

# Human Enteric $\alpha$ -Defensin 5 Promotes *Shigella* Infection by Enhancing Bacterial Adhesion and Invasion

Dan Xu<sup>1,2,3†</sup>, Chongbing Liao<sup>1,2†</sup>, Bing Zhang<sup>1</sup>, W. David Tolbert<sup>3</sup>, Wangxiao He<sup>1,2,3</sup>,  
Zhijun Dai<sup>4</sup>, Wei Zhang<sup>1</sup>, Weirong Yuan<sup>3</sup>, Marzena Pazgier<sup>3</sup>, Jiankang Liu<sup>1</sup>, Jun Yu<sup>5</sup>,  
Philippe J. Sansonetti<sup>6</sup>, Charles L. Bevins<sup>7</sup>, Yongping Shao<sup>1,2\*</sup> and Wuyuan Lu<sup>1,2,3\*</sup>

<sup>1</sup>Key Laboratory of Biomedical Information Engineering of the Ministry of Education, School of  
Life Science and Technology, Xi'an Jiaotong University, Xi'an, China.

<sup>2</sup>Center for Translational Medicine, Frontier Institute of Science and Technology, Xi'an Jiaotong  
University.

<sup>3</sup>Institute of Human Virology and Department of Biochemistry and Molecular Biology, University  
of Maryland School of Medicine, Baltimore, Maryland, USA.

<sup>4</sup>The Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine.

<sup>5</sup>Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow,  
Scotland, UK.

<sup>6</sup>Institut Pasteur, Unité de Pathogénie Microbienne Moléculaire, 75724 Paris, France.

<sup>7</sup>Department of Microbiology and Immunology, University of California, School of Medicine,  
Davis, California, USA.

†These authors contributed equally to this work.

\*Correspondence to: [wlu@ihv.umaryland.edu](mailto:wlu@ihv.umaryland.edu) (lead contact) or

[yongping.shao@xjtu.edu.cn](mailto:yongping.shao@xjtu.edu.cn)

21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36

## SUMMARY

*Shigella* is a Gram-negative bacterium that causes bacillary dysentery worldwide. It invades the intestinal epithelium to elicit intense inflammation and tissue damage, yet the underlying mechanisms of its host selectivity and low infectious inoculum remain perplexing. Here we have reported that *Shigella* co-opts human  $\alpha$ -defensin 5 (HD5), a host defense peptide important for intestinal homeostasis and innate immunity, to enhance its adhesion to and invasion of mucosal tissues. HD5 promoted *Shigella* infection *in vitro* in a structure-dependent manner. *Shigella*, commonly devoid of effective host-adhesion apparatus, preferentially targeted HD5 to augment its ability to colonize the intestinal epithelium through interactions with multiple bacterial membrane proteins. HD5 exacerbated infectivity and *Shigella*-induced pathology in a culture of human colorectal tissues and three animal models. Our findings illuminate how *Shigella* exploits innate immunity by turning HD5 into a virulence factor for infection, unveiling a mechanism of action for this highly proficient human pathogen.

## INTRODUCTION

37  
38 Intestinal colonization and epithelial adhesion is a crucial early event in the  
39 pathogenesis of many enteropathogens, which can then enable bacterial invasion of  
40 host epithelial cells and disseminated infection (Cossart and Sansonetti, 2004;  
41 Sonnenberg, 2000; Pizarro-Cerdá and Cossart, 2006). Most enterobacteria use  
42 fimbriae, an adhesive filamentous organelle protruding from the outer-membrane  
43 surface of Gram-negative bacteria, for host attachment (Choudhury et al., 1999; Kline et  
44 al., 2009; Li et al., 2009). Paradoxically, *Shigella*, the etiological agent of bacillary  
45 dysentery, lacks such adhesion machinery in general, yet it is a remarkably infectious  
46 and contagious enteropathogen that invades and elicits intense inflammation and tissue  
47 damage of the colorectal epithelium (Carayol and Tran Van Nhieu, 2013; Perdomo et  
48 al., 1994; Phalipon and Sansonetti, 2007; Schroeder and Hilbi, 2008). Despite a  
49 continued search for mechanisms of adhesion, the question of how *Shigella* has  
50 acquired extraordinary infectivity without a highly efficient and more general host  
51 adhesion apparatus remains unanswered. In studying the mode of action of  
52 antimicrobial peptides against *Shigella*, we found that when the human enteric  $\alpha$ -  
53 defensin 5 (HD5), an abundant and important host protective molecule produced by  
54 Paneth cells of the small intestine (Bevins and Salzman, 2011), binds *Shigella*, it  
55 augments infectivity via enhanced bacterial adhesion to and subsequent invasion of  
56 epithelial cells and tissues. We posited that *Shigella* subverts innate host defense to  
57 colonize and destroy the intestinal epithelium by turning HD5 into a molecular  
58 accomplice that imparts its infectivity and host selectivity.

59

## RESULTS

60

61 **HD5 promotes *Shigella* infection of epithelial cells *in vitro*.** Antimicrobial peptides,  
62 expressed primarily in phagocytes and epithelia, play critical roles in host immune  
63 defense against pathogenic infection often through microbicidal activity (Bevins and  
64 Salzman, 2011; Ganz, 2003; Lehrer and Lu, 2012; Selsted and Ouellette, 2005; Zasloff,  
65 2002). To study the role of antimicrobial peptides in *Shigella* pathogenesis, we tested a  
66 panel of six human and two murine defensin peptides against the *Shigella flexneri* strain  
67 Sf301 in an *in vitro* antibacterial activity assay (Ericksen et al., 2005; Mastroianni and  
68 Ouellette, 2009), including human neutrophil alpha-defensin 1 (HNP1), enteric alpha-  
69 defensins 5 and 6 (HD5 and HD6), beta-defensins 2 and 3 (HBD2 and HBD3), the  
70 cathelicidin peptide LL-37, and murine alpha-defensins (cryptdins) 3 and 4 (Crp3 and  
71 Crp4). While most of the human antimicrobial peptides displayed varying but weak  
72 bactericidal activity at low micromolar concentrations, HD6 and the two cryptdins  
73 showed little killing (Fig. S1A). Weak antibacterial activity was also observed for HD5  
74 with the *Shigella sonnei* strain Ss86 and five clinical isolates (Fig. S1B). To test whether  
75 this antibacterial activity correlated with the ability of these peptides to inhibit *Shigella*  
76 infection *in vitro*, we quantified bacterial adhesion, invasion and intracellular replication  
77 in a conventional infection assay using HeLa cells (Fig. S2). When added to Sf301, two  
78 human alpha-defensins, HNP1 and HD5, enhanced *Shigella* adhesion to and invasion  
79 of HeLa cells in an inoculum-dependent manner, with the enteric alpha-defensin HD5  
80 being markedly more active than its neutrophil counterpart HNP1 (Fig. 1A-B). In fact,  
81 HD5 at 2  $\mu$ M enhanced bacterial adhesion or invasion by more than 30-fold. Our

82 subsequent study thus focused on HD5 not only for its site of abundant expression  
83 relevant to *Shigella* infection, but also for its superior infection-enhancing activity.

84 Dissection of time-dependent cellular events in *Shigella* infection further revealed  
85 that HD5 acted early, predominantly at the bacterial infection step (adhesion and  
86 invasion) rather than on intracellular replication (Fig. 1C-D). Similar results were  
87 obtained for HD5 with *Shigella sonnei* and also multiple clinical isolates (Fig. S1C-D).  
88 Consistent with these findings, immunofluorescence and scanning electron microscopy  
89 (SEM) studies showed that HD5-treated GFP-expressing or unlabeled Sf301 clustered  
90 on the surface of HeLa cells within 10 min of bacteria and HeLa cocubation (Fig. 1E).  
91 To verify the viability of adhered *Shigella*, we transformed mCherry-labeled Sf301 (red)  
92 with a reporter plasmid that expresses GFP (green) when the type 3 secretion system  
93 (T3SS) is activated upon host cell contact (Campbell-Valois et al., 2014), an obligate  
94 step in *Shigella* invasion, and found that HD5 treatment turned many clustered bacteria  
95 yellow (colocalized green and red) within 60 min of cocubation with HeLa cells (Fig.  
96 1F). Without host cells, HD5 alone failed to change the color of bacteria as illustrated in  
97 Figs. 1F and S1G, suggesting that it did not activate the T3SS directly. Furthermore,  
98 HD5 was found to promote strong adhesion of *Shigella* even when the type III secretion  
99 system (T3SS), responsible for bacterial invasion and virulence, was inactivated either  
100 transcriptionally at low growth temperature (Maurelli et al., 1984) or genetically by  
101 deletion of *spa33* encoding an essential component of the T3SS (Morita-Ishihara et al.,  
102 2006) (Fig. S1E). In addition, HD5-enhanced *Shigella* infection was not restricted to  
103 HeLa cells, albeit the standard *in vitro* infection model for *Shigella* (Philpott et al., 2000),  
104 as a wide variety of epithelial cell lines of different species and/or tissue origins were

105 found equally susceptible (Fig. S1F and Table S1). Taken together, these results  
106 indicate that HD5 promoted *Shigella* infection *in vitro* by enhancing bacterial adhesion to  
107 epithelial cells, leading to increased bacterial invasion.

108 **HD5 promotes *Shigella* infection *in vivo*.** The impact of HD5 on the *in vivo*  
109 invasiveness of *Shigella* was extensively examined in three different animal models: (1)  
110 cornea infection in guinea pigs (the classic "Sereny test") (Sereny, 1955), (2) colon  
111 infection in guinea pigs (Arena et al., 2015; Shim et al., 2007), and (3) ileum and colon  
112 infection in mice (Sawasvirojwong et al., 2013). For the Sereny test, Sf301 was  
113 inoculated in the eye at a density of  $1 \times 10^6$  CFU/eye, together with HD5 at 0, 4, and 8  
114  $\mu\text{M}$ ; the severity of infection graded from 1 to 3 was scored daily for one week (Fig.  
115 S3A). Eye infection worsened over time with the symptoms developing much more  
116 rapidly in the HD5-treated groups than the control group. As shown in Fig. 2A, in the  
117 absence of HD5, three out of fifteen animals developed full-blown keratoconjunctivitis  
118 and one showed signs of mild irritation, after one week. By sharp contrast, in the  
119 presence of 4  $\mu\text{M}$  HD5 the number of animals with high grade keratoconjunctivitis  
120 increased to 6 in just three days (Fig. S3A) and to 11 (of 15) in one week, and three  
121 animals died in the group treated with 8  $\mu\text{M}$  HD5. Of note, treatment with HD5 alone at  
122 8  $\mu\text{M}$  had no adverse effects on the animals (Fig. 2A). These data support that HD5  
123 facilitated bacterial adhesion to and invasion of corneal epithelial cells in guinea pigs.

124 In a second guinea pig model, anaesthetized animals were inoculated  
125 intrarectally with  $1 \times 10^8$  CFU of HD5-treated,  $1 \times 10^8$  CFU of mock-treated,  $1 \times 10^9$  CFU of  
126 mock-treated GFP-expressing Sf301 in 200  $\mu\text{l}$  medium, or medium alone at equal  
127 volume (negative control). Animals were euthanized at 4, 8, 24 and 48 h post-challenge,

128 and the distal 10 cm of colon tissue was harvested for quantitative fluorescence imaging  
129 of *Shigella* infection as previously described (Arena et al., 2015). Analysis of tissue-  
130 associated bacteria revealed that HD5-treated *Shigella* achieved a much greater early  
131 adhesion and colonization (at both 4 and 8 h post-challenge time points), when  
132 compared to the corresponding  $1 \times 10^8$  CFU of mock-treated bacteria, but also when  
133 compared to a higher inoculum ( $1 \times 10^9$  CFU) of mock-treated bacteria (Figs. 2B-C, S3B).  
134 SEM imaging corroborated these results by showing extensive colonization of colonic  
135 crypts by HD5-treated *Shigella* (Fig. 2D).

