Shigella IpaB and IpaD displayed on *L. lactis* bacterium-like particles induce protective immunity in adult and infant mice

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Abstract

Shigella spp. are among the enteric pathogens with the highest attributable incidence of moderate-to-severe diarrhea in children under 5 years of age living in endemic areas. There are no vaccines available to prevent this disease. In this work, we investigated a new *Shigella* vaccine concept consisting of non-living, self-adjuvanted, *Lactococcus lactis* bacterium-like particles (BLP) displaying *Shigella* invasion plasmid antigen (Ipa) B and IpaD and examined its immunogenicity and protective efficacy in adult and newborn/infant mice immunized via the nasal route. Unique advantages of this approach include the potential for broad protection due to the highly conserved structure of the Ipas and the safety and practicality of a probiotic-based mucosal/adjuvant delivery platform. Immunization of adult mice with BLP-IpaB and BLP-IpaD (BLP-IpaB/D) induced high levels of Ipa-specific serum IgG and stool IgA in a dose-dependent manner. Immune responses and protection were enhanced by BLP delivery. Vaccine-induced serum antibodies exhibited opsonophagocytic and cytotoxic neutralizing activity, and IpaB/D IgG titers correlated with increased survival post-challenge. Ipa-specific antibody secreting cells were detected in nasal tissue and lungs, as well as IgG in bronchoalveolar lavage. Bone marrow cells produced IpaB/D-specific antibodies and contributed to protection after adoptive transfer. The BLP-IpaB/D vaccine conferred 90% and 80% protection against *S. flexneri* and *S. sonnei,* respectively. Mice immunized with BLP-IpaB/D as newborns also developed IpaB and IpaD serum antibodies; 90% were protected against *S. flexneri* and 44% against *S. sonnei*. The BLP-IpaB/D vaccine is a promising candidate for safe, practical and potentially effective immunization of children against shigellosis.

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INTRODUCTION

A variety of new vaccine approaches are being explored to improve the safety and effectiveness of pediatric immunization. Methods that allow administration of vaccines through mucosal routes are highly desirable, as they are more practical, less invasive and easier to implement than parenteral injection, the route typically used for routine immunization. Effective mucosal vaccines that can prevent the devastating burden of childhood diarrhea in less developed areas of the world would make a substantial contribution to public health. The recent Global Enteric Multicenter Study (GEMS), led by the Center for Vaccine Development at the University of Maryland, identified *Shigella* as one of the organisms associated with the largest incidence of diarrheal disease in children under 5 years of age.[1] When incidence of disease was stratified by age, toddlers 11–23 months of age were found to be the most affected group.[1] In addition to unacceptably high mortality rates, repeated bouts of diarrheal disease throughout childhood can result in impaired development and long-term disability.[2,3] Aiming to identify an effective pediatric prophylactic tool to substantially reduce this burden, we focused on *Shigella* invasion plasmid antigens (Ipas), which are components of the Type III secretion system, as potential candidates for the development of a broadly protective subunit-based *Shigella* vaccine. We have recently shown that adjuvanted *Shigella* IpaB and IpaD were able to induce robust cross-protective immunity in mice immunized via mucosal[4,5] or parenteral[6,7] routes. The purpose of this study was to investigate the use of non-living *Lactococcus lactis* bacterium-like particles (BLP) as an adjuvant and vaccine display system for mucosal delivery of *Shigella* IpaB and IpaD that could potentially be used to immunize susceptible children. The *L. lactis* BLP consist of peptidoglycan (PGN) shell particles devoid of intracellular content that are produced by heat-acid treatment of *L. lactis*. They have the same shape and size as the living *L. lactis*.[8] Antigens are displayed on the particle surface as a fusion protein containing a PGN binding domain that attaches non-covalently and with high avidity to the PGN wall.[8,9] Because *L. lactis* is a generally regarded as a safe (GRAS) food additive, the BLP are likely to be safe for immunization of children through a mucosal route. The BLP PGN is a Toll-like receptor 2 (TLR-2) agonist[10] and serves as a mucosal adjuvant.[11] Because of their larger size (+/− 1–2 μm), the particles interact more efficiently with mucosal antigen-presenting cells (APC) and facilitate vaccine uptake. Conceptually, this approach would be highly advantageous because it combines safety, strong immunogenic capacity and ease of delivery for effective and practical immunization early in life. A precedent exists for efficient mucosal immunization of newborn mice with *Yersinia pestis* LcrV displayed on *L. lactis* BLP, which induced mucosal and systemic immunity and afforded complete protection against systemic plague infection.[10] Likewise, the BLP technology has been successfully tested in adult mice as a vaccine delivery system for protection against respiratory syncytial virus[12], malaria[13] and *Streptococcus pneumoniae.*[14]

Serving as adjuvants, the BLP have been shown to enhance the immunogenicity[15] and protective efficacy[16,17] of intranasally (i.n.) delivered influenza virus vaccines.

Herein, we examined the in vivo distribution of i.n. delivered *L. lactis* BLP and the immunogenicity and protective capacity of combined BLP displaying IpaB or IpaD (BLP-IpaB/D). Increasing doses of BLP-IpaB/D and IpaB/D alone were tested in adult and newborn mice, and a thorough characterization of the mucosal and systemic immune responses was performed, including a detailed analysis of serum antibodies along with their functional capacity and association with protection.

