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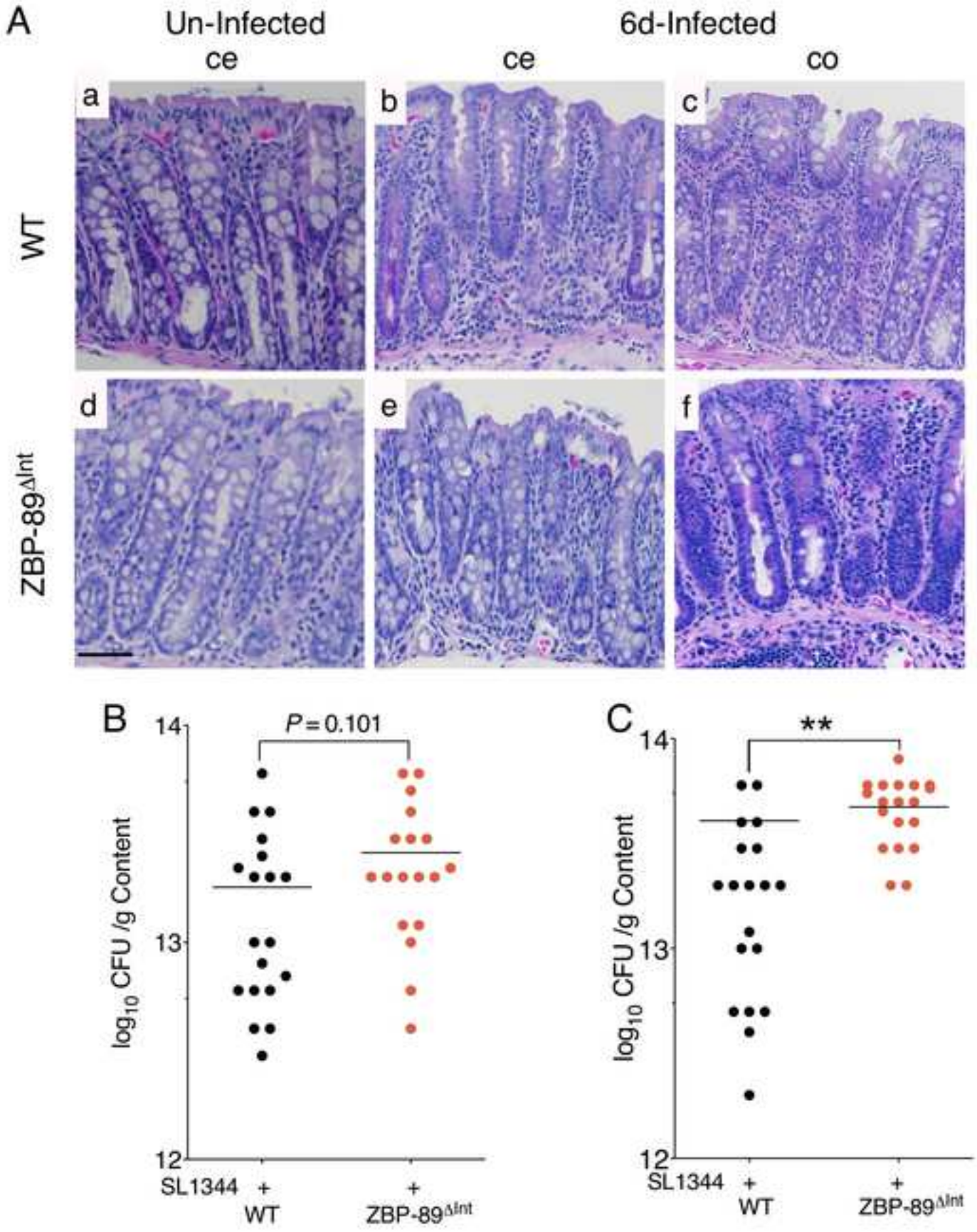