136         Histological changes by bacterial infection in the colon at the later time points (24  
137 h and 48 h) were examined by HE staining (Fig. 2E-G). All *Shigella*-inoculated animals  
138 showed some evidence of histopathology at 24 h and 48 h post-challenge, albeit to  
139 different extents. Without HD5 treatment,  $1 \times 10^8$  CFU of Sf301 caused mild disruption of  
140 luminal surface adjacent to the initial inoculating site 24 h post-challenge, whereas  
141  $1 \times 10^9$  CFU caused much more severe tissue damage during the same time period. HD5  
142 potentiated the tissue damage caused by  $1 \times 10^8$  CFU of Sf301, characterized by  
143 thickened submucosa and disruption of mucosal and submucosal layers, with edema,  
144 erosion, and crypt distortion comparable to  $1 \times 10^9$  CFU. By 48 h after initial inoculation,  
145 most of the histopathology caused by  $1 \times 10^8$  CFU of Sf301 inoculum had healed, and the  
146 integrity of mucosa was almost restored. In contrast, the infection by  $1 \times 10^8$  CFU of HD5-  
147 treated Sf301 bacteria showed worsened pathology at 48 h, comparable to infection  
148 with 10-fold higher inoculum ( $1 \times 10^9$  CFU) Sf301 *Shigella*. The initial infection loci  
149 expanded and there was nearly complete destruction of mucosal and submucosal  
150 layers. Moreover, rather than a single major infection locus caused by Sf301 bacteria in

151 each animal of the mock treated groups, multiple (3-5) infection loci were identified in  
152 colonic tissue of each animal infected by HD5-treated *Shigella* 48 h post-challenge.  
153 These data are consistent with the conclusion that HD5-treated *Shigella* disseminated  
154 more extensively along the colonic tissue mucosa within 8 h after initial inoculation than  
155 did mock-treated Sf301 bacteria. Of note, core body temperature of challenged animals,  
156 a representative sign of bacillary dysentery, showed a dramatic HD5-dependent effect.  
157 While inoculation of  $1 \times 10^8$  CFU of mock-treated Sf301 *Shigella* resulted in a marginal  
158 increase in body temperature within 48 h,  $1 \times 10^8$  CFU of HD5-treated bacteria caused  
159 severe fever in inoculated animals (an average increase by 1.3 °C) from 24 h,  
160 comparable to the high-inoculum ( $1 \times 10^9$  CFU) group (Fig. 2H).

161 To extend the findings from the two guinea pig models, we adopted a well-  
162 established murine ileal and colonic loop model to investigate the impact of HD5 on the  
163 *in vivo* colonization and pathogenesis of *Shigella* (Sawasvirojwong et al., 2013).  
164 Analysis of tissue-associated bacteria in mouse colonic and ileal loops confirmed that  
165 HD5-treated *Shigella* was much more robust in adhesion and colonization than mock-  
166 treated bacteria (Fig. 2I-J). Taken together, our findings from three different animal  
167 models provide compelling evidence that HD5 can promote *Shigella* infection *in vivo* by  
168 enhancing bacterial adhesion to and invasion of epithelial tissues.

169 **HD5 exacerbates *Shigella*-elicited human tissue damage *ex vivo*.** Human colorectal  
170 explants were cultured as an *ex vivo* model to further investigate the role of HD5 in  
171 pathogenesis with human tissue (Fig. S3C). SEM analysis revealed that HD5  
172 significantly enhanced the adhesion of clustered *Shigella* cells to the colonic mucosa  
173 (Fig. 3A-B). Histologically, human colonic tissues mock-treated with medium or treated



174 with HD5 alone at 8  $\mu$ M displayed an intact luminal epithelial layer and normal tissue  
175 architecture (Fig. 3C-D). In the absence of HD5, samples inoculated with *Shigella*  
176 ( $1 \times 10^6$  CFU) showed disruption of the luminal surface of epithelia, but the tissue  
177 architecture was largely maintained (Fig. 3C-D). Co-inoculation of *Shigella* and HD5 led  
178 to a complete destruction of not only the epithelium, but also its underlying tissue  
179 structure (Fig. 3D). These results indicate that *Shigella* was capable of invading the  
180 human colonic epithelium and did so much more efficiently in the presence of HD5, thus  
181 further corroborating the proposed model of pathogenesis.

182 To provide additional support of the *ex vivo* study, we established an *in vitro*  
183 infection model using a polarized epithelial monolayer of human Caco-2 cells as  
184 previously described (Mounier et al., 1992). As shown in Fig. 4A, addition of 8  $\mu$ M HD5  
185 to the Caco-2 monolayer had limited impact over a period of 24 h on its integrity and  
186 permeability as measured by trans-epithelial electrical resistance (TEER). While  
187 *Shigella* alone was slowly destructive, a dramatically accelerated disintegration of the  
188 monolayer was evident in the presence of HD5. In fact, at 6 h, HD5 treatment increased  
189 intracellular CFU by over 300-fold (compared with the mock treatment group) due  
190 presumably to highly efficient bacterial invasion and cell-cell spreading of amplifying  
191 *Shigella* (Fig. 4B). These results are consistent with fluorescence and SEM imaging  
192 studies showing that HD5 potentiated *Shigella* destruction of the tight junction of the  
193 epithelium (Fig. 4C).

#### 194 **Human luminal fluids from the small intestine promote *Shigella* infection *in vitro*.**

195 When maximally secreted, the concentration of HD5 in the lumen of the small intestine  
196 is estimated to be in the millimolar range (Ayabe et al., 2000; Ghosh et al., 2002;

197 Ouellette, 2011). However, unstimulated the quantity of HD5 is far lower in aspirated  
198 small intestinal fluids from healthy donors undergoing routine screening colonoscopy, in  
199 part due to the large volumes of electrolyte solution to enable sampling. When such  
200 clinical aspirates collected from 17 individuals were analyzed directly, none were found  
201 active in enhancing *Shigella* adhesion to and invasion of HeLa cells *in vitro*. When  
202 concentrated and tested for activity in the infection assay, the ileal fluids became active  
203 in promoting *Shigella* infection *in vitro* (Fig. 4D-E). The infection-enhancing activity was  
204 largely neutralized by anti-HD5 serum, indicating that HD5 in human luminal fluids from  
205 the small intestine contributed to *Shigella* infection *in vitro*. In addition, if prior to  
206 concentration exogenous HD5 (4  $\mu$ M) was added to the intestinal fluid samples, they  
207 gained the ability to promote *Shigella* infection (Fig. S3E), albeit to various degrees.  
208 These data support that the activity shown for 1-8  $\mu$ M concentrations of HD5 in the *in*  
209 *vitro* and *in vivo* models of *Shigella* infection reported in the current investigation reflects  
210 similar activity reasonably anticipated during clinical infection in the human intestine.

211 **Structural basis of HD5-enhanced *Shigella* infection.** The primary structures of  
212 epithelial defensins vary remarkably from species to species (Bevins and Salzman,  
213 2011; Ganz, 2003; Lehrer and Lu, 2012; Selsted and Ouellette, 2005). To elucidate the  
214 structural basis underlying HD5-promoted *Shigella* infection of host cells, we functionally  
215 analyzed a panel of 23 analogs of HD5 in an infection assay using Sf301 and HeLa  
216 cells (Fig. 5A-B). Our data showed that: (1) the native tertiary structure of HD5 was  
217 absolutely required, as replacement of the six Cys residues by isosteric aminobutyric  
218 acid (Abu) to remove the three intra-molecular disulfide bonds of HD5 abolished  
219 adhesion and invasion enhancement; (2) while native HD5 readily dimerizes, the

220 dimerization-debilitating analog E21Me-HD5, where the amide peptide bond is  
221 methylated at Glu21 to impair HD5 dimerization (Rajabi et al., 2012), was largely  
222 inactive in promoting *Shigella* infection; (3) Ala replacement of bulky hydrophobic  
223 residues, particularly those in the C-terminal region of HD5, such as Leu26, Tyr27 and  
224 Leu29, was functionally detrimental; (4) Arg28 was a key residue for activity, although  
225 the other cationic Arg residues were functionally dispensable. Because Tyr27 and  
226 Arg28 appeared to be critically important residues, we crystallized both Y27A-HD5 and  
227 R28A-HD5 and determined their structures to 1.75 and 2.4 Å, respectively (Fig. 5C-D).  
228 The functionally inactive Y27A-HD5 existed as a monomer (Fig. 5E), consistent with the  
229 fact that this residue, but not Arg28, is part of a contiguous hydrophobic core (along with  
230 Leu29) mediating HD5 dimerization. The R28A-HD5 formed a canonical dimer similar to  
231 wild-type HD5 (Fig. 5F), but was inactive, indicating dimerization alone was not  
232 sufficient for activity. Rather, for the importance of Arg28, mutational and structural  
233 analysis identified two amphipathic surfaces in the HD5 dimer (Fig. 5G), comprising  
234 residues Leu16, Val19, Leu26 and Arg28, which likely operate in tandem in interactions  
235 with molecular and cellular targets of the defensin. Taken together, these results  
236 highlight hydrophobicity and selective cationicity that are structurally segregated in a  
237 stable dimer as the most important molecular determinants of HD5 function. Of note,  
238 these structure-function determinants are in agreement with previous studies of  
239 antibacterial and antiviral activities of HD5 (Lehrer and Lu, 2012; Rajabi et al., 2012;  
240 Tenge et al., 2014).

241 **Fimbria deficiency in *Shigella* confers its sensitivity to HD5-mediated**  
242 **enhancement in bacterial infection.** Type I fimbriae are the major components that

243 impart host adhesiveness for many enterobacteria such as *E. coli* and *Salmonella*  
244 (Edwards and Puente, 1998; Jones et al., 1995; Pizarro-Cerdá and Cossart, 2006), yet  
245 they are conspicuously absent from most *Shigella* strains, including Sf301 and many  
246 clinical isolates, due to independent mutations in the *fim* gene clusters (Bravo et al.,  
247 2015; Snellings et al., 1997). To investigate the role of fimbriae in HD5-enhanced  
248 *Shigella* infection, we restored fimbria production in Sf301 by expressing the *fim*  
249 cassette from *E. coli* JM103 (Fig. 6A). While fimbria-expressing Sf301 showed much  
250 stronger basal adhesion to HeLa cells than wild-type Sf301, its improved adhesion  
251 capacity became largely insensitive to HD5 treatment (Fig. 6B). Results paralleling  
252 these were found for *E. coli* BL21, the fimbria-deficient counterpart of JM103 of the  
253 same genetic background, as well as the *fimA*-deleted strain JM103  $\Delta$ *fimA* (Fig. 6C).  
254 Similar to observations with *Shigella*, the fimbria-expressing *E. coli* showed stronger  
255 basal adhesion to HeLa cells, but the enhanced adhesion became largely insensitive to  
256 HD5 treatment. Consistent with these results, HD5 promoted a substantial increase in  
257 adhesion to HeLa cells of the mutant strain SNP494 of *Salmonella enterica* serovar  
258 Typhimurium, whose fimbria and flagella were deleted (Chu et al., 2012), despite a  
259 lower basal adhesion level as compared with its wild type counterpart IR715 (Fig. S4H-  
260 I). Taken together, our findings demonstrate that although fimbria deficiency in *Shigella*  
261 conferred its poor ability to adhere intrinsically, this deficiency greatly augmented the  
262 ability for HD5 to mediate adhesion to host epithelial cells.

