P84	Mouse	1
CD19	PerCP-Cy5.5	eBioscience	eBio1D3	Mouse	1
NK1.1	PerCP-Cy5.5	eBioscience	PK136	Mouse	1
CD138	PerCP-Cy5.5	Biolegend	281-2	Mouse	1
IL17A	BUV395	eBioscience	TC11-18H10.1	Mouse	1
CD45	BV605 or BV785	Biolegend	30-F11	Mouse	1
CD16/32	–	BD	2.4G2	Mouse	2.5
CD45	BV605	Biolegend	HI30	Human	0.25
CD14	AF700	Biolegend	M5E2	Human	2.5
CD16	BV421	Biolegend	3G8	Human	0.4
HLA-DR	APC-e780	eBioscience	LN3	Human	0.12
CD3	PerCP-Cy5.5	Biolegend	UCHT1	Human	0.5
CD19	PerCP-Cy5.5	Biolegend	SJ25C1	Human	0.25
CD20	PerCP-Cy5.5	Biolegend	2H7	Human	0.25
CD11c	BV711	Biolegend	3.9	Human	0.5
Hepcidin	AF647	Amgen	2.7	Human	2

Intestinal iron imaging

Ceca from DSS-treated mice were excised, fat was carefully removed, and cecal content was removed by flushing extensively with 25% (V/V) OCT compound. Samples were frozen in 100% OCT and 5-µm sections were cut. A Teledyne Cetac Technologies LSX-213 G2+ laser ablation system was connected to an Agilent Technologies 8900 ICP-MS by Tygon® tubing. A flow of 0.75 liters/min He gas carried ablated material from the ablation chamber and was mixed with 0.55 liters/min Ar via a y-piece prior to the ICP-MS. A 50-µm diameter laser beam was rastered at a speed of 100 µm/s. Laser fluence and repetition rate was optimized for ablation of the tissue whilst preventing ablation of the underlying glass slide (0.05 J/cm^2, 20 Hz). A 3.0 ml/min flow of H_2 in the ICP-MS octopole reaction system (ORS®) was used to minimize interferences and improve signal stability. Following LA-ICP-MS imaging, ablation line-specific .csv files generated by the MassHunter Workstation software (Agilent) were read into RStudio (version 0.98.1102), and counts/second (CPS) values for each element were coerced into a list of m by n element-specific matrices, where m = number of ablation lines and n = number of ablation spots per ablation line. For each matrix, row-specific gas blank CPS medians (which here corresponded to the median values in pixels (ablation spots) collected during the first 10 s of each ablation line) were subtracted from each pixel in the corresponding row. Gas-blank corrected matrices were converted to the RasterStack format using the R package 'raster', where each $X_{m\ x\ n}$ matrix was converted to a RasterLayer with spatial extent ($x_{min} = 0$, $x_{max} = n$, $y_{min} = 0$, $y_{max} = m$). ICP-MS imaging was repeated in two independent laboratories, and equivalent results were observed. For matched hematoxylin and eosin staining, 5-µm serial sections were cut and stained according to manufacturer recommendations using an H&E staining kit (Abcam).

Bone marrow-derived dendritic cell assays

Bone marrow-derived DCs were generated by culturing bone marrow cells in the presence of 20 ng/ml GM-CSF for 8–10 days. Culture media was replaced with fresh media every 3 days. Frequencies of $CD11c^+$ cells were ≥95%. All cell incubations were performed at 37°C and 5% CO2. For TLR, cytokine, and heat-killed *E. coli* stimulations, the stimulus was applied for 24 hours before RNA isolation. For live *E. coli* stimulation, bacteria were added (MOI=50) for 2 hours and then washed away and killed by the addition of gentamicin. Heat-killed bacteria were added at a concentration corresponding to MOI=50. All cytokines were added at a concentration of 20 ng/ml, and TLR ligands were added at 100 ng/ml (LPS, Pam3CSK4), 500 ng/ml (flagellin), or 10 μg/ml (CpG). All TLR ligands were purchased from Invivogen, except LPS (Sigma, E. coli O111:B4, IEX-purified).

Ex vivo cell stimulation

Mouse spleens were chopped into small pieces and digested for 30 min at 37°C with shaking in digestion buffer (1 mg/ml Collagenase D, 20 μg/ml DNaseI, and 2% FCS in HEPES-buffered RPMI 1640), and passed through a 70-μm cell strainer. Erythrocytes were lysed in ACK lysis buffer. $CD11c^+$ cells were pre-selected with magnetic beads (Miltenyi), and cDC2s were sorted and cultured for 16 hours in myeloid culture media (RPMI/Pen/Strep/10%FCS). 2×10^5 cells per well were stimulated in 1 ml with media alone, or media supplemented with either 500 ng/ml LPS (Sigma, E. coli O111:B4, IEX-purified) or heat-killed *E. coli* equivalent to 5×10^8 CFU/ml.

Hepcidin protein analyses

Tissues from naïve- or DSS-treated C57BL/6 mouse tissues were homogenized and hepcidin protein was determined by a quantitative ELISA assay (Intrinsic Lifesciences).

Gene expression profiling and quantitative real-time PCR

RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA isolated from tissues of DSS-treated animals was further purified by precipitating in 2.5 M Lithium chloride on ice for 30 minutes, centrifuging at 14,000 × g for 30 min at 4°C, washing sequentially in 100% and 70% ethanol, and resuspending in purified water. cDNA was generated using Superscript reverse transcriptase II (Invitrogen). Quantitative real-time PCR was performed using SYBR green chemistry (Invitrogen) or TaqMan on the ABI 7500 real-time PCR system (Applied Biosystems). For qPCR measurements, each condition was compared to the mock-treated group or the $Myd88^{-/-}$ group by an unpaired two-tailed Student's *t*-test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). Mouse *Hamp* qPCR primers were purchased from ThermoFisher (reference # Mm00519025_m1). Samples were normalized to either *Actb* (Figs. 1D, 3A, S1B, and S2) or *Hprt1* (all other figures), and displayed as fold change over PBS-treated BMDCs or naive mice. For microarray analysis, BMDCs in antibiotic-free media were treated with *E. coli* (MOI=50) or an equivalent volume of PBS for 2 hours. Cells were washed extensively and plated in media containing gentamicin (100 μg/ml). After 4 subsequent days of culture, cells were lysed directly in TRIzol. RNA was isolated, amplified, reverse-transcribed to cDNA and hybridized to an Affymetrix GeneChip (Mouse Gene 1.0ST). Relative expression data was normalized by Z-score transformation.

RNA sequencing

RNAseq library preparation and sequencing were performed by the Genomics and Epigenomics Core Facility at Weill Cornell Medicine. Libraries were prepared from sorted DCs using the Clontech SMARTer® Ultra® Low Input RNA Kit V4 (Clontech Laboratories), and were sequenced on an Illumina HiSeq 4000 instrument, producing 50-bp single-end reads. Reads were demultiplexed using Illumina CASAVA v1.8.2 and adapters were trimmed using FLEXBAR v2.4 (*25*). Reads were aligned to the GRCm38/mm10 mouse genome using STAR v2.3.0 (*26*), and aligned reads were counted at the gene level using the R package Rsubread (*27*). Differential expression and principal components analysis were performed using DESeq2 v1.22.2 (*28*), using only genes having 50 or more counts in at least two samples. Differential expression p-values were corrected for multiple comparisons using a previously described method (*29*) using a false discovery rate of 0.1. PCA plots were generated using the 500 genes with the highest variance after applying the DESeq2 varianceStabilizingTranformation function. Heatmaps were produced using the Morpeus software (https://software.broadinstitute.org/morpheus/).