RESULTS

Shigella IpaB and IpaD displayed on *L. lactis* BLP

IpaB and IpaD PGN anchor fusion proteins (PA) were successfully produced and attached to the *L. lactis* BLP. The SDS-PAGE analysis of the vaccine preparations revealed bands near the 87 KDa and 64 KDa expected for the IpaB-PA fusion protein loaded onto the BLP and IpaB alone, respectively (Figure 1a). Bands in the proximity of the theoretical sizes of 61 KDa and 38 KDa were seen for IpaD-PA loaded on the BLP and IpaD alone, respectively (Figure 1b). The IpaB and IpaD displayed on the BLP were recognized by specific monoclonal antibodies as shown by immunofluorescence images (Figure 1c); no signal was detected for the BLP alone.

In vivo distribution of *L. lactis* BLP

To assess the in vivo distribution of i.n. delivered BLP and identify mucosal tissues potentially involved in immunologic priming, fluorescently labeled BLP (fBLP) were administered to adult mice at a concentration equivalent to the highest dose used in the vaccine studies and following the same immunization procedures (described below). Mice that received unlabeled BLP served as negative controls. Whole body images were obtained every 2–4 h for 2 days. The fBLP were seen in the nasal cavity immediately after vaccination and most of the inoculum remained there for the next 2 h (Figure 2a and b); a small fraction (10%) was still detected in this compartment 4 h after inoculation but had completely disappeared at the 12 h time point. The majority of the particles reached the gastrointestinal tract (most likely due to swallowing reflex) 4 h after inoculation, with a fraction remaining for up to 24 h (Figure 2a and b). Although not evident from the whole body images, the fBLP also localized in the lung, as demonstrated by images of dissected tissue 24 h after inoculation (Figure 2a). Almost all of the fBLP had been cleared from the body 2 days after vaccination (Figure 2a and b).

Protective immunity induced by intranasal immunization with BLP-IpaB/D in adult mice

The immunogenicity and protective efficacy of the BLP-IpaB/D in comparison with IpaB/D (the proteins alone) was first examined in a dose-escalation experiment. Adult mice were immunized i.n. with increasing dosage levels of each of the vaccines on days 0, 14 and 28, as described in Materials and Methods and in the legend to Figure 3. Immunization with BLP-IpaB/D resulted in robust serum IgG responses to both IpaB and IpaD, which increased in a dose-dependent manner (Figure 3a). Mice that received the maximum (Max) dose of

BLP-IpaB/D (the largest amount that could be given in the 30-μl inoculum) were the first to respond; this group exhibited significant levels of IpaB and IpaD-specific IgG, above all other groups, after the first immunization. Mice that received the high, medium and low doses of BLP-IpaB/D responded only after the second vaccination. The influence of the vaccine dose in the magnitude of serum IgG responses was particularly noticeable at this time point (Figure 3a). Although titers increased and reached a plateau after the 3rd vaccination in all groups, the recipients of the BLP-IpaB/D Max dose remained the best responders throughout. The BLP-IpaB/D Max was also the only group that developed significant and sustained IpaB-specific IgA responses in stool (Figure 3b). Stool IgA responses against IpaD were also detected but did not follow a dose-response pattern. No antibodies were detected in serum or stool from BLP and PBS controls. Importantly, the IgG responses to IpaB and IpaD markedly improved when displayed on the BLP as compared to the proteins given alone. BLP-IpaB/D recipients achieved ~3 and 5 $\log_{10}$ higher IpaB and IpaD serum IgG titers, respectively, than those that received IpaB and IpaD alone. In fact only the group that received the max dose of IpaB and IpaD alone developed a response above the negative controls. Fecal IgA responses were absent following immunization with IpaB/D alone.

One month after vaccination all mice were subjected to a lethal pulmonary challenge with fully virulent *S. flexneri* strain 2a 2457T. Mice immunized with BLP-IpaB/D had the highest survival rates: 90% for those that received the maximum dose and 60% in the high and medium dose groups (Figure 3c). Protection was dramatically reduced when IpaB and IpaD were given alone: less than half (40%) of the mice that received the maximum dose survived and only 10% of those that received the high dose (statistically indistinguishable from the unvaccinated controls) survived. When challenged with wild-type *S. sonnei*, which together with *S. flexneri 2a* cause the majority of endemic pediatric shigellosis in the developing world, the BLP-IpaB/D Max dose afforded 80% protection; none of the other vaccine groups exhibited significant protection against this strain (Figure 3c). Negligent protection against *S. sonnei* and *S. flexneri* (20%) was seen occasionally in mice that received BLP alone, whereas all unvaccinated (PBS) controls succumbed to infection 6 to 8 days after challenge (Figures 3c and d). Results were consistent when different batches of vaccine were tested in separate experiments both in terms of kinetics of antibody production and BLP-immune enhancement (Figure 3d), as well as protective efficacy (Figure 3e).

IpaB- and IpaD antibodies and prediction of protection by titer and functional capacity

A detailed statistical analysis was performed to establish potential associations between vaccine-induced antibody responses (IgG, IgG1 and IgG2a in serum and IgA in stool) and protection against disease. First, survival rates post challenge were compared among mice immunized with BLP-IpaB/D or IpaB/D classified as responders vs. non-responders. Serum IgG titers 50 EU ml^{-1} and stool IgA titers 10 EU ml^{-1} were considered the threshold for a positive response (corresponding to 4 times the lower limits of detection, which was also 4 times the titer for all unvaccinated controls) (Table 1). The proportion of survivors was significantly higher among mice that had developed positive responses for IgG and IgG2a against both IpaB and IpaD in serum and IgA against IpaB in stool, compared to non-responders ($P<0.05$, two-sided Fisher exact test). Furthermore, there was a significant

association between survival and the magnitude of serum ELISA titers for each type of antibody measured (*P*<0.05, likelihood ratio test in logistic regression) (Table 1). Vaccinated mice that were protected from *S. flexneri* infection (i.e. healthy survivors that maintained at least 80% of body weight) had significantly higher serum IgG titers anti-IpaB and IpaD than those that succumbed to infection at the time of challenge (Figure 4a). A further analysis of vaccine-induced antibodies and probability of survival using logistic regression confirmed the association between IpaB- and IpaD-specific IgG and protection (*P*<0.001). The models indicated that an IpaB titer ~ 1×10^6 EU ml^{-1} predicted at least an 80% chance of survival (60% if the titer dropped to 1×10^5 EU ml^{-1}) and an IpaD titer ~ 1×10^5 EU ml^{-1} predicted at least 60% probability of survival (Figure 4b).