263 **HD5 targets multiple bacterial membrane proteins to promote *Shigella* adhesion**  
264 **to host cells.** To better understand the mechanism of HD5-mediated adherence, we  
265 found that in the presence of HD5, *Shigella* preferably attached to the periphery of

266 adhered, but not suspended, host cells, where the dynamic cell-substratum contacts  
267 occur (Fig. S4A-C). siRNA silencing studies coupled with immunofluorescence staining  
268 suggest that integrins are involved as host factors in HD5-promoted *Shigella* adhesion  
269 (Fig. S4D-G), consistent with their known ability to interact with  $\alpha$ -defensins (Chavakis  
270 et al., 2004; Economopoulou et al., 2005). These findings notwithstanding, HD5  
271 primarily targeted the pathogen rather than host cells to enhance infection. Although  
272 HD5 can bind to both host and bacterial cells efficiently (Fig. S5A), HD5 was  
273 substantially more effective in promoting bacterial infection when pre-incubated with  
274 Sf301, compared to pre-incubation with HeLa cells (Fig. 5D). SEM, TEM and  
275 immunogold-TEM studies revealed that HD5, but not its unstructured analog Abu-HD5,  
276 bound to the *Shigella* surface and formed patches of an “adhesive” structure, leading to  
277 the clustering of *Shigella* cells and their adhesion to host cells (Fig. 6E-F, S5B).

278         To identify the bacterial targets with which HD5 directly interacts, we first  
279 investigated whether HD5, a known lectin capable of binding to glycosylated proteins  
280 (Lehrer et al., 2009), could interact with the bacterial LPS to promote host adhesion by  
281 characterizing several LPS truncation mutants of Sf301. Our data (Fig. S6A-C),  
282 however, showed that LPS was not targeted by HD5 for bacterial adhesion promotion.  
283 We next focused on potential proteinaceous targets of HD5 on the *Shigella* surface.  
284 Trypsin is the proteolytic processing enzyme of pro-HD5 and mature HD5 is stable to its  
285 hydrolytic activity (Ghosh et al., 2002). Pre-treatment of Sf301 with trypsin, while  
286 maintaining bacterial viability, lost HD5-augmented host adhesion capacity (Fig. S6D-  
287 E), consistent with a proteinaceous target. HD5 enhanced bacterial adhesion to host  
288 cells within minutes of co-incubation, thus likely targeting preexisting surface

289 components without involving the *de novo* protein synthesis and/or membrane shuttling  
290 machinery – a notion also supported by an adhesion assay using heat and  
291 paraformaldehyde-inactivated Sf301 (Fig. S6G-H).

292         Using the  $\lambda$  red mutagenesis system (Datsenko and Wanner, 2000), we  
293 genetically ablated Spa33, an essential component of the T3SS (Morita-Ishihara et al.,  
294 2006), IcsA, an autotransporter protein reported to function as an adhesin in  
295 hyperadhesive *Shigella flexneri* mutants lacking the T3SS component IpaD  
296 (Brotcke Zumsteg et al., 2014), and three most abundant outermembrane proteins,  
297 OmpA, OmpC and OmpF (Ambrosi et al., 2012; Bernardini et al., 1993). Deletion of  
298 Spa33, IcsA, OmpA or OmpC in Sf301 led uniformly to a moderate drop in HD5-  
299 mediated adhesion (Fig. 6G), except for OmpF whose expression in Sf301 was  
300 undetectable (Fig. S6F). As expected, a double genetic ablation of a combination of  
301 Spa33, OmpA and OmpC, and  $\Delta$ OmpA $\Delta$ OmpC in particular, further reduced bacterial  
302 adhesion. OmpC expression via plasmids not only restored adhesion of the OmpC-null  
303 mutant above that of the wild-type, but also increased HD5-mediated adhesion of the  
304 wild-type, OmpA-null and OmpF-null strains (Fig. 6G). Thus, our data indicate that  
305 multiple bacterial surface proteins collectively contributed to HD5-mediated adhesion.

306

307

## DISCUSSION

308 The human  $\alpha$ -defensin HD5 contributes to innate host defense against enteropathogens  
309 in the gut (Bevins and Salzman, 2011; Chu et al., 2012; Salzman et al., 2003), and  
310 helps maintain intestinal homeostasis by forming a chemical barrier that segregates the  
311 gut microbiota from host epithelium to limit tissue inflammation and microbial

312 translocation (Belkaid and Hand, 2014; Bevins and Salzman, 2011). Our biochemical  
313 and structural data, mechanistic and functional studies at the molecular and cellular  
314 levels, and *in vivo* and *ex vivo* findings all support that *Shigella* exploits HD5 for  
315 virulence and host infection. These results highlight that host defense factors such as  $\alpha$ -  
316 defensins, which are vitally host protective (Bevins and Salzman, 2011; Selsted and  
317 Ouellette, 2005; Zasloff, 2002), can be important contributors to pathogenesis when  
318 exploited by pathogens. Our work thus provides a noteworthy example of how immunity  
319 can serve as a “double-edged sword” in health and disease (Hansson and Libby, 2006).

320         Our findings not only shed light on how *Shigella* adheres to and invade host cells  
321 despite its lack of fimbriae, but also offer a clue on host-range selectivity of *Shigella*  
322 infection. Enteropathogens such as *Salmonella* and *E. coli* have a sophisticated  
323 adhesion apparatus, including fimbriae, flagella and other adhesins to ensure efficient  
324 bacterial colonization of the intestinal epithelium and subsequent invasion and infection  
325 (Donnenberg, 2000; Pizarro-Cerdá and Cossart, 2006). *Shigella*, on the contrary, lacks  
326 such adhesion machinery in general (Bravo et al., 2015; Schroeder and Hilbi, 2008) and  
327 has a poor inherent ability to colonize host epithelium *in vitro*, confounding its  
328 extraordinary *in vivo* infectivity – 10-100 bacterial cells are sufficient to induce clinical  
329 symptoms in humans (DuPont et al., 1989). Despite recent reports of two adhesion  
330 molecules of *Shigella*, IcsA and MAM, which operate only in specific biological settings  
331 (Brotcke Zumsteg et al., 2014; Mahmoud et al., 2016), no efficient and general *Shigella*  
332 adhesin has been reported thus far. In fact, *Shigella* had long been thought to initially  
333 breach intestinal epithelial barriers through M cell-mediated transcytosis, followed by  
334 dissemination to epithelial cells from the basolateral side (Carayol and Tran Van Nhieu,

335 2013; Cossart and Sansonetti, 2004; Phalipon and Sansonetti, 2007). Our findings  
336 provide a compelling alternative hypothesis that secreted HD5 enables a mode of direct  
337 and active invasion by *Shigella* from the apical surface of the intestinal epithelium. We  
338 propose that *Shigella*, while in transit, encounters HD5 molecules in the small intestine  
339 and becomes highly adhesive and invasive as it reaches more distal sites in the colon.  
340 Since HD5 is expressed by Paneth cells in the small intestine (Bevins and Salzman,  
341 2011), its high local concentration at the luminal surface of the small intestine is likely  
342 lethal to *Shigella*, which might then largely restrict bacterial adhesion and invasion to the  
343 downstream colonic epithelium where HD5 is greatly reduced in abundance. Moreover,  
344 the finding that Concanavalin A, a lectin that interacts with diverse host proteins  
345 containing mannose carbohydrates, failed to inhibit HD5-enhanced *Shigella* infection *in*  
346 *vitro* suggests a limited role played by mucin and/or surface glycoproteins in attenuating  
347 this HD5 activity *in vivo*. This direct invasion model reinforces a recent finding that  
348 *Shigella* primarily targets colonic crypts during the initial stages of mucosal invasion  
349 (Arena et al., 2015).

350 It is plausible that *Shigella* may have undertaken a different evolutionary  
351 trajectory from *Salmonella* or *E. coli* to manifest its pathogenicity. Hijacking a host  
352 innate immune molecule to facilitate bacterial adhesion and invasion might provide  
353 evolutionary advantage for *Shigella*, as lack of adhesive appendages such as fimbriae  
354 or flagella (Bravo et al., 2015) should help the pathogen evade host immune  
355 surveillance and thrive in the gut with significantly less anabolic burden. However, such  
356 a strategy that depends on a specific host factor would restrict host range, especially  
357 when targeting an epithelial  $\alpha$ -defensin, where primary structures vary markedly from



358 species to species. While it is not unusual for a microbial pathogen to exploit host  
359 components to promote its pathogenicity, our findings contribute a striking example of  
360 this phenomenon.

361 In addition, human neutrophil granular proteins containing  $\alpha$ -defensins HNPs 1-4  
362 can enhance *Shigella* adhesion *in vitro* at sub-lethal concentrations (Eilers et al., 2010),  
363 in accordance with our results on HNP1. Furthermore, both HD5 and HD6 have been  
364 reported to enhance HIV-1 infectivity *in vitro* by promoting virion attachment to target  
365 cells (Rapista et al., 2011). However, both the molecular mechanisms and physiological  
366 implications of those findings remain to be determined. A very recent report found that a  
367 mouse adenovirus to promotes its host entry in a receptor-independent manner by  
368 binding to mouse alpha-defensins (cryptdins), resulting in enhanced enteric viral  
369 infection (Wilson et al., 2017) and suggesting that pathogen exploitation of defensins for  
370 infection is not restricted to humans.

371 The precise molecular details underlying HD5-enhanced *Shigella* adhesion to  
372 epithelial cells and tissues need to be further clarified. HD5 is capable of binding to  
373 diverse molecular, bacterial and viral targets in a multivalent, somewhat promiscuous  
374 fashion (Lehrer et al., 2009; Lehrer and Lu, 2012; Rajabi et al., 2012; Tenge et al.,  
375 2014). It may serve as a bridging molecule to directly crosslink bacterial and host cells  
376 as reported for HD5 and other mammalian defensins (Lehrer et al., 2009; Leikina et al.,  
377 2005). Alternatively, by clustering *Shigella* cells, HD5 could endow the pathogen with a  
378 much-enhanced ability to adhere through multivalent high-avidity interactions between  
379 bacterial and host surface proteins. Cell-cell contact activates the T3SS, leading to  
380 *Shigella* invasion orchestrated by bacterial virulent effector proteins delivered by the

381 T3SS into host cells (Cossart and Sansonetti, 2004; Donnenberg, 2000; Hauser, 2009;  
382 Pizarro-Cerdá and Cossart, 2006). In the absence of an efficient host-adhesion  
383 apparatus of its own, HD5-promoted *Shigella* adhesion and colonization could  
384 potentiate T3SS activation, thus indirectly facilitating bacterial invasion and infection.  
385 Whether or not HD5 is capable of directly enabling *Shigella* invasion remains to be  
386 examined, though. Of note, shortening the LPS of *Shigella* increases accessibility of its  
387 T3SS tip to the host cell surface, thus augmenting T3SS activation and bacterial  
388 invasion (West et al., 2005). Although HD5 did not target LPS directly to promote  
389 adhesion, the possibility that HD5 perturbs the LPS structure for enhanced T3SS  
390 activation and *Shigella* invasion cannot be formally excluded.