Colon tissue fractionation for RNA extraction

Colon tissue was removed, cleaned from remaining fat tissue, and opened longitudinally before being washed in ice-cold PBS. Dissociation of epithelial cells was performed by incubation on a shaker at 37°C in PBS containing 2% FBS, 5 mM EDTA, and 1 mM DTT two times for 20 min. After each step, samples were vortexed and the epithelial cell fraction was passed through a 100-µm filter and collected. Afterwards, remaining tissue was chopped into small pieces and enzymatic digestion was performed on a shaker at 37°C for 40 min in RPMI 1640 media containing 5% FBS, 0.4 U/ml dispase, 1 mg/ml collagenase III, and 20 µg/ml DNaseI. Dissociated leukocytes were passed through a 70-µm filter and collected. Remaining tissue (stromal fraction) was washed twice in ice-cold PBS and then homogenized in Trizol. Collected epithelial cells and leukocytes were further enriched by a 20%/40% or 40%/80% Percoll gradient, respectively, before homogenization in Trizol.

Fecal 16S and *Bifidobacteria* colonization analyses

16S rRNA gene sequencing methods were adapted from the methods developed for the NIH-Human Microbiome Project and the Earth Microbiome Project. Briefly, bacterial genomic DNA was extracted using MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories). The 16S rDNA V4 region was amplified by PCR and sequenced in the MiSeq platform (Illumina) using the 2 × 250 bp paired-end protocol. The 16S rRNA gene pipeline data incorporates phylogenetic and alignment-based approaches to maximize data resolution. The read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090, allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at the first base with Q5. In addition, a quality filter was applied to the resulting merged reads and reads with >0.05 expected errors were discarded. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm44. OTUs were mapped to an optimized version of the SILVA Database containing only the 16S V4 region to determine taxonomies. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A rarefied OTU table

from the output files generated in the previous two steps was used for downstream analyses of α-diversity, β-diversity, and phylogenetic trends. Differential abundance testing was performed using DESeq2 with default parameters after exporting the data using the phyloseq function phyloseq_to_deseq2. The resulting p-values were corrected for multiple comparisons. Significant clustering of samples by genotype was assessed using PERMANOVA (*30*) with 10,000 permutations, as implemented in the vegan R package (*31*). For analysis of *Bifidobacteria* colonization by qPCR, fecal DNA was purified using the PowerSoil kit (Qiagen). *Bifidobacteria* was quantified using a species-specific primer pair (F: 5′-CGGGTGAGTAATGCGTGACC-3′, R: 5′-TGATAGGACGCGACCCCA-3′) normalized to universal 16S primers (UniF340: 5′-ACTCCTACGGGAGGCAGCAGT-3′; UniR514: 5′-ATTACCGCGGCTGCTGGC-3′).

Colon tissue bacterial colonization

Mice were given 3.5% DSS for 7 days, and colon tissue was isolated at necropsy and fat was removed. Tissue was cut longitudinally and feces and luminal content was removed by gentle scraping. Tissues were washed 4 times sequentially in ice-cold sterile PBS by vortexing for 30 seconds. Tissues were then weighed and homogenized by bead-beating for 3 minutes at a frequency of 30/s. Tissue homogenate was then plated on aerobic or anaerobic brain heart infusion agar (BHI) (Sigma). Plates were incubated 96 hours at 37°C before counting CFU.

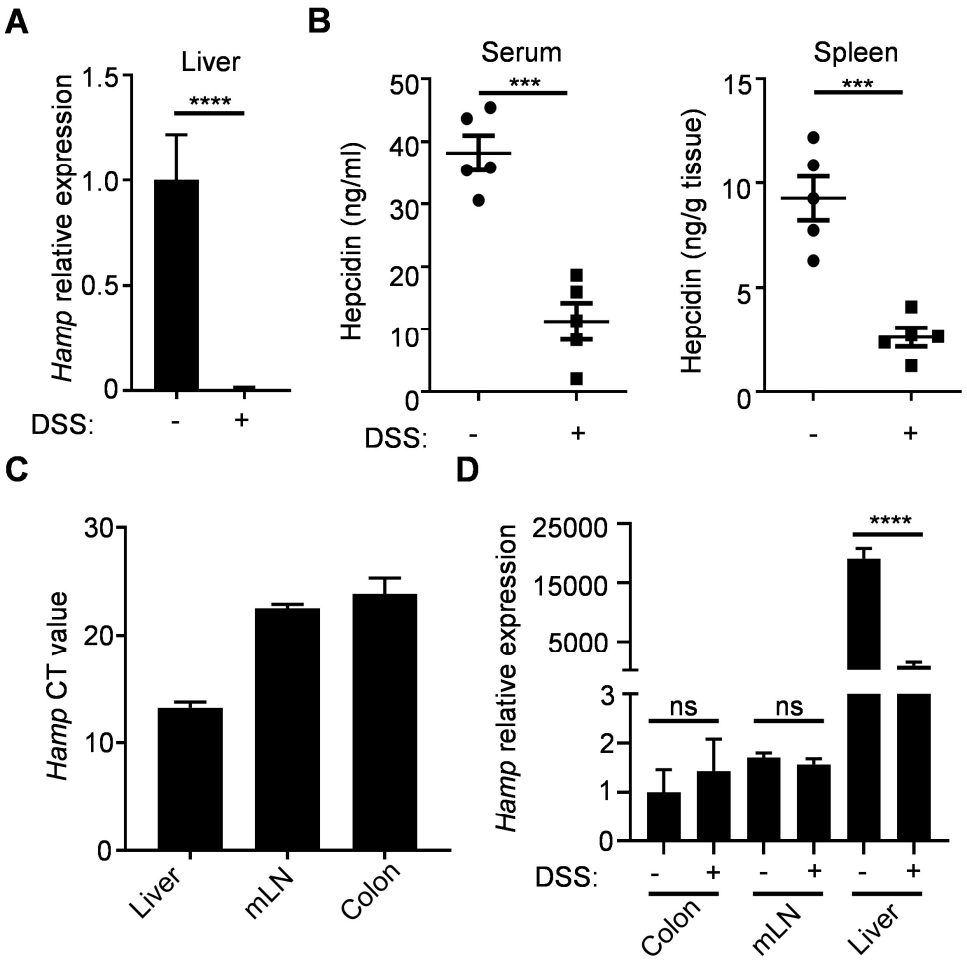


Fig. S1. Hepcidin is expressed in the intestine and associated lymphoid tissues. Mice were given normal drinking water or DSS for 7 days and liver hepcidin expression (A) and systemic protein levels (B) were measured. Hepcidin expression was analyzed in naive tissues (C, D) or DSS-treated mouse tissues (D), and data were analyzed by unpaired two-tailed Student's *t*-test (***:$p<0.001$; ****:$p<0.0001$). In (D), all data are normalized to the naïve colon expression level. Representative data are shown from two independent experiments, each with n=5 per group. Data are shown as the mean ± SEM.

