

1 **NAIP–NLRC4-deficient mice are susceptible to shigellosis**

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20 **Abstract**

21 Bacteria of the genus *Shigella* cause shigellosis, a severe gastrointestinal disease that is a major
22 cause of diarrhea-associated mortality in humans. Shigellosis develops upon oral ingestion of as few
23 as 100 bacteria, but million-fold higher doses fail to cause disease in mice. The lack of a
24 physiologically relevant mouse model of shigellosis has impeded our understanding of this important
25 human disease, but why mice are resistant is unknown. Here we show that in human cells, but not in
26 mice, *Shigella* evades detection by the NAIP–NLRC4 inflammasome, an immune sensor present in
27 intestinal epithelial cells (IECs). We find that NAIP–NLRC4-deficient mice are highly susceptible to
28 oral *Shigella* infection and recapitulate the clinical features of human shigellosis, including bacterial
29 replication in IECs and neutrophilic inflammation of the colon. Confirming a role for bacterial
30 replication in IECs in our new model, a *Shigella* mutant lacking IcsA, a factor required for cell-to-cell
31 spread among IECs, is attenuated in otherwise susceptible NAIP–NLRC4-deficient mice. Although
32 inflammasome-mediated cell death is widely held to promote *Shigella* infection and pathogenesis, we
33 instead demonstrate that IEC-specific NAIP–NLRC4-induced cell death is sufficient to protect the host
34 from shigellosis. Thus, NAIP–NLRC4-deficient mice are a physiologically relevant and experimentally
35 tractable model for shigellosis. More broadly, our results suggest that the lack of an inflammasome
36 response in IECs may help explain the extreme susceptibility of humans to shigellosis.

37

38 **Introduction**

39 *Shigella* is a genus of Gram-negative enterobacteriaceae that causes ~269 million infections and
40 ~200,000 deaths annually, a quarter of which are of children under the age of five (Khalil et al., 2018).
41 Disease symptoms include fever, abdominal cramping, and inflammatory diarrhea characterized by
42 the presence of neutrophils and, in severe cases, blood (Kotloff et al., 2018). There is no approved
43 vaccine for *Shigella* and antibiotic resistance continues to rise (Ranjbar and Farahani, 2019). *Shigella*
44 pathogenesis is believed to be driven by bacterial invasion, replication, and spread within colonic
45 IECs. *Shigella* virulence requires a plasmid-encoded type III secretion system (T3SS) that injects ~30
46 effectors into host cells (Schnupf and Sansonetti, 2019; Schroeder and Hilbi, 2008). The virulence
47 plasmid also encodes IcsA, a bacterial surface protein that nucleates host actin at the bacterial pole to
48 propel the pathogen through the host cell cytosol and into adjacent epithelial cells (Bernardini et al.,
49 1989; Goldberg and Theriot, 1995).

50 A major impediment to studying *Shigella* is the lack of experimentally tractable *in vivo* models
51 that accurately recapitulate human disease after oral inoculation. Although the infectious dose for
52 humans is as low as 10-100 bacteria (DuPont et al., 1969; DuPont et al., 1989), mice are resistant to
53 high doses of oral *Shigella* challenge (Freter, 1956; McGuire and Floyd, 1958). Rabbits, guinea pigs,
54 zebrafish, piglets, and macaques have been used as models (Islam et al., 2014; Jeong et al., 2010;
55 Mostowy et al., 2013; Ranallo et al., 2014; Shim et al., 2007; West et al., 2005; Yum and Agaisse,
56 2019; Yum et al., 2019) but the cost and/or limited tools in these systems impair detailed studies of
57 pathogenesis. Oral streptomycin administration and other treatments facilitate *Shigella* colonization of
58 the mouse intestinal lumen by ablating the natural colonization resistance provided by the microbiome
59 (Freter, 1956; Martino et al., 2005; Medeiros et al., 2019). However, antibiotic-treated mice do not
60 present with key hallmarks of human disease, likely due to the failure of *Shigella* to invade and/or
61 establish a replicative niche within the mouse intestinal epithelium.

62 Inflammasomes are cytosolic multi-protein complexes that initiate innate immune responses
63 upon pathogen detection or cellular stress (Lamkanfi and Dixit, 2014; Rathinam and Fitzgerald, 2016).
64 The NAIP–NLRC4 inflammasome is activated when bacterial proteins, such as flagellin or the rod and
65 needle proteins of the T3SS apparatus, are bound by NAIP family members. Importantly, the *Shigella*
66 T3SS inner rod (MxiL) and needle (MxiH) proteins are both potent agonists of human and mouse
67 NAIPs (Reyes Ruiz et al., 2017; Yang et al., 2013). Activated NAIPs then co-assemble with NLRC4 to
68 recruit and activate the Caspase-1 (CASP1) protease (Vance, 2015; Zhao and Shao, 2015). CASP1
69 then cleaves and activates the pro-inflammatory cytokines IL-1 β and IL-18 and the pore-forming
70 protein Gasdermin-D (Kayagaki et al., 2015; Shi et al., 2015), initiating a lytic form of cell death called
71 pyroptosis. We and others recently demonstrated that activation of NAIP–NLRC4 in IECs further
72 mediates the cell-intrinsic expulsion of infected epithelial cells from the intestinal monolayer (Rauch et

73 al., 2017; Sellin et al., 2014). In the context of *Shigella* infection, it is generally accepted that
74 inflammasome-mediated pyroptosis of infected macrophages promotes pathogenesis by initiating
75 inflammation, and by releasing bacteria from macrophages, allowing the bacteria to invade the
76 basolateral side of intestinal epithelial cells (Ashida et al., 2014; Lamkanfi and Dixit, 2010; Schnupf
77 and Sansonetti, 2019). However, it has not been possible to test the role of inflammasomes in the
78 intestine after oral *Shigella* infection due to the lack of a genetically tractable model. Here we develop
79 the first oral infection mouse model for *Shigella* infection and demonstrate a specific host-protective
80 function for inflammasomes in intestinal epithelial cells.
81

82 Results

83 *Shigella* suppresses the human NAIP–NLRC4 inflammasome

84 The *Shigella* T3SS effector OspC3 inhibits cytosolic LPS sensing by the human Caspase-4 (CASP4)
85 inflammasome, but does not bind to the mouse ortholog, Caspase-11 (CASP11) (Kobayashi et al.,
86 2013). We reasoned that inflammasome inhibition may be a general strategy used by *Shigella* to
87 establish infection, and that such inhibition might occur in a host-specific manner. To test this
88 hypothesis, we compared inflammasome-dependent cell death following *Shigella* infection of mouse
89 C57BL/6 (B6) bone marrow-derived macrophages (BMMs) and human PMA-differentiated THP1 cells.
90 Infection with the wild-type (WT) *Shigella flexneri* strain 2457T but not the avirulent BS103 strain
91 (which lacks the virulence plasmid) resulted in CASP1-dependent cell death in both mouse
92 (Sandstrom et al., 2019) and human cells (Figure 1A,B). Cell death was negligible in *Shigella*-
93 infected mouse *Nlrc4*^{−/−} BMMs (Figure 1A). In contrast, *Shigella* infection induced similar levels of cell
94 death in WT and *NLRC4*^{−/−} THP1 cells, indicating that NLRC4 is not a major contributor to *Shigella*-
95 induced CASP1 activation in human cells (Figure 1B).

96 To confirm prior reports (Yang et al., 2013) that the *Shigella* T3SS MxiH (needle) protein is a
97 potent agonist of the human NAIP–NLRC4 inflammasome, and that THP1 cells express a functional
98 NAIP–NLRC4 inflammasome (Kortmann et al., 2015; Reyes Ruiz et al., 2017), we produced
99 recombinant LFn-MxiH for cytosolic delivery to cells via the protective antigen (PA) channel, as
100 previously described (Rauch et al., 2017; Rauch et al., 2016; von Moltke et al., 2012). Treatment of
101 THP1 cells with LFn-MxiH+PA (“NeedleTox”), but not PA alone, induced a CASP1- and NLRC4-
102 dependent cell death (Figure 1C). We also confirmed that *Shigella* MxiH and MxiL (rod) activate
103 human NAIP–NLRC4 in a reconstituted inflammasome assay (Reyes Ruiz et al., 2017; Tenthorey et
104 al., 2014) (Figure 1D). These results confirm that the *Shigella* rod and needle proteins are capable of
105 activating the human NAIP–NLRC4 inflammasome. Moreover, since we observe robust activation of
106 NAIP–NLRC4 in mouse cells (Figure 1A), our data indicate that the *Shigella* T3SS needle and rod
107 proteins are delivered to the cytosol during infection. However, the presence of other inflammasomes
108 in THP1 cells obscures our ability to determine if *Shigella* activates human NLRC4.

109 To eliminate cell death induced by the AIM2 and PYRIN inflammasomes, we used THP1
110 *AIM2*^{−/−} cells treated with colchicine, an inhibitor of PYRIN (Gao et al., 2016). Interestingly, WT
111 *Shigella* infection did not induce pyroptosis of colchicine-treated *AIM2*^{−/−} THP1 cells (Figure 1E).
112 Thus, although human THP1 cells express functional NAIP–NLRC4, and *Shigella* rod and needle
113 proteins can activate NAIP–NLRC4, we nevertheless observe no such activation during *Shigella*
114 infection. We therefore hypothesized that *Shigella* might antagonize the human NAIP–NLRC4
115 inflammasome. To test this hypothesis, *AIM2*^{−/−} colchicine-treated THP1 cells were either uninfected
116 or infected with the *Shigella* WT or BS103 strains for one hour, and then treated with NeedleTox to
117 induce NAIP–NLRC4-dependent cell death. Interestingly, NeedleTox-induced pyroptosis was
118 significantly reduced in cells previously infected with WT but not avirulent BS103 *Shigella* (Figure 1E).
119 As a control, treatment with nigericin, an agonist of the NLRP3 inflammasome, induced cell death
120 similarly in cells that were uninfected or infected with either WT or BS103 *Shigella* strains. Thus, WT
121 *Shigella* infection appears to specifically suppress activation of the human but not the mouse NAIP–
122 NLRC4 inflammasome. Future studies will be required to address the mechanism of *Shigella*
123 antagonism of human NAIP–NLRC4, as well as the mechanism of *Shigella* activation of AIM2 and/or
124 PYRIN.