391         Finally, although *Shigella* is highly infectious in humans at an extremely low  
392 inoculum, it does not readily infect other animals. In fact, no suitable animal model is  
393 available to accurately recapitulate the pathogenesis of *Shigella* (Phalipon and  
394 Sansonetti, 2007). Mice express abundant quantities of  $\alpha$ -defensins (cryptdins) in the  
395 intestine (Ouellette, 2011), yet they are relatively very resistant to oral *Shigella*  
396 challenge. While this resistance may be due at least in part to the lack of IL-8 (Singer  
397 and Sansonetti, 2004), the finding that mouse cryptdins, in contrast to HD5, are  
398 incapable of promoting *Shigella* adhesion and colonization suggests an alternative  
399 explanation for host specificity of this important human enteropathogen. Whether the  
400 lack of expression in other animals of an ortholog of HD5 capable of enhancing *Shigella*  
401 pathogenesis is sufficient to confer resistance to infection warrants additional  
402 investigation.

403

404 **AUTHOR CONTRIBUTIONS**

405 WL, YS and DX conceived and designed the study. DX, CL, BZ, WDT, WH, WZ, WY  
406 and MP performed the experiments. ZD provided human colorectal tissue samples and  
407 performed histological analysis. JY, PJS and CLB provided bacterial strains, helped with  
408 study design, and edited the manuscript. DX, YS and WL wrote the paper. All authors  
409 read and approved the manuscript.

410

411 **ACKNOWLEDGMENTS**

412 This work was supported in part by Program 985 of XJTU (to WL) and grants from the  
413 Natural Science Foundation of China (31401211 & 31770146 to DX), and the National  
414 Institutes of Health of USA (R01GM106710 and R01CA219150 to WL and  
415 R37AI032738 to CLB).

416

417 **DECLARATION OF INTERESTS**

418 The authors declare no competing interests.

419

420 **REFERENCES**

421

422 Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd,  
423 J.J., Hung, L.-W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). PHENIX: a  
424 comprehensive Python-based system for macromolecular structure solution. *Acta*  
425 *Crystallographica Section D* 66, 213-221.

426 Ambrosi, C., Pompili, M., Scribano, D., Zagaglia, C., Ripa, S., and Nicoletti, M. (2012).  
427 Outer Membrane Protein A (OmpA): A New Player in *Shigella flexneri* Protrusion  
428 Formation and Inter-Cellular Spreading. *PLoS ONE* 7, e49625.

429 Arena, E.T., Campbell-Valois, F.-X., Tinevez, J.-Y., Nigro, G., Sachse, M., Moya-Nilges,  
430 M., Nothelfer, K., Marteyn, B., Shorte, S.L., and Sansonetti, P.J. (2015a). Bioimage  
431 analysis of *Shigella* infection reveals targeting of colonic crypts. Proceedings of the  
432 National Academy of Sciences 112, E3282-E3290.

433 Arena, E.T., Campbell-Valois, F.X., Tinevez, J.Y., Nigro, G., Sachse, M., Moya-Nilges,  
434 M., Nothelfer, K., Marteyn, B., Shorte, S.L., and Sansonetti, P.J. (2015b). Bioimage  
435 analysis of *Shigella* infection reveals targeting of colonic crypts. Proc Natl Acad Sci U S  
436 A 112, E3282-3290.

437 Ayabe, T., Satchell, D.P., Wilson, C.L., Parks, W.C., Selsted, M.E., and Ouellette, A.J.  
438 (2000). Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response  
439 to bacteria. Nat Immunol 1, 113-118.

440 Belkaid, Y., and Hand, T.W. (2014). Role of the microbiota in immunity and  
441 inflammation. Cell 157, 121-141.

442 Bernardini, M.L., Sanna, M.G., Fontaine, A., and Sansonetti, P.J. (1993). OmpC is  
443 involved in invasion of epithelial cells by *Shigella flexneri*. Infection and Immunity 61,  
444 3625-3635.

445 Bevins, C.L., and Salzman, N.H. (2011). Paneth cells, antimicrobial peptides and  
446 maintenance of intestinal homeostasis. Nat Rev Microbiol 9, 356-368.

447 Bravo, V., Puhar, A., Sansonetti, P., Parsot, C., and Toro, C.S. (2015). Distinct  
448 Mutations Led to Inactivation of Type 1 Fimbriae Expression in *Shigella spp.* PLoS ONE  
449 10, e0121785.

450 Brotcke Zumsteg, A., Goosmann, C., Brinkmann, V., Morona, R., and Zychlinsky, A.  
451 (2014). IcsA Is a *Shigella flexneri* Adhesin Regulated by the Type III Secretion System  
452 and Required for Pathogenesis. Cell Host & Microbe 15, 435-445.

453 Brunger, A.T. (1997). Free R value: Cross-validation in crystallography. In Methods in  
454 Enzymology (Academic Press), pp. 366-396.

455 Campbell-Valois, F.X., Schnupf, P., Nigro, G., Sachse, M., Sansonetti, P.J., and Parsot,  
456 C. (2014). A fluorescent reporter reveals on/off regulation of the *Shigella* type III  
457 secretion apparatus during entry and cell-to-cell spread. Cell Host Microbe 15, 177-189.

458 Carayol, N., and Tran Van Nhieu, G. (2013). Tips and tricks about *Shigella* invasion of  
459 epithelial cells. Curr Opin Microbiol 16, 32-37.

460 Chart, H., Smith, H.R., La Ragione, R.M., and Woodward, M.J. (2000). An investigation  
461 into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5alpha and  
462 EQ1. J Appl Microbiol 89, 1048-1058.

463 Chavakis, T., Cines, D.B., Rhee, J.S., Liang, O.D., Schubert, U., Hammes, H.P., Higazi,  
464 A.A., Nawroth, P.P., Preissner, K.T., and Bdeir, K. (2004). Regulation of

465 neovascularization by human neutrophil peptides (alpha-defensins): a link between  
466 inflammation and angiogenesis. *FASEB J* 18, 1306-1308.

467 Chen, V.B., Arendall, W.B., III, Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral,  
468 G.J., Murray, L.W., Richardson, J.S., and Richardson, D.C. (2010). MolProbity: all-atom  
469 structure validation for macromolecular crystallography. *Acta Crystallographica Section*  
470 *D* 66, 12-21.

471 Choudhury, D., Thompson, A., Stojanoff, V., Langermann, S., Pinkner, J., Hultgren,  
472 S.J., and Knight, S.D. (1999). X-ray Structure of the FimC-FimH Chaperone-Adhesin  
473 Complex from Uropathogenic *Escherichia coli*. *Science* 285, 1061-1066.

474 Chu, H., Pazgier, M., Jung, G., Nuccio, S.P., Castillo, P.A., de Jong, M.F., Winter, M.G.,  
475 Winter, S.E., Wehkamp, J., Shen, B., et al. (2012). Human alpha-defensin 6 promotes  
476 mucosal innate immunity through self-assembled peptide nanonets. *Science* 337, 477-  
477 481.

478 Cossart, P., and Sansonetti, P.J. (2004). Bacterial Invasion: The Paradigms of  
479 Enteroinvasive Pathogens. *Science* 304, 242-248.

480 Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes  
481 in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of*  
482 *Sciences* 97, 6640-6645.

483 Donnenberg, M.S. (2000). Pathogenic strategies of enteric bacteria. *Nature* 406, 768-  
484 774.

485 DuPont, H.L., Levine, M.M., Hornick, R.B., and Formal, S.B. (1989). Inoculum Size in  
486 Shigellosis and Implications for Expected Mode of Transmission. *Journal of Infectious*  
487 *Diseases* 159, 1126-1128.

488 Economopoulou, M., Bdeir, K., Cines, D.B., Fogt, F., Bdeir, Y., Lubkowski, J., Lu, W.,  
489 Preissner, K.T., Hammes, H.P., and Chavakis, T. (2005). Inhibition of pathologic retinal  
490 neovascularization by alpha-defensins. *Blood* 106, 3831-3838.

491 Edwards, R.A., and Puente, J.L. (1998). Fimbrial expression in enteric bacteria: a  
492 critical step in intestinal pathogenesis. *Trends in Microbiology* 6, 282-287.

493 Eilers, B., Mayer-Scholl, A., Walker, T., Tang, C., Weinrauch, Y., and Zychlinsky, A.  
494 (2010). Neutrophil antimicrobial proteins enhance *Shigella flexneri* adhesion and  
495 invasion. *Cellular Microbiology* 12, 1134-1143.

496 Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and  
497 development of Coot. *Acta Crystallographica Section D* 66, 486-501.

498 Ericksen, B., Wu, Z., Lu, W., and Lehrer, R.I. (2005). Antibacterial activity and specificity  
499 of the six human {alpha}-defensins. *Antimicrob Agents Chemother* 49, 269-275.

500 Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*  
501 3, 710-720.

502 Ghosh, D., Porter, E., Shen, B., Lee, S.K., Wilk, D., Drazba, J., Yadav, S.P., Crabb,  
503 J.W., Ganz, T., and Bevins, C.L. (2002). Paneth cell trypsin is the processing enzyme  
504 for human defensin-5. *Nat Immunol* 3, 583-590.

505 Hansson, G.K., and Libby, P. (2006). The immune response in atherosclerosis: a  
506 double-edged sword. *Nat Rev Immunol* 6, 508-519.

507 Hauser, A.R. (2009). The type III secretion system of *Pseudomonas aeruginosa*:  
508 infection by injection. *Nat Rev Microbiol* 7, 654-665.

509 Jin, Q., Yuan, Z., Xu, J., Wang, Y., Shen, Y., Lu, W., Wang, J., Liu, H., Yang, J., Yang,  
510 F., et al. (2002). Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity  
511 through comparison with genomes of *Escherichia coli* K12 and O157. *Nucleic Acids Res*  
512 30, 4432-4441.

513 Jones, C.H., Pinkner, J.S., Roth, R., Heuser, J., Nicholes, A.V., Abraham, S.N., and  
514 Hultgren, S.J. (1995). FimH adhesin of type 1 pili is assembled into a fibrillar tip  
515 structure in the Enterobacteriaceae. *Proceedings of the National Academy of Sciences*  
516 92, 2081-2085.

517 Kline, K.A., Fälker, S., Dahlberg, S., Normark, S., and Henriques-Normark, B. (2009).  
518 Bacterial Adhesins in Host-Microbe Interactions. *Cell Host & Microbe* 5, 580-592.

519 Labrec, E.H., Schneider, H., Magnani, T.J., and Formal, S.B. (1964). Epithelial cell  
520 penetration as an essential step in the pathogenesis of bacillary dysentery. *Journal of*  
521 *Bacteriology* 88, 1503-1518.

522 Lehrer, R.I., Jung, G., Ruchala, P., Andre, S., Gabius, H.J., and Lu, W. (2009).  
523 Multivalent binding of carbohydrates by the human alpha-defensin, HD5. *J Immunol*  
524 183, 480-490.