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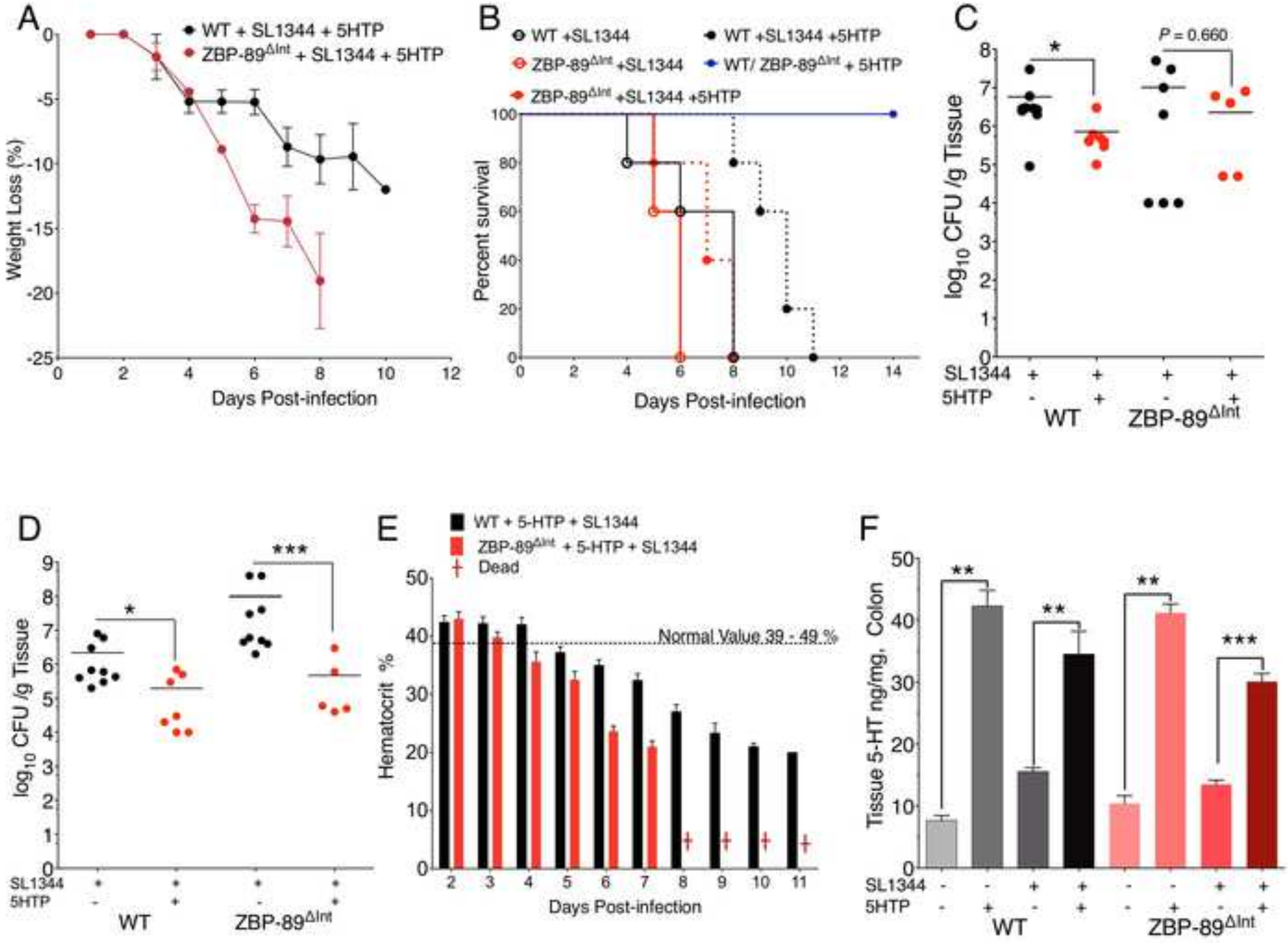
