
Figures and figure supplements

NAIP–NLRC4-deficient mice are susceptible to shigellosis

Patrick S Mitchell et al

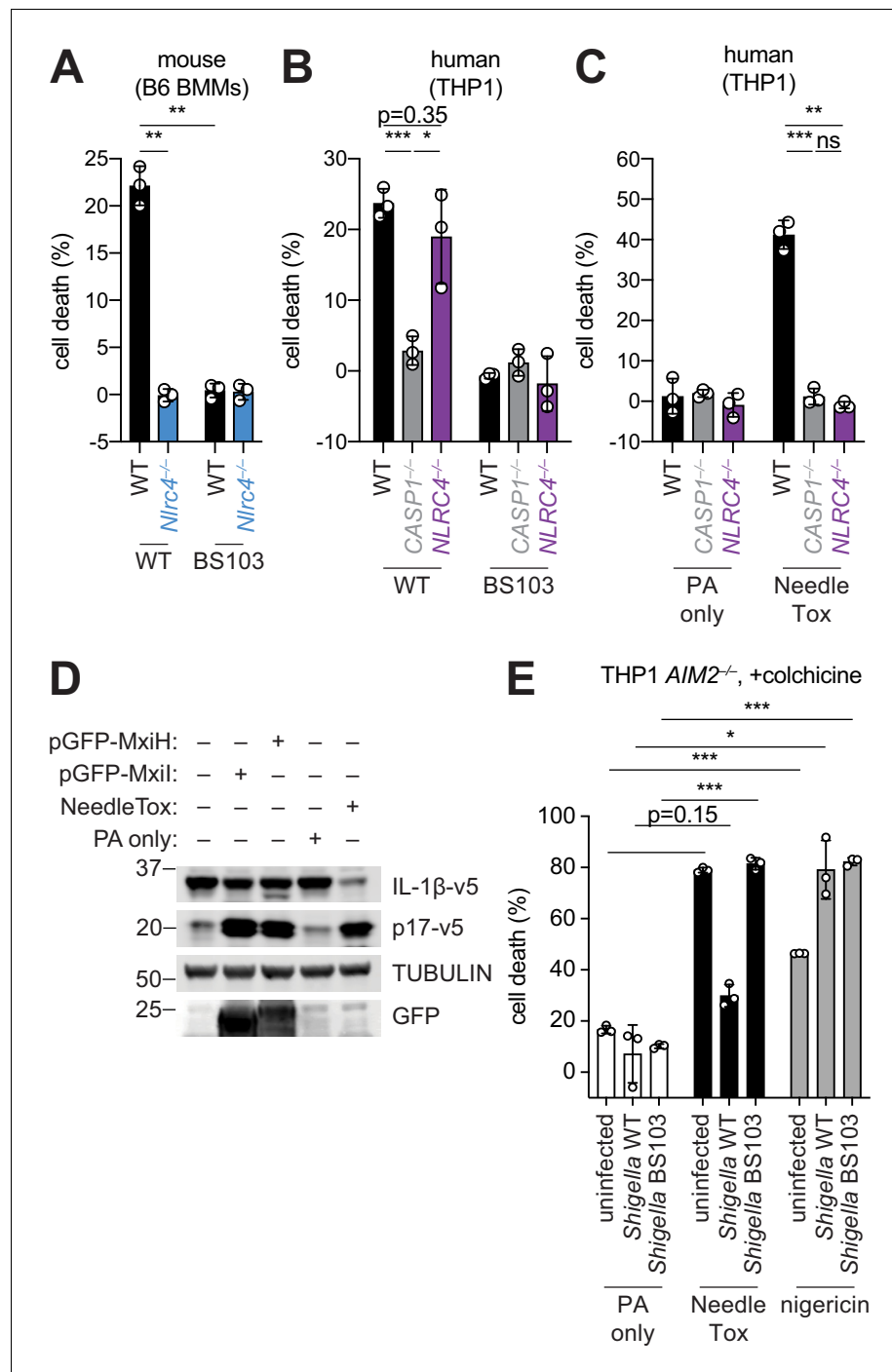


Figure 1. *Shigella* infection appears to suppress the NAIP–NLRC4 inflammasome. (A) *Shigella* infection (MOI 10) of C57BL/6 WT or *Nlrc4*^{-/-} bone-marrow-derived macrophages (BMMs). Cell death was measured 30 min post-infection (after spinfection, invasion, and washes) by propidium iodide uptake and reported as percent death relative to 100% killing by treatment with Triton X-100. (B) Cell death of *Shigella* infected THP1 WT, *CASP1*^{-/-} or *NLRC4*^{-/-} cells as in (A). Cell death was measured 30 min post-infection. (C) Cell death of THP1 WT, *CASP1*^{-/-} or *NLRC4*^{-/-} cells treated with 10 μ g/mL PA alone or in combination with 10 μ g/mL LFn-MxiH ('NeedleTox'). Cell death was measured 4 hr post-challenge. (D) Human NAIP–NLRC4 inflammasome reconstitution in 293T cells. Inflammasome activation was measured by CASP1-dependent processing of pro-IL-1 β to p17 by co-transfection of an empty vector, pGFP-MxiH or pGFP-MxiI, or by treatment with 10 μ g/mL PA alone or in combination with 10 μ g/mL LFn-MxiH. (E) Colchicine (1 μ M)-treated *AIM2*^{-/-} THP1 cells were either left uninfected or infected for 1 hour

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(after spinfection, invasion, and washes) with WT or BS103 Shigella (MOI 10), and then treated with 10 µg/mL PA alone, PA + 1.0 µg/mL LFn-MxiH ('NeedleTox'), or 10 µM nigericin. Cell death was measured by PI staining and is reported as cell death relative to TX-100-treated controls per infection type. Data are representative of at least three independent experiments. Mean ± SD is shown, unpaired t-test with Welch's correction: * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, ns = not significant ($p > 0.05$).