525 Lehrer, R.I., and Lu, W. (2012). alpha-Defensins in human innate immunity. *Immunol*  
526 *Rev* 245, 84-112.

527 Leikina, E., Delanoe-Ayari, H., Melikov, K., Cho, M.-S., Chen, A., Waring, A.J., Wang,  
528 W., Xie, Y., Loo, J.A., Lehrer, R.I., and Chernomordik, L.V. (2005). Carbohydrate-  
529 binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins.  
530 *Nat Immunol* 6, 995-1001.

531 Li, Y.-F., Poole, S., Nishio, K., Jang, K., Rasulova, F., McVeigh, A., Savarino, S.J., Xia,  
532 D., and Bullitt, E. (2009). Structure of CFA/I fimbriae from enterotoxigenic *Escherichia*  
533 *coli*. *Proceedings of the National Academy of Sciences* 106, 10793-10798.

534 Mahmoud, R.Y., Stones, D.H., Li, W., Emara, M., El-domany, R.A., Wang, D., Wang, Y.,  
535 Krachler, A.M., and Yu, J. (2016). The Multivalent Adhesion Molecule SSO1327 plays a  
536 key role in *Shigella sonnei* pathogenesis. *Molecular Microbiology* 99, 658-673.

537 Mastroianni, J.R., and Ouellette, A.J. (2009). Alpha-defensins in enteric innate  
538 immunity: functional Paneth cell alpha-defensins in mouse colonic lumen. *J Biol Chem*  
539 284, 27848-27856.

540 Maurelli, A.T., Blackmon, B., and Curtiss, R. (1984). Temperature-dependent  
541 expression of virulence genes in *Shigella* species. *Infection and Immunity* 43, 195-201.

542 McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and  
543 Read, R.J. (2007). Phaser crystallographic software. *Journal of Applied Crystallography*  
544 40, 658-674.

545 Molloy, M.P., Herbert, B.R., Slade, M.B., Rabilloud, T., Nouwens, A.S., Williams, K.L.,  
546 and Gooley, A.A. (2000). Proteomic analysis of the *Escherichia coli* outer membrane.  
547 *European Journal of Biochemistry* 267, 2871-2881.

548 Morita-Ishihara, T., Ogawa, M., Sagara, H., Yoshida, M., Katayama, E., and Sasakawa,  
549 C. (2006). *Shigella* Spa33 Is an Essential C-ring Component of Type III Secretion  
550 Machinery. *Journal of Biological Chemistry* 281, 599-607.

551 Mounier, J., Vasselon, T., Hellio, R., Lesourd, M., and Sansonetti, P.J. (1992). *Shigella*  
552 *flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole.  
553 *Infection and Immunity* 60, 237-248.

554 Murshudov, G.N., Vagin, A.A., and Dodson, E.J. (1997). Refinement of Macromolecular  
555 Structures by the Maximum-Likelihood Method. *Acta Crystallographica Section D* 53,  
556 240-255.

557 Otwinowski, Z., and Minor, W. (1997). [20] Processing of X-ray diffraction data collected  
558 in oscillation mode. In *Methods in Enzymology* (Academic Press), pp. 307-326.

559 Ouellette, A. (2011). Paneth cell  $\alpha$ -defensins in enteric innate immunity. *Cell. Mol. Life*  
560 *Sci.* 68, 2215-2229.

561 Pazgier, M., Ericksen, B., Ling, M., Toth, E., Shi, J., Li, X., Galliher-Beckley, A., Lan, L.,  
562 Zou, G., Zhan, C., et al. (2013). Structural and functional analysis of the pro-domain of  
563 human cathelicidin, LL-37. *Biochemistry* 52, 1547-1558.

564 Perdomo, O.J., Cavillon, J.M., Huerre, M., Ohayon, H., Gounon, P., and Sansonetti,  
565 P.J. (1994). Acute inflammation causes epithelial invasion and mucosal destruction in  
566 experimental shigellosis. *J Exp Med* 180, 1307-1319.

567 Phalipon, A., and Sansonetti, P.J. (2007). *Shigella*'s ways of manipulating the host  
568 intestinal innate and adaptive immune system: a tool box for survival? *Immunol Cell Biol*  
569 85, 119-129.

570 Philpott, D.J., Edgeworth, J.D., and Sansonetti, P.J. (2000). The pathogenesis of  
571 *Shigella flexneri* infection: lessons from in vitro and in vivo studies. Philos Trans R Soc  
572 Lond B Biol Sci 355, 575-586.

573 Pizarro-Cerdá, J., and Cossart, P. (2006). Bacterial Adhesion and Entry into Host Cells.  
574 Cell 124, 715-727.

575 Rajabi, M., Ericksen, B., Wu, X., de Leeuw, E., Zhao, L., Pazgier, M., and Lu, W.  
576 (2012). Functional Determinants of Human Enteric  $\alpha$ -Defensin HD5: CRUCIAL ROLE  
577 FOR HYDROPHOBICITY AT DIMER INTERFACE. Journal of Biological Chemistry 287,  
578 21615-21627.

579 Rapista, A., Ding, J., Benito, B., Lo, Y.-T., Neiditch, M., Lu, W., and Chang, T. (2011).  
580 Human defensins 5 and 6 enhance HIV-1 infectivity through promoting HIV attachment.  
581 Retrovirology 8, 45.

582 Salzman, N.H., Ghosh, D., Huttner, K.M., Paterson, Y., and Bevins, C.L. (2003).  
583 Protection against enteric salmonellosis in transgenic mice expressing a human  
584 intestinal defensin. Nature 422, 522-526.

585 Sawasvirojwong, S., Srimanote, P., Chatsudthipong, V., and Muanprasat, C. (2013). An  
586 Adult Mouse Model of *Vibrio cholerae*-induced Diarrhea for Studying Pathogenesis and  
587 Potential Therapy of Cholera. PLoS Negl Trop Dis 7, e2293.

588 Schroeder, G.N., and Hilbi, H. (2008). Molecular Pathogenesis of *Shigella* spp.:  
589 Controlling Host Cell Signaling, Invasion, and Death by Type III Secretion. Clinical  
590 Microbiology Reviews 21, 134-156.

591 Selsted, M.E., and Ouellette, A.J. (2005). Mammalian defensins in the antimicrobial  
592 immune response. Nat Immunol 6, 551-557.

593 Sereny, B. (1955). Experimental shigella keratoconjunctivitis; a preliminary report. Acta  
594 Microbiol Acad Sci Hung 2, 293-296.

595 Shim, D.H., Suzuki, T., Chang, S.Y., Park, S.M., Sansonetti, P.J., Sasakawa, C., and  
596 Kweon, M.N. (2007). New animal model of shigellosis in the Guinea pig: its usefulness  
597 for protective efficacy studies. J Immunol 178, 2476-2482.

598 Singer, M., and Sansonetti, P.J. (2004). IL-8 is a key chemokine regulating neutrophil  
599 recruitment in a new mouse model of *Shigella*-induced colitis. J Immunol 173, 4197-  
600 4206.

601 Snellings, N.J., Tall, B.D., and Venkatesan, M.M. (1997). Characterization of *Shigella*  
602 type 1 fimbriae: expression, FimA sequence, and phase variation. Infection and  
603 Immunity 65, 2462-2467.

604 Szyk, A., Wu, Z., Tucker, K., Yang, D., Lu, W., and Lubkowski, J. (2006). Crystal  
605 structures of human  $\alpha$ -defensins HNP4, HD5, and HD6. Protein Science 15, 2749-2760.



606 Tenge, V.R., Gounder, A.P., Wiens, M.E., Lu, W., and Smith, J.G. (2014). Delineation of  
607 Interfaces on Human Alpha-Defensins Critical for Human Adenovirus and Human  
608 Papillomavirus Inhibition. PLoS Pathog 10, e1004360.

609 Tsilingiri, K., Barbosa, T., Penna, G., Caprioli, F., Sonzogni, A., Viale, G., and Rescigno,  
610 M. (2012). Probiotic and postbiotic activity in health and disease: comparison on a novel  
611 polarised ex-vivo organ culture model. Gut 61, 1007-1015.

612 Wei, G., Pazgier, M., de Leeuw, E., Rajabi, M., Li, J., Zou, G., Jung, G., Yuan, W., Lu,  
613 W.Y., Lehrer, R.I., and Lu, W. (2010). Trp-26 imparts functional versatility to human  
614 alpha-defensin HNP1. J Biol Chem 285, 16275-16285.

615 West, N.P., Sansonetti, P., Mounier, J., Exley, R.M., Parsot, C., Guadagnini, S.,  
616 Prevost, M.C., Prochnicka-Chalufour, A., Delepierre, M., Tanguy, M., and Tang, C.M.  
617 (2005). Optimization of virulence functions through glucosylation of Shigella LPS.  
618 Science 307, 1313-1317.

619 Wilson, S.S., Bromme, B.A., Holly, M.K., Wiens, M.E., Gounder, A.P., Sul, Y., and  
620 Smith, J.G. (2017). Alpha-defensin-dependent enhancement of enteric viral infection.  
621 PLoS Pathog 13, e1006446.

622 Wu, Z., Ericksen, B., Tucker, K., Lubkowski, J., and Lu, W. (2004). Synthesis and  
623 characterization of human alpha-defensins 4-6. J Pept Res 64, 118-125.

624 Wu, Z., Hoover, D.M., Yang, D., Boulegue, C., Santamaria, F., Oppenheim, J.J.,  
625 Lubkowski, J., and Lu, W. (2003). Engineering disulfide bridges to dissect antimicrobial  
626 and chemotactic activities of human beta-defensin 3. Proc Natl Acad Sci U S A 100,  
627 8880-8885.

628 Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature 415, 389-  
629 395.

630

631

## FIGURE LEGENDS

632 **Fig. 1. HD5 promotes *Shigella* infection of epithelial cells *in vitro*.** (A, B) The effects  
633 of eight antimicrobial peptides at sub-lethal concentrations on *Shigella flexneri* Sf301  
634 adhesion (A) to and invasion (B) of HeLa cells during bacterial infection. (C, D) The  
635 effects of HD5 treatment on Sf301 invasion (C) and proliferation (D) when added during  
636 initial infection (co-incubation) or after invasion (post-infection). Adhesion, invasion and  
637 proliferation assays were performed as described in Methods. Experimental details are  
638 illustrated in Fig. S2. Data are shown as mean  $\pm$  SD of at least three independent  
639 experiments. Statistical significance was calculated (for peptide-treated samples  
640 compared to vehicle controls (0  $\mu$ M)) using a one-way ANOVA (Dunnett's multiple  
641 comparison Test), and p values are as follows: \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.  
642 (E) Fluorescence microscopy (left panels) and scanning electron microscopy (right  
643 panels) analysis of Sf301 adhesion to HeLa cells in the absence (control) or presence of  
644 4  $\mu$ M HD5 (MOI=50:1). GFP-expressing bacteria are green,  $\beta$ 1 integrin is red, and  
645 nuclei are blue (DAPI). For fluorescent images, the scale bars represent 50  $\mu$ m; for  
646 SEM images, the bars represent 10  $\mu$ m. (F) Confocal microscopy images of HeLa cells  
647 infected for 60 min with mCherry-labeled WT Sf301 harboring a GFP-expressing  
648 reporter plasmid (Campbell-Valois et al., 2014) in presence or absence of 4  $\mu$ M HD5.  
649 HeLa cells are counterstained with DAPI, and GFP expression is induced upon  
650 activation of type 3 secretion system triggered by bacterial cell contact with the host.  
651 Note that red and green overlay gives rise to yellow. The scale bars represent 50  $\mu$ m.  
652

