

1 **Characterization of a novel fusion protein of IpaB and IpaD of *Shigella* and its potential**
2 **as a pan-*Shigella* vaccine**

3 **Running title: IpaB/IpaD fusion protein vaccine against shigellosis**

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22

23 **Abstract.**

24 Shigellosis is an important disease in the developing world where about 90 million people
25 become infected with *Shigella spp.* each year. We previously demonstrated that the type three
26 secretion apparatus (T3SA) proteins IpaB and IpaD are protective antigens in the mouse lethal
27 pulmonary model. In order to simplify vaccine formulation and process development, we now
28 evaluate a vaccine design that incorporates both of these previously tested *Shigella* antigens
29 into a single polypeptide chain. To determine if this fusion protein (DB Fusion) retains the
30 antigenic and protective capacities of IpaB and IpaD, we immunized mice with the DB Fusion
31 and compared the immune response to that elicited by the IpaB/IpaD combination vaccine.
32 Purification of the DB Fusion required co-expression with IpgC, the IpaB chaperone, and after
33 purification it maintained the highly α -helical characteristics of IpaB and IpaD. The DB Fusion
34 also induced comparable immune responses and retained the ability to protect mice against *S.*
35 *flexneri* and *S. sonnei* in the lethal pulmonary challenge. It also offered limited protection against
36 *S. dysenteriae* challenge. Our results show the feasibility of generating a protective *Shigella*
37 vaccine comprised of the DB Fusion.

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39 Keywords: Shigella, vaccine, fusion protein, dmLT

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44 **Introduction.**

45 Shigellosis is a severe gastrointestinal disease caused by *Shigella* spp. This disease is
46 characterized by fever, bloody diarrhea and tenesmus. Recent calculations estimate the annual
47 rate of *Shigella* infections at ~90 million with 100,000 deaths per year (1). An important number
48 of these infections occur in children under five years old living in developing countries (2). Other
49 at-risk populations include military personnel deployed abroad (3) and refugees (4). Implications
50 of this disease include severe impairment of child development and nutrition (5), as well as an
51 important mortality index (2).

52 Four different species of *Shigella* have been described (6): *S. flexneri*, *S. sonnei*, *S. boydii* and
53 *S. dysenteriae*. Modifications in the O-antigen give rise to about 50 serotypes (6, 7). The
54 predominance of specific serotypes varies both geographically (8) and during the course of a
55 single outbreak (9), complicating the epidemiology of shigellosis. Immune responses against
56 *Shigella* during natural infection are predominantly serotype-specific, in part due to the high
57 immunodominance of bacterial LPS (10, 11). This dominance results in poor cross-reaction
58 between different *Shigella* serotypes, thus opening the possibility of subsequent reinfections by
59 *Shigella* bearing different O-antigens.

60 *Shigella* infection requires the use of a highly conserved type three secretion system (T3SS)
61 encoded on a virulence plasmid present in all *Shigella* species. After crossing the intestinal
62 barrier via M cells, *Shigella* is taken up by macrophages, however, it escapes these phagocytes
63 by inducing apoptosis mediated by the T3SS translocator IpaB (12). *Shigella* then uses the
64 T3SS to inject protein effectors via the basolateral side of epithelial cells to promote bacterial
65 entry. The pathogen lyses the resulting phagosome, replicates, and moves spreads to adjacent
66 cells. Control of type III secretion occurs at the needle tip of the secretion apparatus needle by
67 the activity of IpaD along with IpaB (13, 14).