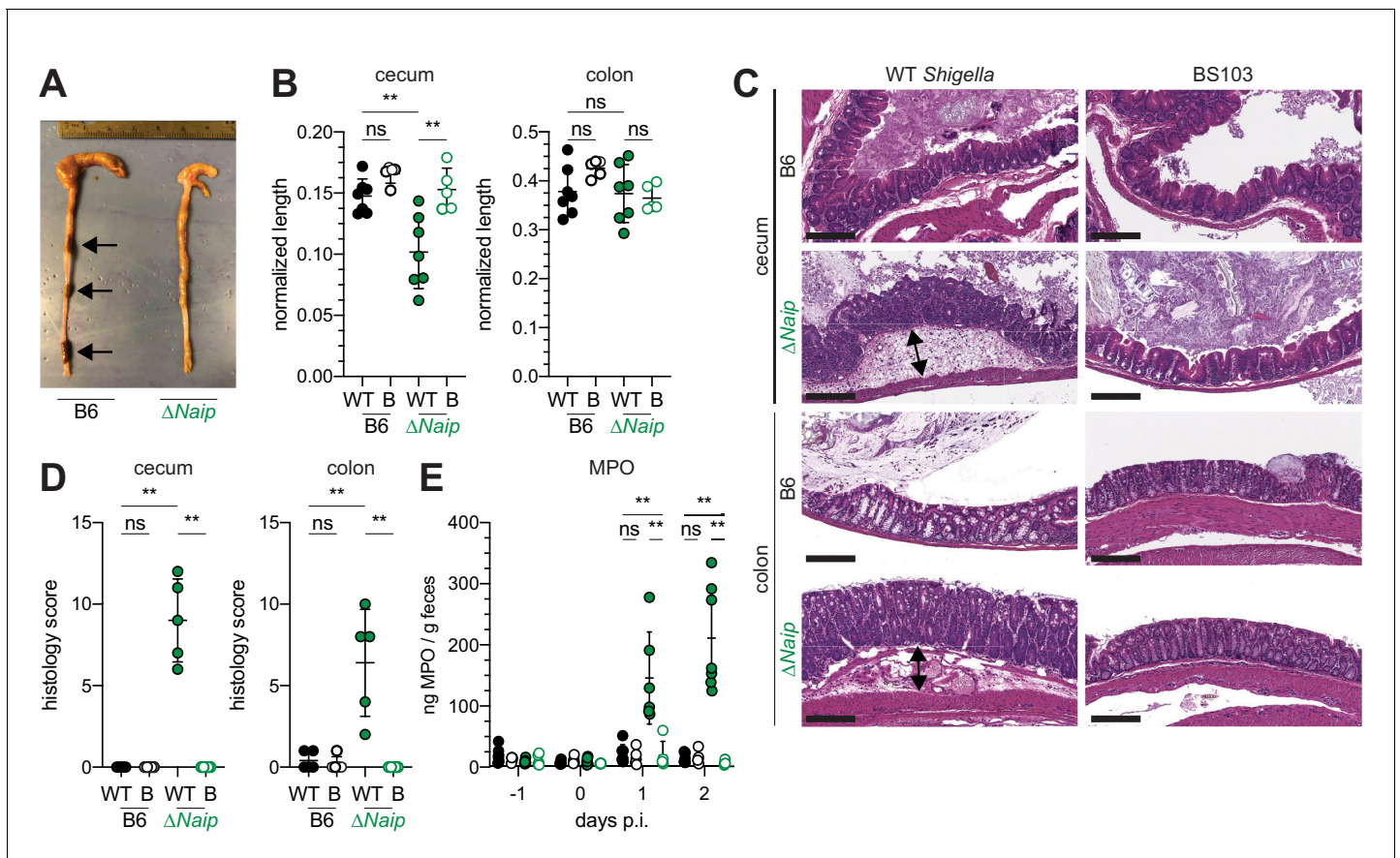


Figure 2. *Shigella*-infected B6.Δ*Naip* mice exhibit intestinal inflammation. (A–E) B6.WT and B6.Δ*Naip* (green) mice (lacking expression of all *Naip* genes) treated orally with 25 mg streptomycin sulfate were orally challenged the next day with 5×10^7 CFU of WT or BS103 ('B', non-invasive) *Shigella*. Endpoint harvests were performed at 48 hr post-infection (p.i.). (A) Representative images of the cecum and colon dissected from B6.WT and B6.Δ*Naip* mice. Note cecum tissue thickening (size reduction), macroscopic edema, and loose stool (absence of arrows). (B) Quantification of cecum and colon lengths. Values were normalized to mouse weight prior to infection; cecum length (cm) / mouse weight (g). WT, wild-type *Shigella* (filled symbols); B, BS103 (open symbols). (C) Representative images of H&E stained cecum and colon tissue from infected mice. Scale bar, 200 μ m. (D) Blinded quantification of histology score (cumulative) for tissues in (C). Edema, hyperplasia, inflammatory infiltrate, and epithelial cell death were scored from 0 to 4. The final score is the sum of individual scores from each category. (E) MPO levels measured by ELISA from feces of B6.WT and B6.Δ*Naip* mice collected -1 through 2 days p.i. (B, D, E) Each symbol represents one mouse. Filled symbols, WT *Shigella*; open symbols, BS103. Data are representative of two independent experiments. Mean \pm SD is shown in (B,D,E), Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant ($p > 0.05$).

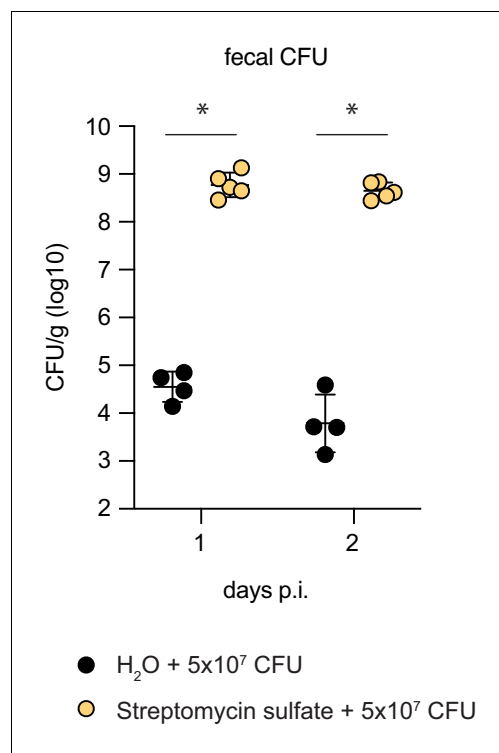


Figure 2—figure supplement 1. Antibiotic pre-treatment followed by oral route *Shigella* infection permits substantial luminal colonization. CFU determination per gram (g) feces of B6 WT mice that were treated orally with 25 mg streptomycin sulfate (yellow) or water (black) and orally challenged the next day with 5×10^7 CFU of WT *Shigella*. Feces were collected 1 and 2 days post-infection (p.i.). Data are representative of three experiments. Each symbol represents one mouse. Mann-Whitney test, * $p < 0.05$.