653 **Fig. 2. HD5 promotes *Shigella* infection *in vivo*.** (A) Sereny test of *Shigella* infection  
654 using guinea pigs. Hartley guinea pigs (6-8 weeks of age) were inoculated with  $10^6$   
655 CFU/eye of mid-log phase Sf301 either in the absence (n=15) and presence of HD5  
656 (either 4  $\mu$ M (n=14) or 8  $\mu$ M (n=14)). A control group (n=6) was inoculated with HD5  
657 alone (8  $\mu$ M). Animals were observed and scored for the development of conjunctivitis  
658 over 7 consecutive days. Eye pathology was independently scored by three individuals  
659 (blinded to treatment group) on a scale of 0-3, grade 0 (no disease or mild irritation),  
660 grade 1 (mild conjunctivitis or late development and/or rapid clearing of symptoms),  
661 grade 2 (keratoconjunctivitis without purulence), and grade 3 (fully developed  
662 keratoconjunctivitis with purulence) as indicated in Fig. S3A. The data are  
663 representative of three independent experiments. Each point represents a single  
664 animal. Statistical significance compared with inoculation of  $10^6$  CFU in the absence of  
665 HD5 was determined using a Mann-Whitney test, \*p < 0.05, \*\*p < 0.01. Please also see  
666 Fig. S3A for daily scoring. (B-H) Colon infection model with guinea pigs. Hartley guinea  
667 pigs (6-8 weeks of age) were inoculated intrarectally by  $1 \times 10^8$  CFU of HD5-treated (8  
668  $\mu$ M),  $1 \times 10^8$  CFU of mock-treated,  $1 \times 10^9$  CFU of mock-treated GFP-expressing Sf301 or  
669 medium alone, with 20 animals in each of the three treatment groups and 8 in the  
670 negative control group. Animals were monitored for 48h, and the distal 10 cm of colon  
671 tissue from groups of euthanized animals was harvested for analysis at 4, 8, 24 and 48  
672 h post-challenge. (B) Confocal microscopy images of representative colon sections at 4  
673 h post-challenge, counterstained with DAPI. The scale bars represent 50  $\mu$ m. Please  
674 also see Fig. S3B for images at 8h and 24h post-challenge. The ten most bacteria-  
675 enriched fields from each experimental group were analyzed at 4 h (n=3), 8 h (n=3), 24

676 h (n=5) and 48 h (n=5) post-challenge, followed by automated enumeration of individual  
677 bacteria (**C**). Results are representative of three independent experiments and are  
678 shown as mean  $\pm$  SD. Statistical significance in comparison with  $1 \times 10^8$  CFU of mock-  
679 treated group at each time point was calculated using a one-way ANOVA (Dunnett's  
680 multiple comparison Test), and p values are as follows: \*p < 0.05, \*\*p < 0.01. (**D**) SEM  
681 analysis of bacterial infection of the colonic mucosa at 2h post-challenge. The scale  
682 bars represent 5  $\mu$ m. (**E**) Histopathology analysis of representative colon sections at 24  
683 h and 48 h post-challenge by HE staining. The scale bars represent 100  $\mu$ m. Colon  
684 histopathology scores at 24 h (**F**) or 48 h (**G**) (n=5, each) were assigned as follows: 0,  
685 intact colonic architecture, no acute inflammation or epithelial injury; 1, focal minimal  
686 acute inflammation; 2, focal mild acute inflammation; 3, severe acute inflammation with  
687 multiple crypt abscesses and/or focal ulceration; 4, severe acute inflammation, multiple  
688 crypt abscesses, epithelial loss, and extensive ulceration. Results are representative of  
689 three independent experiments. Indicated are mean  $\pm$  SEM. Each point represents a  
690 single animal. Statistical significance in comparison with  $1 \times 10^8$  CFU of mock-treated  
691 group was determined using a Mann-Whitney test, \*p < 0.05, \*\*p < 0.01. (**H**) Core body  
692 temperature of animals 48 h post-challenge. Results are representative of three  
693 independent experiments. Indicated are mean  $\pm$  SD. Each point represents a single  
694 animal (n=9). Statistical significance between indicated groups was determined using a  
695 one-way ANOVA (Tukey's multiple comparison Test), \*p < 0.05, \*\*p < 0.01. (**I-J**) Ileum  
696 and colon infection model in mice. For the colon lops (**I**), a small abdominal incision was  
697 made in fasted, anesthetized mice and two separate loops of colon were isolated by  
698 suture (2-3 cm in length). For each animal, one isolated colonic loop was instilled with

699 1x10<sup>7</sup> CFU of HD5-treated Sf301, and the second loop with mock-treated Sf301 (both in  
700 100 µl medium). For the ileal loop model (**J**), the same experimental approach was  
701 employed except that the two sutured loops were with the distal ileum. Two-hours post  
702 inoculation, mice were euthanized, and loops were harvested for quantitative  
703 fluorescence imaging of *Shigella* infection. The scale bars represent 50 µm. Results are  
704 shown as mean ± SD. Statistical significance in comparison with group challenged with  
705 1x10<sup>7</sup> CFU of mock-treated bacteria was calculated using a t-test, and "\*\*\*" indicates p <  
706 0.05.

707

708 **Fig. 3. HD5 promotes *Shigella* infection *ex vivo*.** (**A**) SEM analysis of the bacterial  
709 adhesion (red arrows) to human colonic mucosa 30 min after inoculation of 10<sup>6</sup> CFU  
710 Sf301 in the absence (control) and presence of HD5 (8 µM). The scale bars represent  
711 10 µm. (**B**) SEM analysis of bacterial invasion of human colonic tissue at 2 h post-  
712 inoculation in presence of 8 µM HD5. Clustered bacteria are indicated by red circles in  
713 the upper panel (the scale bar represents 100 µm), and individual bacteria by red  
714 arrows in the lower panel (the scale bar represents 5 µm). (**C, D**) Analysis of  
715 histopathology of human colorectal explants 2 h after the inoculation either with or  
716 without Sf301 in either the absence or presence of HD5 (8 µM). Untreated (control) and  
717 HD5-treated specimens are included for comparison. Experimental details are  
718 described in Methods. Colon histopathology scores (**C**) were assigned as described  
719 above. Results are representative of three independent experiments. Indicated are  
720 mean ± SEM. Each point represents a colon sample. Statistical significance was  
721 determined using a Mann-Whitney test, \*p < 0.05. Representative images of HE staining

722 (the scale bars represent 100  $\mu\text{m}$ ) and SEM analysis (the scale bars represent 500  $\mu\text{m}$ )  
723 of colonic mucosa at 2h post-challenge are shown in **D**. Please also see Fig. S3C for  
724 experimental procedure.

725

726 **Fig. 4. (A-C)** HD5 potentiates destruction of the epithelium by Shigella. (A) Trans-  
727 epithelial electrical resistance (TEER) analysis of epithelial integrity of the monolayer of  
728 polarized Caco-2 cells infected with Sf301 in either the absence or presence of HD5 (8  
729  $\mu\text{M}$ ). Percent TEER values were normalized against values of each treatment group at  
730 time 0. Data are mean  $\pm$  SD from at least three independent experiments. Statistical  
731 significance was determined using a two-way ANOVA, \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . (B)  
732 Intracellular CFU of the polarized Caco-2 monolayer 6 h post-inoculation of Sf301 in  
733 either the absence or presence of HD5 (8  $\mu\text{M}$ ). Data are mean  $\pm$  SD from at least three  
734 independent experiments. Statistical significance was determined using a t test \*\*\*\*,  $p <$   
735 0.0001. (C) Disruption of the tight junction (as shown by immunofluorescence) and  
736 impairment of the integrity (as shown by SEM) of the epithelium. Polarized Caco-2 cells  
737 grown in transwell inserts were infected with Sf301 in either the absence or presence of  
738 HD5 (8  $\mu\text{M}$ ) for 6 h, followed by immunostaining with antibody against the tight junction  
739 marker ZO-1 (green). Nuclei are stained with DAPI (blue). The scale bars represent 50  
740  $\mu\text{m}$ . Invading bacteria in the polarized Caco-2 monolayer are indicated by red arrows in  
741 the SEM image. The scale bars represent 2  $\mu\text{m}$ . (D, E) Effects of concentrated ileal fluid  
742 aspirates on *Shigella* Sf301 adhesion (D) and invasion (E) in comparison with DMEM  
743 either without or with HD5 (4  $\mu\text{M}$ ). Anti-HD5 antiserum was added to ileal fluids and  
744 HD5-containing DMEM at a dilution titer of 1:100 to examine its neutralizing activity

745 against *Shigella* infection promoted by endogenous HD5. Data are shown as mean  $\pm$   
746 SD of at least three independent experiments. Statistical significance between indicated  
747 groups was determined using a one-way ANOVA (Tukey's multiple comparison test),  
748 and p values are as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001.

749

750 **Fig. 5. Structural determinants of HD5 function. (A, B)** Activities of native and  
751 alanine mutants of HD5 (**A**) and Abu-HD5 (**B**) on *Shigella* adhesion and invasion (for  
752 one hour). The adhesion and invasion assays were as in Fig. 1A, and data are  
753 expressed as the number of intracellular (A, B) and adhering (B) bacteria in HeLa cells  
754 relative to the input. Data are shown as mean  $\pm$  SD of at least three independent  
755 experiments. Statistical significance in comparison with wildtype HD5 in **A** and in  
756 comparison with solvent control group in **B** was determined using a one-way ANOVA  
757 (Dunnett's multiple comparison Test), and p values are as follows: \*p < 0.05, \*\*p < 0.01,  
758 \*\*\*p < 0.001. (**C, D**) The 2Fo-Fc electron density map contoured at 1.0 $\sigma$  of molecule A  
759 of Y27A-HD5 (**C**) and R28A-HD5 crystal (**D**) and the superimposition of defensin  
760 molecules present in the asymmetric units of analogs' crystals with the wildtype HD5  
761 monomers (shown in grey, from PDB code: 1ZMP (Szyk et al., 2006)). Side chains of  
762 cysteines forming disulfide bridges and mutated residues are shown as sticks.  
763 Structural analysis of Y27A-HD5 and R28A-HD5 analogs confirms that both mutant  
764 monomers assume the same fold as the wildtype HD5 monomer with no major changes  
765 to the overall structure and the network of three disulfide bridges. When superimposed,  
766 the root-mean-square deviations (RMSD) between 128 equivalent main chain atoms of  
767 wildtype HD5 and Y27A-HD5 and R28A-HD5 are in the range of 0.91-1.33 Å and 0.35-

768 0.95 Å, respectively. (E, F) Crystal structures of the Y27A-HD5 monomer (E, green) and  
769 the R28A-HD5 dimer (F, yellow) superimposed on the wildtype HD5 dimer in grey (PDB  
770 code: 1ZMP). Mutated residues and alanine substitutions are shown as spheres. (G)  
771 Key functional residues of HD5 forming putative binding surfaces for interactions with  
772 bacterial and host proteins. Positively charged Arg28 residues are colored in blue, and  
773 hydrophobic residues Leu16, Val19, and Leu26 in green. Shades of green depict  
774 differences in activity with residues in light green being less important than those in dark  
775 green. Important residues not depicted in this view are Tyr27 and Leu29.