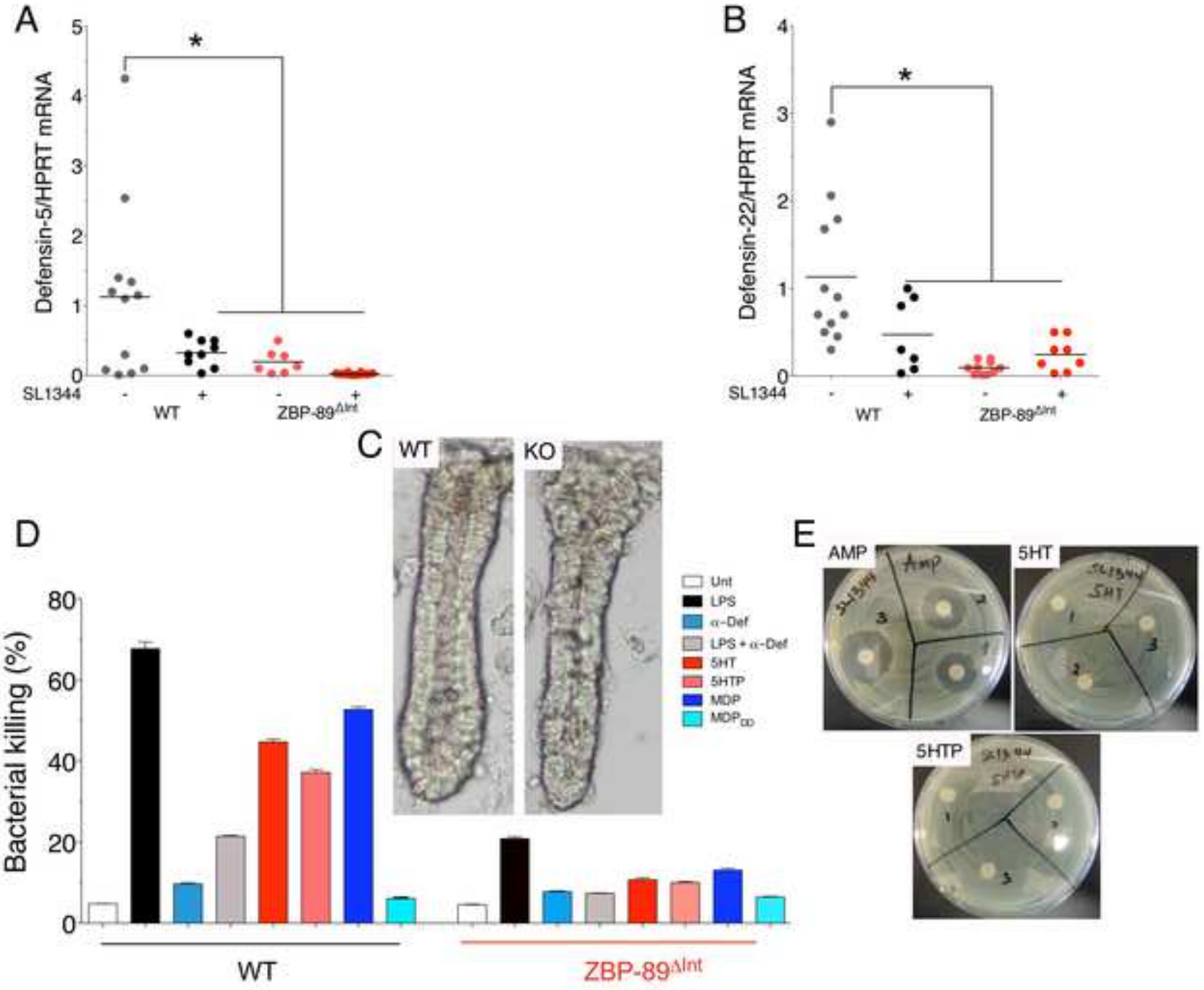
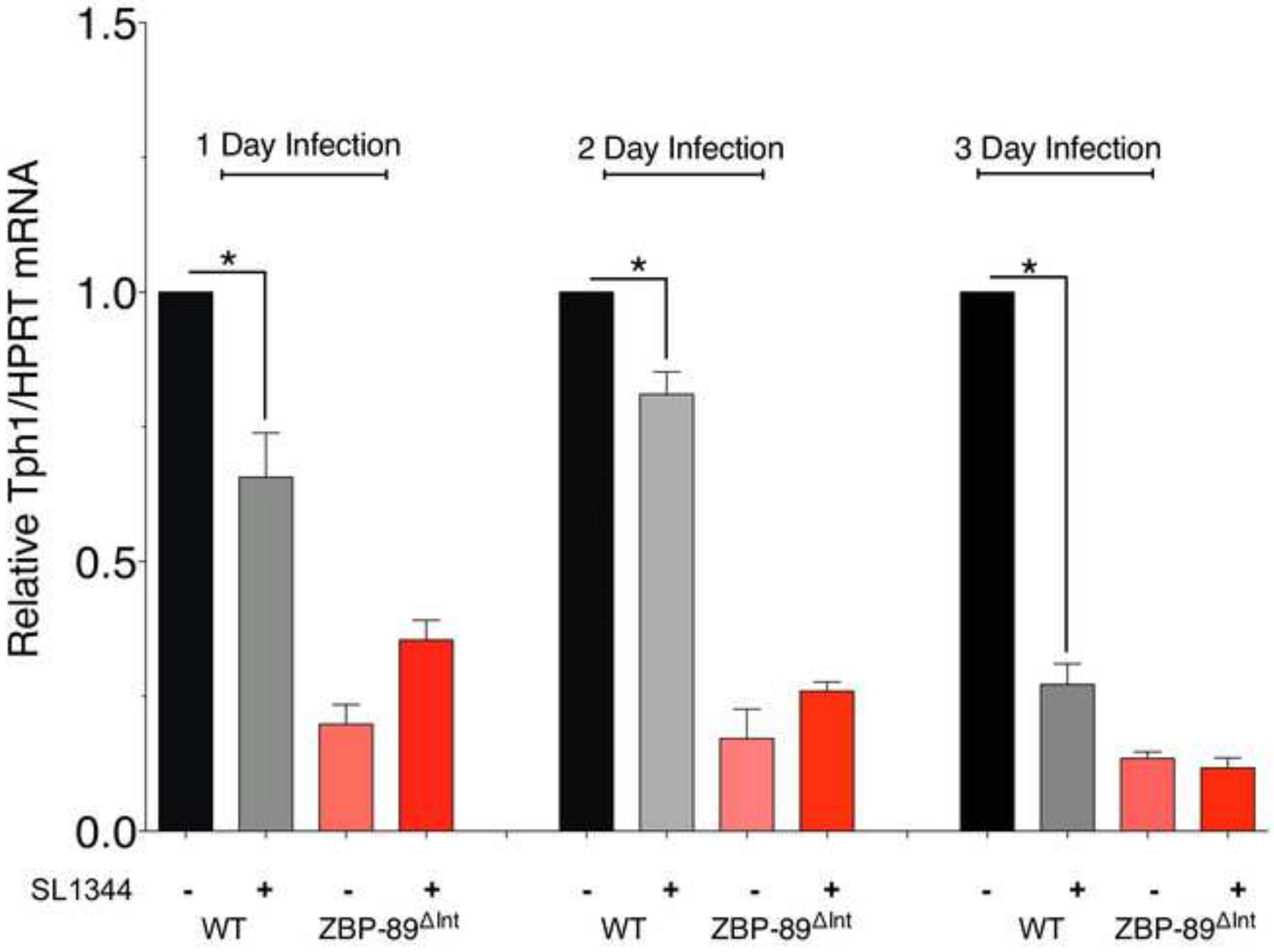