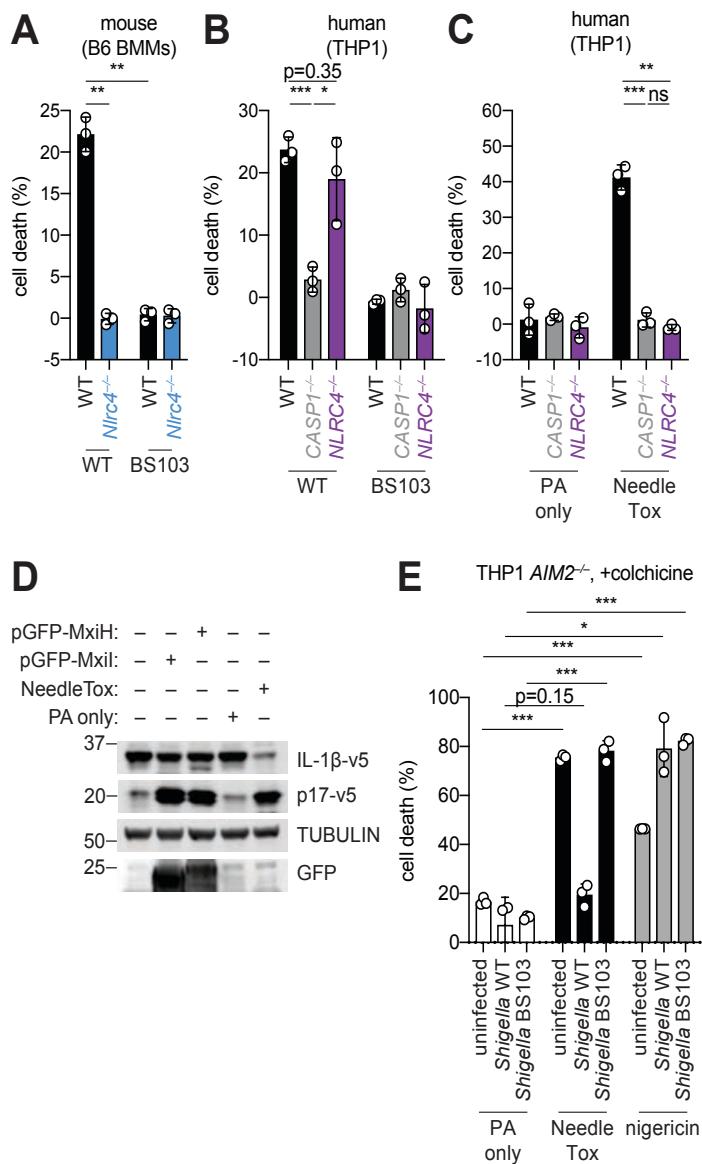


Figure 1. *Shigella* infection suppresses the NAIP–NLRC4 inflammasome.

(A) *Shigella* infection (MOI 10) of C57BL/6 WT or *Nlrc4*^{-/-} bone marrow derived macrophages (BMMs). Cell death was measured 30 minutes 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 minutes 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 hours 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-Mxil, or by treatment with 10 μ g/mL PA alone or in combination with 10 μ g/mL LFn-MxiH.

(E) Colchicine-treated *AIM2*^{-/-} THP1 cells were either left uninfected or infected for 1 hour (after spinfection, invasion, and washes) with WT or BS103 *Shigella* (MOI 10), and then treated with 10 μ g/mL PA alone, PA + 1.0 μ g/mL LFn-MxiH (“NeedleTox”), or 10 μ M 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 \pm SD is shown in (A–C,E), unpaired t-test with Welch’s correction: *P < 0.05, **P < 0.01, ***P < 0.001.

127 **B6.Naip-deficient mice are susceptible to shigellosis**

128 The mouse NAIP–NLRC4 and CASP11 inflammasomes protect the intestinal epithelium from
129 *Salmonella* (Rauch et al., 2017; Sellin et al., 2014). Thus, the above experiments led us to
130 hypothesize that the failure of *Shigella* to antagonize mouse inflammasomes might explain the
131 inborn resistance of mice versus humans to *Shigella* infection. A prediction from this hypothesis is
132 that mice deficient in inflammasomes might be susceptibility to oral *Shigella* challenge.

133 To test this, we first pretreated B6 WT mice orally with streptomycin antibiotic. Consistent with
134 prior studies (Freter, 1956; Martino et al., 2005; Medeiros et al., 2019), we found that antibiotic pre-
135 treatment followed by oral infection allows for robust *Shigella* colonization of the intestinal lumen and
136 feces compared to water-only controls (**Figure S1**). However, these high luminal bacterial loads
137 ($>10^8$ CFU/g feces) do not cause overt disease (**Figure 2** and **Figure S2**). To determine if
138 inflammasomes contribute to the resistance of mice to *Shigella*, we orally challenged WT and
139 *Casp1/11*^{−/−} mice with 5×10^7 CFU of *Shigella*. CASP1 has been previously reported to drive acute
140 inflammation and *Shigella* clearance during mouse lung infection via the processing and release of IL-
141 1 β and IL-18 (Sansonetti et al., 2000). Mice that lack CASP1 and/or CASP11 are also more
142 susceptible to oral infection with *Salmonella* Typhimurium (Crowley et al., 2020). In contrast, B6 WT
143 and *Casp1/11*^{−/−} mice were similarly resistant to *Shigella* infection, showing no signs of intestinal
144 inflammation or disease (**Figure S2A–C**). Thus, neither of the primary caspases associated with the
145 canonical or non-canonical inflammasome are essential for resistance to *Shigella* in the mouse
146 intestine.

147 The NAIP–NLRC4 inflammasome can recruit Caspase-8 (CASP8) in the absence of CASP1,
148 an event which leads to non-lytic cell death and delayed IEC expulsion (Rauch et al., 2017). We
149 reasoned that this compensatory capacity of CASP8 may account for the resistance of *Casp1/11*^{−/−}
150 mice to *Shigella* infection. Thus, to directly test if the mouse NAIP–NLRC4 inflammasome mediates
151 resistance to shigellosis, we orally infected streptomycin-pretreated B6 WT and Δ *Naip* mice with 5×10^7
152 CFU of *Shigella*. Δ *Naip* mice (also called *Naip1-6*^{ΔΔ} mice (Rauch et al., 2016)) harbor a large
153 chromosomal deletion that eliminates expression of all mouse *Naip* genes. Remarkably, *Shigella*-
154 infected Δ *Naip* but not WT mice exhibited clear signs of disease (**Figure 2**). At two days post-
155 challenge, Δ *Naip* mice had altered stool consistency (**Figure 2A**), cecum shrinkage, and thickening of
156 the cecum and colon tissue (**Figure 2A,B**). Histological analysis of primary sites of infection (cecum,
157 colon) revealed edema, epithelial hyperplasia, epithelial sloughing, and inflammatory infiltrate
158 (predominantly neutrophils and mononuclear cells in the submucosa and mucosa) exclusively in
159 Δ *Naip* mice (**Figure 2C,D**). In contrast, we did not observe any indicators of inflammation in Δ *Naip*
160 mice infected with the avirulent BS103 strain (**Figure 2C,D**).

161 A defining feature of human shigellosis is the presence of neutrophils in patient stools (Raqib
162 et al., 2000). The levels of myeloperoxidase (MPO, a neutrophil marker) were low or undetectable in
163 the feces of mice following antibiotic treatment (**Figure 2E**), indicating that microbiota disruption did
164 not itself promote neutrophilic inflammation. Following *Shigella* infection, however, fecal MPO from
165 Δ *Naip* mice dramatically increased (**Figure 2E**). In contrast, MPO levels remained low in both B6 WT
166 and *Casp1/11*^{−/−} mice (**Figure S2D**) or Δ *Naip* mice infected with the avirulent BS103 strain (**Figure**
167 **2E**). These results indicate that NAIP–NLRC4-deficient mice experience robust neutrophilic infiltrate
168 consistent with human shigellosis.