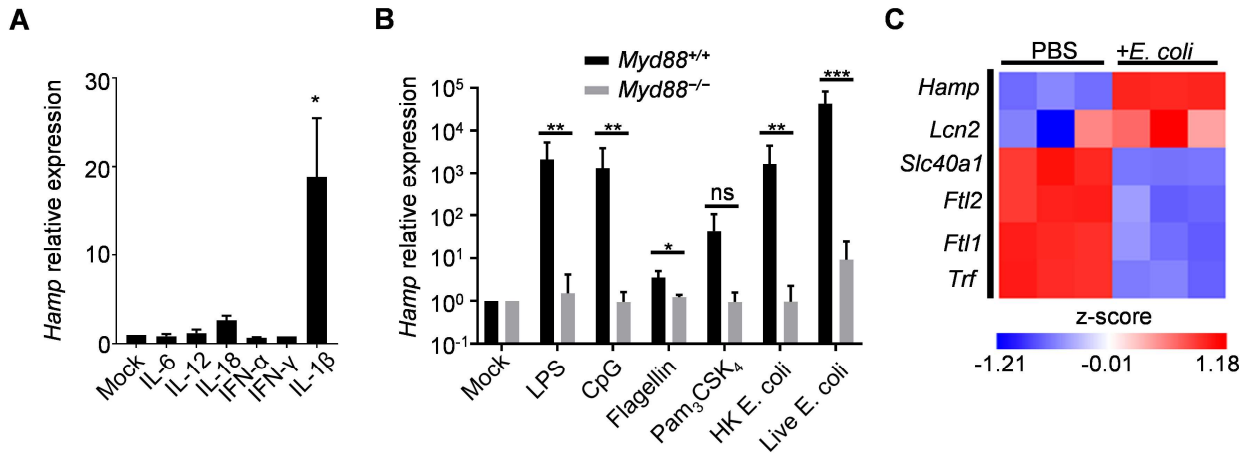


Fig. S2 Dendritic cells express hepcidin in response to microbial stimulation. Bone marrow-derived DCs were exposed to a panel of inflammatory cytokines and *Hamp* expression was determined by qPCR (A). TLR ligands or live or heat-killed bacteria were used to stimulate cells and *Hamp* expression was determined by qPCR (B). Bone marrow-derived DCs exposed to live *E. coli* or PBS were analyzed by an expression microarray (C). Data are shown as the mean ± SEM. Statistics comparing genotypes used unpaired two-tailed Student's *t*-test (*:p<0.05; **:p<0.01; ***:p<0.001)

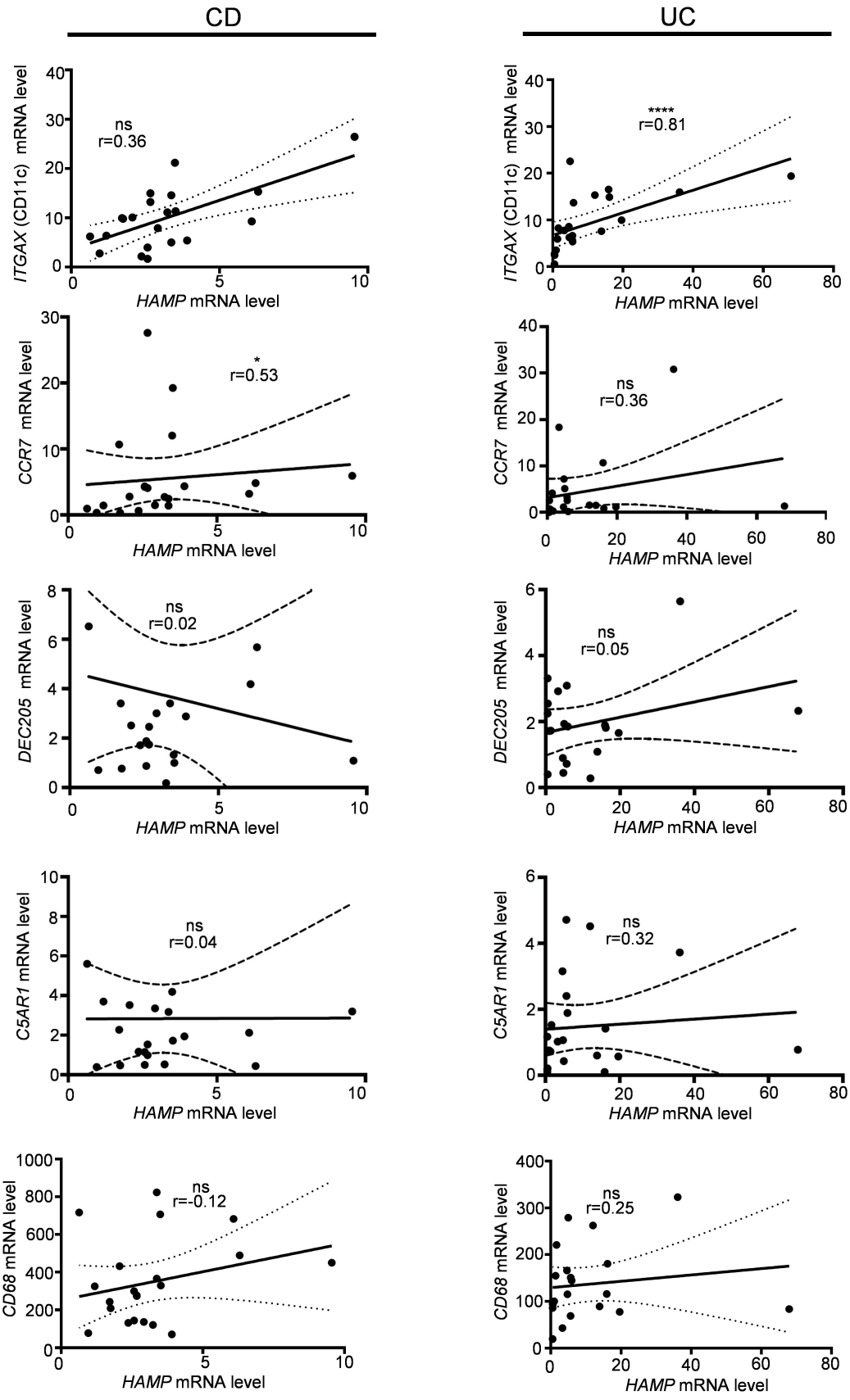

CD
UC
ITGAX (CD11c) mRNA level
HAMP mRNA level
ns
r=0.36
****
r=0.81
CCR7 mRNA level
*
r=0.53
ns
r=0.36
DEC205 mRNA level
ns
r=0.02
ns
r=0.05
C5AR1 mRNA level
ns
r=0.04
ns
r=0.32
CD68 mRNA level
ns
r=-0.12
ns
r=0.25

Fig. S3 Hepcidin expression correlates with DC markers in the intestine of IBD patients. RNA was isolated from affected tissue samples of CD or UC patients, and hepcidin expression was measured by qPCR. Hepcidin (*HAMP*) mRNA levels were analyzed for correlation with DC (*ITGAX, CCR7, DEC205*) and macrophage (*C5AR1, CD68*) marker mRNA levels in patient samples by Spearman correlation analysis (*:$p<0.05$; ****:$p<0.0001$).