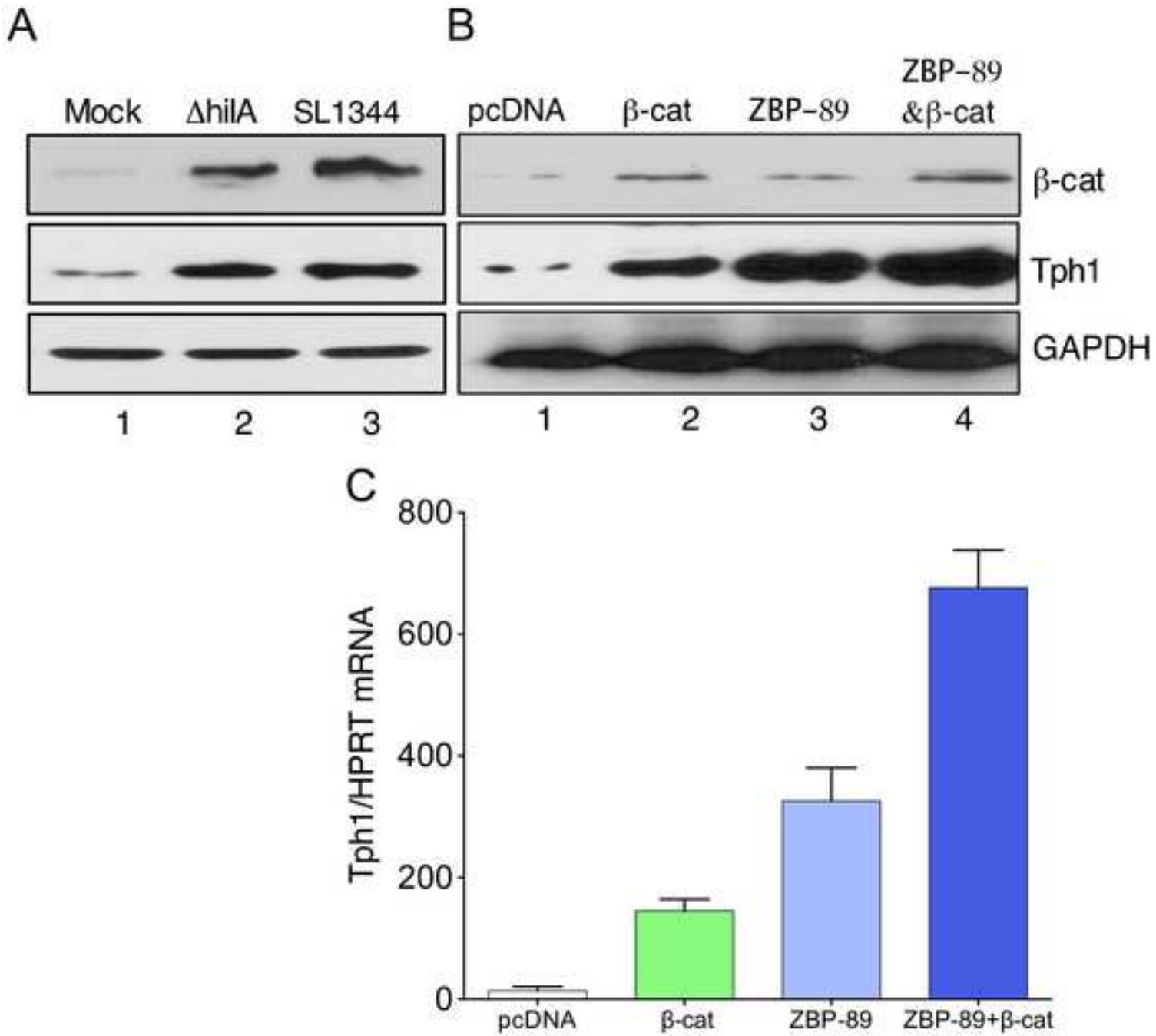
Suppl. Figure 3



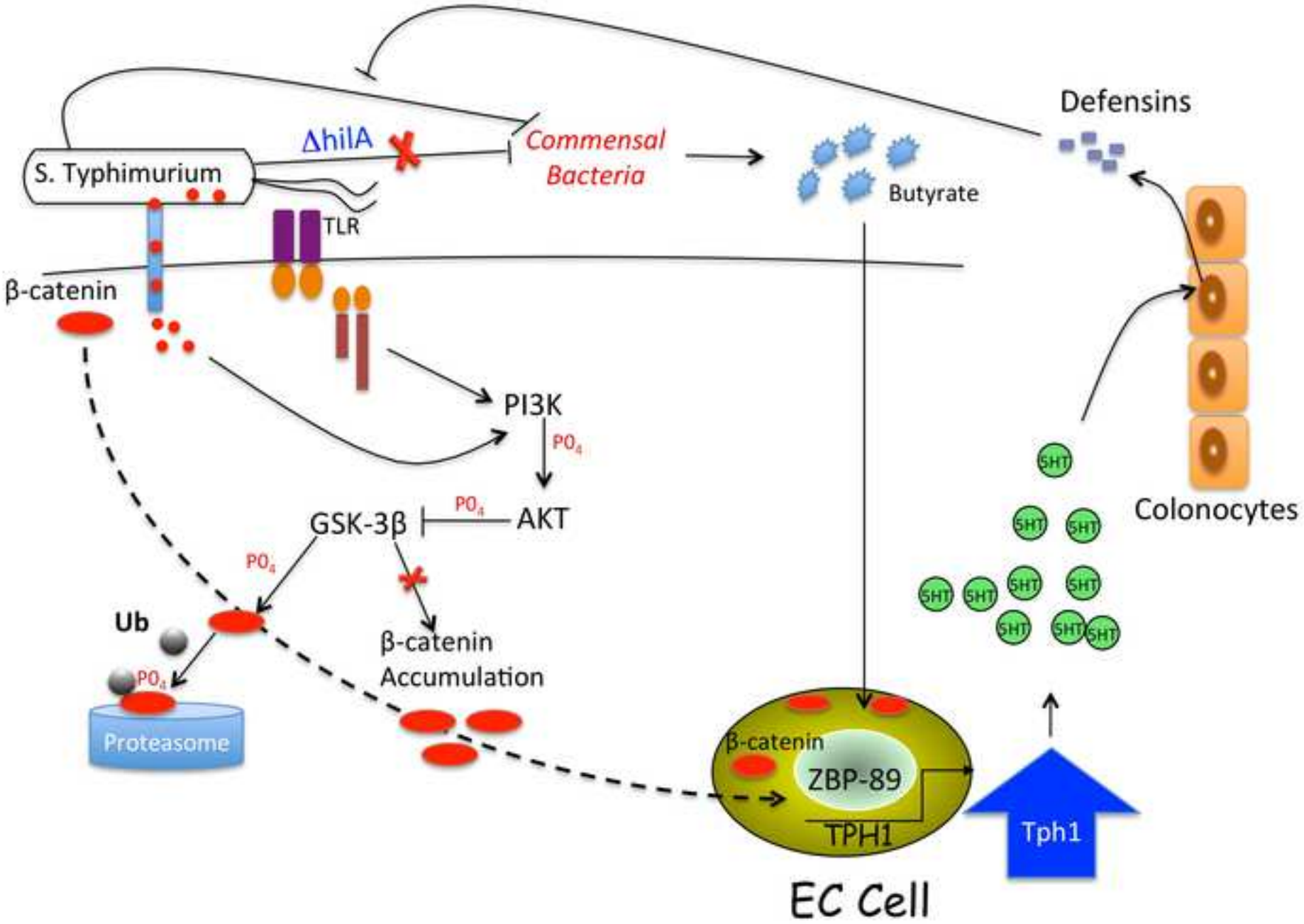
Suppl. Figure 4



Suppl. Figure 5



Suppl. Figure 6



Supplemental Methods

ES Cell Karyotyping and Chimera Genotyping

The University of Michigan Transgenic Animal Model Core electroporated Bruce4 (C57BL/6 derived) embryonic stem (ES) cells with the pLM10-iKO-FinalTV targeting vector (Intrexon Corporation, Blacksburg, VA). G418 was used to select ES cells expressing the targeting vector. Homologous recombination occurred in 18 of 480 ES clones (3.75% success rate). After ES cell karyotyping, 4 clones were deemed suitable for injection into blastocysts. Twenty-four chimeras were generated (16 males and 8 females) from a total of 431 blastocysts injected. Germline transmission of the targeted allele was achieved by breeding male chimeras (derived from ES clone 6073) to C57BL/6^{C2J} females. Genomic PCR using the Expand Long Template PCR System (Roche Indianapolis, IN) was performed according to the manufacturer's instructions to genotype the targeted ES cell clones, chimeric founders and progeny. Genotyping primer sequence pairs were:

ZBP89-5124F: AGACCTACGACCCACAGGGTGG;

ZBP89-7050R: GGCTTCTCTCCACTGTGAGTT.