68 Despite longstanding efforts a *Shigella* vaccine is still not available (7, 15). Several groups have
69 explored different approaches in the search of a *Shigella* vaccine. These include live/attenuated
70 strains of *Shigella* (16), LPS-protein conjugates (17) mixtures of subunit components (18), and
71 recombinant proteins (19). Our hypothesis is that a vaccine comprised of highly conserved
72 protein antigens would provide broad, serotype-independent protection, thus bypassing the
73 need to consider multiple serotypes as is needed for vaccines that target LPS or O-antigen. We
74 have previously demonstrated the protective efficacy of *S. flexneri* 2a-derived IpaB and IpaD,
75 components of the T3SS (20), when included in a vaccine formulation incorporating the novel
76 mucosal adjuvant, dmLT, and delivered intranasally (20), orogastrically (21), or intramuscularly
77 (22) using monophosphoryl lipid A (MPL) and alum hydroxide as adjuvants. The IpaB/IpaD
78 subunit vaccine elicited a strong systemic immunity, with the presence of antibody secreting
79 cells in various compartments, as well as eliciting specific cytokine secreting cells. Protection is
80 achieved against the homologous strain, *S. flexneri*, and a heterologous strain, *S. sonnei* in the
81 mouse pneumonia model. Given that children in low resource countries are a primary target of a
82 *Shigella* vaccine, the ultimate vaccine formulation must be inexpensive. To reduce the cost of
83 an IpaB- and IpaD-based vaccine and simplify manufacture and formulation, we created a
84 genetically fused IpaD-IpaB protein (DB Fusion). The approach of using a fusion of protective
85 antigens has been explored successfully in other subunit vaccines, including LcrV, an IpaD
86 homolog (23-25). In this study, the DB Fusion elicited immune responses of a similar magnitude
87 to those generated by a combination of separate IpaB and IpaD proteins. Interestingly, higher
88 cytokine levels were detected when cells from mice immunized with the DB Fusion were
89 stimulated. In addition, mice were protected in the lethal pulmonary challenge (26, 27) using *S.*
90 *flexneri*, *S. sonnei* and *S. dysenteriae*. Therefore, this novel fusion protein represents an
91 efficient alternative for vaccination against shigellosis in humans.

93 **Materials and methods.**

94 **Materials.** pET plasmids, ligation mix and competent *E. coli* were from EMD Millipore
95 (Billerica, MA). Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were
96 from New England Biolabs (Ipswich, MA). HisTrap Crude FF IMAC columns and Q FF anion
97 exchange columns were from GE Healthcare (Piscataway, NJ). The OPOE was from Enzo Life
98 Sciences (Farmingdale, NY).

99

100 **Generation of plasmids for expression of the DB Fusion in *E.coli*.** *ipaD* was amplified from
101 D/pET15b (28) by PCR using a 5' primer with NdeI restriction site and a 3' primer with XhoI
102 restriction site while *ipaB* was amplified from B/pET15b (29) using a 5' primer with XhoI site and
103 a 3' primer with BamHI site. The PCR products were digested by the appropriate restriction
104 endonucleases and ligated into pET28b. The ligation reaction was used to transform *E.coli*
105 NovaBlue. The resulting DB/pET28b and *ipgC/pACYCDuet-1* were co-transformed into *E.coli*
106 Tuner(DE3) for co-expression (Figure 1A).

107 **Protein purification and sample preparation.** DB/pET28b+*ipgC/pACYCDuet-1*/Tuner(DE3)
108 were grown in Auto-Induction media (30) containing kanamycin (50 µg/ml) and chloramphenicol
109 (25 µg/ml) for 16-18 hours. Purification of the DB Fusion was as previously described for IpaB
110 (20, 31). Briefly, bacteria were collected by centrifugation, resuspended in IMAC binding buffer
111 containing protease inhibitors (Roche, Basel, Switzerland), lysed, the suspension clarified by
112 centrifugation and the supernatant containing the DB Fusion/IpgC complex purified using by
113 IMAC. After further purification using a Q FF anion exchange chromatography column, OPOE
114 was added to 0.5% to release the IpgC. The his-tagged DB Fusion was separated from IpgC
115 using IMAC column with OPOE at 0.5% in all buffers and dialyzed into PBS with 0.5% OPOE.
116 IpaB and IpaD were purified (32). Protein concentrations were determined by A_{280} (33)

117 **Circular dichroism.** Far-UV CD spectra were collected (34). Briefly, a Jasco J-815
118 spectropolarimeter fitted with a Peltier temperature controller (Jasco Inc., Easton, MD) was used
119 to collect spectra from 190 nm to 260 nm through a 0.1 cm path length quartz cuvette. Samples
120 were kept at 10°C and scanned at 50 nm/min with a 1 nm spectral resolution and a 2-second
121 data integration time. All spectra are an average of three measurements. Secondary structure
122 thermal stability was determined by monitoring CD signal at 222 nm as the temperature was
123 increased from 10 to 90°C. The temperature ramp rate was 15°C/hour and data were collected
124 every 2.5°C. All protein solutions were made to 0.5 mg/ml in phosphate citrate buffer pH 7.4
125 with 0.5% OPOE included for IpaB and the DB Fusion. CD signals were converted to mean
126 residue molar ellipticity.