776

777 **Fig. 6. Bacterial determinants of HD5-promoted *Shigella* infection.** (A) TEM  
778 analysis of fimbriae expression in Sf301. The Fim cassette from *E. coli* JM103 was  
779 expressed from arabinose-inducible pBAD vector in Sf301. The scale bars represent 1  
780 µm. (B) Influence of fimbriae-expression on the adhesion and invasion of Sf301 in the  
781 absence or presence of HD5. Data are shown as mean ± SD of at least three  
782 independent experiments. Statistical significance was determined using a two-way  
783 ANOVA, and p values are as follows: \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001. (C) Host  
784 cell-adhesion activity of fimbriated and non-fimbriated *E. coli* strains in the absence or  
785 presence of HD5. Adhesion assays to HeLa cells were carried out with fimbriated *E. coli*  
786 (JM103), its fimbriaedeficient mutant (JM103 Δfim) and non-fimbriated *E. coli* (BL21).  
787 TEM images of these strains are shown on the right panel. The scale bars represent  
788 500 nm. Statistical significance between indicated groups was determined using a t test,  
789 and p values are as follows: \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001. (D) Either Sf301  
790 bacteria, or the HeLa cells, were pre-treated with HD5 at the indicated concentrations



791 for 30 min, washed once with DMEM, and the adhesion and invasion assays were  
792 performed. Data are shown as mean  $\pm$  SD of at least three independent experiments.  
793 Statistical significance compared with solvent control (0  $\mu$ M) group at each time point  
794 was determined using a one-way ANOVA (Dunnett's multiple comparison test), and p  
795 values are as follows: \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. (E) SEM and TEM  
796 analysis of Sf301 treated with HD5 or its linear analogue (Abu-HD5). The scale bars  
797 represent 2  $\mu$ m for SEM and 500 nm for TEM. (F) Immunogold-TEM analysis of HD5  
798 localization in Sf301-HeLa interaction in presence of HD5. HD5 was labeled by ~12 nm  
799 colloidal gold particles and TEM were performed as described in Methods. B, bacterium;  
800 C, Cell. The scale bars represent 500 nm. Please also see Fig. S5B for more  
801 Immunogold-TEM images showing HD5 bridging single bacterium to host and HD5  
802 clustering multiple bacteria. (G) Relative adhesion ability of different Sf301 mutants in  
803 the presence of 4  $\mu$ M HD5. *spa33*, *icsA*, *ompA*, *ompC*, *ompF* genes and some  
804 combinations of two were ablated as described in Methods. *Shigella* strains were  
805 transformed with pBAD plasmids carrying the OmpC coding sequence and induced with  
806 10 mM L-arabinose for OmpC expression. Please also see Fig. S6F for SDS-PAGE  
807 analysis of the genetic ablations and recompletions. Adhesion assays were performed  
808 as in Fig. 1A. Data are normalized to the input and shown as the percentage of the  
809 adherent bacteria of wild-type Sf301 in the presence of 4  $\mu$ M HD5. Data are shown as  
810 mean  $\pm$  SD of at least three independent experiments. Statistical significance compared  
811 with wild type was determined using a one-way ANOVA (Dunnett's multiple comparison  
812 test), and p values are as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001.  
813

814

## **STAR Methods section**

815

Wuyuan Lu (wlu@ihv.umaryland.edu) is the lead contact author responsible for

816

providing reagents upon request.

817

### *Ethics Approval*

818

All the animals used in this study were acquired from the Experimental Animal Center of

819

Xi'an Jiaotong University. The animal studies were approved by the Committee on

820

Animal Research and Ethics, Xi'an Jiaotong University. All the animals were maintained

821

in animal care facilities in the School of Life Science and Technology, and provided with

822

food and water ad libitum. The use of healthy colonic tissues from patients undergoing

823

surgery for colon cancer and the use of human small intestinal fluid aspirates from

824

healthy donors undergoing routine screening colonoscopy were approved by the Ethics

825

Committee of the Second Affiliated Hospital of Xi'an Jiaotong University School of

826

Medicine. Informed consent was obtained from all patients

827

828

### *Reagents*

829

All peptides used in this study were chemically synthesized, correctly folded and highly

830

purified as previously described (Pazgier et al., 2013; Rajabi et al., 2012; Szyk et al.,

831

2006; Wei et al., 2010; Wu et al., 2004; Wu et al., 2003). Anti-human integrin  $\beta$ 1

832

monoclonal antibody (MAB1959Z, P5D2) and anti-human integrin  $\alpha$ 5 $\beta$ 1 monoclonal

833

antibody (MAB1969, JBS5) were purchased from Millipore. Anti-human integrin  $\beta$ 5

834

monoclonal antibody (#3629, D24A5) was purchased from Cell Signaling Technology.

835 All common chemicals and reagents were purchased from Sigma-Aldrich unless  
836 indicated otherwise.

837

### 838 *Bacterial strains*

839 Bacterial strains used in this study are listed in Table S2. *Shigella* strains were cultured  
840 aerobically at 37 °C in Tryptic Soy (TS) broth (Aoboxing, Beijing, China) or on TS agar  
841 plates with addition of 0.1% Congo Red. *E. coli* strains in this study were cultured in  
842 Luria-Bertani (LB) broth (Aoboxing, Beijing, China) or on LB agar plates. Antibiotics  
843 (Sigma) were used as follows: ampicillin 200 µg/ml; kanamycin 100 µg/ml.

844

### 845 *Strain construction*

846 Genetic ablation of Bacterial genes was performed using the λ Red recombination  
847 system (Datsenko and Wanner, 2000). Briefly, bacterial cells transformed with pKD46  
848 were grown in the presence of L-arabinose to induce the expression of the lambda Red  
849 recombinase. A linear PCR product, amplified using the primers listed in Table S2,  
850 containing a kanamycin-resistance cassette (KRC) flanked by FLP and 50 bp of the 5'-  
851 and 3'-end homologous sequences of the target gene was electroporated into the  
852 bacterial cells and kanamycin was used to select the transformants. The plasmid pKD46  
853 was eliminated by incubation at 37 °C. To cure the kanamycin maker, pCP20 was  
854 introduced into kanamycin-resistant cells to elicit the recombination of flanking FLP  
855 sequences at both ends of the kanamycin cassettes. PCR screening for cured colonies  
856 were performed using specific primers listed in Table S2. For OmpC and fimbriae re-

857 expression experiments, the OmpC coding sequence from Sf301 and the fimbriae-  
858 expression cassette from *E. coli* JM103 (encoding FimA, I, C, D, F, G, H) were  
859 separately cloned into Nco I and Xho I sites of pBAD vector using the primers listed in  
860 Table S2. Bacterial cells transformed with pBAD-OmpC or pBAD-Fim were cultured in  
861 LB with 10 mM L-arabinose to induce the expression of OmpC or production of fimbriae  
862 in Sf301.

863

#### 864 *Bactericidal assays*

865 Different bacterial strains ( $\sim 10^6$  CFU) were treated with the indicated concentrations of  
866 peptide in 500  $\mu$ l DMEM (without serum) for 40 min at 37 °C with mild agitation. After  
867 washing, the bacteria were diluted and plated. Bacterial viability was determined by  
868 colony counting and normalized against the viability observed with mock (PBS)  
869 treatment. Results are reported as the mean percentage of input bacteria of three  
870 independent experiments  $\pm$  SD.

871

#### 872 *In vitro adhesion and invasion assay*

873 The cell lines and their culture medium used in this study are listed in Table S1. One  
874 day before the assays, cells were seeded into 24-well plates at a density of  $\sim 10^5$  cells  
875 per well. One hour before the infection, cell culture medium was changed into serum-  
876 free medium and  $\sim 10^6$  CFU Sf301 from mid-exponential phase was added to the cells  
877 together with a titration of HD5 (0-8  $\mu$ M). Bacteria were centrifuged (2000 rpm, 10 min,  
878 RT) onto HeLa cells (MOI 10:1, or indicated MOI) to synchronize the infection. For the

879 adhesion assay, after washing, the cells were lysed with 0.1 % Triton/H<sub>2</sub>O and the CFU  
880 were enumerated after plating. For invasion and proliferation assays, bacteria/HeLa  
881 mixtures were incubated for 40 min after centrifugation and then washed, treated with  
882 gentamicin-containing (25 µg/ml) medium for another 1 hour (invasion) or 4 hours  
883 (proliferation) before being lysed for plating. Adhesion was defined as the total number  
884 of HeLa cell-associated bacteria and is shown as the percentage of input. Invasion and  
885 proliferation were defined as the total number of intracellular bacteria in cells  
886 (extracellular bacteria were killed by gentamicin, a cell-impermeable antibiotic). Average  
887 results of three independent experiments are reported as mean ± SD. For pre-treatment  
888 experiments, cells or bacteria were pre-incubated with HD5 at the indicated  
889 concentrations for 30 min, washed once with DMEM and then mixed to allow infection.  
890 Adhesion assays, invasion assays and proliferation assays were performed as stated  
891 above. Schematic illustration of the assays is shown in Fig. S2.

892

### 893 *Sereny test*

894 Female specific pathogen-free Hartley guinea pigs, aged 6–8 weeks, weighing 120–250  
895 g, were inoculated via conjunctival route with 10<sup>6</sup> CFU/eye of mid-log phase Sf301 in  
896 the absence or presence of 4 µM or 8 µM HD5 as described (Labrec et al., 1964), with  
897 15 animals in each group. The protocol was approved by the Committee on Animal  
898 Research and Ethics of Xi'an Jiaotong University. Inoculated animals were observed  
899 and scored for 7 consecutive days for the development of conjunctivitis. Eye tissues  
900 were scored by three individuals (DX, YS, YC) who were kept unaware of the treatment  
901 group on a scale of 0–3 defined as follows: grade 0 (no disease or mild irritation), grade

902 1 (mild conjunctivitis or late development and/or rapid clearing of symptoms), grade 2  
903 (keratoconjunctivitis without purulence), grade 3 (fully developed keratoconjunctivitis  
904 with purulence). Statistical significance was calculated using a Mann-Whitney test.

905

#### 906 *Colon infection model in guinea pigs*

907 Female pathogen-free Hartley guinea pigs, aged 6–8 weeks, weighing 120–250 g, were  
908 fasted for 24 h and anesthetized by intraperitoneal injection of nembutal (30 mg/kg).

909 Animals were inoculated intrarectally with either  $1 \times 10^8$  CFU of HD5-treated,  $1 \times 10^8$  CFU  
910 of mock-treated (low-inoculum positive control) or  $1 \times 10^9$  CFU (high-inoculum positive  
911 control) of mock-treated GFP-expressing Sf301 in 200  $\mu$ l medium. Animals inoculated

912 with medium containing 8  $\mu$ M HD5 at equal volume served as negative controls. HD5-  
913 treated *Shigella* bacteria were prepared as following:  $1 \times 10^8$  CFU of Sf301 were

914 incubated with 8  $\mu$ M HD5 in 50ml DMEM for 20 minutes followed by centrifugation for  
915 10-min. Most supernatant was decanted, leaving 200  $\mu$ l to resuspend the bacteria.