The wild-type allele generated a 596 bp fragment; whereas, the targeted allele corresponded to a 1926 bp fragment. RT-PCR was performed using Platinum Taq DNA Polymerase (Invitrogen). Mouse ZBP-89 primer pairs included

Exon 6-F: $^{5'}$GGCATGTCTTCATTCATAGAGG;

Exon 8-R: $^{5'}$CTCATACCACATTCATCACAGC.

Five samples from each genotype were analyzed in triplicate. PCR products were purified and submitted for DNA sequencing (University of Michigan DNA Sequencing Core).

RT-qPCR Primer Pairs:

Total RNA was isolated from mouse tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA). The samples were treated with DNase 1 (Invitrogen) before cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). HPRT primer pairs were as follows:

F: $^{5'}$AGTCCCAGCGTCGTGATTAGC; R: $^{5'}$ATAGCCCCCCTTGAGCACACAG.

The primers for Tph1 were:

F: $^{5'}$GCAGCATGATCTCGATGTTGTC and

R:$^{5'}$GGGTGACATTCCCCATGAAGAG; and for Tph2 were:

F: $^{5'}$GAGTTGCTCCACGCTTTGC; R: $^{5'}$ACACTCAGTCTACATCCATCCC.

Cytokine mRNA levels were determined by RT-qPCR using the following primer pairs:

IL1β- F: $^{5'}$CAACCAACAAGTGATATTCTCCATG,

R: $^{5'}$GATCCACACTCTCCAGCTGCA;

IFNγ- F: $^{5'}$TCAAGTGGCATAGATGTGGAAGAA,

R: $^{5'}$TGGCTCTGCAGGATTTTCATG;

TNFα- F: $^{5'}$CATCTTCTCAAAATTCGAGTGACAA,

R: $^{5'}$TGGGAGTAGACAAGGTACAACCC;

IL-17A- F: $^{5'}$GCTCCAGAAGGCCCTCAGA;

R: $^{5'}$AGCTTTCCCTCCGCATTGA;

IL-4- F: ^{5}'ACAGGAGAAGGGACGCCAT;

R: 5'GAAGCCCTACAGACGAGCT-CA;

IL-23p19- F: 5'AAGTTCTCTCCTCTTCCCTGTCGC 3';

R: 5'TCTTGTGGAGCAGCAGATGTGAG;

IL-6: F: 5'GAGGATACCACTCCCAACAGACC;

R: 5'AAGTGCATCATCGTTGTTCATACA.

Immunohistochemistry and Epithelial Separation

Colon and cecal samples from each mouse were removed and fixed in 10% neutral buffered formalin solution overnight and then placed in 75% ethanol. Fixed tissues were embedded in paraffin and cut into 5 μm sections. Tissues were de-paraffinized, rehydrated and stained with hematoxylin and eosin. Pathological scores were assigned in a blinded fashion consisting of the presence and extent of inflammation using validated systems [1, 2].

To compare *Tph1* and *Tph2* mRNA gene expression in the epithelium versus the mesenchyme, the small intestine and colon from each mouse was cut longitudinally then placed in cold PBS. The fragments were transferred to a culture dish containing 2 ml of cold Cell-Recovery Solution (BD Biosciences, USA) and incubated for 8 h at 4^{o}C. Intestine and colon tissue sections were shaken by hand in their individual containers to separate epithelial sheets from the intact submucosa, mesenchyme and smooth muscle layers. The mesenchymal sections were rinsed in sterile PBS to remove adherent epithelial cells before processing for RNA analysis.

Immunohistochemistry and Morphometry

Briefly, the scoring system was performed as follows: rare inflammatory cells in the lamina propria = 0, increased inflammation in lamina propria only = 1, extension into the submucosa = 2, transmural extension = 3. Antigen retrieval

was performed by boiling tissue sections in a microwave oven for 10 min with 10 nM sodium citrate buffer (pH 6.0). Nonspecific binding was blocked for 30 min with 20% donkey serum in PBS-T. Tissue sections were incubated at 4°C overnight with rabbit anti-5HT (1:500; ImmunoStar), chicken anti-GFP (1:1000; Abcam), rat anti-E-cadherin (1:500, Invitrogen) or rabbit anti-ZBP-89 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. After washing, the sections were incubated with 1:500 Cy3 donkey anti-rabbit, Alexa 488 donkey anti-rabbit or Alexa 647 rabbit anti-rat (Jackson Immunolabs) for 1 h at room temperature. The nuclei were counterstained with DAPI. Immunohistochemical studies on 5HT-expressing enterochromaffin cells, were performed on formalin-fixed sections. Endogenous peroxide was blocked by incubating with peroxidase-blocking reagent (Abcam) for 15 min. After washing, antigen retrieval was performed. The sections were then incubated with 1% bovine serum albumin in PBS-T to block nonspecific binding. The sections were incubated with rabbit anti 5-HT antibody (1:500; ImmunoStar) for 1 h at room temperature, then with biotinylated goat anti-rabbit IgG (H-L) for 10 min at room temperature, prior to staining with hematoxylin for 1 min. The numbers of 5-HT-expressing enterochromaffin cells were counted blindly and expressed per high power field.