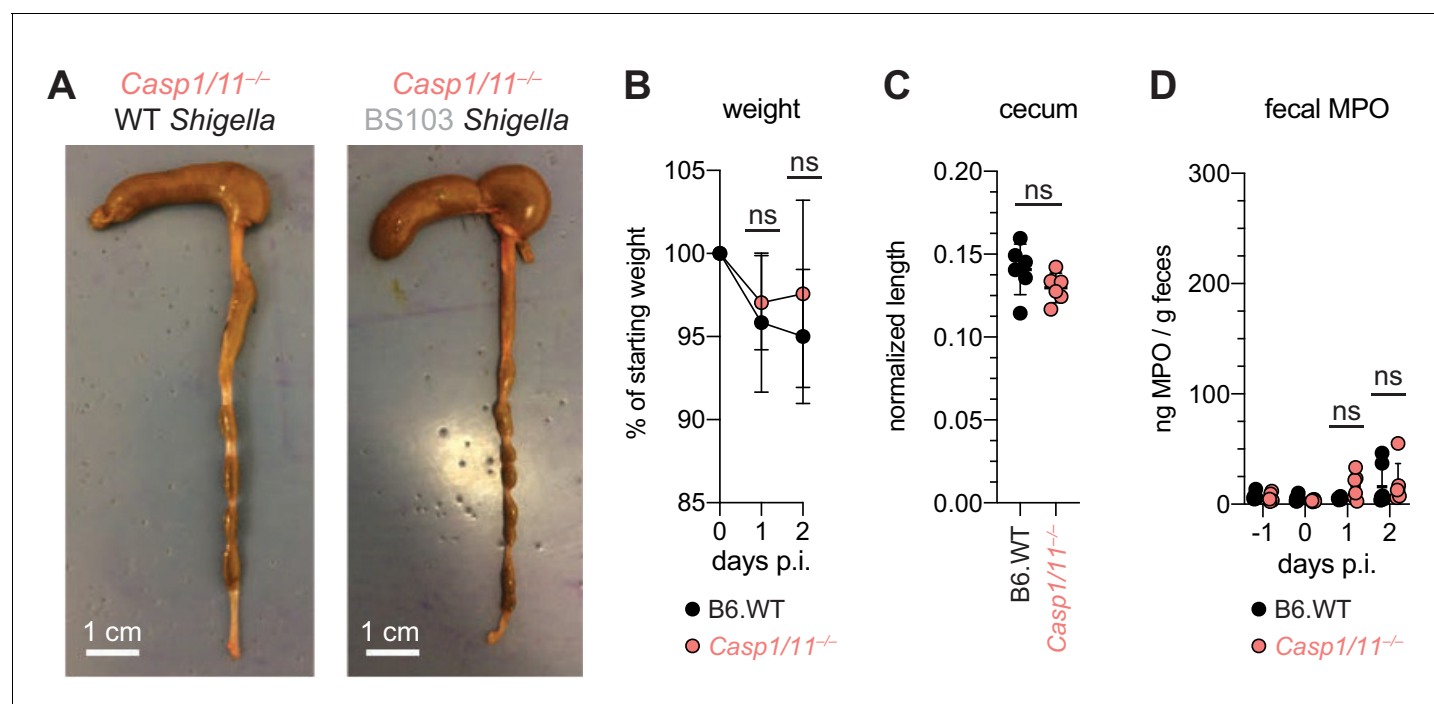


Figure 2—figure supplement 2. B6.*Casp1/11^{-/-}* mice are resistant to oral *Shigella* challenge. (A–D) B6.WT (black) and B6.*Casp1/11^{-/-}* (peach) mice were treated orally with 25 mg streptomycin sulfate and were orally challenged the next day with 5×10^7 CFU of either WT or BS103 (avirulent) *Shigella*. (A) Representative images of the cecum and colon dissected at 2 days post-infection (p.i.). (B) Mouse weights. (C) Quantification of cecum and colon lengths. Values were normalized to mouse weight prior to infection; cecum length (cm)/mouse wt. (D) MPO levels measured by ELISA from feces of B6.WT and B6.*Casp1/11^{-/-}* mice collected –1 through 2 days p.i. Each symbol represents one mouse. Data are representative of three independent experiments. Mean \pm SD is shown in (B,C,D), Mann-Whitney test, * $p < 0.05$, ns = not significant ($p > 0.05$).

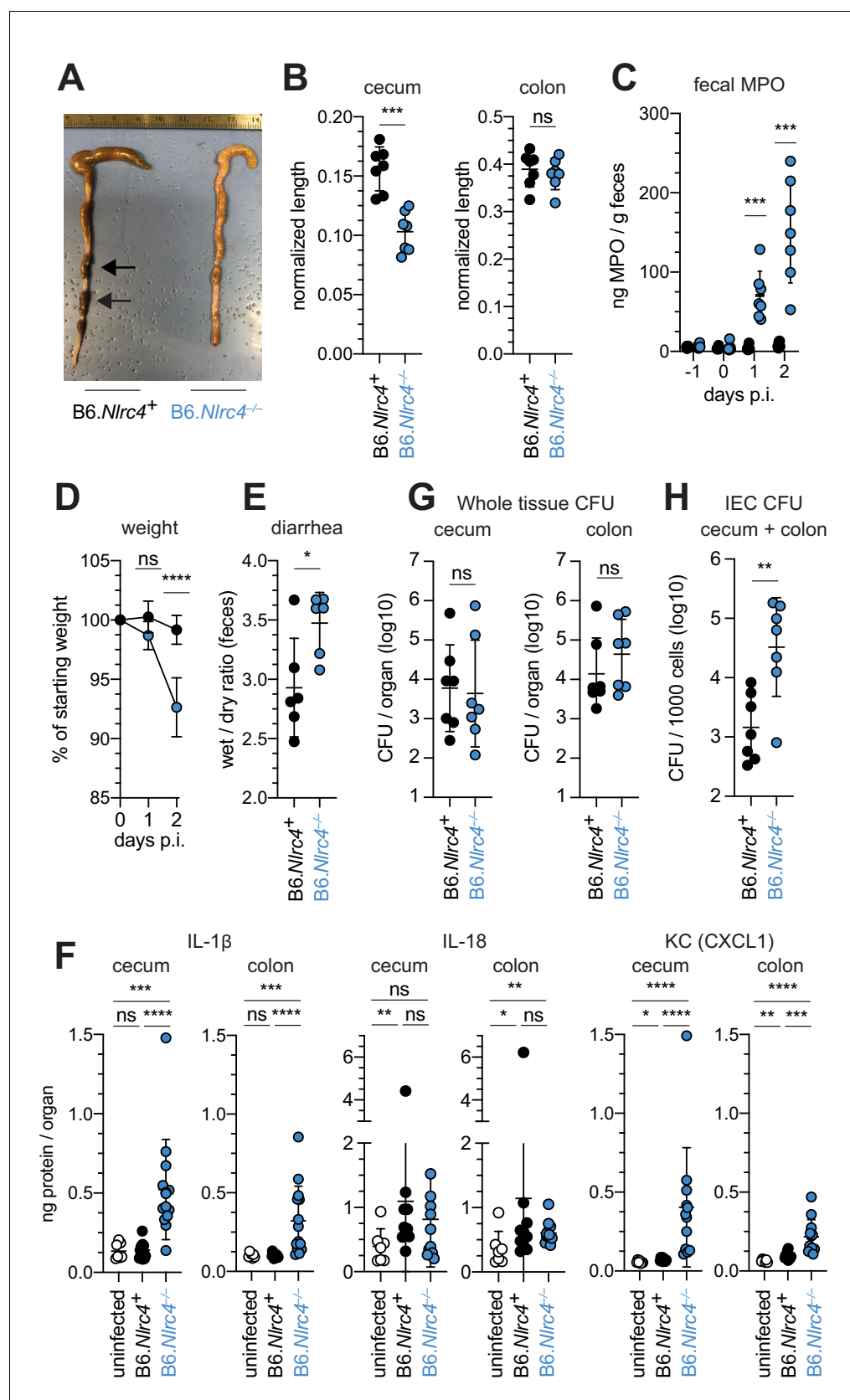


Figure 3. *Shigella*-infected B6.Nlrp4^{-/-} mice exhibit intestinal inflammation and bacterial colonization of IECs. (A–E) B6.Nlrp4^{+/+} and B6.Nlrp4^{-/-} littermates were cohoused with B6.WT mice for a minimum of three weeks. Mice