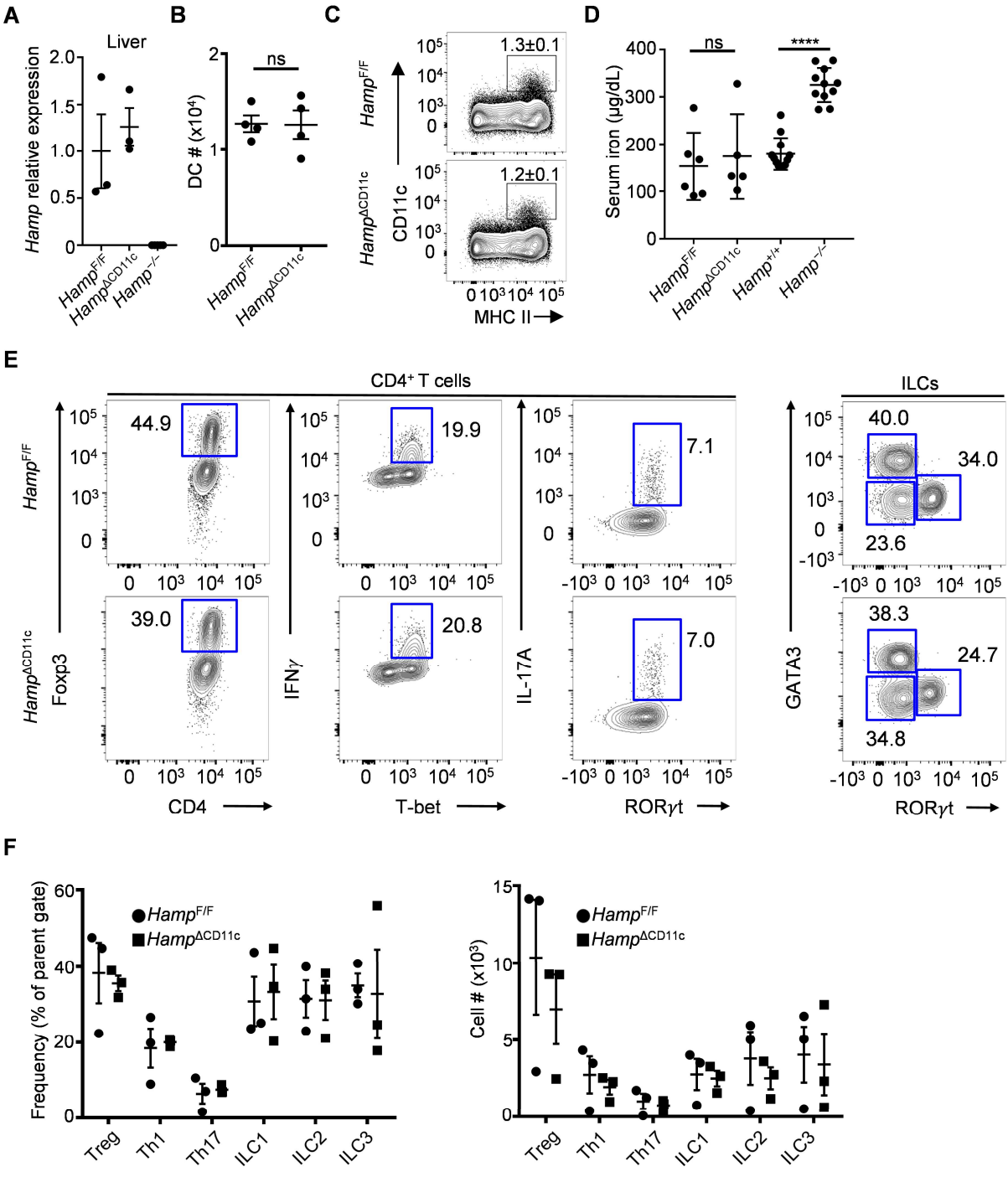
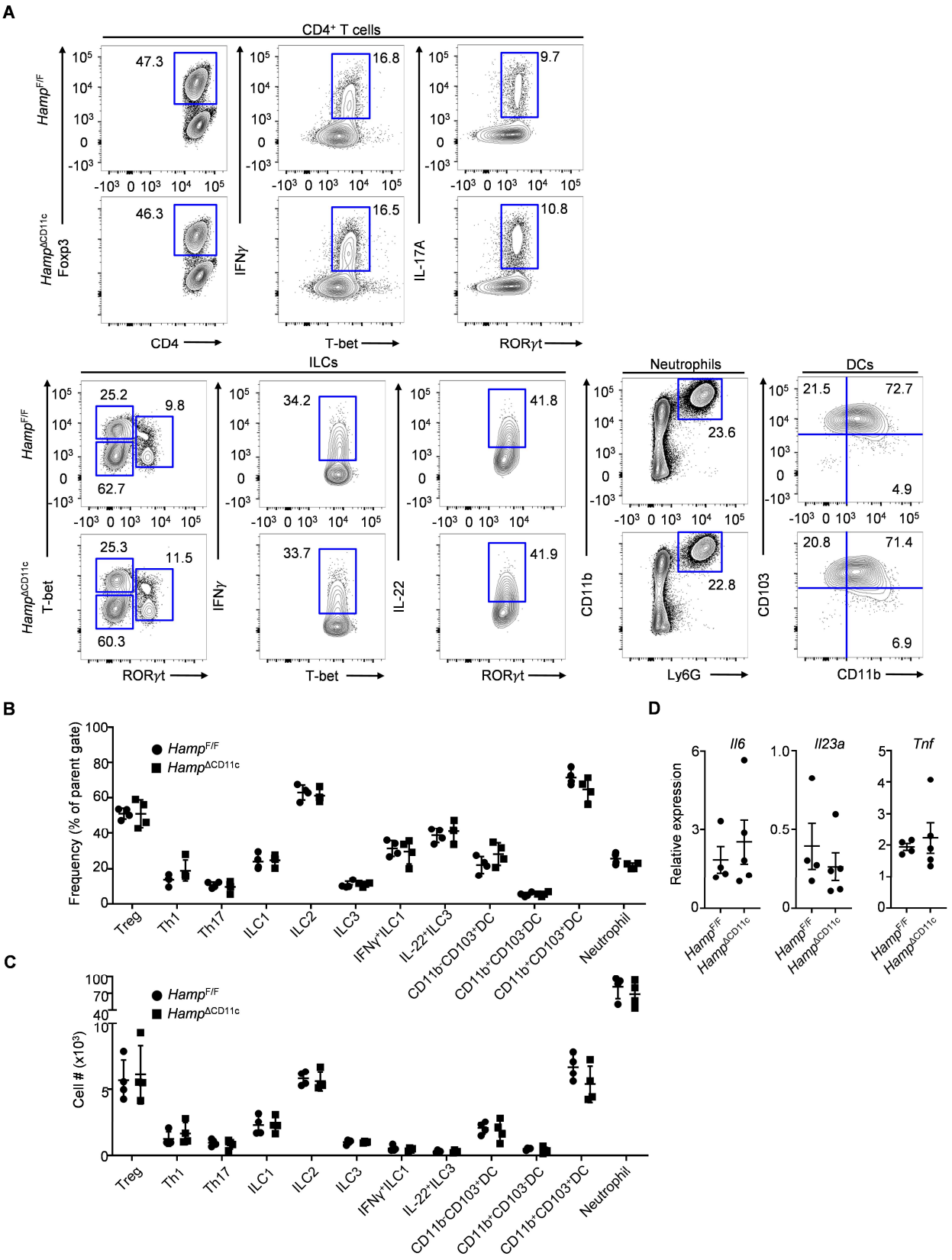


Fig. S4 DC-derived hepcidin does not impact systemic iron or immune cell homeostasis in naïve mice. Hepcidin expression was determined by qPCR in mice exposed to DSS for 7 days (A). Colonic lamina propria DCs were quantified in absolute numbers (B) or as a percentage of live CD45$^+$ cells (C). Colon lamina propria cells were isolated and pre-gated for single, live, CD45$^+$ events, and analyzed by surface markers to elucidate DC, CD4 T cell, and ILC cell populations (C, E). In (C), representative staining is shown and the DC frequency among live CD45$^+$ cells is shown as a pooled mean ± SEM from two independent trials (with n=3 mice per trial). In (E), representative staining is shown for T cells and ILCs. Serum iron was measured by

a ferrozine-based colorimetric assay (D). Cell subset frequencies and total numbers (F) are shown from one representative experiment (of two independent trials) with n=3 mice. Cell populations among the two genotypes were compared by an unpaired two-tailed Student's *t*-test, and no significant differences were found (using p=0.05 as a cutoff). Serum iron levels were compared by an unpaired two-tailed Student's *t*-test between control mice and knockouts (****:p<0.0001).



1 **Fig. S5 DC-derived hepcidin does not impact innate or adaptive immunity during intestinal**

damage and inflammation. Mice were given 3.5% DSS for 7 days, and colon lamina propria lymphocytes were analyzed by flow cytometry, pre-gating on live, single, $CD45^+$ events. Representative gating is shown in (A). Cell subset frequencies (B) and total numbers (C) are shown from one representative experiment (of two independent trials) with n=4 individual mice. Cell populations among the two genotypes were compared by an unpaired two-tailed Student's *t*-test, and no significant differences were found (using p=0.05 as a cutoff). Macrophages were also sorted from littermate mice given 3.5% DSS for 7 days, and analyzed for inflammatory gene expression by qPCR (D). Expression was normalized to the marker gene *Hprt1* and was compared between the two genotypes by an unpaired two-tailed Student's *t*-test, and no significant differences were found (using p=0.05 as a cutoff). Data shown are representative of two independent experiments with n=3-5 mice per group.