127 **Mice and immunizations.** Six to eight week old female BALB/c mice (Charles River
128 Laboratories, Wilmington, MA) were used. Mice were anesthetized and vaccinated intranasally
129 using 30 μ l (20). IpaB (13 μ g) combined with IpaD (7 μ g) or the DB Fusion (20 μ g) were
130 admixed with dmLT (2.5 μ g). These doses represent equimolar concentrations of antigens. Mice
131 that received adjuvant or vehicle alone were included as controls. Vaccine was delivered at day
132 0, 14 and 28.

133 **Specific IgG antibodies.** Antibodies specific for IpaB and IpaD were determined by ELISA (20).
134 Briefly, 96 well plates coated with IpaB or IpaD (1 μ g/ml in PBS) were blocked overnight with
135 PBS with 10% milk. Each well was incubated with serum for 1h at 37°C. After washing the
136 plates with PBS Tween (0.05%), secondary antibody (KPL, Gaithersburg, MD) was added for 1h
137 at 37°C. HRP substrate was added and reaction stopped with H₃PO₄. Endpoint titers were
138 calculated and represented as ELISA units per ml (EU ml⁻¹).

139 **Stool IgA.** Fresh fecal samples (3-5 pellets/mouse) were collected. Each sample was
140 resuspended in 10% (w/v) PBS with 0.2% NaN₃. The supernatant was clarified by centrifugation

141 and PMSF added to 1 mM. IgA levels were determined by ELISA with an anti-IgA antibody
142 (Southern Biotech, Birmingham, AL). Endpoint titers were calculated as above.

143 **Antibody secreting cells (ASCs).** Antibody secreting cells were determined (20). Briefly, cell
144 suspensions were obtained by homogenizing through a nylon mesh (BD Biosciences, San
145 Diego, CA) the organs from five mice per group. Samples were incubated with 5 µg/ml of IpaB
146 or IpaD for 24h at 37°C. After washing with PBS Tween, antibodies against IgG or IgA were
147 added. Trueblue (KPL) was used as a substrate in an agarose overlay. Spots were counted
148 under a stereomicroscope by 2 individuals and a mean of quadruplicate wells was expressed as
149 specific ASCs per 10⁶ cells.

150 **IFN-γ ELISpot.** Splenocytes were collected from five mice per group at day 56. Cells were
151 incubated for 48h at 37°C with 5 µg/ml IpaB or IpaD in plates coated with antibodies against
152 IFN-γ. An ELISPOT assay was performed (BD Biosciences). Spots were counted as above and
153 expressed as Spot Forming Cells (SFC) per 10⁶ cells.

154 **Cytokine determinations.** Splenocytes (obtained at day 56) were incubated with 10 µg/ml
155 IpaB, IpaD or PBS for 48h at 37°C. Secreted interleukin 17 (IL-17) levels were measured using
156 the DuoSet ELISA development kit (22) or using a Th1/Th2 multiplex cytokine plate (Meso
157 Scale Discovery, Gaithersburg, Md).

158 **Challenge.** *Shigella flexneri* 2457T, *Shigella sonnei* 53G and *Shigella dysenteriae* serotype
159 Sd1617 were grown overnight at 37°C in tryptic soy agar with 0.05% Congo red. Ten colonies
160 were picked and grown in tryptic soy broth (EMD Milipore) at 37°C in agitation until ABS₆₀₀ ~1.
161 Bacteria were centrifuged and resuspended in PBS. On day 56, mice were challenged by
162 delivering *Shigella* intranasally (26, 27). The doses administered in 30µl were 6 x10⁶ CFUs for
163 *S. flexneri*, 2.1 x10⁶ CFUs for *S. sonnei* and 7.5 x10⁶ CFUs for *S. dysenteriae*. Changes in
164 health and weight loss were closely monitored for 14 days. Mice that became too sick or

165 remained below 80% of their starting weight for more than 48h were humanely euthanized.
166 Animals were housed and handled in agreement with Oklahoma State University Institutional
167 Animal Care and Use Committee (Protocol #AS-10-6).