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were infected with only WT *Shigella* as described for **Figure 2**. Endpoint harvests were performed 48 hr post-infection (p.i.). B6.*Nlrc4*^{+/-} and B6.WT mice are collectively referred to as B6.*Nlrc4*⁺. **(A)** Representative images of the cecum and colon dissected from B6.*Nlrc4*⁺ and B6.*Nlrc4*^{-/-} mice. Note the cecum tissue thickening (size reduction), macroscopic edema, and loose stool (absence of arrows). **(B)** Quantification of cecum and colon lengths. Values were normalized to mouse weight prior to infection; cecum length (cm) / mouse weight (g). **(C)** MPO levels measured by ELISA from feces of B6.*Nlrc4*⁺ and B6.*Nlrc4*^{-/-} mice collected -1 through 2 days p.i. **(D)** Mouse weights from 0 through 2 days p.i. Each symbol represents the mean for all mice of the indicated condition. **(E)** Quantification of feces weights before and after dehydration at 2 days p.i. A larger ratio indicates diarrhea. **(F)** IL-1 β , IL-18, and KC levels measured by ELISA from tissue of B6.*Nlrc4*⁺ and B6.*Nlrc4*^{-/-} mice collected 2 days p.i. **(G)** CFU determination from gentamicin-treated whole tissue homogenates from the cecum or colon of infected mice. **(H)** CFU determination from the IEC enriched fraction of gentamicin-treated cecum and colon tissue (combined). **(B,C,E-H)** Each symbol represents one mouse. Data are representative of three independent experiments. Mean \pm SD is shown in **(B-F)**. Geometric mean \pm SD is shown in **(F, G)**. Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant ($p > 0.05$).

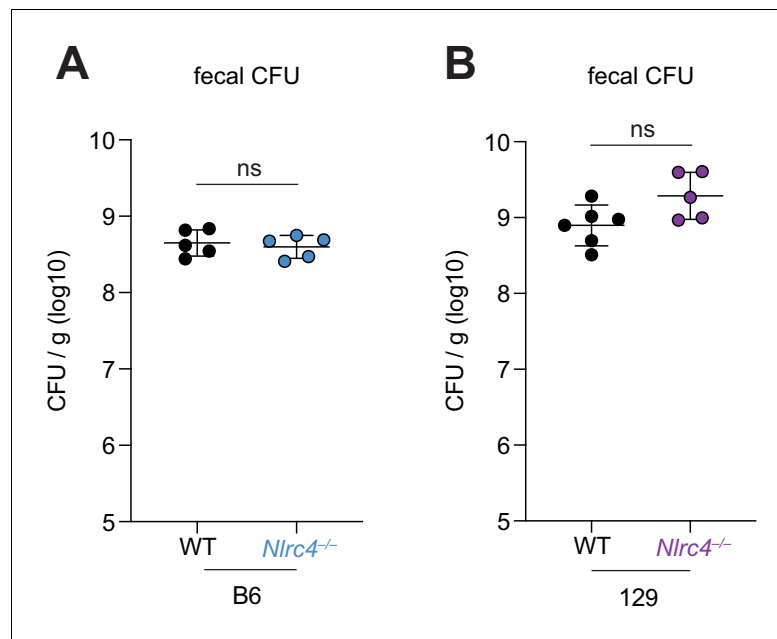


Figure 3—figure supplement 1. Luminal colonization by *Shigella* is similar between WT and NAIP–NLRC4-deficient mice. (A) CFU determination per gram (g) feces from B6 and B6.*Nlrc4*^{-/-} *Shigella* infected mice 2 days post-infection. (B) CFU determination per gram (g) feces from 129 and 129.*Nlrc4*^{-/-} *Shigella* infected mice 2 days post-infection. Each symbol represents one mouse. Mann-Whitney test, *p < 0.05.