Determination of plasma and tissue 5-HT

The 5HT was quantified in either plasma or whole colon homogenates from the VillinCre x ZBP-89$^{FL/FL}$ mice using an ELISA kit according to the manufacture's protocol (Immunotech, Marseille, France).

DNA Affinity Precipitation (DAPA)

Nuclear extracts from STC-1 cells were prepared using detergent extraction. Biotinylated oligonucleotide probes corresponding to the mouse *Tph1* promoter sequence at -123 bp upstream from the cap site were purchased from Integrated DNA Technologies (Coralville, IA). The sense strand sequence was $^{5'}$CGCGCTGTTCCCG CCCCCTCCTCGCCCTCC and the two base pair mutation introduced into the DNA element site used to disrupt binding was $^{5'}$CGCGCTGTTCCCGCTACCTCCTCGCCCTCC. DNA affinity precipitation (DAPA) was performed by incubating the STC-1 nuclear extracts with the biotinylated probe for 16 h then pulling down protein complexes binding to the biotinylated probe with protein A agarose beads as previously described [3, 4]. The non-biotinylated WT and 2bp mutant competitor oligonucleotides were added at twenty times the molar concentration of the probe prior to adding the probe. The protein complexes were resolved on a 10% SDS-PAGE, transferred to PDVF membrane for western blot analysis with ZBP-89 antibody (Santa Cruz Biotechnology).

Cell Culture and Transient Transfections

Mouse STC-1 enteroendocrine cells were grown in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin plus 100μg/ml streptomycin). For transfection, cells were seeded onto Matrigel-coated plates at a density of $1x10^6$ cells /well in twelve–well plates. After twenty-four hours at 37^oC incubation, plasmids were transfected into STC-1 cells using Lipofectamine 2000 (Invitrogen) reagent. To knockdown ZBP-89 levels, STC-1 cells were

plated (200,000 cells/mL) onto 6 well plates in complete DMEM media for 24h prior to replacing the media with serum-free Opti-MEM (Invitrogen) for 1h. Duplex small interfering RNA (100 nM siRNA, Santa Cruz Biotechnology) against ZBP-89 or a scrambled sequence or a mouse β-catenin expression vector in pcDNA was transfected into cells using Fugene (Promega) according to the manufacturer's protocol. siRNA duplexes were removed after 6h and replaced with DMEM media for 24-48h. Cells were harvested and whole cell extracts prepared for immunoblot analysis. The time course of *Tph1* mRNA and protein was performed in three separate experiments using STC-1 cells. The cells were serum-starved for 2 h prior to treatment with 2.5 mM butyrate for up to 48 h then were collected at the indicated times for total RNA or protein analysis. *Tph1* mRNA levels were quantified by RT-qPCR. In a separate experiment, β-catenin (using 1:500 dilution of rabbit anti-mouse, Abcam), Tph1 and ZBP-89 protein levels were determined from the same gel by western blot using GAPDH as a loading control.

WT^{SL1344} or $ST^{\Delta hilA}$ co-culture with STC-1 cells

STC-1 cells were seeded into 12 well plates at 50-75 % confluency and cultured in serum-free media containing antibiotics for 24 h prior to ST^{SL1344} or $ST^{\Delta hilA}$ inoculation. ST^{SL1344} and $ST^{\Delta hilA}$ were quantified then resuspended in (HBSS+) (Sigma Chemical, St. Louis, MO). The STC-1 cells were washed with PBS and maintained in a serum-free and antibiotic-free media for the duration of the co-culture experiment with ST^{SL1344} or $ST^{\Delta hilA}$.

Tissue Protein Analysis by Western Blot

Whole cell protein extracts were prepared from mouse tissues using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL), supplemented with Complete Mini Protease Inhibitors (Roche) according to the manufacturer's recommendations. Lysates were solubilized in Laemmli sample buffer (Bio-Rad Laboratories) at 95°C for 5 min, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). The membrane was blocked using Detector Block (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and probed with rabbit polyclonal ZBP-89 (1:1000, Santa Cruz Biotechnology) or mouse GAPDH antibodies (Chemicon, Temecula, CA).

Salmonella Strains and Gavage

Salmonella enteric serovar Typhimurium SL1344 is a streptomycin-resistant wild-type strain enteric pathogen. *S. typhimurium* strain VV341 is an isogenic mutant of the SL1344 strain and contains a deletion of the *hilA* locus located within the *Salmonella* Pathogenicity Island 1 (*SPI-1*), which regulates mucosal invasion (gift from Dr. Beth A. McCormick from University of Massachusetts Medical School, Worcester MA) [5]. GFP-tagged *S. typhimurium* SL1344 (FPV25) was constructed by inserting an *Eco*RI-*Hind*III fragment containing the promoterless *gfp*mut3 locus [6] into plasmid pED350 [7]. All bacteria were grown in a non-agitated microaerophilic culture by inoculating in 10 ml of Luria-Bertani (LB) broth then incubating overnight (~16 h) at 37^{o}C as previously described [8]. Bacterial supernatants of *S. typhimurium* were obtained by incubating bacteria in HBSS containing Ca^{2+} and Mg^{2+} supplemented with 10 mM HEPES, pH 7.4 (HBSS+) (Sigma Chemical, St. Louis, MO) for 1 h at 37^{o}C.

Oral gavage with 7.5 mg/mouse of streptomycin diluted in 100 μl of sterile HBSS+ was performed according to Grassl et al [9]. Twenty-four hours after streptomycin treatment, the mice were gavaged with ~1x 10^7 colony-forming units (CFU) of *S. typhimurium SL1344* or the Δ*hilA SPI-1* mutant suspended in 100 μl of sterile HBSS or with 100 μl of sterile HBSS alone. The mice were allowed free access to food and water during the entire experimental protocol. At the indicated times after inoculation, mice were euthanized and their ileum, cecum, colon, spleen and liver were removed for analysis. A mortality curve was generated by comparing the percent of eighteen WT versus twelve ZBP-$89^{\Delta Int}$ mice that survived infection with 10^7 CFU WT *S. typhimurium SL1344* strain. The mice were weighed daily. Fecal blood was determined by hemoccult analysis and hematocrits were determined on blood collected at the time of euthanization. Mice showing respiratory distress after gavage or significant body weight loss (10% or more) during the course of the experiment were euthanized.