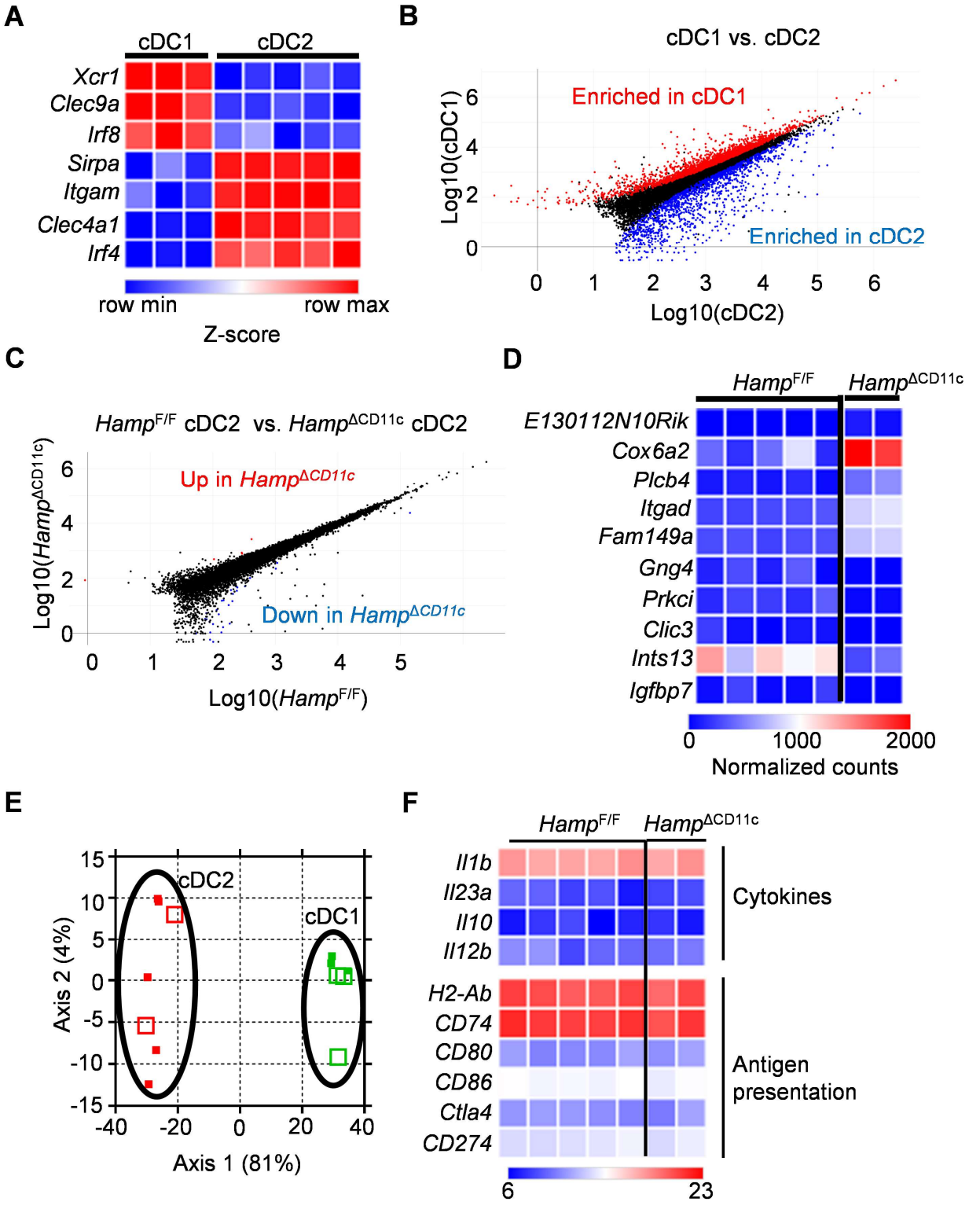


Fig. S6 Comparison of gene expression in DC subsets during intestinal inflammation. cDC1s and cDC2s were sorted from colon lamina propria of control *Hamp*$^{F/F}$ mice (n=3 for cDC1 and n=5 for cDC2) and littermate *Hamp*$^{\Delta CD11c}$ mice (n=3 for cDC1 and n=2 for cDC2) exposed to 3.5% DSS for 7 days, transcription was analyzed globally by RNAseq, and samples

were analyzed for differentially-expressed genes between DC subsets. Canonical cDC1 and cDC2 marker genes were significantly different in agreement with previous reports (A). Differentially expressed genes between cDC1s and cDC2 from *Hamp*$^{F/F}$ mice are highlighted in (B). Differentially expressed genes between cDC2s from *Hamp*$^{F/F}$ and littermate *Hamp*$^{\Delta CD11c}$ mice are highlighted in (C). Five genes were found to be significantly upregulated in *Hamp*$^{\Delta CD11c}$ cDC2s versus *Hamp*$^{F/F}$ cDC2s. These genes plus the top five significantly downregulated genes in *Hamp*$^{\Delta CD11c}$ (aside from *Hamp)* are shown in (D). Principal components analysis is shown (E; *Hamp*$^{F/F}$ cDC1s: Green squares, filled; *Hamp*$^{\Delta CD11c}$ cDC1s: Green squares, unfilled; *Hamp*$^{F/F}$ cDC2s: Red squares, filled; *Hamp*$^{\Delta CD11c}$ cDC2s: Red squares, unfilled). Transcript levels of selected genes are shown for cDC2s plotted as Log2(normalized counts) (F). Z-scores of Log2(normalized counts), normalized by row, are shown in (A), whereas Log10(normalized counts) are shown in (B, C), and normalized counts are shown in (D).

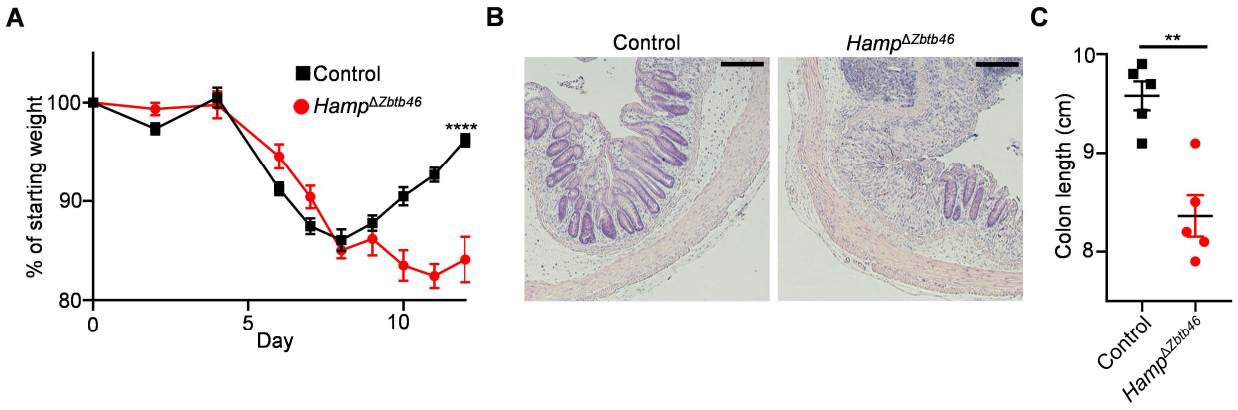


Fig. S7 DC-derived hepcidin is essential for mucosal healing after intestinal inflammation. Mice were given DSS in drinking water for 7 days, and DSS-induced disease and recovery was monitored by weight loss (A), H&E staining of distal colon (B), and colon shortening (C). Data in (A-C) are representative of two independent trials with n=5 per group. Data in (C) were analyzed by unpaired two-tailed Student's *t*-test. In (A), weights at sacrifice, normalized to starting weight, were analyzed by unpaired two-tailed Student's *t*-test. For all statistical tests, **:p<0.01; ****:p<0.0001.