Given the observed correlation between protection against disease and vaccine-induced humoral responses, we investigated the functional capacity of IpaB and IpaD antibodies in facilitating bacterial uptake by phagocytic cells and preventing *Shigella*-induced cytotoxicity. Antibodies induced by BLP-IpaB/D and IpaB/D exhibited opsonophagocytic activity (OPA) seen by the increased uptake of serum-opsonized *S. flexneri* 2a 2457T by mouse macrophages, compared to a non-opsonized organism or organisms opsonized with non-immune serum (Figure 4c). The highest OPA responses were seen in the group immunized with BLP-IpaB/D (Figure 4c). These results prompted us to examine the IgG subclass profile induced by the two vaccine treatments to test the hypothesis that the higher OPA could be associated with an increased proportion of antibodies with higher binding capacity for phagocytic cells. Indeed, higher IgG2a levels were produced after immunization with BLP-IpaB/D as compared to IpaB/D alone (Figure 4d). Additionally, we measured the capacity of vaccine-induced antibodies to neutralize macrophage cytotoxicity in the presence of wild-type *Shigella*. Sera from mice immunized with BLP-IpaB/D and IpaB/D prevented *Shigella*-induced cell death compared to non-immune sera from mice that received PBS (Figure 4e).

Mucosal ASC, antibodies and innate immune mediators in bronchoalveolar fluid (BALf)

To further assess the mucosal responses induced by BLP-IpaB/D immunization, we measured the frequency of IpaB- and IpaD-specific ASC in the nasal-associated lymphoid tissue (NALT) and lung, as well as the levels of IpaB/D-specific IgG and IgA in BALf, one week after the 3rd and final immunization (Figures 5a and b). High numbers of IpaB-specific IgG ASC were detected in the NALT of BLP-IpaB/D vaccinated mice (Figure 5a). An even higher IpaB-specific ASC response was found in the lungs (Figure 5b). Likewise, IpaD-specific IgG ASC were detected in the lungs, albeit at much lower numbers. IpaB and IpaD-specific IgA ASC were induced in response to BLP-IpaB/D, but their magnitude was marginal compared to the IgG ASC. In all cases, the ASC responses in the BLP-IpaB/D group surpassed those of mice that received IpaB/D alone. Elevated IpaB- and IpaD-specific IgG titers were found in BALf, and, similar to the ASC, there was a trend of higher responses in the BLP-IpaB/D recipients compared to those immunized with IpaB/D alone (Figure 5c). No specific antibodies or ASC were detected in the BLP and PBS controls. A summary of serum and mucosal antibody responses in all groups and the corresponding protection levels is given in Table 2. From a large panel of cytokines measured by microarrays (IFN-γ, IL-10, IL-12, IL-2, IL-4, IL-5, KC, and TNF-α) IL-1β, IL-12 and the

neutrophil-attracting chemokine, keratinocyte chemoattractant (KC), were found to be elevated in the BALf of vaccinated mice (Figure 5d). The highest levels were found in the group immunized with BLP-IpaB/D, but positive responses (above the unvaccinated PBS controls) were also found in the IpaB/D and BLP groups (Figure 5d).

Systemic ASC responses and bone marrow adoptive transfer protection

We also examined the presence of antigen-specific ASC in the spleen and in the bone marrow (BM) as potential sources of circulating antibodies and long-lived plasma cells, respectively. IpaB- and IpaD-specific IgG and IgA ASC were found in both tissues mainly in response to BLP-IpaB/D vaccination, whereas no ASCs were detected after vaccination with IpaB/D alone (Figures 6a and b). In most instances, the levels of IgG ASC surpassed those of IgA ASC. BM cells from BLP-IpaB/D recipients adoptively transferred to naïve mice conferred moderate, yet significant protection against lethal *Shigella* infection; 40% of the BM recipients survived the pulmonary challenge (Figure 6c).

Antibody responses and protective efficacy of BLP-IpaB/D in newborn mice

Considering that infants and toddlers would be the main targets of a *Shigella* vaccine and that immune responses may be influenced by age and immune development, we examined the protective capacity of BLP-IpaB/IpaD in mice immunized as newborns and infants. Seven-day-old mice were administered BLP-IpaB/D or IpaB/D via the nasal route and identical booster doses were given on days 14, 21 and 28 after birth. A series of preliminary studies was performed to determine optimal doses and number of booster immunizations needed to maximize protection. Because the serum IgG anti-IpaD levels were consistently low, the amount of IpaD was increased to the highest that could be included in the vaccine inoculum (24 μg in 10 μl). A three-dose regimen with BLP-IpaB/D, which was highly successful in adults, provided only ~40% protection in mice immunized as newborns (data not shown) and hence a fourth dose was added after weaning (day 28). Immunization with BLP-IpaB/D during the neonatal/infant period resulted in significant levels of IpaB- and IpaD-specific serum IgG. As observed in adult mice, antibody responses markedly increased when the Ipas were displayed on the BLP. Immunization with BLP-IpaB/D elicited serum IgG responses against both IpaB and IpaD, whereas lower IpaB- and barely significant (and delayed) IpaD-specific IgG was produced in response to IpaB/D alone (Figure 7a). Newborn/infant immunization with BLP-IpaB/D afforded 90% protection against *S. flexneri* 2a and 44% against *S. sonnei*, which in both cases was significant above the unvaccinated controls (Figure 7b). Protection was greatly reduced upon immunization with IpaB/D alone; only 30% of these mice survived *S. flexneri* challenge and only 11% survived infection with *S. sonnei*.

