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

Running title: IpaB/IpaD fusion protein vaccine against shigellosis

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Abstract.

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

Keywords: Shigella, vaccine, fusion protein, dmLT

Introduction.

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

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

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

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

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

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

Specific IgG antibodies. Antibodies specific for IpaB and IpaD were determined by ELISA (20). Briefly, 96 well plates coated with IpaB or IpaD (1 µg/ml in PBS) were blocked overnight with PBS with 10% milk. Each well was incubated with serum for 1h at 37^{o}C. After washing the plates with PBS Tween (0.05%), secondary antibody (KPL, Gaithesburg, MD) was added for 1h at 37^{o}C. HRP substrate was added and reaction stopped with H_3PO_4. Endpoint titers were calculated and represented as ELISA units per ml (EU ml^{-1}).

Stool IgA. Fresh fecal samples (3-5 pellets/mouse) were collected. Each sample was resuspended in 10% (w/v) PBS with 0.2% NaN_3. The supernatant was clarified by centrifugation

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

Antibody secreting cells (ASCs). Antibody secreting cells were determined (20). Briefly, cell suspensions were obtained by homogenizing through a nylon mesh (BD Biosciences, San Diego, CA) the organs from five mice per group. Samples were incubated with 5 μg/ml of IpaB or IpaD for 24h at 37°C. After washing with PBS Tween, antibodies against IgG or IgA were added. Trueblue (KPL) was used as a substrate in an agarose overlay. Spots were counted under a stereomicroscope by 2 individuals and a mean of quadruplicate wells was expressed as specific ASCs per 10^6 cells.

IFN-γ ELISpot. Splenocyes were collected from five mice per group at day 56. Cells were incubated for 48h at 37°C with 5 μg/ml IpaB or IpaD in plates coated with antibodies against IFN-γ. An ELISPOT assay was performed (BD Biosciences). Spots were counted as above and expressed as Spot Forming Cells (SFC) per 10^6 cells.

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

Challenge. *Shigella flexneri* 2457T, *Shigella sonnei* 53G and *Shigella dysenteriae* serotype Sd1617 were grown overnight at 37°C in tryptic soy agar with 0.05% Congo red. Ten colonies were picked and grown in tryptic soy broth (EMD Milipore) at 37°C in agitation until ABS_{600} ~1. Bacteria were centrifuged and resuspended in PBS. On day 56, mice were challenged by delivering *Shigella* intranasally (26, 27). The doses administered in 30μl were 6 x10^6 CFUs for S. *flexneri*, 2.1 x10^6 CFUs for *S. sonnei* and 7.5 x10^6 CFUs for *S. dysenteriae*. Changes in health and weight loss were closely monitored for 14 days. Mice that became too sick or

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

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

Results.

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

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

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

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

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

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

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

Discussion.

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

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

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

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

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Figure legends.

Figure 1. Biophysical analysis of DB Fusion. (A) Construct harboring the IpaD/B fusion. Restriction sites used for cloning are mentioned along a map of pET28b. (B) A comparative SDS-PAGE with IpaB, IpaD and DB Fusion proteins is shown with molecular weight markers indicated to the left. (C) The CD spectra for IpaB in 0.5% OPOE, IpaD in PBS, and DB Fusion prepared in 0.5% OPOE all indicate predominantly α-helical content with the mean residue molar ellipticity ($[q]_R$) values. (D) Thermal unfolding of the secondary structure of DB Fusion, 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 10^6 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 10^6 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-

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

Figure 7. Challenge. Mice were vaccinated at days 0, 14 and 28 with the indicated treatments. After 56 days, $6x10^6$ CFU of *S. flexneri* 2457T (A), $2.1x10^6$ CFU of *S. sonnei* 53G (B) or $7.5x10^6$ CFU of *S. dysenteriae* 1617 (C) were administered intranasally. Survival was followed for 14 days. #P<0.05 compared to survival of mice vaccinated with PBS using Log-rank test.

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