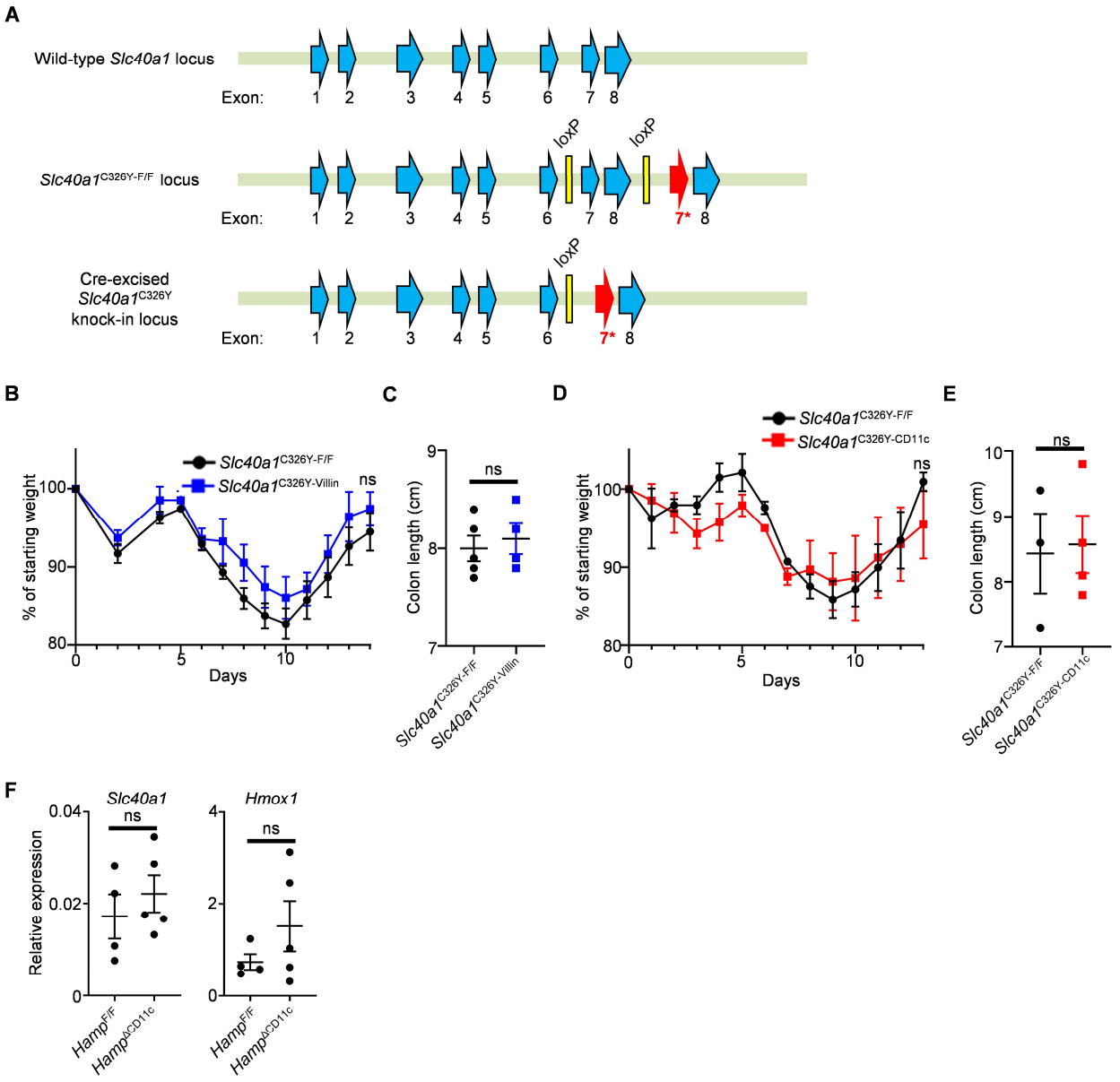


Fig. S8 Hepcidin does not act on DCs or intestinal epithelial cells to promote mucosal healing. The genetic structure of the *Slc40a1*$^{C326Y\text{-floxed}}$ mouse strain is illustrated (A). Mice were given DSS in drinking water for 7 days and disease was monitored by weight development and colon shortening (B-E). Macrophages were sorted from colon lamina propria of mice exposed to 3.5% DSS for 7 days, and transcription of iron-related genes was analyzed by qPCR (F). Data in (C, E and F) were analyzed by unpaired two-tailed Student's *t*-test. In (B) and (D), weights at sacrifice, normalized to starting weight, were analyzed by unpaired two-tailed Student's T test. Data are shown as the mean ± SEM. Data in (B-F) are representative of at least two independent experiments with (n=3-5) per group.