DISCUSSION

The recent estimates of moderate-to-severe diarrhea attributable to *Shigella* spp. in children living in Asia and sub-Saharan Africa[1] re-emphasize the urgent need for effective preventive strategies to reduce mortality rates and alleviate an overall disease burden that results in long-lasting health impairment. Herein, we provide the first report of successful immunization of adult and newborn/infant mice with *L. lactis* BLP displaying *Shigella* IpaB

and IpaD. The vaccine was well tolerated when administered via the nasal route as early as one week after birth and induced robust immune responses. Most importantly, it was highly protective against lethal challenge with *S. flexneri* 2a and *S. sonnei* 53G, the two most prevalent endemic serotypes in the developing world as reported by the GEMS[18] and previous large-scale studies.[19,20] Immunogenicity and protection were dose-dependent and consistent in multiple experiments using different vaccine lots. The safety and suitability of the BLP-IpaB/D vaccine for mucosal delivery and its capacity to induce strong cross-protective immunity in young hosts make it unique and appealing for the immunization of toddlers and young children, who would benefit the most from a practical and effective prophylactic approach.

The BLP exhibited distinct adjuvant properties and contributed to the protective immunity achieved. Display of IpaB and IpaD on the *L. lactis* particles significantly increased the magnitude of systemic and mucosal antibody and ASC responses, innate immune mediators in mucosal secretions and importantly, the survival rate post challenge. It is noteworthy that robust serum IgG responses to IpaD, typically the less immunogenic of the two proteins, were only elicited when it was displayed on the BLP. Similarly, stool IgA was detected only in mice that received BLP-IpaB/D, not in the IpaB/D recipients. This immune enhancement could be explained by a more efficient B cell activation and antigen delivery to APC, and/or by the inherent adjuvant properties of the BLP.[21,22] Displayed on the BLP surface, the proteins are less likely to suffer degradation and, due to the microbial size and surface charge of the particles, they are more easily captured by activated APC than soluble proteins alone.[10,23] We have shown that the *L. lactis* BLP bind TLR2 in vitro and activate adult and neonatal dendritic cells (from both mice and humans), enhance their maturation and capacity for antigen presentation and stimulate the production of pro-inflammatory cytokines including TNF-α and IL-6.[10] Similarly, the interaction of BLP with TLR2 in vivo has been associated with the induction of mucosal IgA responses, as well as splenic IgG ASC and IFN-γ-secreting T cells following co-administration of a split influenza virus vaccine.[11]

The immunological effectors and the precise mechanisms by which IpaB and IpaD mediate protection against *Shigella* lethal pulmonary infection in the mouse model are not fully understood. Presumably, antibodies produced locally and/or transudated from circulation impede bacterial-host cell contact (and inhibit translocation of virulence factors) at the mucosal epithelial interface, thereby preventing tissue damage and bacterial spread.[4,6] Antibodies may also facilitate uptake of organisms by phagocytic cells. A detailed statistical analysis of BLP-IpaB/D and IpaB/D-induced antibodies and rates of survival from multiple experiments revealed a categorical association between a serum antibody response to each of the proteins and protection against lethal experimental challenge. Survival was also associated with the magnitude of serum IgG (and IgG2a) ELISA titers; the stronger the antibody response, the higher the likelihood of vaccinated mice withstanding the lethal infection, with threshold serum IpaB- and IpaD-specific IgG titers of 1×10^5 EU ml^{-1} predicting at least 60% survival.

A new and critical observation was the capacity of IpaB and IpaD antibodies generated through vaccination to reduce *Shigella*-induced macrophage cytotoxicity and facilitate their uptake of bacteria through opsonophagocytosis. These functional properties are important to

prevent tissue damage and further microbial spread during *Shigella* infection, and might represent mechanisms of host defense in vivo. Antibodies generated by the BLP-IpaB/D vaccine elicited higher OPA activity (P<0.01), which could be explained by the larger proportion of IgG2a induced, compared to the Ipas alone. Unlike IgG1, IgG2a binds the FcγRI receptor on mouse macrophages and phagocytic cells[24] with high affinity and is therefore more efficient at activating these cells and promoting microbial clearance. In line with these results, we and others had shown that the BLP promote more balanced Th1/Th2 responses to the carried antigens compared with the same antigens given alone or admixed with alum, which generate a distinct Th2-biased IgG1 response[10,17] that is unsuited for the elimination of intracellular pathogens, particularly in the Th2-prevailing environment early in life.[25]

Among the systemic effectors induced by BLP-IpaB/D immunization, it is worth mentioning the IpaB and IpaD-specific BM ASC (representing long-lived plasma cells or their plasmablasts precursors) that partly protected naïve hosts, as this suggests the potential for this vaccine to elicit effectors that can mediate long-lasting protective immunity. In addition, the BLP-IpaB/D generated potent mucosal immune responses largely represented by IpaB and IpaD-specific IgG ASC in the NALT and lungs, and high levels of IpaB and IpaD-specific IgG in BALf. Some of the antibodies detected in the BALf were absent in serum (i.e., IgA and IpaD-specific IgG in IpaB/D recipients), which suggests that vaccine-induced local ASC produce some of the mucosal antibodies detected. An interesting observation was the predominance of IgG responses in mucosal tissues (NALT and lung ASC, and BALf) as opposed to IgA, which was still sufficient to confer high levels of protection. In humans, serum IgG specific for *Shigella* LPS has been associated with serotype-specific protection [26–30] and it has been proposed that these antibodies transudate the mucosal epithelial barrier and neutralize and/or kill the organisms in the gut lumen.[31] The relative contribution of serum and mucosal antibodies in protection against shigellosis and the mechanisms involved remain to be elucidated.