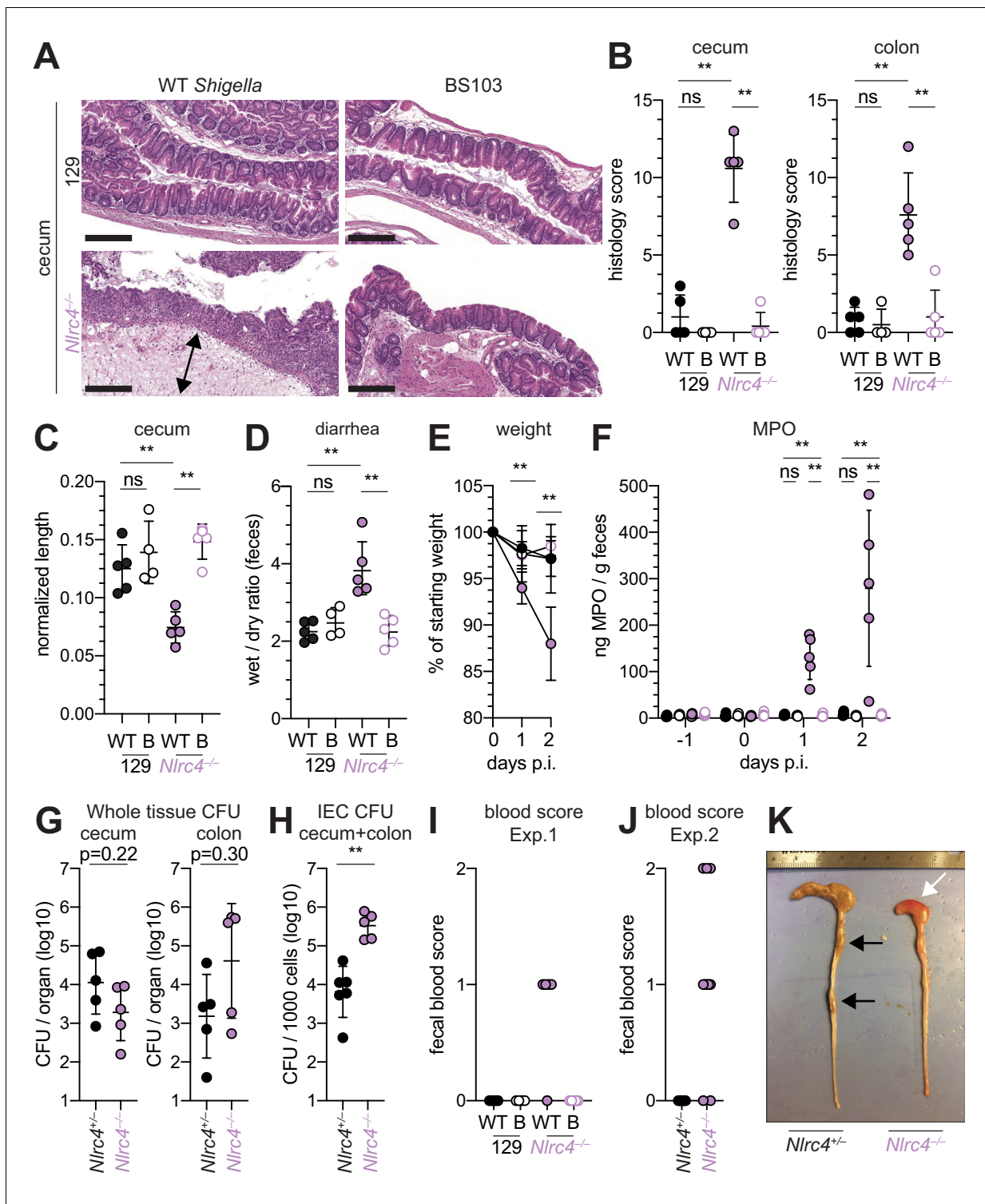


Figure 4. *Shigella*-infected 129.Nlr4^{-/-} mice exhibit hallmarks of severe human shigellosis. (A–H) 129.Nlr4^{+/+} and 129.Nlr4^{-/-} littermates were infected as described for **Figure 2**. Endpoint harvests were performed at 48 hr post-infection (p.i.). (A) Representative images of H&E stained cecum and colon tissue from infected mice. Scale bar, 200 μ m. (B) Blinded quantification of histology score (cumulative) for tissues in (A). Edema, hyperplasia, inflammatory infiltrate, and epithelial cell death were scored from 0 to 4. The final score is the sum of individual scores from each category. (C) Quantification of cecum and colon lengths. Values were normalized to mouse weight prior to infection; cecum length (cm) / mouse weight (g). (D) Quantification of feces weights before and after dehydration at 2 days p.i. A larger ratio indicates diarrhea. (E) Mouse weights at 0 through 2 days p.i. Each symbol represents the mean for all mice of the indicated condition. Statistics refer to both WT *Shigella*-infected 129.Nlr4^{+/+} and 129.Nlr4^{-/-} mice. Figure 4 continued on next page

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and WT versus BS103 *Shigella*-infected 129.*Nlrc4*^{-/-} mice at both 1 and 2 days p.i. All other comparisons were non-significant. (F) MPO levels measured by ELISA from feces of 129.*Nlrc4*^{+/-} and 129.*Nlrc4*^{-/-} mice collected -1 through 2 days p.i. (G) CFU determination from gentamicin-treated whole tissue homogenates from the cecum or colon (H) CFU determination from the IEC enriched fraction of gentamicin-treated cecum and colon tissue (combined). (I,J) Fecal blood scores from feces at two days p.i. 1 = occult blood, 2 = macroscopic blood. (I and J) show scores from two representative experiments. (K) Representative images of the cecum and colon dissected from 129.*Nlrc4*^{+/-} and 129.*Nlrc4*^{-/-} mice. Note the cecum tissue thickening (size reduction), macroscopic edema, and loose stool (absence of arrows), and vascular lesions and bleeding. (B–D,F–J) Each symbol represents one mouse. Filled symbols, WT *Shigella*; open symbols, BS103. Data are representative of three independent experiments. Mean ± SD is shown in (B, C, D, E, F). Geometric mean ± SD is shown in (G and H). Mann-Whitney test, *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant (p > 0.05).