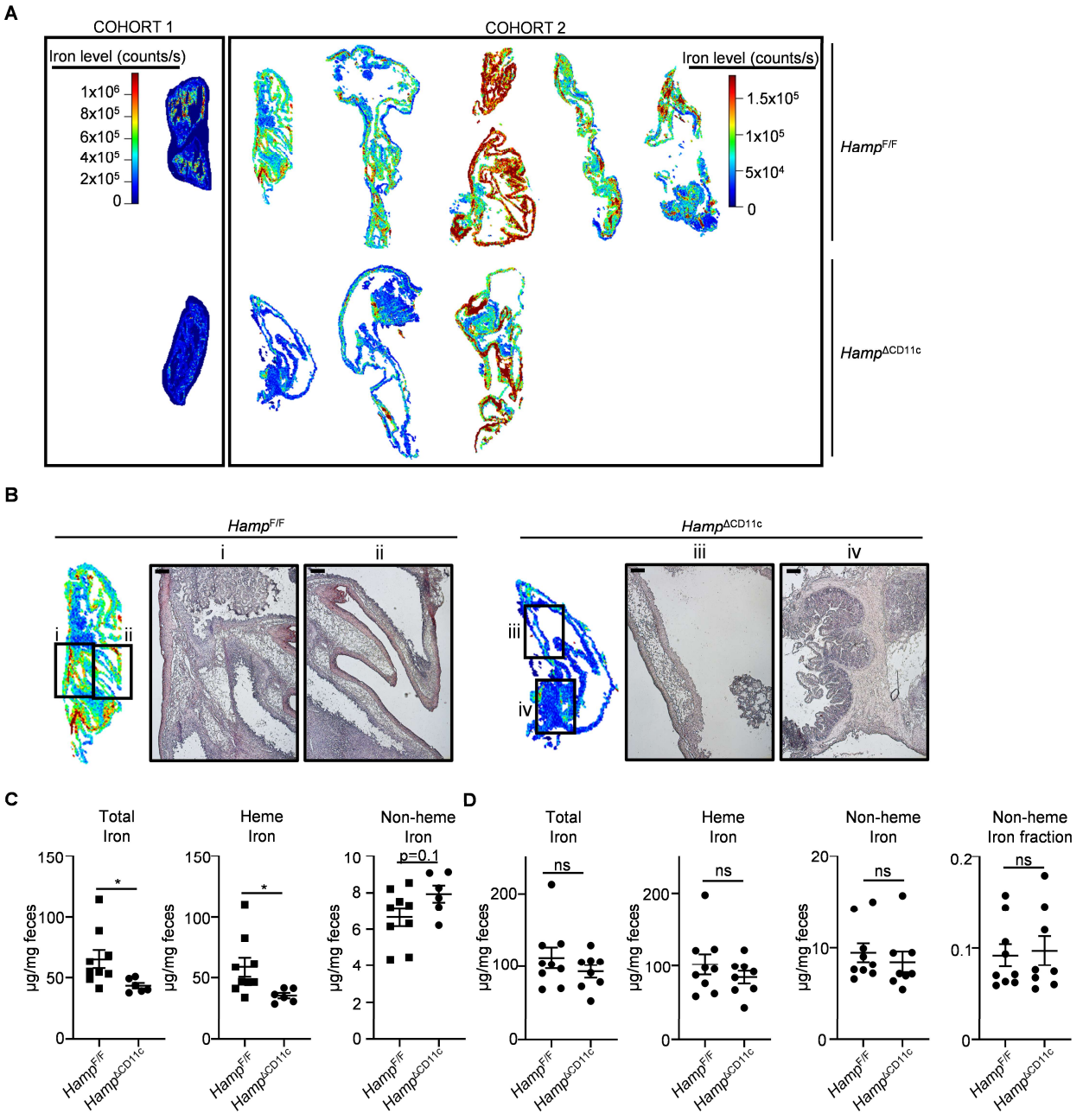


Fig. S9 DC-derived hepcidin modulates colon iron localization in DSS-treated mice. Two independent cohorts (n=1-5 per group) of mice were given 3.5% DSS for 7 days, ceca were collected and thoroughly flushed, and iron levels were determined by quantitative mass spectrometry (A). Each individual *Hamp*$^{\Delta CD11c}$ cecum is displayed in the bottom row, and *Hamp*$^{F/F}$ ceca are displayed in the top row. Representative H&E staining, confirming that iron signals co-localize with cecal epithelium and lamina propria but not lumen, are shown in expanded panels in (B), with labels (i)-(iv) corresponding to inset labels. Scale bars in (B) are 200 µm. Colon contents were collected from either mice given 3.5% DSS for 7 days (C) or naïve mice (D). Non-heme-bound iron and heme-bound iron were measured, and total iron and non-heme-iron fraction were calculated. All experiments utilized littermate *Hamp*$^{\Delta CD11c}$ and control

Hamp$^{F/F}$ mice. Data in (C,D) are shown as mean ± SEM, and are shown pooled from two independent experiments (n=3-5 per group). Data were analyzed by unpaired two-tailed Student's *t*-test (*:$p<0.05$).

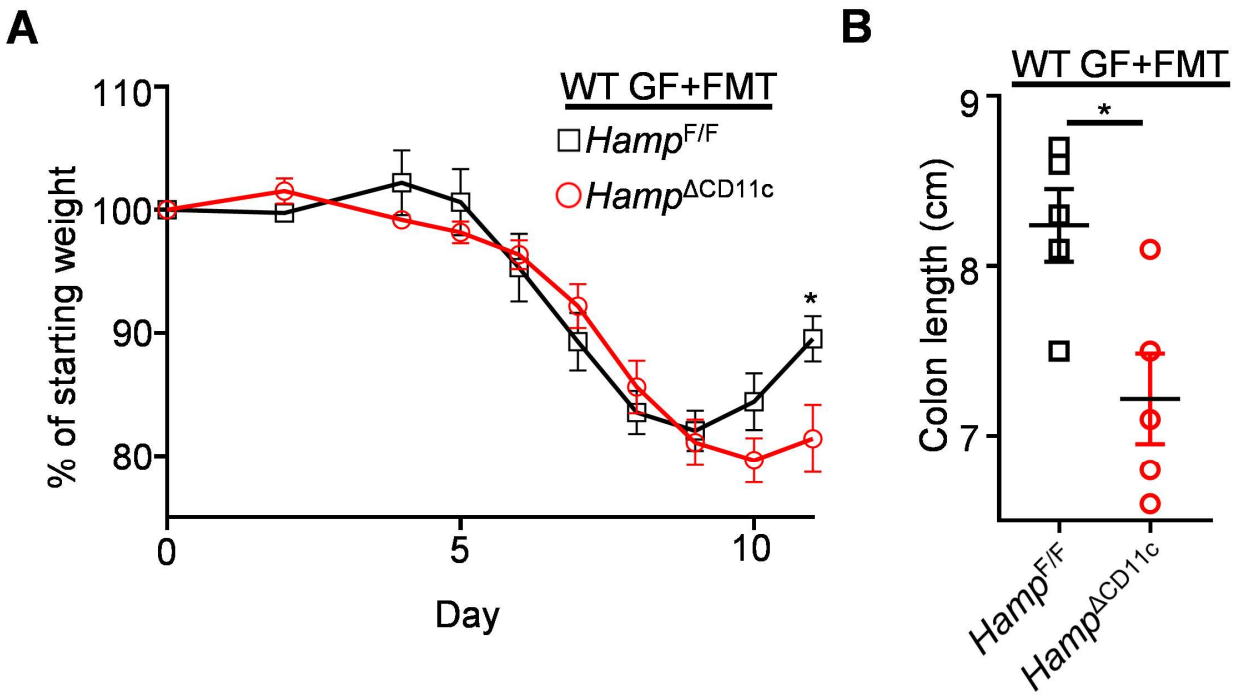


Fig. S10 Mice lacking DC-derived hepcidin exhibit a transmissible dysbiosis that inhibits mucosal healing. Fecal microbiota from *Hamp*$^{\Delta CD11c}$ or littermate control mice were transferred to germ-free wild-type C57BL/6 recipients by a single gavage. DSS administration (for 7 days) was then initiated 6 weeks after transfer. Representative weight loss (A) and colon length data (B) were quantified. Weights at sacrifice, normalized to starting weight, as well as colon length, were analyzed by unpaired two-tailed Student's *t*-test (*:$p<0.05$). Data are shown as mean ± SEM and are representative of three independent experiments with n=4-5 per group.