Another relevant observation was the presence of innate immune mediators (IL-12, IL-1β and mKC) in lung secretions from BLP-IpaB/D recipients. IL-12 plays a major role in host defense against intracellular pathogens by activating macrophages and inducing IFN-γ production. Likewise, KC (CXCL1), a chemokine produced by macrophages and neutrophils (via TLR2 activation pathway), recruits and activates phagocytic cells. *Shigella* IpaB has been attributed with initiating the inflammatory response through caspase-1, by promoting secretion of IL-1β.[32] In a concerted manner, these molecules can facilitate bacterial clearance synergizing with host adaptive immune effectors. This is, to the best of our knowledge, the first demonstration of these cytokines/chemokines being present in mucosal secretions following immunization with a *Shigella* or BLP vaccine.

In an attempt to identify the tissues involved in vaccine uptake, we tracked the in vivo distribution of i.n. delivered fBLP. The particles localized in the NALT the first few hours after inoculation and subsequently in the gut; they were also found in the lungs for up to at least 24 hours. Antigen-specific ASC were detected in NALT and lung, and it is therefore reasonable to assume their involvement in immunological priming. The 30 μl inoculum volume administered is consistent with particles spreading from the nasal cavity into the

lungs, as we have observed in mice given live bacterial vaccines[33]. Others have similarly reported >10 μl intranasal instillations dispersing into the bronchial region as well as the gastrointestinal tract.[34] However, in humans, delivery of a BLP vaccine would be directed to the nasal cavity targeting the NALT as the primary inductive site. The fBLP presumably reached the gut through the swallowing reflex and this likely represents the route excretion. However, some immune activation might have occurred there as well, as suggested by the presence of IgA in stool, while completely absent in circulation. Since *Shigella* is an enteric pathogen, immunological priming in the gut and induction of local (intestinal) immunity would be an advantageous feature of any new vaccine. We had previously reported that oral delivery of IpaB and IpaD resulted in lower protective efficacy than intranasal vaccination[4] and therefore this route was not pursued in the BLP-IpaB/D studies. Since experimental infection of mice with *Shigella* involves nasal/pulmonary exposure (as opposed to enteric in humans), oral vaccination adds a confounding factor to the proper evaluation of vaccine candidates in this model.

An additional important benefit of the BLP technology is that it allows for successful mucosal (even intranasal) immunization, without the need for potentially reactogenic adjuvants. In previous work from our group, IpaB/D delivered i.n., admixed with a double mutant of the *Escherichia coli* heat labile toxin (dmLT), afforded similar protection to that described here for the BLP-IpaB/D, using a smaller dose. However, due to safety concerns, an enterotoxin-derived adjuvant would not be suitable for use in humans.[35] Parenteral immunization with the IpaB/D plus dmLT was likewise effective in mice[6] and potentially suitable for use in children; a drawback is that it would still require special tools (i.e. microneedles) and might not be as practical as mucosal delivery. The BLP were well tolerated when given to 15 human adults via the nasal route (1.25 mg of BLP in 250 μl) mixed with a commercial influenza vaccine.[21] If equally safe in children, this route might provide an alternative to oral vaccination, which is known to yield less than desired immune responses to routine vaccines in developing countries.[36] Supportive of this concept are the results of Mallet et al[37] and Orr et al[38], whereby intranasal immunization with a proteosome-based *S. sonnei* and *S. flexneri* vaccine induced homologous protection in mice and in the guinea pig keratoconjunctivitis model, respectively. While none of these models accurately represent the course of *Shigella* infection in humans, these results confirm the potential of a mucosal/ intranasal subunit vaccine approach.

Pre-clinical studies of *Shigella* vaccine candidates have traditionally involved immunization of adult hosts. Two recent reports investigated protection of young animals through passive protection from maternally derived antibodies.[39,40] Gnobiotic piglets have been used to evaluate live vaccine strains.[41] Ours is, to the best of our knowledge, the first demonstration of substantial cross protective immunity in mice immunized as newborns with a protein-based vaccine. Although the protection against *S. sonnei* was lower than anticipated, it could be improved through optimization of vaccine dose and regimen; it may also reflect a more stringent challenge procedure.

In conclusion, the BLP-IpaB/D vaccine is a promising candidate for immunization of young children against shigellosis. Advantages of this vaccine over other approaches include its safety, suitability for mucosal delivery (which makes it a practical for large scale use),

strong immunogenicity and broad protective capacity. Our results warrant further assessment of this concept in humans.