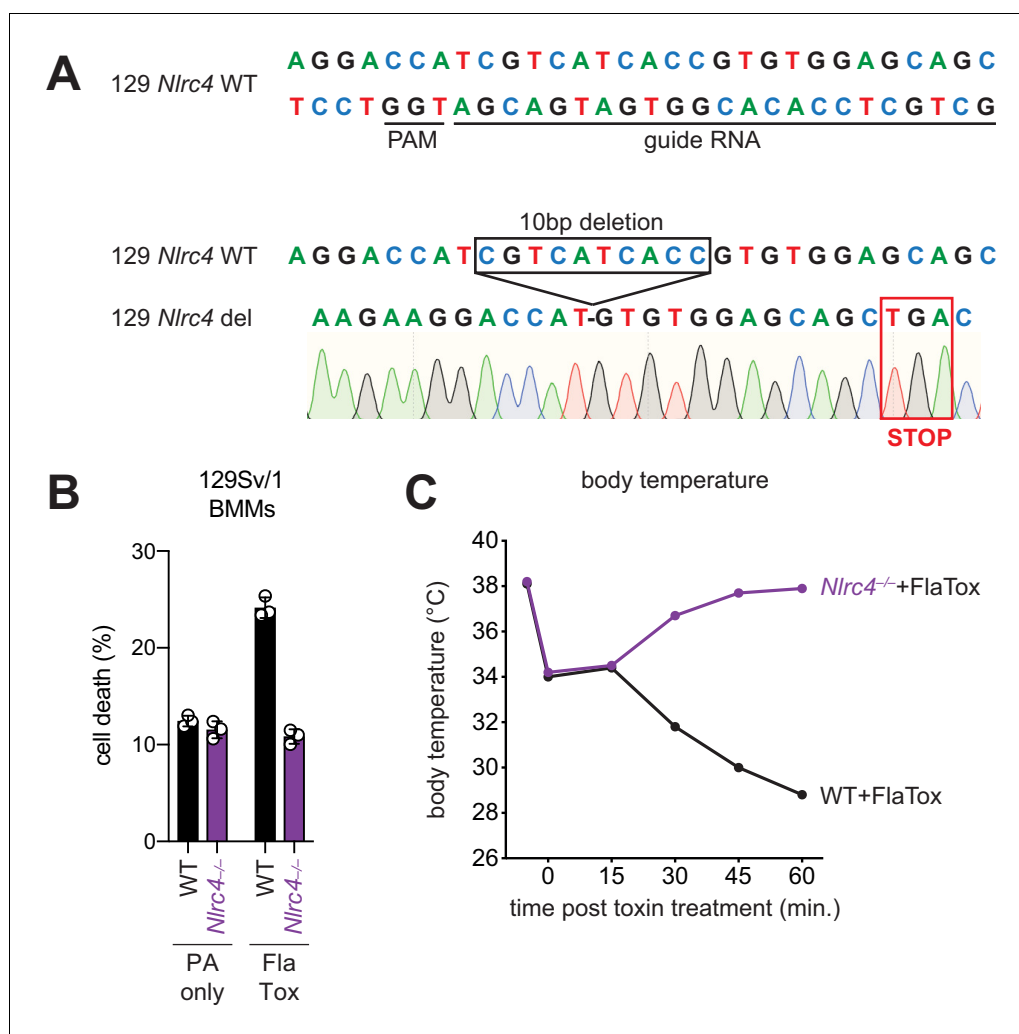


Figure 4—figure supplement 1. Construction and functional characterization of *Nlrc4* knockout mice on the 129S1/SvImJ genetic background. (A) The targeted wild-type *Nlrc4* sequence (chromosome 17, NC_000083.6, exon 5) aligned to the *Nlrc4* guide RNA. The protospacer adjacent motif (PAM) is indicated. Below is a schematic of the Sanger sequencing verified product of CRISPR/Cas9-editing (129 *Nlrc4* del), which results in a 10 base pair deletion and an in-frame early TGA stop codon in exon 5 of *Nlrc4*. (B) Quantification of cell death in 129 WT or *Nlrc4*^{-/-} bone-marrow-derived macrophages (BMMs) treated with 10 µg/mL PA alone or PA + 10 µg/mL LFn-FlaA (LFn fused to *Legionella pneumophila* flagellin, 'FlaTox'). Cell death was measured 30 min post-infection by propidium iodide uptake and reported as percent death relative to 100% killing by treatment with Triton X-100. (C) WT or 129.*Nlrc4*^{-/-} mice were injected intravenously with 0.2 µg/g body weight PA + 0.1 µg/g body weight LFn-FlaA and body temperature was monitored for the indicated times (minutes) post-treatment. The initial temperature decrease in all mice is due to isoflurane treatment.

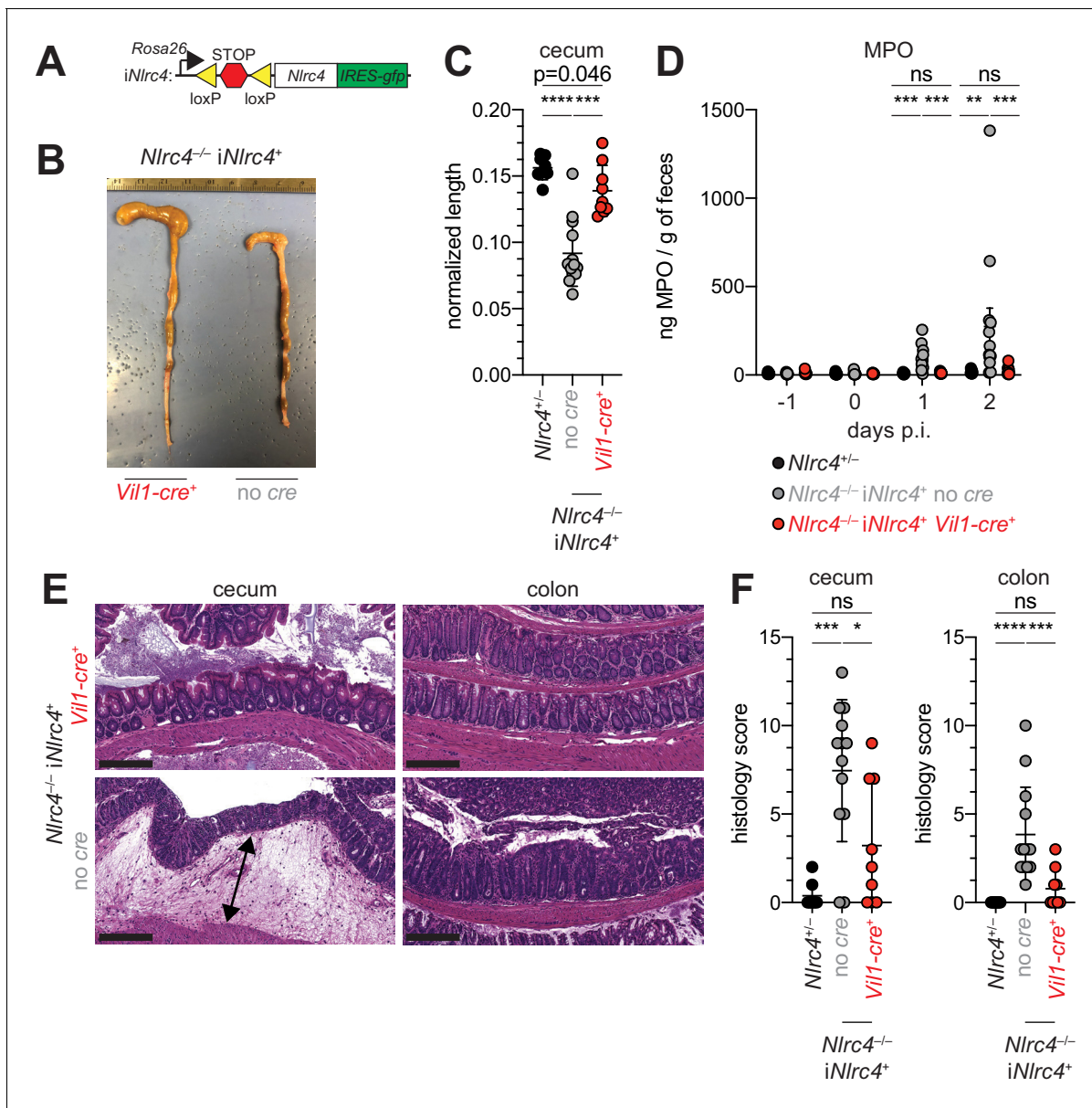


Figure 5. NLRC4 expression in IECs is sufficient to prevent shigellosis. (A) Schematic of the B6 Rosa26 locus containing the iNlrc4 cassette, as described previously (Rauch et al., 2017). (B–F) Vil1-cre positive (+) or negative Nlrc4^{-/-} iNlrc4 littermates, or iNlrc4^{+/+} mice were orally infected with 5x10⁷ CFU of WT *Shigella* 24 hr after oral streptomycin treatment. Endpoint harvests were done 48 hr post-infection (p.i.). (B) Representative images of the cecum and colon dissected from iNlrc4 Nlrc4^{-/-} Vil1-cre positive or negative mice. (C) Quantification of cecum length reduction normalized to the weight of the animal prior to infection; cecum length (cm) / mouse weight (g). (D) MPO levels measured by ELISA of feces collected -1 through 2 days p.i. (E) Representative images of H&E stained cecum and colon tissue from infected mice. Scale bar, 200 μ m. (F) Blinded quantification of histology score (cumulative) for cecum and colon tissue. Data are representative of two independent experiments. Mean \pm SD is shown in (C, D, F), Mann-Whitney test, *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant (p > 0.05). (C,D,F) Each symbol represents one mouse.