168 **Statistical analysis.** GraphPad Prism 5.04 was used to generate graphics and statistical
169 comparisons. Differences were analyzed using t-test. Survival plots were analyzed using Log
170 rank tests. A p value of less than 0.05 was considered significant for all comparisons. Vaccine
171 efficacy was calculated by using the formula $\text{efficacy} = (\text{ARU} - \text{ARV})/\text{ARU} \times 100$, where
172 ARU=attack rate in unvaccinated group and ARV= attack rate in vaccinated group (35).

173

174 **Results.**

175 **DB Fusion protein is expressed and folded.** Following co-expression with the *Shigella*
176 chaperone protein IpgC, DB Fusion was isolated using the mild nonionic detergent OPOE,
177 resulting in a dominant 101.2 kDa product comprised of both IpaD and IpaB (Fig. 1B). Like
178 IpaB, the isolated DB Fusion remains soluble in buffer containing 0.5% OPOE. Far-UV circular
179 dichroism (CD) measurements of IpaD, IpaB, and DB Fusion all resulted in spectra exhibiting
180 dominant minima at 208 and 222 nm, characteristic of proteins with highly α -helical secondary
181 structures (Fig. 1C). This suggests that the fusion maintained a proper and organized secondary
182 structure following purification and separation from IpgC. The secondary structure thermal
183 stabilities for all three proteins were determined using CD spectroscopy by monitoring mean
184 residue molar ellipticity at 222 nm as a function of temperature. The resulting plots indicated a
185 transition at $\sim 58^\circ\text{C}$ for IpaB and two transitions at 60°C and 80°C for IpaD (Fig. 1D), which is in
186 agreement with previously published data (13, 34). Interestingly, the thermal unfolding curve for
187 the DB Fusion protein exhibits characteristics intermediate to both IpaD and IpaB with a major
188 transition at 60°C and a minor one around 78°C . Furthermore, the DB Fusion mean residue

189 molar ellipticity values for both the far-UV scans and the thermal unfolding curves lie between
190 those for IpaD and IpaB alone, further suggesting that the IpaD and IpaB domains of the fusion
191 protein both maintain a substantial portion of their original structural characteristics.

192 **DB Fusion protein generates antibody titers similar to the combination of IpaB and IpaD.**

193 Mice were vaccinated intranasally three times at days 0, 14 and 28, and serum IgG titers
194 against IpaB and IpaD determined by ELISA (Fig. 2 A, B). The antibody titers against IpaB and
195 IpaD elicited by the DB Fusion in the presence of dmLT were comparable to those generated by
196 vaccination with IpaB and IpaD with dmLT. The peak antibody levels and the kinetics follow a
197 very similar pattern, with no significant differences observed over time. In both cases, IpaD
198 responses were delayed until day 28 (after two immunizations). Although the DB Fusion protein
199 administered without dmLT is able to generate detectable antibodies against IpaB and IpaD,
200 adjuvant is required for generation of consistent titers higher than 10^3 - 10^4 EU/ml. No specific
201 IgG was detected in mice immunized with PBS.

202 To assess the intestinal mucosal antibody responses, fecal IgA antibody titers were determined
203 by ELISA (Fig. 2 C, D). Both the DB Fusion and the combination of IpaB and IpaD administered
204 with dmLT elicited specific IgA titers in stool. IgA antibodies against IpaB were detected in the
205 group immunized with DB Fusion with adjuvant at day 28, one time point ahead of the group
206 that received IpaB and IpaD. The stool IgA titer was tenfold higher for IpaB than for IpaD in the
207 DB Fusion group and the IpaD antibodies were not detected until day 42 rather than at day 28
208 as was the case for the IpaB antibodies. No stool IgA specific for these proteins was detected in
209 the group immunized with the DB Fusion without dmLT or in the group immunized with PBS.