Quantitative Bacterial Cultures

Mouse liver and spleen were collected at the time of necropsy and processed using quantitative cultures as previously described [10]. The ileum, cecum and colon contents were weighed, homogenized in sterile saline, serially diluted in saline and then cultured on LB agar with 100 μg/ml streptomycin.

Tissue Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined on tissue scrapings from the cecum and colon collected at the time of euthanization and homogenized in normal saline. The amount of peroxide converted was normalized to protein [11].

Leukocyte cell counts

Leukocyte counts were determined from the blood samples collected at the time of euthanization. A thin film of blood was made on a microscopic slide, allowed to dry and then stained with Diff-Quik Stain (modified Giemsa) (Polysciences, Inc. Eppelheim, Germany).

Short Chain Fatty Acid Determination

For SCFA determination, fecal matter from the colon was weighed then flash-frozen in liquid nitrogen. Frozen samples were transferred to the University of Michigan Metabolic Core for extraction according to a previously published protocol [12], and then gas chromatography-mass spectrometry analysis was performed (GC-MS, Agilent 6890, Wilmington, DE). The fecal samples were thawed in the extraction solvent comprised of 3% 1M HCl (v/v) then homogenized by a probe sonicator. After centrifugation to remove cellular debris, the supernatant was transferred to a new eppendorf tube for extraction with diethyl ether. After phase separation, the upper ether layer was transferred to an autosampler vial for GC-MS analysis using ^{13}C-labeled internal standards. GC separation was performed using a ZB-Wax plus column (0.25µm x 0.25mm x 30m). A single quadrupole mass spectrometer (Agilent, 5973 inert MSD) was used to identify and quantify the SCFAs using the Agilent Chemstation software. The acquired GC-MS data was processed on the Agilent Mass Hunter quantitative analysis software (version B.04). Retention times (Rts) and characteristic masses of SCFAs were used for peak identification and quantification. Internal standards and external calibration curves were used to

calculate the concentrations in the samples. Absolute quantities of SCFAs (acetate, butyrate and propionate) in the mouse fecal samples were normalized to the sample mass.

Colonic crypt isolation

Adult mouse colon was dissected and rinsed with ice-cold PBS. Crypts were isolated by chelation as described [13] with some modifications. Briefly, 6 cm of the colon was flushed with ice-cold PBS and cut longitudinally and then subsequently into 1 mm fragments. The fragments were shaken in a 50-ml conical tube with 10 ml of 1 mM EDTA in Ca^{++}- Mg^{++}- free PBS for 5 min. Tissue fragments were resuspended in cold EDTA buffer using a transfer pipette. To isolate intestinal crypts, the tissue fragments were allowed to settle by gravity for 1 min, and then the supernatant was removed to check under a light microscope. Intact crypts were eluted at 5 min intervals, and then pelleted by centrifugation (200g for 3 min at 4^{o}C) to separate crypts from single cells. The buffer was gently aspirated and the pelleted crypts were re-suspended in 100 μL - 500 μL of media containing RSpondin 1 [14]. Crypt viability was verified by trypan blue exclusion.

Bacteria-Killing Assay

Bactericidal assays were performed from crypt-enriched fractions using ~2000 isolated crypts/well suspended in 100 μL of media. Crypts were stimulated with 1 μg/mL of LPS, LPS plus 12 μg of rabbit anti- pan-defensin antibody/μl (gift from Dr. Andy Ouellette, USC), 1 mg/mL MDP, 5HT, 5-HTP, or MDP_{DD} for 30 min at 37^{o}C then removed by centrifugation at 200g for 10 min. Supernatants were

collected and incubated with 1 x 10^3 cfu of SL1344 strain for 1 h at 37^{o}C and then plated in serial dilutions up to 10^{-3}. Plates were incubated overnight at 37^{o}C and bacterial colonies were counted. The percent killing was calculated using the following formula: *Percent killing = {1- (cfu in stimulated samples) x dilution/(cfu in unstimulated control x dilution)} x 100* [15].

5HT / 5HTP Antimicrobial Test

Whatman Filter paper #3 was punched into disks. The disks were autoclaved for 15 min then impregnated with ampicillin (AMP) (0.5 μg; 10 μg; 20 μg), 5HT or 5HTP (0.5 mg/mL; 1 mg/mL; 2 mg/mL). The disks were allowed to air dry. Impregnated disks were placed on *S. typhimurium* a culture plate inoculated with SL1344. Zones of inhibition were recorded after 16-18 h of incubation at 37^{o}C.

Experimental Protocol

5-hydroxytryptophan (5HTP) (Sigma-Aldrich) was administered by oral gavage at a dose of 50 mg/kg twice daily for 8 days starting 2 days before the induction of colitis[16].