METHODS

Preparation of vaccines and antigens

Recombinant IpaB and IpaD were purified by affinity and size exclusion chromatography as previously described.[5,42] To be loaded onto the *L. lactis* BLP particles (formerly called Gram Enhancement Matrix particles), each protein was expressed as a fusion containing the peptidoglycan anchoring (PA) domain (IpaB-PA and IpaD-PA). The plasmid construction, expression and purification of IpaD-PA has been described elsewhere.[43] IpaB-PA was produced by inserting the DNA sequence for PA 5′ to the *ipaB* sequence in pACYC-Duet as previously described.[44] The purification of IpaB-PA was the same as for IpaB. *L. lactis* BLP were produced by boiling *L. lactis* whole cells in 10% trichloroacetic acid for 30 min, followed by extensive washing in PBS, as previously described.[8] One mg of BLP contains approximately 8×10^9 particles. To produce BLP-IpaD, *L. lactis* cell-free culture supernatants containing IpaD-PA and BLP were mixed under gentle rotation for 30 min at room temperature as described elsewhere.[8] To produce BLP-IpaB, BLP were incubated with purified IpaB-PA for 30 min with gentle rocking at room temperature. Removal of excess IpaB or IpaD-PA was achieved by three sequential centrifugation of the particles, removal of the supernatants, and resuspension. The resulting vaccine preparations BLP-IpaB and BLP-IpaD (along with IpaB and IpaD alone) were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS PAGE). Samples were diluted 1:2 in Laemmli sample buffer® (BioRad, Hercules, CA) containing 5% 2β-Mercaptoethanol, boiled at 95°C for 10 min and loaded (1 μg/well) in a 4–12% pre-casted Mini-PROTEAN TGX Gel® (BioRad, Hercules, CA). The separated proteins were detected by Coomassie staining (EZ blue™) (Sigma, St. Louis, MO). The amount of IpaB-PA and IpaD-PA bound to the BLP was determined by SDS PAGE using IpaB-PA or bovine serum albumin as standard, respectively. The BLP-IpaB/D and IpaB/D vaccine components were stored at −80°C until use.

The BLP-IpaB and BLP-IpaD were further analyzed by dual color immunofluorescence. Briefly, suspensions containing 100 μl of BLP-IpaB and BLP-IpaD were washed with ultrapure water (Milli-Q, Millipore, Billerica, MA), resuspended in PBS containing 2% Bovine serum albumin (BSA, Sigma, St. Louis, MO) and incubated for one hour at room temperature with biotinylated mouse anti-IpaB or anti-IpaD monoclonal antibodies (a gift from Dr. Ed Oaks). After washing, BLP-IpaD samples were incubated with anti-mouse Alexa Fluor® 488 (Molecular Probes, Grand Island, NY) and BLP-IpaB samples with Streptavidin Alexa Fluor® 598 (Molecular Probes, Grand Island, NY); 1:50 and 1:200, dilutions, respectively, in PBS containing 2% BSA for one hour at room temperature. After washing, samples were resuspended in 100 μl of ultrapure water, and a 10-μl sample was air dried onto a microscope slide and applied ProLong® Gold antifade reagent (Life Technologies, Eugene, OR) along with a coverslip. Images were acquired at 100× magnification on a Zeiss Axio Imager Z.1 fluorescence microscope using ZEN Imaging Software (Carl Zeiss Microscopy, Oberkochen, Germany).

In vivo tracking of *L. lactis* BLP

The BLP particles were labeled with Alexa Fluor® 790 Succinimidyl Esters (NHS esters) purchased from Molecular Probes, Grand Island, NY, through chemical binding to non-protonated aliphatic amine groups on the particle surface. Briefly, the BLP (15 mg) were washed and resuspended in 0.1M NaHC0$_3$ pH 8.3, and incubated with 1 μg of Alexa Fluor® 790 for 1 h at 4°C in the dark and with agitation. Adequate labeling (>80%) was confirmed by flow cytometry. The fluorescent BLP (fBLP) were washed three times in PBS and resuspended to a final concentration of 10 mg ml^{-1} in PBS containing 0.01% thimerosal (Sigma-Aldrich, St. Louis, MO). Adult female Balb/c mice (Charles River Laboratories, Wilmington, MA) were placed in an anesthesia induction chamber (Piramal Healthcare, Mumbai, Maharashtra, India) dispensing Isoflurane (Fluriso™, VETone®, Boise, ID) 2.5% and, once fully anesthetized, 30 μl of fBLP (0.3mg) were administered i.n. The mice were then moved to the imaging chamber of a Xenogen IVIS-200 system (Perkin Elmer, Waltham, MA) and placed lying on their backs on a heated shelf surface to maintain body temperature. Isoflurane (0.5%) was administered through nose cones during imaging. Pictures were taken and analyzed using Living Image® Software Version 4.3.1 (Perkin Elmer), creating gates or Regions of Interest (ROIs). The fluorescent intensity (Epi-fluorescence) of the fBLP was measured as radiant efficiency using the flowing equation: $\left[\left(\text{photons} \, \text{s}^{-1} \, \text{cm}^{-2} \, \text{steradian}^{-1} \right) \text{per} \left(\mu \text{W} \, \text{cm}^{-2} \right) \right]$. The background was defined as the ROI (nasal tissue, lungs or intestine) of mice that received unlabeled BLP.