169 **B6.Nlrc4**^{−/−} mice are susceptible to shigellosis

170 To confirm that the NAIP–NLRC4 inflammasome confers resistance to *Shigella*, to account for
171 potential microbiota-associated phenotypes, and to further characterize the disease phenotype, we
172 next infected streptomycin-pretreated B6.*Nlrc4*^{+/−} and B6.*Nlrc4*^{−/−} littermates, as well as B6 WT mice
173 that had been co-housed for three weeks prior to inoculation (*Nlrc4*^{+/−} and B6 WT mice are hereby
174 referred to collectively as *Nlrc4*⁺). Consistent with our prior results in Δ *Naip* mice, we observed
175 thickening of the intestinal mucosa (**Figure 3A**), cecum shrinkage (**Figure 3A,B**), increased fecal
176 MPO levels (**Figure 3C**), and acute weight loss (**Figure 3D**) in *Shigella*-infected B6.*Nlrc4*^{−/−} mice but
177 not B6.*Nlrc4*⁺ littermates or co-housed mice. B6.*Nlrc4*^{−/−} mice also had diarrhea, which was apparent

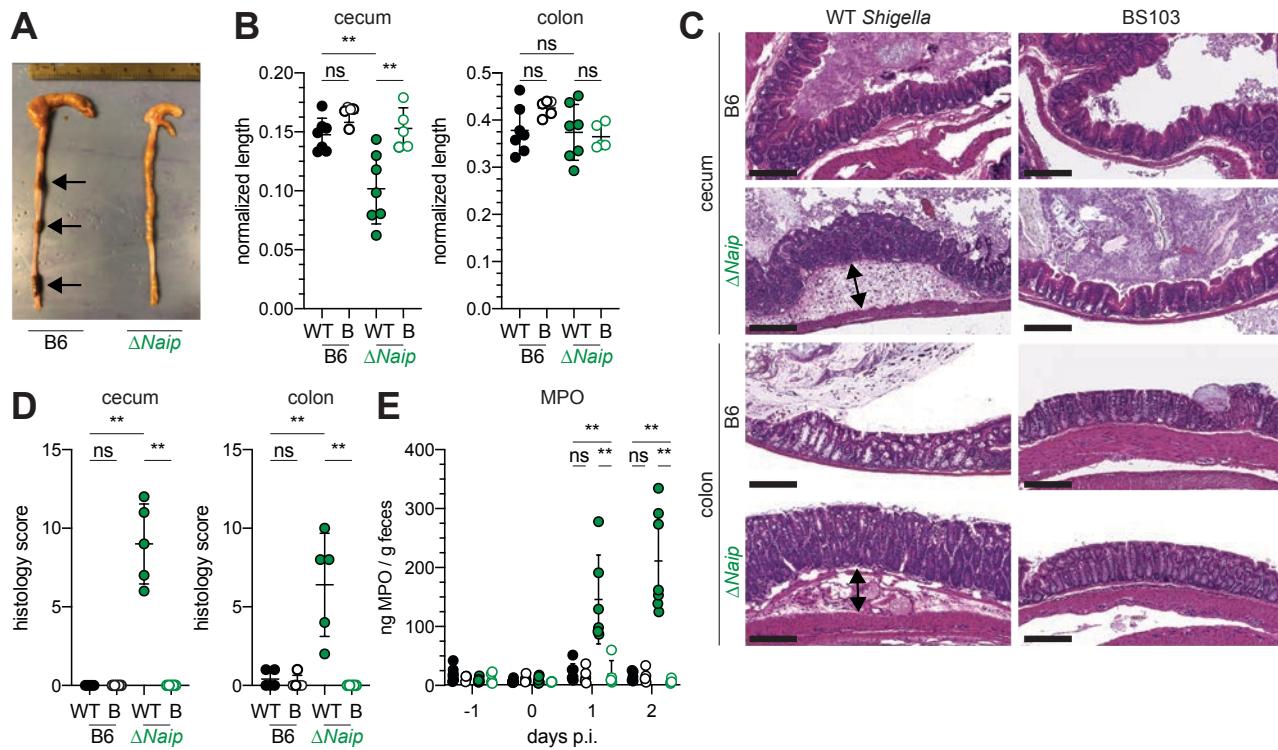


Figure 2. *Shigella*-infected B6.Δ*Naip* mice exhibit intestinal inflammation.

(A–E) B6.WT and B6.Δ*Naip* (green) mice (lacking expression of all *Naip* genes) treated orally with 25mg streptomycin sulfate were orally challenged the next day with 5×10^7 CFU of WT or BS103 (non-invasive) *Shigella*. Endpoint harvests were performed at 48 hours 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 0-4. 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.

179 by visual inspection of luminal contents and measured by the wet-to-dry ratio of fecal pellets (Figure
 180 3E). Thus, B6.*Nlrc4*^{-/-} mice phenocopy the disease susceptibility of B6.Δ*Naip* mice, and strongly
 181 suggest that the NAIP–NLRC4 inflammasome mediates the resistance of mice to *Shigella* infection.

182 Surprisingly, despite the clear differences in disease between *Shigella* infected B6.*Nlrc4*⁺ and
 183 B6.*Nlrc4*^{-/-} mice, we found no significant difference in the bacterial burdens of cecum or colon tissue
 184 (Figure 3F). To more directly measure the intracellular colonization of IECs, the primary replicative
 185 niche for *Shigella*, we enriched IECs from the ceca and colons of infected B6.*Nlrc4*⁺ and B6.*Nlrc4*^{-/-}
 186 mice (see Methods). We found an ~20-fold difference in colonization in enriched IECs between
 187 B6.*Nlrc4*⁺ and B6.*Nlrc4*^{-/-} mice (Figure 3G), indicating that disease in our model correlates with
 188 invasion of and replication in IECs. Importantly, we observed no CFU differences in feces at the time
 189 of harvest (Figure S3A), excluding the possibility that differences in IEC CFU were caused by
 190 differences in luminal *Shigella* density. These data suggest that *Shigella* colonizes the intestinal
 191 tissue during infection of either genotype but can only invade the epithelium and provoke disease in
 192 NAIP–NLRC4-deficient mice.

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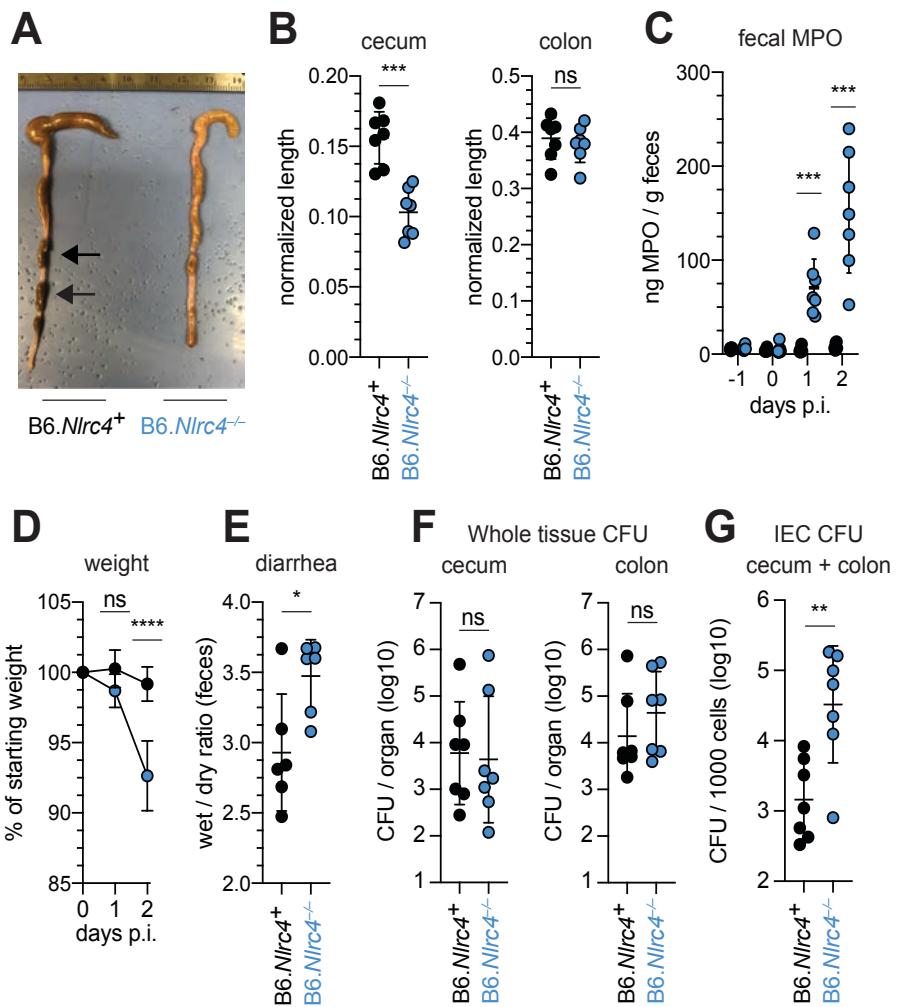


Figure 3. *Shigella*-infected B6.Nlrc4^{-/-} mice exhibit intestinal inflammation and bacterial colonization of IECs.

(A-E) B6.Nlrc4^{+/+} and B6.Nlrc4^{-/-} littermates were cohoused with B6.WT mice for a minimum of three weeks. Mice were infected as described for Figure 2. Endpoint harvests were performed 48 hours 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 feces weights before and after dehydration at 2 days p.i. A larger ratio indicates diarrhea.

(C) CFU determination from gentamicin-treated whole tissue homogenates from the cecum or colon of infected mice.

(D) CFU determination from the IEC enriched fraction of gentamicin-treated cecum and colon tissue (combined).

(E-G) Each symbol represents one mouse. Data are representative of three independent experiments. Mean \pm SD is shown in (B,D,E), Mann-Whitney test, *P < 0.05, **P < 0.01, ***P < 0.001.