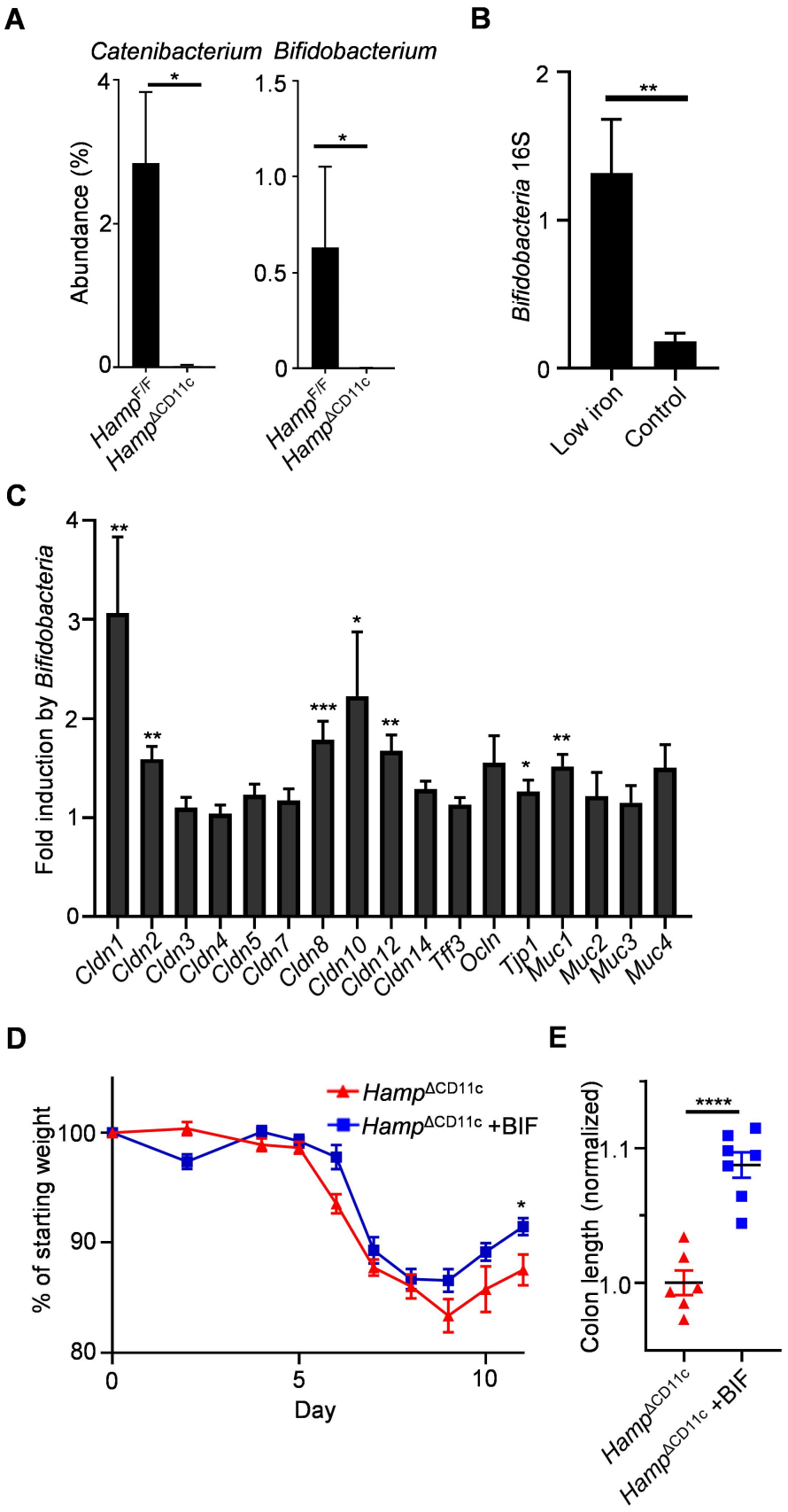


Fig. S11 DC-derived hepcidin promotes colonization with iron-sensitive and tissue-

protective *Bifidobacteria*. Fecal microbiota of naïve mice were analyzed by 16S rRNA gene sequencing and differential abundance testing was performed. Abundance for the only two differentially-abundant genera, *Catenibacterium* and *Bifidobacterium*, is displayed as a percentage of total bacterial 16S (A). Unpaired two-tailed Student's *t*-test was used to compare samples (*:$p<0.05$). Wild-type mice were placed on iron-modified diets at 3.5 weeks of age and maintained for 4 weeks, and *Bifidobacteria* colonization was determined by qPCR and normalized to day 0 of the modified diet administration (B). Data shown are representative of two independent experiments with n=5 per group, and data were analyzed by the Mann–Whitney *U* test (**:$p<0.01$). In (C), mice were given *Bifidobacteria* by oral gavage or no treatment 3X/week for two weeks and gene expression in colon tissue was examined by qPCR. Data from two independent experiments, each n=5, were pooled, and gene expression was normalized to the "no treatment" group. Unpaired two-tailed Student's t-test was used to compare the *Bifidobacteria*-treated group to the control group (*:$p<0.05$; **:$p<0.01$; ***:$p<0.001$). Mice were given *Bifidobacteria* by oral gavage or no treatment 3X/week for two weeks and then given DSS for 7 days, and disease was monitored by weight loss (D) and colon length (E). Data shown are pooled from two independent experiments each with n=3-4 per group. Weights at sacrifice, normalized to starting weight, as well as colon length, were analyzed by unpaired two-tailed Student's *t*-test (*:$p<0.05$; ****:$p<0.0001$). Colon length was also normalized to the "no treatment" group within each independent experiment. All data shown are depicted as mean ± SEM.

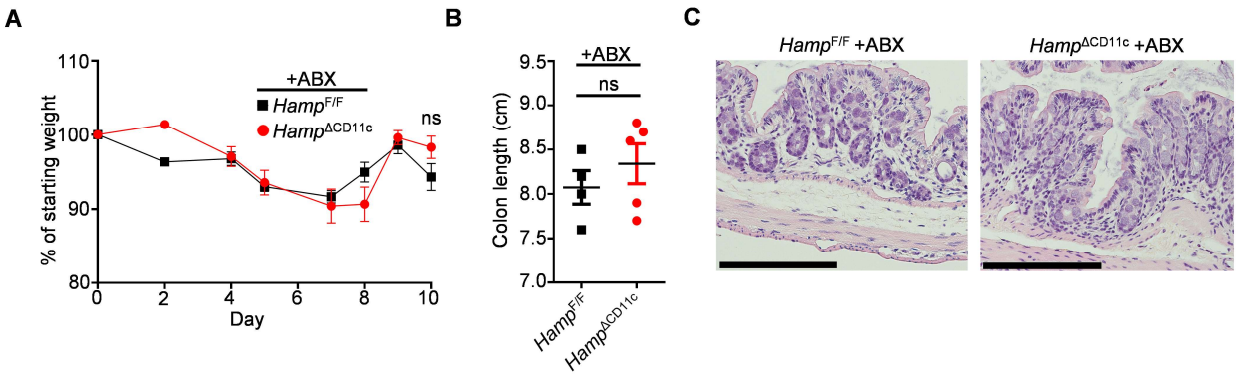


Fig. S12 Antibiotic treatment normalizes mucosal healing in mice lacking DC-derived hepcidin. Mice on a broad-spectrum antibiotic cocktail were given DSS for 7 days, and disease was monitored by weight loss (A), colon length (B), and colon histology (C). Scale bars are 200 μm. All data are representative of four to five individual mice (n=4-5) replicated in three independent experiments. Data are shown as the mean ± SEM.

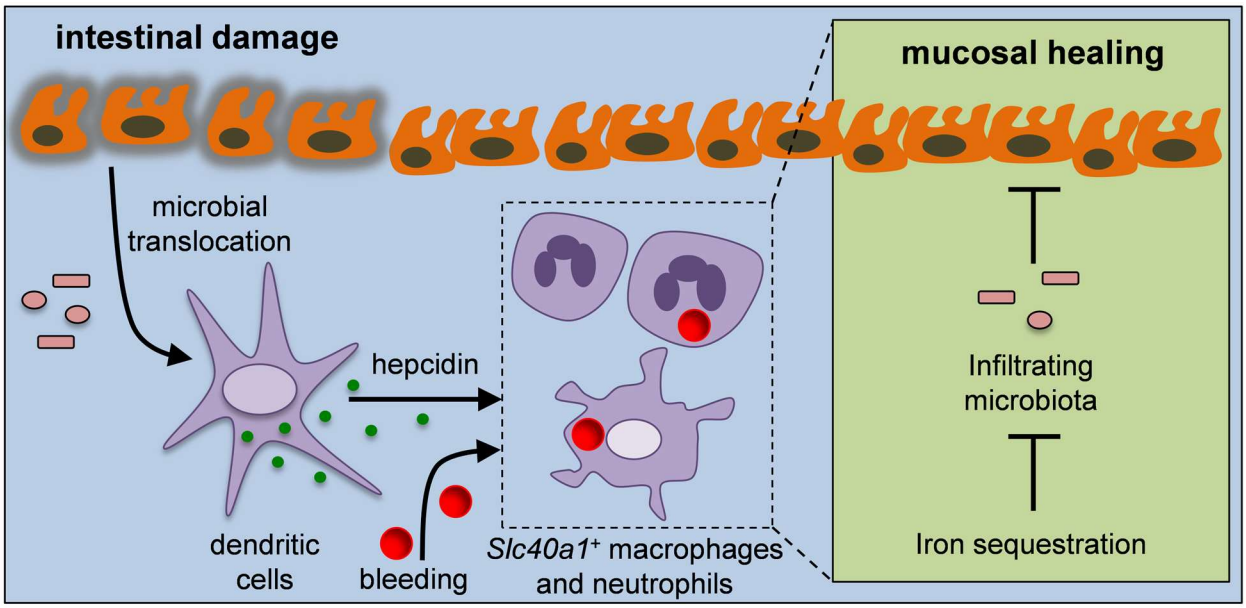


Fig. S13 DC-derived hepcidin sequesters iron from the microbiota to promote mucosal healing. Here we mechanistically define a novel pathway of mucosal healing in the mammalian intestine. In brief, upon intestinal damage there is intestinal bleeding and an influx of microbes that activate DCs to produce hepcidin. DC-derived hepcidin targets ferroportin-expressing macrophages and neutrophils in the large intestine in order to block iron release into the extracellular compartment. This subsequently limits tissue-infiltrating microbes following intestinal damage, supporting optimal mucosal healing in the gut.

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