210 **The DB Fusion protein generates antibody secreting cells (ASCs).** At day 56, the frequency
211 of IgG and IgA secreting cells specific for each antigen was determined by ELISpot. In the lungs
212 (Fig. 3A), the frequency of ASCs specific for IpaB was higher for DB Fusion+dmLT, especially

213 for IgA secreting cells. This tendency was also observed for ASCs specific for IpaD. Only IgG
214 secreting cells specific for IpaB were detected in lungs from mice immunized with the DB Fusion
215 without adjuvant. In spleens (Fig. 3B), we found a higher frequency of IgA secreting cells
216 specific for IpaB than IgG secreting cells and when the groups that received IpaB+IpaD and the
217 DB Fusion are compared, no major differences are observed. In general, the responses against
218 IpaB were higher than responses against IpaD in spleens. The DB Fusion without dmLT failed
219 to elicit ASCs in the spleens. Finally, the frequencies of ASCs in the bone marrow (Fig. 3C)
220 specific for IpaB were higher in the group that received IpaB+IpaD+dmLT, while for IpaD a
221 higher IgA response was observed in the group that received the DB Fusion+dmLT. For this
222 organ, a more balanced IgG/IgA response was observed.

223 **The DB Fusion protein generates higher frequencies of specific IFN- γ secreting cells.** The
224 frequency of IFN- γ secreting cells was analyzed by ELISpot using cells extracted from spleens
225 of immunized mice at day 56 (Fig. 4). When compared to IpaB+IpaD+dmLT, the DB
226 Fusion+dmLT elicited higher numbers of specific IFN- γ secreting cells. This was more evident
227 for IpaD specific IFN- γ secreting cells where a threefold higher frequency was seen in the group
228 that received DB Fusion+dmLT (~20 spot forming cells/ 10^6 cells for IpaB+IpaD+dmLT compared
229 to ~70 SFC/ 10^6 cells for DB Fusion+dmLT). The DB fusion without dmLT failed to generate IpaB
230 specific IFN- γ secreting cells but managed to elicit a moderate number of IpaD specific IFN- γ
231 secreting cells. No specific IFN- γ secreting cells were detected in mice treated with PBS.

232 **DB Fusion protein generates a distinct profile of cytokine secretion.** Spleen cells were
233 stimulated with IpaB or IpaD and the resulting supernatants analyzed for cytokine secretion. IL-
234 2 levels varied depending on the antigen used for stimulation (Fig. 5A). For IpaB, the group that
235 received the DB Fusion+dmLT showed higher cytokine secretion levels than the group that
236 received IpaB+IpaD+dmLT. The opposite was observed for IpaD, where cells obtained from

237 animals that received IpaB+IpaD+dmLT secreted higher levels of IL-2 than cells obtained from
238 animals that received DB Fusion+dmLT. For IL-4, cells from mice that were vaccinated with
239 IpaB+IpaD+dmLT showed higher cytokine secretion when stimulated with either IpaB or IpaD
240 (Fig. 5B). In contrast, no significant differences were detected in levels of IL-5 secretion
241 between the groups immunized with IpaB+IpaD+dmLT or the DB Fusion+dmLT (Fig. 5C).
242 Secretion of the KC chemokine in response to IpaB was higher in cells from mice that received
243 DB Fusion+dmLT with no differences being observed between these two treatments when IpaD
244 was used to stimulate these cells (Fig. 5D). In the case of TNF- α , significant differences were
245 detected when IpaD was used to stimulate these cells with the DB Fusion+dmLT immunized
246 mice showing a greater response (Fig. 5E). In contrast, no differences in TNF- α secretion were
247 observed with IpaB stimulation. Levels of IL-17 secretion were also measured in response to
248 IpaB and IpaD stimulation of spleen cells (Fig. 6). Cells from mice that received the DB
249 Fusion+dmLT secreted higher amounts of IL-17 in response to IpaB than cells from mice that
250 received IpaB+IpaD+dmLT.

251 **DB Fusion protein protects against *Shigella* homologous and heterologous challenges.**

252 At day 56, vaccinated animals (N=10 per bacterial strain) were challenged with *S. flexneri* 2a, *S.*
253 *sonnei*, or *S. dysenteriae*, and protection was followed for 14 days after infection. For the
254 homologous challenge using *S. flexneri*, mice that received IpaB+IpaD+dmLT showed a
255 protection of 90%, while the mice that received the DB Fusion+dmLT showed a protection of
256 70%. Mice that received the DB Fusion without dmLT showed 20% protection after 14 days. No
257 protection was observed for mice immunized with PBS (Fig. 7A). When *S. sonnei* was used to
258 challenge vaccinated animals, we observed 100% protection in animals vaccinated with
259 IpaB+IpaD+dmLT or the DB Fusion+dmLT. Mice that received the DB Fusion alone showed
260 80% protection, while mice treated with PBS showed 20% protection. These numbers result in a
261 calculated vaccine efficacy of 80% for both groups that received vaccine formulated with dmLT,

262 and 55% for the group that received the DB Fusion alone Fig. 7B). For *S. dysenteriae*, the group
263 that received IpaB+IpaD+dmLT only showed 10% protection, while the group that received the
264 DB Fusion+dmLT showed a protection of 40%. No protection was observed in mice vaccinated
265 with DB Fusion without dmLT or in mice that received PBS (Fig. 7C).