194 **Shigella causes bloody diarrheal disease in 129.Nlrc4^{-/-} mice**

195 To determine whether the role of NAIP–NLRC4 in mediating protection against *Shigella* is robust
 196 across diverse mouse strains, we generated *Nlrc4*^{-/-} mice on the 129S1 genetic background.
 197 129.Nlrc4^{-/-} mice have a 10bp deletion in exon 5 of the *Nlrc4* coding sequence, resulting in loss of
 198 NLRC4 function (Figure S4). Importantly, 129S1 mice are naturally deficient in CASP11, which
 199 responds to cytosolic LPS, and thus 129.Nlrc4^{-/-} mice lack functional NAIP–NLRC4 and CASP11
 200 signaling.
 201

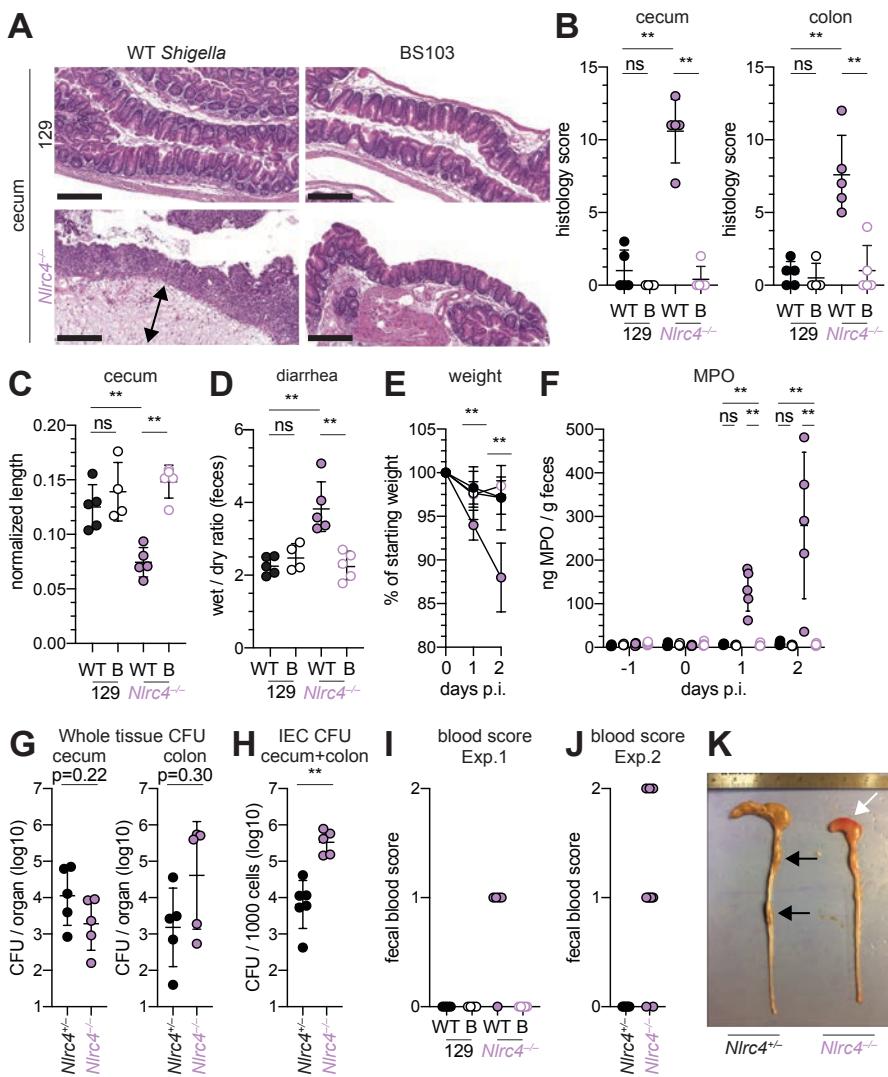


Figure 4. *Shigella*-infected 129.Nlrc4^{−/−} mice exhibit hallmarks of severe human shigellosis.

(A–H) 129.Nlrc4^{+/−} and 129.Nlrc4^{−/−} littermates were infected as described for Figure 2. Endpoint harvests were performed at 48 hours 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 0-4. 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 two 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.Nlrc4^{+/−} and 129.Nlrc4^{−/−} mice 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 \pm SD is shown in (B,D,E), Mann-Whitney test, *P < 0.05, **P < 0.01, ***P < 0.001.

203 Similar to the B6 genetic background, antibiotic-pretreated 129.*Nlrc4*^{-/-} but not 129.*Nlrc4*^{+/+}
204 littermates challenged with WT (or BS103) *Shigella* exhibited severe signs of shigellosis, including
205 pronounced edema, epithelial cell hyperplasia, and disruption of the columnar epithelium of infected
206 tissues (**Figure 4A,B**). 129.*Nlrc4*^{-/-} mice also exhibited dramatic cecum shrinkage and diarrhea
207 (**Figure 4C,D,K**), lost between eight and 18 percent of their starting weight within two days of infection
208 (**Figure 4E**), and exhibited a massive increase in fecal MPO following infection (**Figure 4F**). We found
209 no significant difference in the bacterial colonization of the whole cecum and colon tissue between
210 129.*Nlrc4*^{+/+} and 129.*Nlrc4*^{-/-} mice (**Figure 4G**). However, IECs enriched from infected 129.*Nlrc4*^{-/-}
211 mice again exhibited an ~20-fold higher bacterial burden than IECs enriched from 129.*Nlrc4*^{+/+} mice
212 (**Figure 4H**), despite similar levels of luminal colonization (**Figure S3B**).

213 A hallmark of severe human shigellosis (dysentery) is the presence of blood in patient stools
214 — a phenotype we did not observe in NAIP–NLRC4-deficient mice on the B6 background. We tested
215 129.*Nlrc4*^{-/-} mouse stools for the presence of occult blood and found that 4/5 mice infected with WT
216 *Shigella* had occult blood in their feces (**Figure 4I**). In a subsequent infection, 80% (8/10) of
217 129.*Nlrc4*^{-/-} mice had bloody stool (occult blood only, n=5; macroscopically visible blood, n=3) (**Figure**
218 **4J**). In mice with visible blood, we often observed ruptured blood vessels in the cecum or colon
219 (**Figure 4K**).

221 **Epithelial NLRC4 is sufficient to protect mice from shigellosis**

222 Given the difference in *Shigella* colonization of IECs between WT and NAIP–NLRC4-deficient mice,
223 we next sought to determine if IEC-specific expression of the NAIP–NLRC4 inflammasome is
224 sufficient to protect mice from *Shigella* infection. We thus infected B6 mice that selectively express
225 NLRC4 in IECs. These mice encode a Cre-inducible *Nlrc4* gene on an otherwise *Nlrc4*^{-/-} background
226 and are referred to as iNLRC4 mice (**Figure 5A**) (Rauch et al., 2017). Crosses of iNLRC4 and *Vil1*-
227 *Cre* mice generated animals with selective expression of NLRC4 in Villin⁺ IECs. *Shigella* infected *Vil1*-
228 *Cre*⁺ iNLRC4 mice, but not *Cre*⁻ littermate controls, were protected from intestinal inflammation to a
229 similar extent as co-housed *Nlrc4*^{+/+} mice (**Figure 5B–F**). Thus, NLRC4 expression in IECs is
230 sufficient to prevent shigellosis.

231 To further characterize the role of the NAIP–NLRC4 inflammasome during *Shigella* infection of
232 IECs, we generated intestinal epithelial stem cell-derived organoids from the ceca of 129.WT and
233 129.*Nlrc4*^{-/-} mice, and established a transwell monolayer infection assay (see Methods). We were
234 unable to recover CFUs from WT IEC monolayers infected with WT *Shigella* (**Figure 5G**). In contrast,
235 129.*Nlrc4*^{-/-} IEC monolayers supported replication of WT *Shigella*. The avirulent non-invasive BS103
236 strain was detected sporadically at low levels, independent of NAIP–NLRC4, while a strain lacking
237 IcsA, a protein essential for *Shigella* actin tail formation and cell-to-cell spread (Bernardini et al., 1989;
238 Goldberg and Theriot, 1995), colonized 129.*Nlrc4*^{-/-} IEC monolayers to a lesser extent than WT
239 *Shigella*, consistent with loss of IcsA-mediated cell-to-cell spread. Immunostaining for *Shigella* in
240 infected IEC organoid cultures also revealed intracellular replication and actin tail formation (detected
241 by fluorescent phalloidin) exclusively in *Nlrc4*^{-/-} IEC monolayers infected with WT *Shigella* (**Figure**
242 **5H**). Thus, IEC organotypic infections faithfully recapitulate the NAIP–NLRC4-dependent differences
243 in *Shigella* replication observed *in vivo*. We conclude that the protection mediated by NAIP–NLRC4 is
244 cell-intrinsic and does not require cytokine signaling to additional immune cell populations, although
245 such signaling may have additional effects *in vivo*. Importantly, these data further demonstrate that
246 *Shigella* virulence factors are functional within mouse cells and can initiate invasion and actin-based
247 motility in mouse IECs, as long as the NAIP–NLRC4 inflammasome is absent.