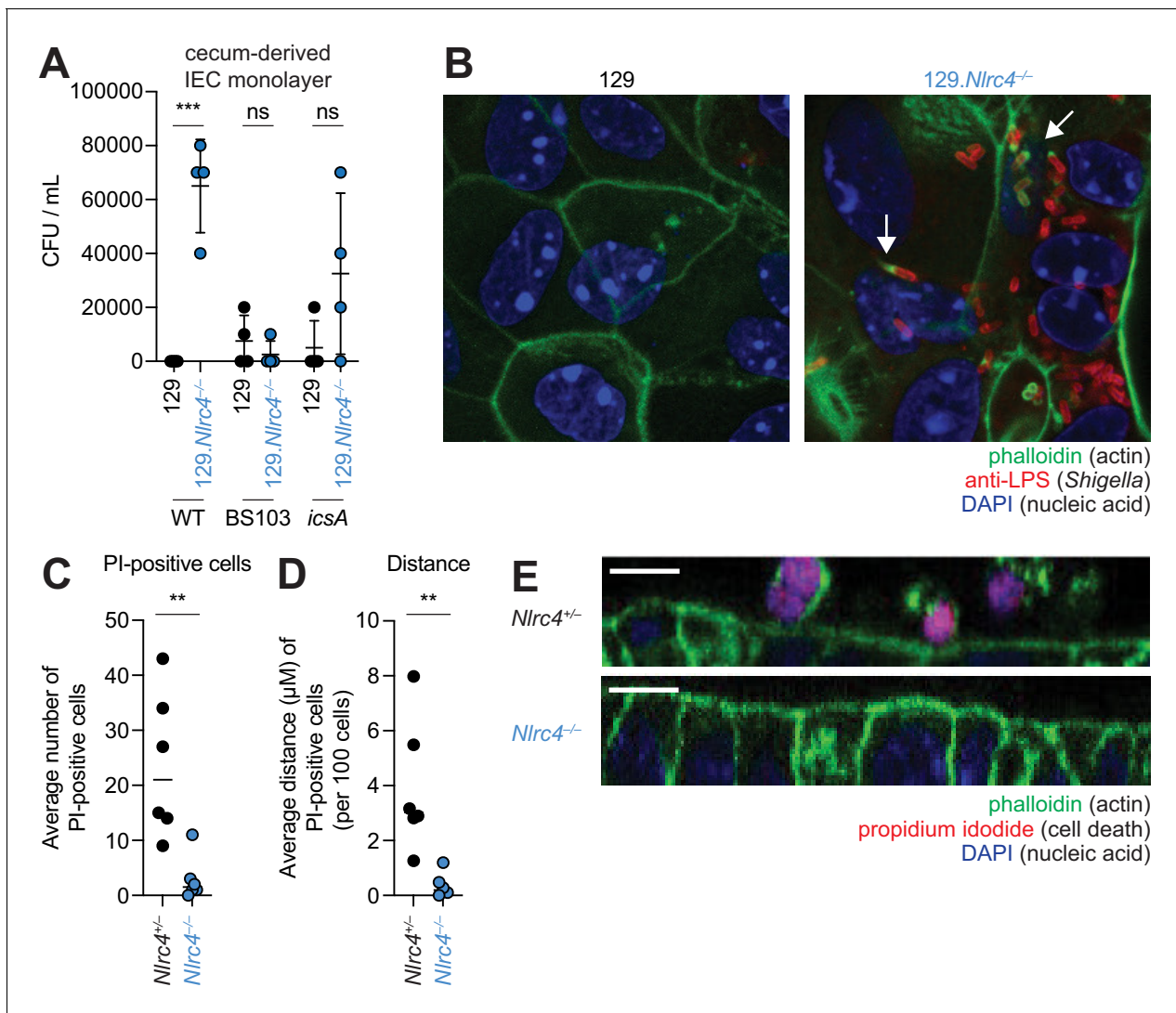


Figure 6. NLRC4 prevents *Shigella* colonization and cell expulsion in IEC monolayer cultures. (A) *Shigella* (WT, BS103, or *icsA*) CFU from transwell culture of WT or 129.Nlrc4^{-/-} cecum-derived IEC monolayers. CFU was determined 8 hr p.i. Each symbol represents one infected monolayer. (B) Immunofluorescent staining of WT *Shigella*-infected transwell cultures of WT or 129.Nlrc4^{-/-} cecum-derived IEC monolayers: green, fluorescent phalloidin (actin); red, anti-*Shigella* LPS, blue, DAPI (nucleic acid). (C,D) Quantification of the number and position of propidium iodide (PI)-positive cells in *Shigella*-infected 129.Nlrc4^{+/-} or 129.Nlrc4^{-/-} cecum-derived IEC monolayers. In (C), each symbol represents the average number of PI-positive cells within an imaged field. In (D), each symbol represents the average distance of PI-positive cells from the lower boundary of the z-stack, per 100 cells. Two fields were counted for three independent slides. (E) A representative XZ-projection of *Shigella*-infected transwell cultures of 129.Nlrc4^{+/-} or 129.Nlrc4^{-/-} cecum-derived IEC monolayers showing expelled PI⁺ cells above the monolayer. Green, fluorescent phalloidin (actin); red, PI (cell death); blue, DAPI (nucleic acid). Mann-Whitney test, **p < 0.01, ***p < 0.001, ns = not significant (p > 0.05).