Immunization, cell adoptive transfer and experimental infection

Female Balb/c adult mice (7–8 weeks old) were immunized i.n. on days 0, 14 and 28 with increasing doses of BLP-IpaB and BLP-IpaD or IpaB and IpaD (alone): 2.5 μg IpaB and 10 μg IpaD, referred to as BLP-IpaB/D-Low; 5 μg IpaB and 20 μg IpaD (BLP-IpaB/D-Med); 10 μg IpaB and 40 μg IpaD (BLP-IpaB/D-High), and 20 μg of IpaB and 53 μg of IpaD (BLP-IpaB/D-Max). The amount of BLP given with each vaccine was consistently 300 μg. The Max dose was the largest amount of BLP-IpaB/D that could be given in the 30 μl inoculum volume. Other groups received equivalent doses of IpaB and IpaD (without BLP), also referred to as IpaB/D-Low, IpaB/D-Med, IpaB/D-High and IpaB/D-Max, respectively. Newborn (7-days old) mice received 2 μg IpaB and 24 μg IpaD alone or displayed onto the BLP (in a 10 μl volume), on days 7, 14, 21 and 28 after birth. In both experiments, unvaccinated controls received BLP alone or PBS. The vaccine inoculum was dispensed half into each nare, with a pipette and under Isoflurane anesthesia, as previously described.[4] Serum and fecal samples were obtained from individual mice before and every two weeks after vaccination. Bronchoalveolar lavage fluid, NALT, spleen and BM cells were collected from subgroups of mice at days 35 or 69 as described before.[4,5] For adoptive transfer experiments, BM cells obtained on day 69 post-vaccination were injected i.v. to adult mice; 6×10^7 cells in 100 μl of PBS were administered to each recipient via the tail vein. One month after the last immunization (day 56) or 48 h after cell adoptive transfer, adult mice were challenged with 6×10^7 CFU virulent *S. flexneri* 2a 2457T (corresponding to ~11 MLD$_{50}$) or with 1.3×10^8 CFU of *S. sonnei* 53G (corresponding to ~6 MLD$_{50}$), as previously described.[4,5] Newborn mice were challenged with 3×10^7 CFU of *S. flexneri* 2a 2457T (corresponding to ~3 MLD$_{50}$) or with 9.35×10^7 CFU of *S. sonnei* 53G. All animal studies

and procedures were approved by the University of Maryland Institutional Animal Care and Use Committee.

Antibody measurements

(i) ELISA IpaB- and IpaD- specific antibodies were measured by ELISA as previously described. [4,5] **(ii) Opsonophagocytosis.** A *Shigella* opsonophagocytic assay was developed following previously described format[45], with modifications. Briefly, J774A.1 mouse macrophages were seeded into 24-well plates ($2{\times}10^5$ cells per well) and grown in Dulbecco's modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% Defined Fetal Bovine Serum (GE Healthcare HyClone, Logan, UT) at 37°C, 5% CO_2, until they reached 90% confluence. *S. flexneri* 2a wild type strain 2457T was grown overnight at 37°C in Tryptic Soy Agar (TSA) (Difco, Becton Dickinson Diagnostics, Sparks, MD) supplemented with 0.02% Congo Red (Sigma-Aldrich), and isolated colonies were sub-cultured in Animal-Product-Free Luria Bertani broth (Lennox) (Athena Enzyme Systems, Baltimore, MD) and grown at 37°C until reaching an optical density measured at a 600 nm wavelength (OD_{600}) of 0.2. Ninety μl bacterial suspension containing ~ 2–$3{\times}10^5$ CFU were incubated with 10 μl of individual serum samples (previously inactivated for 20 min at 56°C) for 20 min at room temperature, in constant rotation. A 10 μl aliquot of opsonized organisms (2–$3{\times}10^4$ CFU) was added to the cell monolayers (in duplicate wells), centrifuged (10 min, $1{,}000 \times g$) and incubated for 45 min. After washing with PBS, 500 μl of fresh medium containing 100 μg ml^{-1} of gentamicin (Gibco) was added for 1 h to remove external organisms. Cells were washed again, lysed with 500μl of PBS containing 0.5% Triton X-100 (Sigma-Aldrich) and supernatants (25 μl aliquots, in triplicate) were cultured overnight at 37°C in TSA-Congo Red to determine viable counts. The percent of phagocytosis was calculated as the ratio of CFU recovered/CFU added to the wells $\times$ 100. **(iii) Neutralization of macrophage cytotoxicity.** *Shigella* is known to induce apoptosis of macrophages.[46] To determine whether serum from immunized animals could prevent macrophage cytotoxicity upon exposure to wild-type organisms, we established a cytotoxicity neutralization assay based on methods previously described.[47–49] A 30 μl suspension of *S. flexneri* 2a strain 2457T, prepared as described above and containing $2{\times}10^5$ CFU, was incubated with 10 μl of mouse serum in 96-wells round bottom plates (in duplicate) for 20 min at room temperature. The content of each well was then transferred to a 96-well plate seeded with J774A.1 macrophages ($3{\times}10^3$ per well, moi: 60) and incubated for 18 h at 37°C, 5% CO2. The plate was centrifuged and lactate dehydrogenase (LDH) release (an indicator of cytotoxicity) was measured in 50 μl of culture supernatants using a colorimetric assay (CytoTox 96, Promega, Madison, WI). Absorbances values at 490 nm were measured using a Multiskan ELISA reader (Thermo Scientific). Cytotoxicity (cell death) was determined as: (experimental LDH release–spontaneous LDH release)/ (maximum LDH release–spontaneous LDH release), where spontaneous LDH release was the LDH activity in supernatants from cells incubated in medium alone and total LDH release the activity in lysed macrophages. Percent neutralization was calculated as 1-cell death $\times$100.

ASC and cytokines

IgA and IgG ASC were measured by ELISpot as previously described.[4,5] Cytokines were measured in alveolar lavages or in culture supernatants from cells stimulated with IpaB or IpaD, using a MSD® Mouse Th1/Th2 Multi-array® tissue culture kit (Meso Scale Discovery, Rockville, MD), as previously described.[4,6]

Statistical analysis

Overall differences over time in % fluorescence and antibody responses (in serum and stool) were assessed by multivariate analysis of variance (MANOVA), using the different each time points measured as variables. Comparisons between vaccinated groups at specific time points were made using one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons test. Survival for serum IgG responders (titers 50 ELISA Units ml^{-1}) and non-responders (<50 ELISA Units ml^{-1}) and stool IgA responders (titers 10 ELISA Units ml−1) and non-responders (<10 ELISA Units ml^{-1}) were analyzed by a Fisher exact test, with the two-sided p-value calculated as twice the one-sided p-value in the direction of the observed difference. Associations between probability of survival and serum IgG titers were evaluated using logistic regression models. Survival after challenge was assessed by Kaplan-Meier curves and log-rank tests. Two-sided P 0.05 was considered statistically significant. GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA), SAS version 9.3 (SAS Institute, Cary, NC) and NCSS 8 (Number Cruncher Statistical Systems, Kaysville, UT) were used for statistical analyses.