248 **IcsA-dependent cell-to-cell spread is required for pathogenesis**

249 The *Shigella* IcsA protein is required for virulence in humans (Collins et al., 2008; Mani et al., 2016;
250 Orr et al., 2005). To test if *icsA* is required for pathogenesis in mice, we infected 129.*Nlrc4*^{-/-} mice
251 with isogenic WT, *icsA* mutant, or BS103 *Shigella* and monitored disease for eight days. Mice infected
252 with WT *Shigella* exhibited weight loss (**Figure 6A**), diarrhea (**Figure 6B**), increases in fecal MPO
253 (**Figure 6C**), and blood in their stool (**Figure 6D**). Signs of disease in WT-infected mice peaked

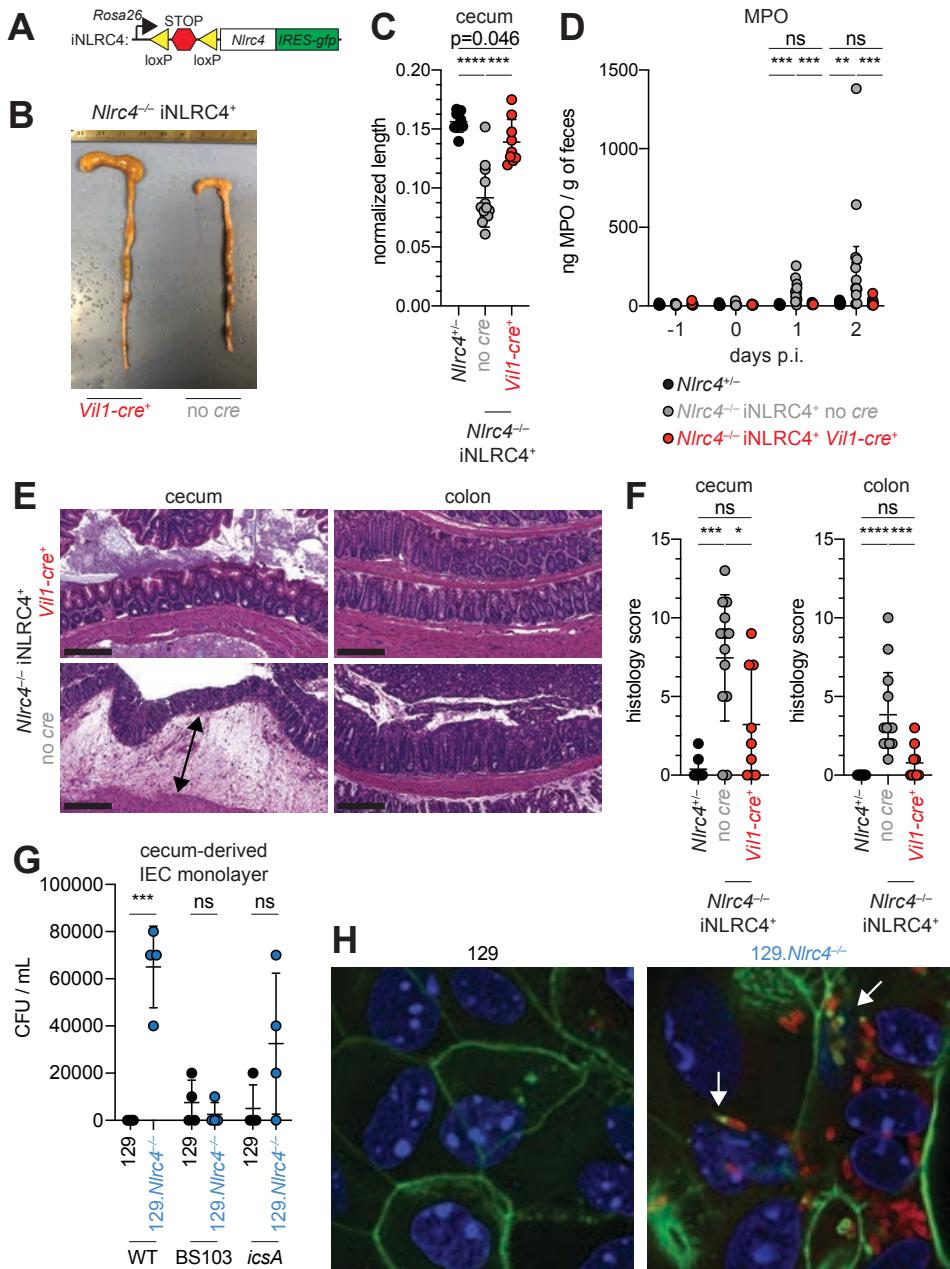


Figure 5. Nlrc4 expression in IECs is sufficient to prevent shigellosis.

(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 Nlrc4+/- mice were orally infected with 5×10^7 CFU of WT *Shigella* 24 hours after oral streptomycin treatment. Endpoint harvests were done 48 hours 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. (C,D,F) Each symbol represents one mouse.

(G) *Shigella* (WT, BS103, or icsA) CFU from transwell culture of WT or 129.Nlrc4-/- cecum-derived IEC monolayers. CFU was determined 8 hours p.i. Each symbol represents one infected monolayer.

(H) 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).

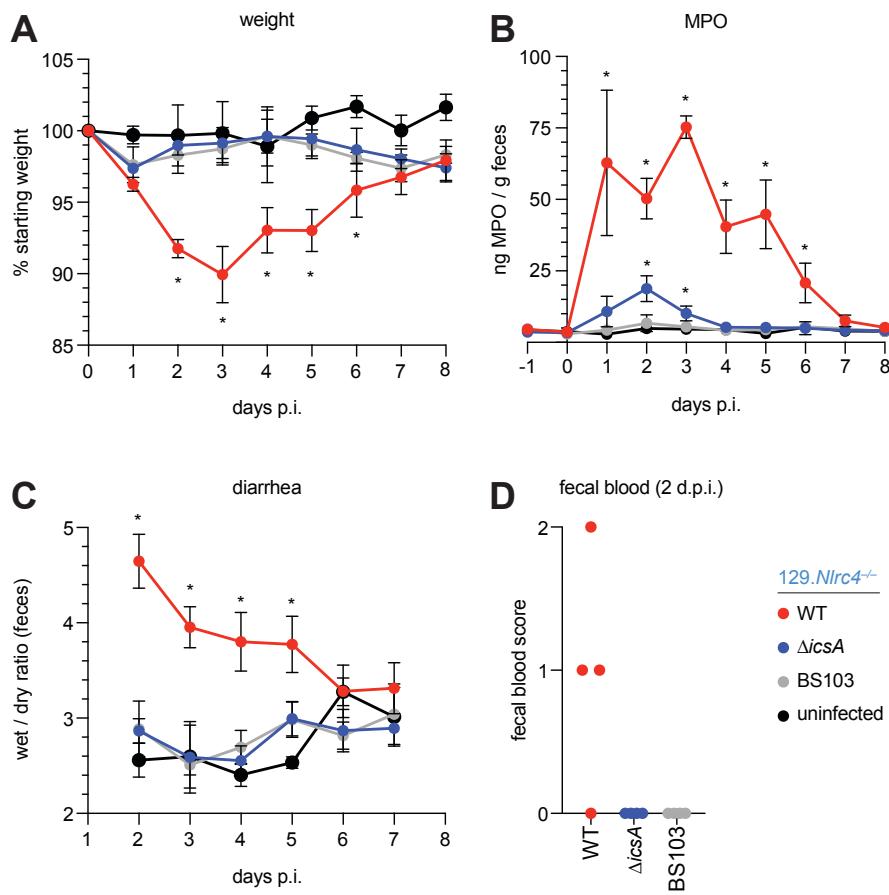


Figure 6. 129.Nlrc4^{-/-} mice are resistant to attenuated *Shigella* strains.

(A–D) 129.Nlrc4^{-/-} littermates were uninfected (black) or inoculated orally with 5 \times 10⁷ 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) MPO levels measured by ELISA from feces collected -1 through 8 days p.i.

(C) Quantification of diarrhea comparing weight of feces before and after dehydration. A larger ratio indicates diarrhea.

(D) Fecal blood scores from feces at two days post-infection (d.p.i.). 1 = occult blood, 2 = macroscopic blood. Each symbol represents feces from one mouse.

(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 + uninfected at the same day.

256 between 2-3 days post-infection with weight loss, stool consistency, and MPO signal returning to
 257 baseline levels at approximately seven days post-infection, consistent with the disease progression
 258 and resolution of human shigellosis. Interestingly, 129.Nlrc4^{-/-} mice infected with icsA mutant *Shigella*
 259 did not experience weight loss, diarrhea, or fecal blood, and largely phenocopied mice infected with
 260 the non-invasive BS103 strain (Figures 6A–D). We did observe a slight but significant increase in
 261 fecal MPO levels at 1-3 days post-infection in these mice (Figure 6B). These results suggest that, as
 262 in humans (Coster et al., 1999; Kotloff et al., 1996), icsA mutants can provoke mild inflammation upon
 263 initial colonization of the intestinal epithelium, but that dissemination of bacteria among IECs is a
 264 critical driver of severe disease.

265

266 Discussion

267 Here we demonstrate that the NAIP–NLRC4 inflammasome is a formidable species-specific barrier to
 268 *Shigella* invasion of the intestinal epithelium. *Shigella* infection of antibiotic pre-treated, NAIP–NLRC4-

269 deficient mice recapitulates key features of human shigellosis, including bacterial invasion of and
270 replication in IECs, severe inflammatory disease at relevant sites (e.g., colon, cecum), and bloody
271 diarrhea. Thus, inflammasome-deficient mice provide the first physiologically relevant mouse model of
272 bacillary dysentery, paving the way for genetic and mechanistic *in vivo* studies of the host factors
273 underlying *Shigella* pathogenesis that have long been elusive.