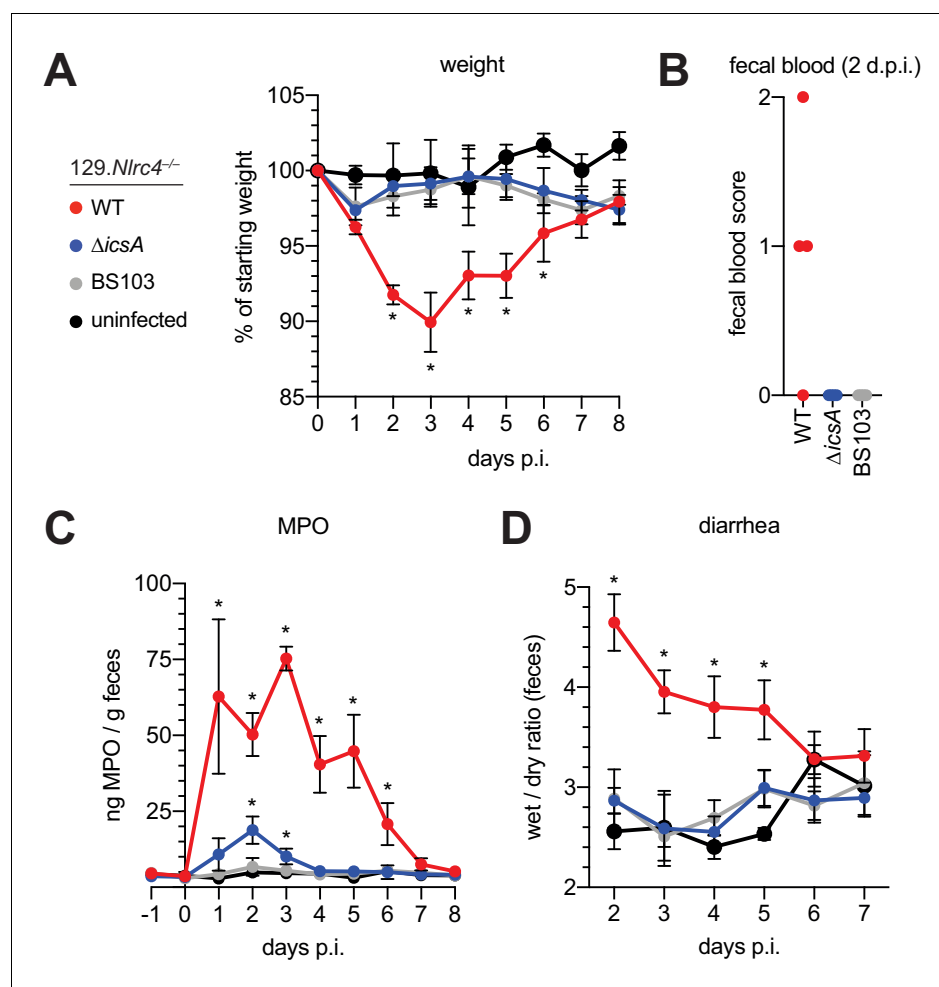


Figure 7. 129.Nlrc4^{-/-} mice are resistant to attenuated *Shigella* strains. (A–D) 129.Nlrc4^{-/-} littermates were uninfected (black) or inoculated orally with 5x10⁷ CFU of WT (red), *icsA* mutant (blue), or BS103 (grey) *Shigella* 24 hours after oral streptomycin treatment and monitored for 8 days post-infection (p.i.). (A) Mouse weights. (B) Fecal blood scores from feces at 2 days post-infection (d.p.i.). 1 = occult blood, 2 = macroscopic blood. Each symbol represents feces from one mouse. (C) MPO levels measured by ELISA from feces collected -1 through 8 days p.i. (D) Quantification of diarrhea comparing weight of feces before and after dehydration. A larger ratio indicates diarrhea. (A–C) Each symbol represents the mean at a specific time point for four individual mice per infection condition. Data are representative of two independent experiments. Mean \pm SEM is shown in (A–C) Mann-Whitney test, *p < 0.05. In (A,B) significance was determined by independently comparing to Day 0 and to BS103 + uninfected at the same day. In (C), significance was determined by comparing to BS103 and uninfected at the same day.

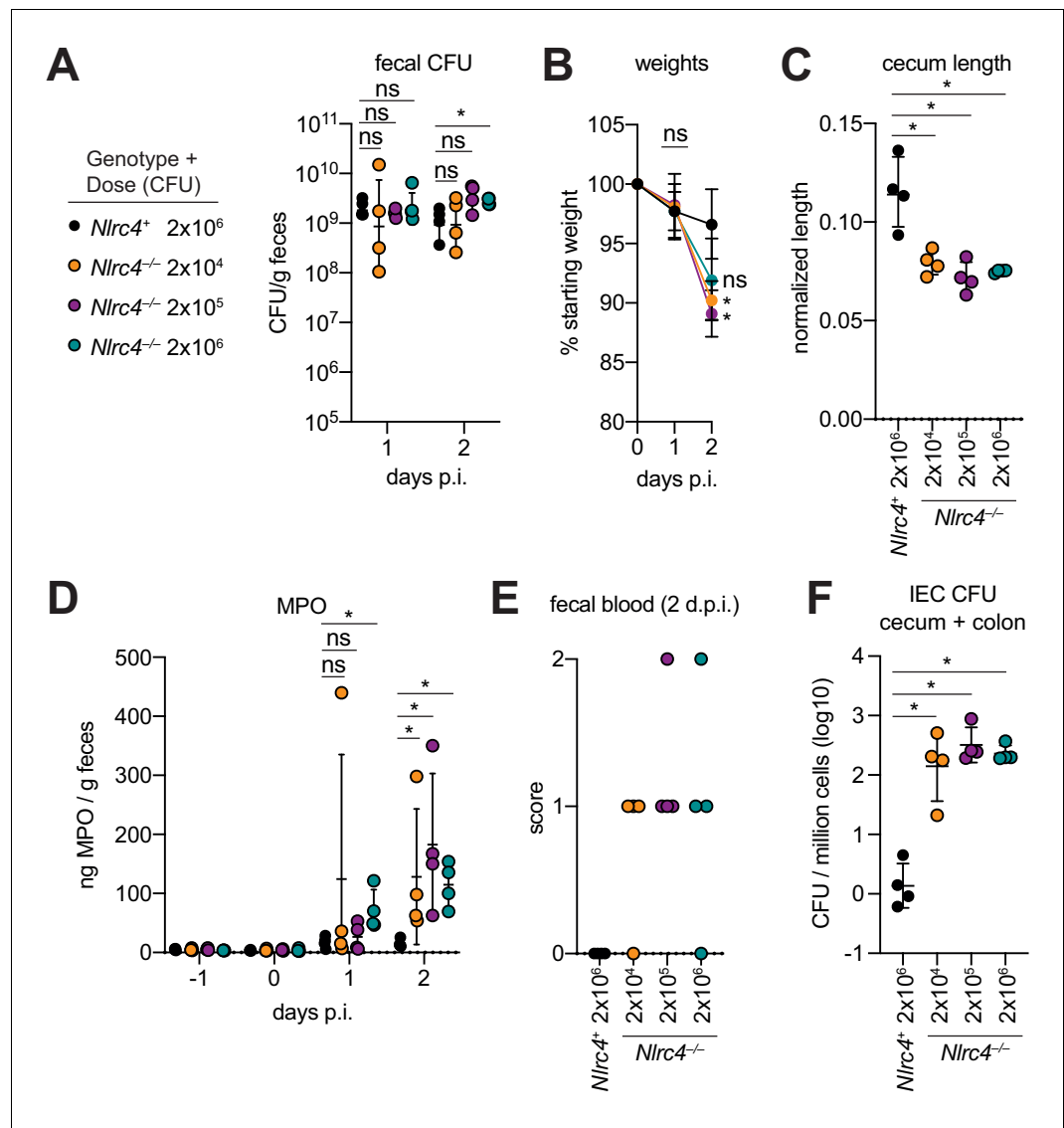


Figure 8. Antibiotic treated *Nlrc4*^{-/-} mice are susceptible to modest infectious doses of *Shigella*. (A–F) 129.*Nlrc4*⁺ mice were inoculated orally with 2x10⁶ (black) and 129.*Nlrc4*^{-/-} littermates were inoculated with 2x10⁶ (teal), 2x10⁵ (purple), or 2x10⁴ (orange) CFU of WT *Shigella* 24 hr after oral streptomycin treatment. Endpoint harvests were done 48 hr post-infection (p.i.). (A) CFU determination from feces. (B) Mouse weights. Each symbol represents the mean at that time point. (C) Quantification of cecum length reduction normalized to the weight of the animal prior to infection; cecum length (cm) / mouse weight (g). (D) MPO levels measured by ELISA from feces collected -1 through 2 days p.i. (E) Fecal blood scores from feces at 2 days post-infection (d.p.i.). 1 = occult blood, 2 = macroscopic blood. (F) CFU determination from the IEC enriched fraction of gentamicin-treated cecum and colon tissue (combined). (A, C–F) Each symbol represents one mouse. Data are representative of two independent experiments. Mean ± SD is shown in (A–D). Geometric mean ± SD is shown in (F). Figure Mann-Whitney 8 test, *p < 0.05, ns = not significant (p > 0.05).