Supplemental Table and Figures Legends

Supplemental Table 1: RT-qPCR analysis of inflammatory cytokines in WT and ZBP-89$^{\Delta Int}$ mice +/- *S. typhimurium*

	WT		**ZBP-89$^{\Delta Int}$**	
Cytokine	**----**	***+Sal***	**----**	***+Sal***
IL-1β	1.03± 0.6	34.9± 5.6	1.02± 0.1	41.6± 2.5
INFγ	0.98± 0.1	15.6± 2.6	1.05± 0.1	13.8± 2.2
TNFα	1.1± 0.2	30.2± 3.8	1.05± 0.1	33.4± 2.6
IL-17	0.99± 0.2	14.7± 3.8	1.75± 0.1	15.8± 2.6
IL-4	1.0± 0.5	14.9± 1.9	4.9± 1.0	29.0± 3.0
IL-23p19	1.0± 0.5	13.7± 2.5	3.2± 0.5	17.7± 2.0
IL-6	0.9± 0.1	40.3± 6.0	1.0± 0.1	52.9± 5.8

Cytokine mRNA levels normalized to HPRT mRNA.
n = 10 mice per condition. ***+Sal***= *S. typhimurium*

Supplemental Figure Legends

Suppl. Figure 1

ZBP-89$^{\Delta Int}$ show increased luminal bacterial loads. Streptomycin gavage (7.5 mg/kg) was performed 24h prior to inoculating with 10^7 CFU/mouse of *Salmonella SL1344* (time 0). (**A**) H&E stains of cecum (ce) and colon (co) from WT and ZBP-89$^{\Delta Int}$ mice before (a, d) and after 6 days of infection (b,c,e,f)**.** Quantitative cultures for *Salmonella SL1344* were performed for WT and ZBP-89$^{\Delta Int}$ mice at the time of euthanization and expressed as CFU/g content using a Log_{10} scale on the Y-axis. Quantitative cultures for (**B**) ileum, (**C**) cecum. Each point represents individual mice from two separate experiments for infected WT and ZBP-89$^{\Delta Int}$ mice. ***P* < 0.01.

Suppl. Figure 2

5-HTP pre-treatment improved survival of infected mice. 5-HTP was administered by oral gavage (50 mg/kg) 24h prior to streptomycin pretreatment and 48 h prior to inoculating with 10^7 CFU/mouse of *Salmonella SL1344*. (**A**) Mouse body weights were obtained at the time of euthanization. (**B**) The number of mice remaining was plotted over time as % survival for two experiments. Log-rank test was used to test the statistical significance in survival between the WT, N = 10 per experiment; and the ZBP-89$^{\Delta Int}$ mice, N = 10 per experiment (*P* = 0.0244). There was also statistical significance in survival between the WT mice treated with 5-HTP, N = 10 per experiment and the ZBP-89$^{\Delta Int}$ mice treated with 5-HTP, N = 10 per experiment (*P* = 0.0084). Solid lines = without 5HTP; dotted lines = with 5HTP. Systemic bacterial loads were determined by quantitative cultures of tissue extracts. CFU/g content was determined at the time of euthanization then plotted as the Log_{10} for WT and ZBP-89$^{\Delta Int}$ mice. (**C**) liver, (**D**) spleen, (**E**) *Hematocrits from infected WT and ZBP-89$^{\Delta Int}$ mice*. Hematocrits were determined on blood from mice drawn at the time of euthanization before and after 5-HTP treatment. N = 8 mice per group, (**F**) 5-HT tissue levels after 5-HTP treatment. N= 20 WT and N= 18 ZBP-89$^{\Delta Int}$ mice. Data represent the mean ± SEM for 2 experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Suppl. Figure 3

Reduced defensin mRNA and colonic crypt killing activity. mRNA levels for *defensin* 5/22 in WT (**A**) and ZBP-89$^{\Delta Int}$ mice (**B**) determined 3 days after inoculating with *S. typhimurium*. (**C**) Phase contrast microscopy of crypts isolated from the colons of WT and ZBP-89$^{\Delta Int}$ mice. (**D**) Crypts isolated from the colon were treated with 1 μg/mL of LPS, anti-defensin antibody alone, LPS plus anti-defensin antibody, 1 mg/mL 5HT, 5-HTP, MDP or MDP_{DD}. Secretions were incubated with *S. typhimurium* 1344 to quantify killing activity by quantitative cultures. N= 3 experiments. **P* < 0.05 was considered significant. (**E**) Disk assay for three concentrations of Ampicillin (AMP) (1 = 0.5 μg; 2 = 10 μg; 3 = 20 μg) or

5HT and 5-HTP (1 = 0.5 mg/mL; 2 = 1 mg/mL; 3 = 2 mg/mL) exposed to *S. typhimurium*.

Suppl. Figure 4
S. typhimurium inhibits Tph1 mRNA levels. mRNA levels for *Tph1* were determined at 1, 2 and 3 days after inoculating WT and ZBP-89$^{\Delta Int}$ mice with *S. typhimurium*. The *Tph1* mRNA levels for uninfected WT mice was set to 1.0 and compared to WT infected and ZBP-89$^{\Delta Int}$ uninfected and infected mice. N=12 *P < 0.05 was considered significant.

Suppl. Figure 5
ST^{SL1344} or ST$^{\Delta hilA}$ induce Tph1 and ZBP-89 and/or β-catenin regulate Tph1. (**A**) Western blot analysis of Tph1 protein after co-culture of mock (lane 1) ST$^{\Delta hilA}$ (lane 2) and ST^{SL1344} (lane 3) with STC-1 cells. (**B**) Western blot and (**C**) RT-qPCR analysis of Tph1 protein and endogenous *mTph1* mRNA after transfecting pcDNA (lane 1), β-catenin (β-cat) expression vector (lane 2), ZBP-89 (lane 3) or co-transfecting ZBP-89 and β-catenin expression vectors (lane 4) into STC-1 cells. GAPDH control for Tph1 western blots. N = 3 independent experiments.

Suppl. Figure 6
Proposed model of S. typhimurium regulation of Tph1 gene expression. WT *S typhimurium* blocks induction of 5HT production from the EC cell in part by reducing luminal butyrate levels and subsequently *ZBP-89* expression, which is required for basal and butyrate-induction of *Tph1* gene expression. Suppression of colonic butyrate levels requires the *hilA* invasion locus since butyrate levels remain normal when mice are infected with the *hilA* deletion mutant. Both *S. typhimurium* strains strongly induce *Tph1* gene expression ex vivo through several possible mechanisms, e.g., activation of TLRs expressed on colonocytes including EC cells or virulence factors injected via the type 3 secretory apparatus. Both mechanisms induce βcatenin activity, which can cooperate with ZBP-89 to induce *Tph1* gene expression.

Supplemental References

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