274 A long-held belief is that *Shigella* exploits inflammasomes to induce pyroptosis. Pyroptotic cell
275 death is presumed to allow bacteria to escape macrophages and invade the basolateral surface of
276 polarized enterocytes (Ashida et al., 2014; Lamkanfi and Dixit, 2010; Schnupf and Sansonetti, 2019).
277 Although we do not directly address this possibility, our experiments suggest that *Shigella* has instead
278 evolved to inhibit the human NAIP–NLRC4 inflammasome to evade intestinal epithelial cell death.
279 Indeed, we find that the NAIP–NLRC4 inflammasome plays a critical role in host defense by limiting
280 *Shigella* replication and spread in IECs. Further supporting this notion, only *Nlrc4*^{−/−} but not WT IEC
281 organoid monolayers are permissive to *Shigella* infection. *Salmonella* infected IECs are expelled from
282 the intestinal epithelial barrier in an NLRC4-dependent manner (Rauch et al., 2017; Sellin et al.,
283 2014). The same mechanism is also likely to occur during *Shigella* infection and suggests that
284 epithelial inflammasomes coordinate the expulsion of infected IECs as a general defense strategy
285 against enteric bacterial pathogens.

286 We only observe bloody diarrhea (dysentery) in *Nlrc4*^{−/−} mice generated on the 129 genetic
287 background. 129 mice naturally harbor a null *Casp11* allele (Kayagaki et al., 2011). Although other
288 genetic differences may contribute in part to the variation in susceptibility to *Shigella* between B6 and
289 129 NAIP–NLRC4-deficient mice, we speculate that both the NAIP–NLRC4 and the *CASP11*
290 inflammasomes mediate protection in IECs against *Shigella* invasion. However, the susceptibility of
291 B6 NAIP–NLRC4-deficient (but *CASP11*⁺) mice, as well as the resistance of *Casp11*^{−/−} mice,
292 suggest that these inflammasomes are not strictly redundant and that NAIP–NLRC4 alone is sufficient
293 to confer resistance to shigellosis in mice. The *Shigella* effector OspC3 antagonizes the human LPS
294 sensor *CASP4* (Kobayashi et al., 2013), and IEC-expressed *CASP4* provides protection against other
295 human bacterial pathogens (Holly et al., 2020; Knodler et al., 2014), underscoring the importance of
296 the LPS sensing pathway during human infection. Similarly, our finding that *Shigella* appears to
297 suppress the human NAIP–NLRC4 inflammasome implies that evasion and/or antagonism of
298 inflammasomes is a general virulence strategy during human infections.

299 There is currently no licensed *Shigella* vaccine, and very limited knowledge of what vaccine-
300 induced immune responses would be desirable to elicit to mediate protection (Barry et al., 2013; Mani
301 et al., 2016). Our new shigellosis model will finally allow the field to leverage the outstanding genetic
302 tools and reagents in the mouse to address fundamental questions about the immune response to
303 *Shigella*. Our finding that NAIP–NLRC4 inflammasome-deficient mice clear the attenuated *Shigella*
304 *icsA* strain, derivatives of which are currently deployed in human vaccine trials (Collins et al., 2008;
305 Coster et al., 1999; Ranallo et al., 2014), speaks to the readiness of our model to support testing and
306 development of *Shigella* therapeutics. More broadly, our results also provide a striking example of
307 how inflammasomes provide an important species-specific barrier against infection.

309 **Methods**

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311 **Cell culture**

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327 **Toxins**

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349 **Infection of cells in culture**

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293T cells were cultured in DMEM supplemented with 10% FBS and 2mM L-glutamine. THP1 cells were cultured in RPMI supplemented with 10% FBS and 2mM L-glutamine. B6 primary BMMs were cultured in RPMI supplemented with 10% FBS, 5% mCSF, 100U/ml penicillin, 100mg/ml Streptomycin and 2mM L-glutamine. THP1 cells were a gift from Veit Hornung, and generated as previously described (Gaidt et al., 2017). Cells were grown in media without antibiotics for infection experiments.

Bacterial strains
All experiments were conducted with the *S. flexneri* serovar 2a WT 2457T strain, or the WT-derived virulence plasmid-cured strain BS103 (Maurelli et al., 1984) or *icsA* mutant (Goldberg and Theriot, 1995; Makino et al., 1986). The *icsA* mutant strain was a gift from Marcia Goldberg. Natural streptomycin resistant strains of 2457T and BS103 were generated by plating cultured bacteria on tryptic soy broth (TSB) plates containing 0.01% Congo Red (CR) and increasing concentrations of streptomycin sulfate. Streptomycin-resistant strains were confirmed to grow indistinguishably from parental strains in TSB broth lacking antibiotics, indicating an absence of streptomycin-dependence.

Toxins
Recombinant proteins for cytosolic delivery of *Shigella* MxiH were produced using the BD BaculoGOLD system for protein expression in insect cells. The MxiH coding sequence was subcloned into pAcSG2-6xHIS-LFn using the primers: PSMpr943 F (BamHI) 5' - GAAAGG GGATCC ATG AGT GTT ACA GTA CCG GAT AAA GAT TGG ACT CTG - 3' and PSMpr944 R (NotI) 5' - GAAAGG GCGGCCGC TTA TCT GAA GTT TTG AAT AAT TGC AGC ATC AAC ATC C - 3'. The PA-6xHIS coding sequence was subcloned from pET22b-PA-6xHIS(Rauch et al., 2016) into pAcSG2 using the primers: PSMpr896 F (XbaI) 5' - GAAAGG CTCGAG ATG GAA GTT AAA CAG GAG AAC CGG TTA TTA AAT GAA TC - 3' and PSMpr897 R (NotI) 5' - GAAAGG GCGGCCGC TCA GTG GTG GTG GTG GTG GTG T - 3'. Constructs were co-transfected with BestBac linearized baculovirus DNA (Expression Systems) into SF9 cells following the manufacturer's protocol to generate infectious baculovirus. Primary virus was amplified in SF9 cells. Recombinant proteins were produced by infecting 2L of High Five cells with 1ml of amplified virus/L cells. Cells were harvested ~60 hours after infection by centrifugation at 500xg for 15 minutes. Cell pellets were resuspended in lysis buffer (50mM Tris pH7.4, 150mM NaCl, 1% NP-40 with protease inhibitors) and lysed on ice using a dounce homogenizer. Samples were then clarified at 24,000xg for 30 minutes and supernatants were batch bound to 1ml nickel resin for 2 hours at 4°C. Samples were column purified by gravity. Resin was washed with 100ml of wash buffer (20mM Tris pH7.4, 400mM NaCl, 20mM imidazole). Sample was eluted with 1ml fractions of elution buffer (20mM Tris pH7.4, 150mM NaCl, 250mM imidazole). Peak elutions were pooled and buffer exchanged into 20mM Tris pH7.4.

Infection of cells in culture
S. flexneri was grown at 37°C on tryptic soy agar plates containing 0.01% Congo red (CR), supplemented with 100µg/ml spectinomycin and 100 µg/ml carbenicillin for growth of the *icsA* strain. For infections, a single CR-positive colony was inoculated into 5ml TSB and grown shaking overnight at 37°C. Saturated cultures were back-diluted 1:100 in 5ml fresh TSB shaking for ~2 hours at 37°C. THP1 and BMM cells were seeded at 100,000 cells per well of a Nunc F96 MicroWell white polystyrene plate. Bacteria were washed three times in cell culture media, then spinfected onto cells for 10 minutes at 500xg. Bacterial invasion was allowed to proceed for an additional 20 minutes at 37°C, followed by three washes in with cell culture media containing 25mg/ml gentamicin. Cells were then maintained in cell culture media containing 2.5mg/ml gentamicin with propidium iodide (Sigma, diluted 1:100 from stock) at 37°C for the duration of the assay (30 minutes to 4 hours). A MOI of 10 was used unless otherwise specified. For suppression assays, PMA-differentiated THP1 cells were infected as described above for 1 hour. Media was then replaced with cell culture media containing

361 2.5mg/ml gentamicin and propidium iodide (1:100) and either 10 μ g/ml PA only, PA with 1.0 μ g/ml LFn-
362 MxiH, or 10 μ M nigericin. PI uptake was measured using a SpectraMax M2 plate reader, and 100%
363 cell death was set by normalizing values of infected wells to cells lysed with 1% Triton X-100 after
364 background subtraction based on media only controls.

365

366 **Establishment, propagation and infection of IECs**

367 Primary intestinal epithelial stem cell-derived organoids from the cecum were isolated and maintained
368 in culture as previously described(Miyoshi and Stappenbeck, 2013). Each transwell monolayer culture
369 was established 1:1 from a confluent enteroid Matrigel (Corning, 356255) 'dome.' Enteroids were
370 disassociated from Matrigel with 0.25% trypsin for 10 minutes, manually disrupted, resuspended in
371 monolayer culture wash media (ADMEM/F12 supplemented with 20% FBS, 1% L-glutamine) and
372 plated on polycarbonate transwells (Corning, 3413) that had been pre-coated for >1 hour at 37°C with
373 1:30 Matrigel:wash media. Monolayer cultures were differentiated for 12-14 days in complete
374 monolayer culture media (monolayer culture wash media mixed 1:1 with LWRN-conditioned media
375 supplemented with 10 μ M Y27632 (Stem Cell), and RANKL (BioLegend) in the absence of SB431542).
376 Two days prior to infection, cells were cultured in antibiotic-free monolayer culture media. Monolayers
377 were treated with 20 μ M EGTA for 15 minutes prior to *Shigella* infection (MOI=10). Bacterial invasion
378 was allowed to proceed for 2 hours, followed by gentamicin washes as described above to both upper
379 and lower compartments, then maintained in monolayer culture media containing 2.5mg/ml
380 gentamicin with propidium iodide (Sigma, diluted 1:100 from stock) at 37°C for the duration of the
381 assay (1 hour for IF and 8 hours for CFU determination). For IF, cells were washed in PBS, fixed in
382 4% paraformaldehyde for 15 minutes, permeabilized in 0.1% Triton X-100 for 15 minutes, blocked in
383 PBS with 2% BSA, 0.1% Tween-20 for 1 hour. Primary antibodies were incubated overnight, followed
384 by 1 hour stain with fluorophore-conjugated secondary antibodies and 10 minute staining with DAPI
385 and fluorophore-conjugated phalloidin. Slides were analyzed on a Zeiss LSM710. Antibodies: anti-
386 *Shigella* (Abcam, ab65282), 488 phalloidin (PHDG1-A, Cytoskeleton Inc.), Alexfluor conjugated
387 secondary antibodies (Invitrogen). To determine bacterial replication in IECs, 8h post-infection
388 monolayers were washed three times with PBS, lysed in 1% Triton X-100, and bacteria were plated
389 for CFU determination.