266

267 **Discussion.**

268 Despite progress using different approaches, a *Shigella* vaccine is still not available. When the
269 main target group for a *Shigella* vaccine is children living in developing countries, variables that
270 impact cost of production should be evaluated to diminish the vaccine cost. Taking this in
271 consideration, we generated a fusion protein consisting of IpaD and IpaB. The DB Fusion
272 shared characteristics of IpaB. DB fusion expression was only achieved in the presence of
273 IpaB's chaperone, IpgC, which is removed using 0.5% OPOE during chromatography
274 purification. Although this indicates that the IpaD portion of the DB Fusion is not sufficient to
275 generate an independently soluble polypeptide, the subsequent purification step allows for a
276 highly pure protein. Therefore, this purification step may be advantageous. Additionally, the DB
277 Fusion maintains a highly α -helical secondary structure in solution with stability similar to that of
278 IpaB. While IpaD undergoes two thermal transitions, these transitions are not seen in the DB
279 Fusion. After three immunizations with equimolar concentrations, the DB Fusion+dmLT was
280 able to elicit serum IgG and stool IgA titers against both IpaB and IpaD at a magnitude similar to
281 that elicited by administering the combination of IpaB and IpaD with dmLT. Therefore,
282 recognition and generation of antibody responses against the components of the DB Fusion
283 remain at comparable levels. The presence of antibody secreting cells in the same organ
284 compartments supports this statement. Both IgG and IgA secreting cells were observed in the
285 lungs, spleens and bone marrow, specific for both IpaB and IpaD. The differences observed in

286 the frequencies of ASCs, however, suggest there are some small differences in how the
287 proteins are able to activate B cells, which could impact the fate and distribution of plasmatic
288 and memory cells. While mice immunized with DB Fusion+dmLT showed higher frequencies of
289 ASCs in the lungs and spleens, a lower frequency was observed in the bone marrow,
290 suggesting differences in effector versus long term memory ratios. Although the DB Fusion
291 without dmLT was able to elicit serum IgG responses against IpaB and IpaD, these responses
292 were of a lower magnitude and highly variable between the individuals. Furthermore, it failed to
293 induce IgA secretion in stool and generation of specific cytokine secreting cells. This highlights
294 the requirement of the dmLT adjuvant for these responses.

295 The analysis of the cytokine secretion profiles elicited by each group showed some differences
296 between the immunized groups. Some cytokine responses were higher when the DB Fusion
297 was used for immunization. In particular, the frequency of IFN- γ secreting cells and IL-17
298 secretion levels were higher in cells obtained from mice immunized with the DB Fusion. Even if
299 dmLT has the capacity of eliciting IL-17 responses by itself (36), the presence of the adjuvant in
300 both formulations indicate the possibility that the fusion could be recognized by the immune
301 system in a different manner than the individual proteins. Most importantly, this demonstrates
302 that the DB Fusion has a unique advantage in the generation of cell mediated immunity, which
303 can be important for control of *Shigella*. Indeed, both IFN- γ and IL-17 have been described as
304 important cytokines during *Shigella* infection (37, 38). The challenge experiments show that
305 both proteins are able to provide heterologous protection. In the case of *S. dysenteriae*, only the
306 DB Fusion with dmLT was able to provide significant protection. This particular challenge is
307 more stringent as we used a strain that expresses Shiga toxin. The ability of the DB Fusion to
308 protect in contrast to the combination of IpaB and IpaD could be related to the cytokine profile
309 elicited by this protein. Even with this tendency of higher protection with higher cytokine
310 secretion, the role of antibodies cannot be ruled out. The protective efficacy of the DB fusion

311 without adjuvant in the *S. sonnei* challenge could then relate to antibodies generated by this
312 protein. Even if humoral responses could be less involved in protection, we still detect a 55%
313 protective efficacy. This is probably only observed for *S. sonnei* given that the challenge dose
314 that was used is lower than for *flexneri* and *dysenteriae*. In conclusion, we provide evidence
315 that a fusion protein comprised of IpaB and IpaD is able to generate immune responses against
316 the two subcomponents, retaining heterologous protection capabilities and generating higher
317 IFN- γ and IL-17 responses, which could be important for protection against shigellosis in
318 humans.