916 Temporally representative samples from colonic tissues were obtained at 4, 8, 24 and  
917 48h post-intrarectal challenge by euthanizing animals using nembutal. The distal 10 cm  
918 of colon was harvested and flushed with 4% (vol/vol) paraformaldehyde (PFA) in

919 1 $\times$ PBS, inverted on wooden skewers, and kept in 4% PFA 1 $\times$ PBS for 1–2 h to complete  
920 fixation of the tissue and then incubated in 1 $\times$ PBS glycine (100 mM) for 30 min to

921 quench the PFA. Tissues were then immersed successively in 15% and 30% (wt/vol)  
922 sucrose at 4 °C overnight. Tissues were removed from the skewers by a longitudinal

923 incision and prepared as Swiss rolls (Arena et al., 2015). Swiss rolls were then

924 embedded in Tissue-Tek OCT compound (Sakura) using a flash-freeze protocol and

925 frozen at  $-80^{\circ}\text{C}$ . These OCT-frozen tissue preparations were cut as 20- $\mu\text{m}$ -thick  
926 transversal sections. Tissues on slides were fixed with 4% PFA for 10 min at room  
927 temperature and permeabilized in PBS/0.2% Triton X-100 for 10 min. The slides were  
928 washed, mounted with Anti-Fade solution (Invitrogen) containing DAPI onto glass slides  
929 and visualized under a Zeiss confocal microscope. *Shigella* infection foci were identified  
930 by GFP fluorescence microscopy. The ten most bacteria-enriched fields from each  
931 group were analyzed, followed by automatic enumeration of individual *Shigella* bacteria  
932 using ImageJ 1.51k software (from <http://imagej.nih.gov/ij>).

933

#### 934 *Murine ileal and colonic loop*

935 Eight-week-old pathogen-free Balb/c mice (weight 20–30 g) were fasted for 24 h and  
936 anesthetized by intraperitoneal injection of nembutal (60 mg/kg). While maintaining the  
937 body temperature at  $37^{\circ}$  using a heating pad, a small abdominal incision was made and  
938 two adjacent loops of either distal ileum or colon were isolated by suture (2–3 cm in  
939 length) in each animal. For each animal, one loop was instilled with  $1 \times 10^7$  CFU of HD5-  
940 treated Sf301 (GFP-expressing), and the other was instilled with mock-treated Sf301  
941 (both in 100  $\mu\text{l}$  medium). HD5-treated *Shigella* bacteria were prepared as following:  
942  $1 \times 10^7$  CFU of Sf301 were incubated with  $8 \mu\text{M}$  HD5 in 5ml DMEM for 20 minutes  
943 followed by centrifugation for 10-min. Most supernatant was decanted leaving 100  $\mu\text{l}$  to  
944 resuspend the bacteria. The ileum (or colon) tissues were obtained at 2h post-  
945 inoculation, processed and analyzed as described above for the colon infection model  
946 of guinea pigs.

947

948 *Human small intestinal fluid aspirates*

949 Ileal aspirates were obtained from healthy individuals who were undergoing routine  
950 screening colonoscopy for colon polyps. Prior to colonoscopy (~24h beforehand),  
951 patients were administered a routine polyethylene glycol-electrolyte solution to purge  
952 the bowel of contents, and the patients remained on clear fluids until the procedure was  
953 completed. During the colonoscopy procedure, the terminal ileum was intubated.  
954 Approximately 5-15 ml of ileal luminal fluid was aspirated and immediately placed on  
955 ice. Specimens were clarified by centrifugation and filtered through a 0.22 µm filter. For  
956 some specimens, the fluid was further concentration by centrifugal filtration. The  
957 aliquots were stored in a freezer at -80° prior to analysis. The clarified fluid was used as  
958 the medium for *Shigella* infection assays.

959

960 *Human colorectal explants*

961 The human colorectal explants were established in accordance to a protocol recently  
962 developed by Tsilingiri et al. (Tsilingiri et al., 2012). Briefly, healthy colonic tissues were  
963 obtained from patients undergoing surgery for colon cancer. Tissue samples,  
964 maintained in Hank's balanced salt solution on ice, were transported to the laboratory  
965 and processed within two hours. The mucosa layer was separated from the underlying  
966 tissues, and then divided into pieces (~2-3 cm by ~2-3 cm) and placed on soft agar (1%  
967 in PRMI 1640 medium) with the mucosal surface facing upward. A sterile cylinder (8  
968 mm in inner diameter) was used to remove small pieces of soft agar under the center of



969 the tissue segments and fresh medium was added to the small holes so that a small dip  
970 was formed at the center of the segment (Fig. S3C). An aliquot ( $5 \times 10^5$  CFU) of *Shigella*  
971 from logarithmic growth culture in 10  $\mu$ l of PRMI 1640 medium was added to the dip of  
972 the colon tissue and the explants were cultured in medium with or without 8  $\mu$ M HD5 at  
973 37 °C for various times (from 30 min to 2 hours) in a cell culture incubator. Bacteria-free  
974 segments served as negative controls for each experiment and time point. Following  
975 incubation, infected explants were fixed in 10% buffered formalin, paraffin embedded  
976 and HE-stained for histopathological examination.

977

#### 978 *Measurement of trans-epithelial electrical resistance (TEER)*

979 Caco-2 cells ( $3 \times 10^4$  cells per well) were grown on 24-well transwell inserts with a 0.4  
980  $\mu$ m pore size (Corning) in 10% FBS DMEM. Five days after seeding, the polarized cells  
981 were placed in serum-free DMEM, and an aliquot of Sf301 bacteria (MOI=50:1) was  
982 inoculated to the apical chamber either with or without HD5. The infected CaCo-2 cells  
983 were incubated for 60 min and then washed, treated with gentamicin-containing (50  
984  $\mu$ g/ml) medium. TEER values of the polarized epithelial monolayer were measured by  
985 Millicell ERS-2 Voltohmmeter (Merck Millipore) for 24 hours. Monolayers with TEER  
986 values within ,800–1200  $\Omega$ .cm<sup>2</sup> were considered to have an appropriate barrier function  
987 and were used in the study.

988

#### 989 *Crystallization, Data collection and Structure Determination*

990 Lyophilized HD5 mutant proteins were dissolved in water (20 mg/ml), mixed in a 1:1  
991 ratio with precipitant solutions composed as listed in Table S4 and equilibrated at 22° in

992 a hanging drop crystallization format. Crystals were flash frozen in liquid nitrogen after a  
993 brief soak in cryoprotectant solution (Table S4). Data were collected at the Stanford  
994 Synchrotron Radiation Light Source (SSRL) beamlines BL7-1 (Y27A mutant crystal form  
995 1) and BL12-2 (Y27A mutant crystal form 2 and the R28A mutant). All data were  
996 processed and reduced with HKL2000 (Otwinowski and Minor, 1997). Structures were  
997 solved by molecular replacement with the program Phaser (McCoy et al., 2007) from  
998 the CCP4 suite based on the coordinates of the 1ZMP wild type HD5 monomer (Szyk et  
999 al., 2006). Refinement was carried out with Refmac (Murshudov et al., 1997) and/or  
1000 Phenix (Adams et al., 2010) and model building was done with COOT (Emsley et al.,  
1001 2010). Data collection and refinement statistics are shown in Table S5. Ramachandran  
1002 statistics were calculated with Molprobit (Chen et al., 2010) and illustrations were  
1003 prepared with Pymol Molecular graphics (<http://pymol.org>). Crystallographic data were  
1004 collected at the Stanford Synchrotron Radiation Lightsource (SSRL), a Directorate of  
1005 SLAC National Accelerator Laboratory and an Office of Science User Facility operated  
1006 for the U.S. Department of Energy Office of Science by Stanford University. The SSRL  
1007 Structural Molecular Biology Program is supported by the US Department of Energy  
1008 Office of Biological and Environmental Research, by the National Institutes of Health  
1009 (NIH) National Center for Research Resources, Biomedical Technology Program  
1010 (P41RR001209), and by the National Institute of General Medical Sciences.

1011

#### 1012 *Immunofluorescence microscopy*

1013 Cells were plated onto glass coverslips and adhesion assays were performed as  
1014 described using Sf301 harboring the GFP-expressing plasmid. Cells were fixed in 3%

1015 paraformaldehyde at room temperature for 15 min followed permeablization with  
1016 0.1% Triton (in PBS) for 3-5min, washed in PBS and blocked with 5% BSA (in PBS) for  
1017 30 min at room temperature. The coverslips were incubated with Actin,  $\alpha$ 5 $\beta$ 1 or  $\beta$ 1  
1018 antibodies at 4 °C overnight followed by PBS washing and incubation with Alexa Fluor  
1019 594 secondary antibody (Invitrogen Molecular Probes, Carlsbad, CA) for 1 h. The  
1020 coverslips were washed, mounted with Anti-Fade solution (Invitrogen) containing DAPI  
1021 onto glass slides and visualized under Zeiss confocal microscope.

1022

### 1023 *Electron microscopy*

1024 For scanning electron microscopy (SEM), adhesion assay was performed in the  
1025 presence of 4  $\mu$ M HD5 (MOI 50:1) as described above. Cells were fixed with  
1026 paraformaldehyde (15min) and glutaraldehyde (overnight). The fixed specimens were  
1027 dehydrated in graded ethanol, critical point dried with CO<sub>2</sub> and coated with gold-  
1028 palladium beads with a diameter of 15 nm. Samples were photographed using a Philips  
1029 XL-30 scanning electron microscope at 20 kV.

1030 For transmission electron microscopy (TEM), bacteria (treated with or without HD5)  
1031 were stained with 1.5% phosphotungstic acid for 90s and examined by TEM (H-7650,  
1032 HITACHI).

1033 For immunogold-TEM, the adhesion assay was performed in the presence of 4  $\mu$ M HD5  
1034 (MOI 50:1) as previously described. Cells were fixed in 3% paraformaldehyde at room  
1035 temperature for 15 min followed permeablization with 0.1% Triton (in PBS) for 3-5min,  
1036 washed in PBS and blocked with 5% BSA (in PBS) for 30 min at room temperature.

1037 Cells were then incubated with rabbit anti-HD5 antibody at 1:100 dilution at 4 °C  
1038 overnight, followed by PBS washing and incubation with 12nm colloidal gold-conjugated  
1039 donkey anti-rabbit IgG (H+L) for 2h at room temperature. Cells were washed and further  
1040 fixed in 3% paraformaldehyde at room temperature for 15 min. Cells were collected by  
1041 scratching and centrifugation. Further fixation was performed in 3% paraformaldehyde  
1042 and 0.25% glutaraldehyde at 4 °C overnight. Cell mass were embedded and cut into  
1043 ultrathin sections as described, colloidal gold particles were recognized as dark spots  
1044 under TEM (H-7650, HITACHI).

1045

#### 1046 *siRNA silencing*

1047 The siRNA oligonucleotides were synthesized by Shanghai GenePharma (Shanghai,  
1048 China) and their sequences are shown in Table S6. Cells were transfected with siRNAs  
1049 according to the recommended procedures of Lipofectamine™2000 Transfection  
1050 Reagent (Invitrogen, Carlsbad, CA).

1051

#### 1052 *Statistical analysis*

1053 The data were collected from at least three independent experiments in triplicate or  
1054 quadruplicate, unless otherwise indicated. Data were combined and represented as  
1055 mean ± SEM or mean ± SD as indicated. Results were analyzed by various statistical  
1056 tests using GraphPad Prism version 7.  $p < 0.05$  was considered statistically significant.

1057 Microscopy images are representative of at least two independent experiments.

1058

1059 *Data availability*

1060 The data that support the findings of this study are available from the corresponding  
1061 author upon request.