390

391 **Reconstituted NAIP–NLRC4 inflammasome activity assays**

392 To reconstitute inflammasome activity in 293T cells, constructs (100ng of each) producing human
393 NAIP, NLRC4, CASP1 and IL-1 β were co-transfected with constructs (200ng of each) producing
394 *Shigella* MxiL, MxiH or empty vector (pcDNA3) using Lipofectamine 2000 (Invitrogen) following the
395 manufacturer's protocol and harvested 24 hours post-transfection. For experiments using recombinant
396 proteins, fresh media containing 10 μ g/ml PA and 1.0 μ g/ml LFn-MxiH was added to cells for 3-4 hours.
397 In all experiments, cells were lysed in RIPA buffer with protease inhibitor cocktail (Roche).

398

399 **Immunoblot and antibodies**

400 Lysates were clarified by spinning at 16,100xg for 10 minutes at 4°C. Clarified lysates were denatured
401 in SDS loading buffer. Samples were separated on NuPAGE Bis-Tris 4-12% gradient gels
402 (ThermoFisher) following the manufacturer's protocol. Proteins were transferred onto Immobilon-FL
403 PVDF membranes at 375mA for 90 minutes and blocked with Odyssey blocking buffer (Li-Cor).
404 Proteins were detected on a Li-Cor Odyssey Blot Imager using the following primary and secondary
405 antibodies: anti-IL-1 β (R&D systems, AF-201-NA), anti-GFP (Clontech, JL8), anti-TUBULIN (Sigma,
406 clone TUB 2.1), Alexfluor-680 conjugated secondary antibodies (Invitrogen).

407

408 **Animal Procedures**

409 All mice were maintained in a specific pathogen free colony until 1-2 weeks prior to infection,
410 maintained under a 12 hour light-dark cycle (7am to 7pm), and given a standard chow diet (Harlan
411 irradiated laboratory animal diet) ad libitum. Wild-type C57BL/6J and 129S1/SvImJ mice were
412 originally obtained from the Jackson Laboratories. 129.Nlrc4 $^{-/-}$ animals were generated by targeting

413 *Nlrc4* via CRISPR-Cas9 mutagenesis. CRISPR/Cas9 targeting was performed by pronuclear injection
414 of Cas9 mRNA and sgRNA into fertilized zygotes, essentially as described previously(Wang et al.,
415 2013). Founder mice were genotyped by PCR and sequencing using the primers: JLR035 F 5'
416 CAGGTCACAGAAG AAGACCTGAATG 3' and JLR036 R 5' CACCTGGACTCCTGGATTGG 3'.
417 Founders carrying mutations were bred one generation to wild-type mice to separate modified
418 haplotypes. Homozygous lines were generated by interbreeding heterozygotes carrying matched
419 haplotypes. B6. Δ Naip mice were generated as described previously (Rauch et al., 2016). B6.*Nlrc4*^{−/−}
420 mice and iNLRC4 mice were generated as described previously(Rauch et al., 2017). iNLRC4 mice
421 were crossed to the *Nlrc4*^{−/−} line and then further crossed to *Vil1*-cre (Jax strain 004586) transgenic
422 lines on a *Nlrc4*^{−/−} background. Animals used in infection experiments were littermates or, if not
423 possible, were co-housed upon weaning. In rare cases when mice were not co-housed upon weaning,
424 mice were co-housed for at least one week prior to infection. Animals were transferred from a SPF
425 colony to an ABSL2 facility at least one weeks prior to infection. All animal experiments complied with
426 the regulatory standards of, and were approved by, the University of California, Berkeley Animal Care
427 and Use Committee.
428

429 ***In vivo Shigella infections***

430 Mouse infections were performed in 6-16 week old mice. Initially, mice deprived of food and water for
431 4-6 hours were orally gavaged with 100 μ L of 250 mg/mL streptomycin sulfate dissolved in water (25
432 mg/mouse) and placed in a cage with fresh bedding. 24 hours later, mice again deprived of food and
433 water for 4-6 hours were orally gavaged with 100 μ L of 5 \times 10⁸ CFU (5 \times 10⁷ CFUs per mouse) of log-
434 phase, streptomycin resistant *Shigella flexneri* 2457T, BS103, or *icsA* mutant 2457T prepared as
435 above and resuspended in PBS. Mouse weights and fecal pellets were recorded or collected daily
436 from one day prior to infection to the day of euthanasia and harvest (usually 2 days post-infection) to
437 assess the severity of disease and biomarkers of inflammation. Infection inputs were determined by
438 serially diluting a fraction of the initial inoculum and plating on TSB plates containing 0.01% CR and
439 100 μ g/mL streptomycin.
440

441 **Fecal CFUs, fecal MPO ELISAs, wet/dry ratio, fecal occult blood**

442 Fecal pellets were collected in 2mL tubes, suspended in 2% FBS in 1mL of PBS containing protease
443 inhibitors, and homogenized. For CFU enumeration, serial dilutions were made in PBS and plated on
444 TSB plates containing 0.01% CR and 100 μ g/mL streptomycin sulfate. For MPO ELISAs, fecal
445 homogenates were spun at 2,000g and supernatants were plated in triplicate on absorbent
446 immunoassay 96-well plates. Recombinant mouse MPO standard, MPO capture antibody, and MPO
447 sandwich antibody were purchased from R&D. Wet/dry ratios were determined by weighing fecal
448 pellets before and after they had been dried in a fume hood. The presence or absence of fecal occult
449 blood in fresh pellets was determined using a Hemoccult blood testing kit (Beckman Coulter).
450

451 **Histology**

452 Mice were euthanized at two days post-infection by CO₂ inhalation and cervical dislocation. Ceca and
453 colons from mice were isolated, cut longitudinally, removed of luminal contents, swiss-rolled, and
454 fixed in methacarn followed by transfer to 70% ethanol. Samples were processed by routine histologic
455 methods on an automated tissue processor (TissueTek, Sakura), embedded in paraffin, sectioned at
456 4 μ m thickness on a rotary microtome, and mounted on glass slides. Sections were stained with
457 hematoxylin and eosin on an automated histostainer and coverslipped. Histopathological evaluation
458 was performed by light microscopy (Olympus BX45, Olympus Corporation) at magnifications ranging
459 from x20 to x600 by a board-certified veterinary pathologist (I.L.B.) who was blinded to the
460 experimental groups at the time of evaluation. Representative images were generated as Tiff files
461 from digitized histology slides scanned on a digital slide scanner (Leica Aperio AT2, Leica
462 Biosystems). Images were taken using freely downloadable software (Image Scope, Leica Aperio,
463 Leica Biosystems) and processed in Adobe Photoshop. Photo processing was confined to global
464 adjustments of image size, white balance, contrast, brightness, sharpness, or correction of lens

465 distortion and did not alter the interpretation of the image. Sample preparation, imaging, and histology
466 scoring was conducted by the Unit for Laboratory Animal Medicine at the University of Michigan.
467

468 **Intestinal CFU determination**

469 To enumerate whole tissue intestinal CFU, ceca and colons from mice were isolated, cut longitudinally
470 and removed of luminal contents, placed in culture tubes containing 400 μ g/mL gentamicin antibiotic
471 in PBS, vortexed, and incubated in this solution for 1-2 hours. Organs were washed 5 times in PBS to
472 dilute the gentamicin, homogenized in 1mL of PBS, serially diluted, and plated on TSB agar plates
473 containing 0.01% CR and 100 μ g/mL streptomycin. To enumerate intracellular CFU from the intestinal
474 epithelial cell fraction of the cecum and colon, organs prepared as above were incubated in RPMI with
475 5% FBS, 2mM L-glutamine, and 400 μ g/ml of gentamicin for 1-2 hours. Tissues were then washed 5
476 times in PBS, cut into 1cm pieces, placed in 15mL of stripping solution (HBSS, 10mM HEPES, 1mM
477 DTT, 2.6mM EDTA), and incubated at 37°C for 25 minutes with gentle agitation. Supernatants were
478 passed through a 100 μ m filter and the remaining pieces of tissue were shaken in a 50mL conical with
479 10 mL of PBS and passed again through the 100 μ m filter. This enriched epithelial cell fraction was
480 incubated in 50 μ g/mL gentamicin for 25 minutes on ice, spun at 300xg at 4°C for 8 minutes, and
481 washed twice by aspirating the supernatant, resuspending in PBS, and spinning at 300xg at 4°C for 5
482 minutes. After the first wash, a fraction of cells were set aside to determine the cell count. After the
483 second wash, the pellet was resuspended and lysed in 1mL of 1% Triton X-100. Serial dilutions were
484 made from this solution and plated on TSB agar plates containing 0.01% CR and 100 μ g/ml
485 streptomycin and CR+ positive colonies were counted following overnight incubation at 37°C.
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487

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646 **Acknowledgements**

647 We thank M. Goldberg for advice and for sharing the *icsA* mutant *Shigella* strain. We are grateful to G.
648 Barton and H. Darwin for comments on the manuscript, and members of the Vance and Barton Labs
649 for discussions. Funding: R.E.V. is an HHMI Investigator and is supported by NIH AI075039 and
650 AI063302; P.S.M. is supported by a Jane Coffin Childs Memorial Fund postdoctoral fellowship. J.L.R.
651 is an Irving H. Wiesenfeld CEND Fellow; E.A.T. is supported by the UC Berkeley Department of
652 Molecular and Cell Biology NIH Training Grant 5T32GM007232-42; C.F.L. is a Brit d'Arbeloff MGH
653 Research Scholar and supported by NIH AI064285 and NIH AI128743; I.R. is supported by the
654 Medical Research Foundation MRF2012.