319

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323

324 **Figure legends.**

325

326 Figure 1. Biophysical analysis of DB Fusion. (A) Construct harboring the IpaD/B fusion.
327 Restriction sites used for cloning are mentioned along a map of pET28b. (B) A comparative
328 SDS-PAGE with IpaB, IpaD and DB Fusion proteins is shown with molecular weight markers
329 indicated to the left. (C) The CD spectra for IpaB in 0.5% OPOE, IpaD in PBS, and DB Fusion
330 prepared in 0.5% OPOE all indicate predominantly α -helical content with the mean residue
331 molar ellipticity ($[\theta]_R$) values. (D) Thermal unfolding of the secondary structure of DB Fusion,
332 IpaB and IpaD as a function of temperature is shown.

333 Figure 2. Serum IgG titer kinetics. Mice were vaccinated three times at time points indicated by
334 arrows. Blood samples were collected and serum was separated. IgG antibodies specific for
335 IpaB (panel A) or IpaD (panel B) were measured by ELISA. The individual titers are represented
336 as EU ml⁻¹, and each point represents mean ± S.D. of 10 mice per group. *P<0.05 comparing
337 groups that received IpaB+IpaD+dmLT and DB Fusion+dmLT using T test. IgA antibodies
338 specific for IpaB (panel C) or IpaD (panel D) were measured by ELISA. The pool titers are
339 represented as EU ml⁻¹, and each point representing pooled samples of ten mice per group.

340 Figure 3. Antibody secreting cells. Immunized mice (N=5 per group) were euthanized at day 56
341 and organs were collected. Single cell suspensions obtained from lungs (A), spleen (B) and
342 bone marrow (C) were incubated in plates with IpaB or IpaD. IgG and IgA secreting cells were
343 detected by ELISpot, and plotted as mean specific ASCs per 10⁶ cells ± S.D. *P<0.05
344 comparing groups that received IpaB+IpaD+dmLT and DB Fusion+dmLT using T test.

345 Figure 4. IFN-γ secreting cells. Splenocytes obtained at day 56 from immunized animals were
346 incubated with 10 µg/ml IpaB and IpaD. IFN-γ secreting cells were determined by ELISpot and
347 spot forming cells (SFC) per 10⁶ cells were calculated and plotted as mean ± S.D. of
348 quadruplicate wells. *P<0.05 comparing groups that received IpaB+IpaD+dmLT and DB
349 Fusion+dmLT using T test.

350 Figure 5. Cytokines. Splenocytes obtained at day 56 from immunized animals were incubated
351 with 10 µg/ml IpaB and IpaD. After 48h, supernatants were collected and levels of cytokine
352 secretion in response to IpaB (left) and IpaD (right) were then measured (in pg/ml) using an
353 MSD cytokine detection plate. Each bar represents mean of quadruplicate wells ± S.D. *P<0.05
354 comparing groups that received IpaB+IpaD+dmLT and DB Fusion+dmLT using T test.

355 Figure 6. IL-17 secretion. Splenocytes obtained at day 56 from immunized animals were
356 incubated with 10 µg/ml IpaB and IpaD. After 48h, supernatants were collected and levels of IL-

357 17 secretion were measured using an ELISA kit. Each bar represents mean of quadruplicate
358 wells \pm S.D. *P<0.05 comparing groups that received IpaB+IpaD+dmLT and DB Fusion+dmLT
359 using T test.

360 Figure 7. Challenge. Mice were vaccinated at days 0, 14 and 28 with the indicated treatments.
361 After 56 days, 6×10^6 CFU of *S. flexneri* 2457T (A), 2.1×10^6 CFU of *S. sonnei* 53G (B) or 7.5×10^6
362 CFU of *S. dysenteriae* 1617 (C) were administered intranasally. Survival was followed for 14
363 days. #P<0.05 compared to survival of mice vaccinated with PBS using Log-rank test.

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