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656 **Competing interests**

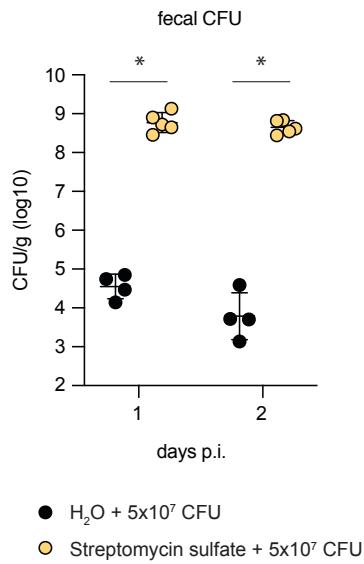
657 R.E.V. has a financial relationship with Aduro BioTech and Ventus Therapeutics and both he and the
658 companies may benefit from the commercialization of the results of this research.

659

660 **Author contributions**

661 P.S.M, J.L.R., and R.E.V. conceived the study, designed the experiments, and wrote the original
662 manuscript; P.S.M and J.L.R. performed the majority of the experiments with contributions from E.A.T
663 and R.A.C.; I.R., L.G., and C.F.L. provided resources; I.R. and C.F.L. contributed to methodology and
664 supervision; P.S.M, J.L.R., R.E.V., I.R., L.G., and C.F.L. edited and reviewed the manuscript.

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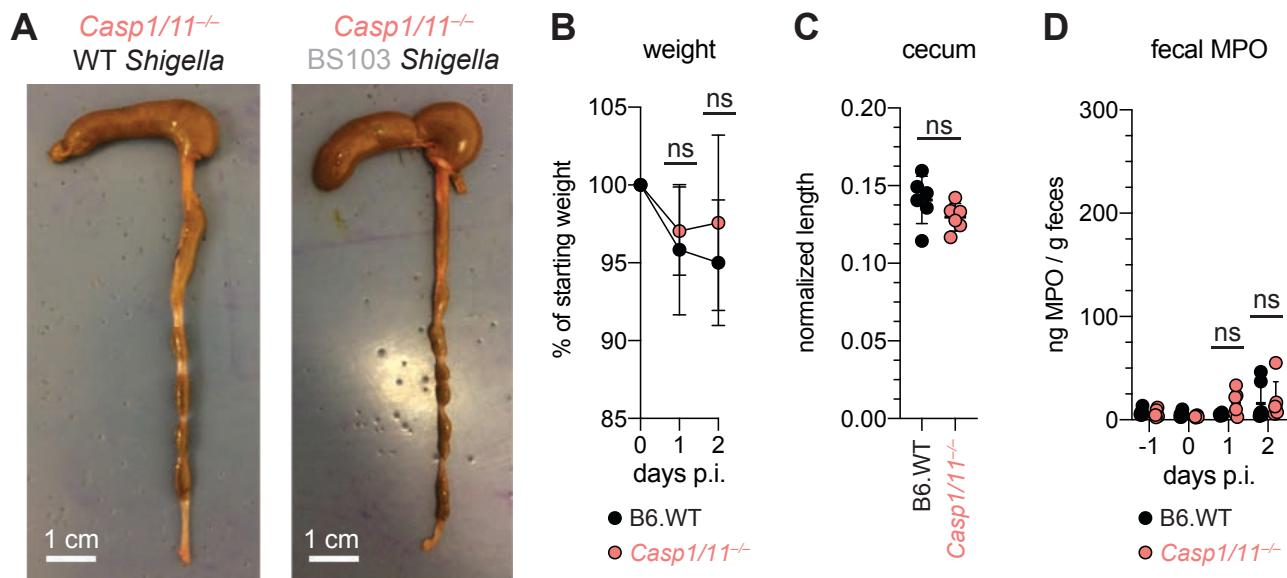


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Figure S1. Antibiotic pre-treatment followed by oral route *Shigella* infection permits substantial lumenal colonization.

CFU determination per gram (g) feces of B6 WT mice that were treated orally with 25mg 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, $^*P < 0.05$.



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675 **Figure S2. B6.Casp1/11^{-/-} mice are resistant to oral *Shigella* challenge.**

676 (A-D) B6.WT (black) and B6.Casp1/11^{-/-} (peach) mice were treated orally with 25mg streptomycin sulfate and were orally
677 challenged the next day with 5×10^7 CFU of either WT or BS103 (avirulent) *Shigella*.

678 (A) Representative images of the cecum and colon dissected at 2 days post-infection (p.i.).

679 (B) Mouse weights.

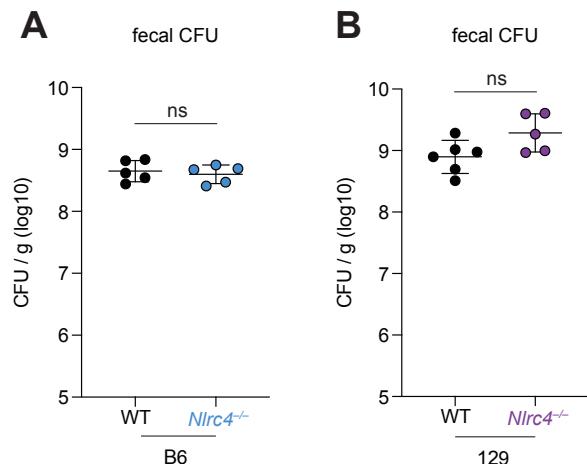
680 (C) Quantification of cecum and colon lengths. Values were normalized to mouse weight prior to infection; cecum length (cm)
681 / mouse weight.

682 (D) MPO levels measured by ELISA from feces of B6.WT and B6.Casp1/11^{-/-} mice collected -1 through 2 days p.i.

683 Each symbol represents one mouse. Data are representative of three independent experiments. Mean ± SD is shown in
684 (B,C,D), Mann-Whitney test, *P < 0.05.

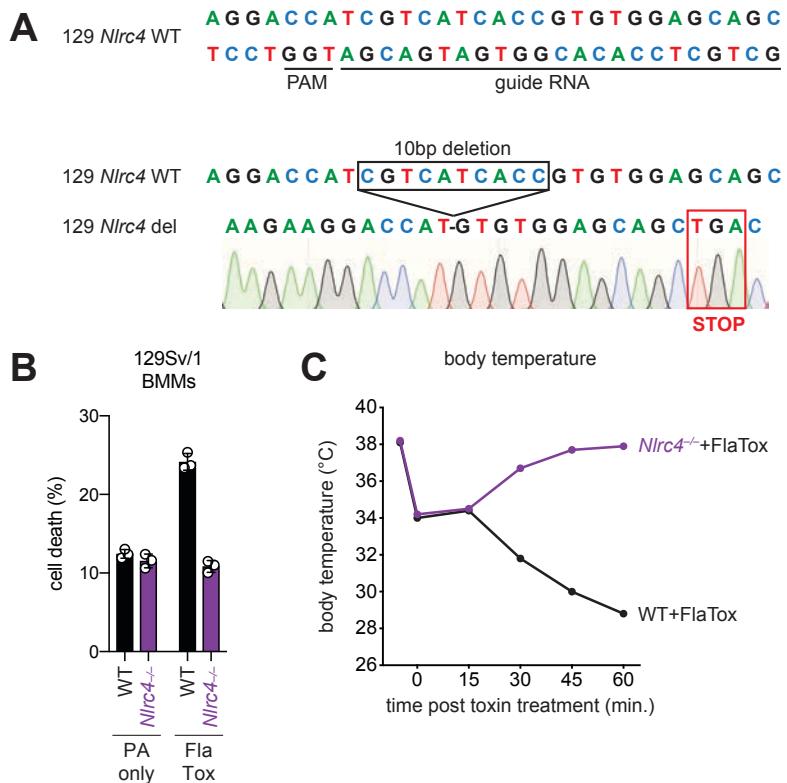
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Figure S3. Luminal colonization by *Shigella* is similar between WT and NAIP–NLRC4-deficient mice.
(A) CFU determination per gram (g) feces from B6 and B6. $Nlrc4^{-/-}$ *Shigella*-infected mice 2 days post-infection.
(B) CFU determination per gram (g) feces from 129 and 129. $Nlrc4^{-/-}$ *Shigella*-infected mice 2 days post-infection.
Each symbol represents one mouse. Mann-Whitney test, * $P < 0.05$.



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Figure S4. Construction and functional characterization of *Nlrc4* knockout mice on the 129S1/SvImJ genetic background.

(A) The targeted wildtype *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 μ g/mL PA alone or PA + 10 μ g/mL LFn-FlaA (LFn fused to *Legionella pneumophila* flagellin, "FlaTox"). Cell death was measured 30 minutes 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 μ g/g body weight PA + 0.1 μ g/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.