Acknowledgments

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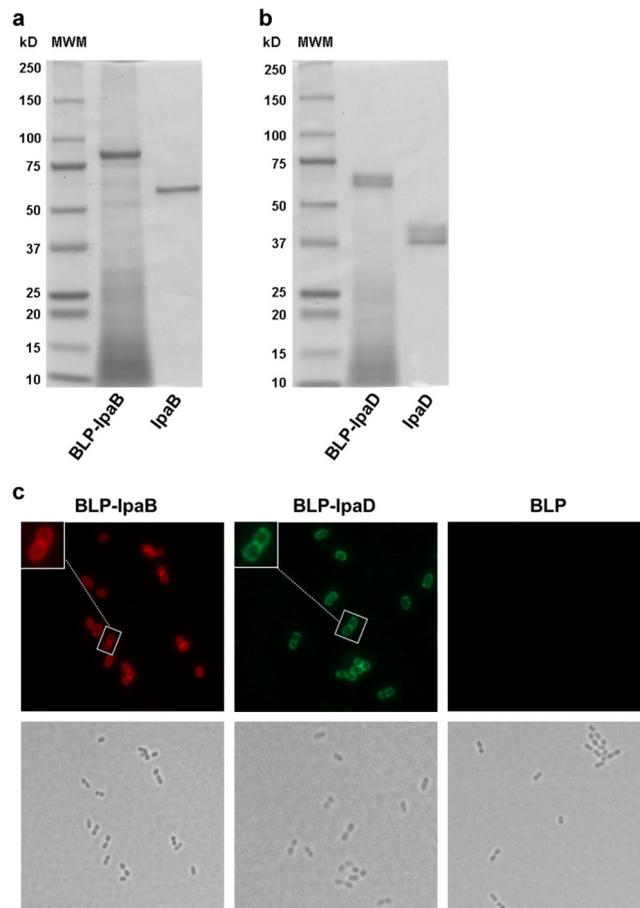
Figure 1. Analysis of BLP-IpaB/D by SDS-PAGE and fluorescence microscopy
(**a**) SDS PAGE analysis of BLP displaying IpaB (BLP-IpaB) or (**b**) IpaD (BLP-IpaD). The size of molecular weight markers (in kDa) is shown on the left (MWM). Proteins were stained with Coomassie blue. (**c**) Immunofluorescence (top) and bright field microscopy images (bottom) of BLP-IpaB, BLP-IpaD and BLP alone. BLP display of IpaB and IpaD was revealed by incubation with specific monoclonal antibodies: biotin labeled anti-IpaB followed by Streptavidin-Alexa Fluor® 594 (red) and unlabeled anti-IpaD followed by anti-mouse Alexa Fluor® 488 (green).
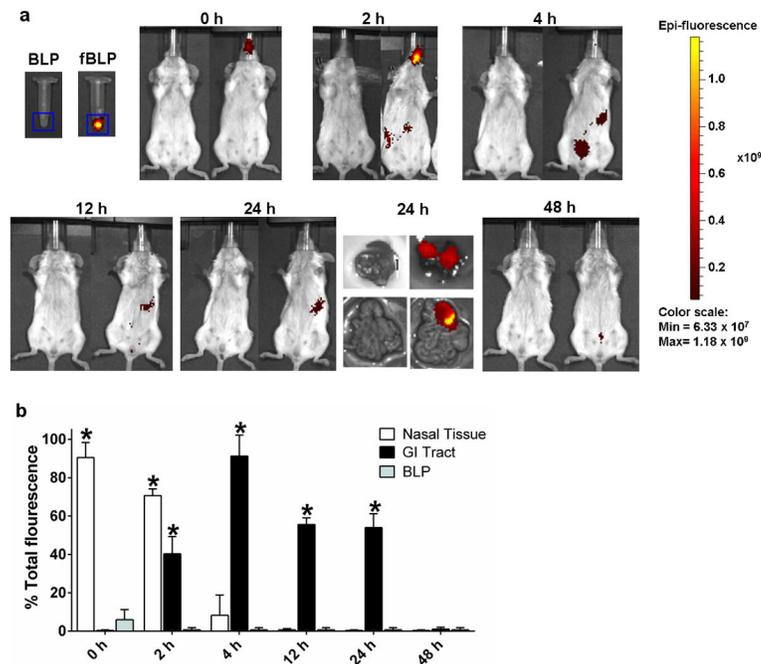
Figure 2. In vivo imaging of *L. lactis* BLP distribution
(**a**) Unlabeled and fluorescent BLP (fBLP) were administered i.n. to adult mice in a 30 μl volume, under anesthesia, and whole body images were obtained at the indicated time points using a Xenogen IVIS-200 imagining system. (**b**) Fluorescence distribution among different organs. Each bar represents mean % of total fluorescence+ SEM from 3 mice per time point; total fluorescence was the radiant efficiency of fBLP in the 30-μl inoculum. Significant overall differences were determined by multivariate analysis of variance (MANOVA), with % fluorescence at each time point as the variable. Comparisons between fBLP and BLP were then made at each individual time point using one-way ANOVA with Dunnett's comparison to a common control (BLP alone); * denotes statistical significance $P<0.05$ compared to unlabeled BLP.
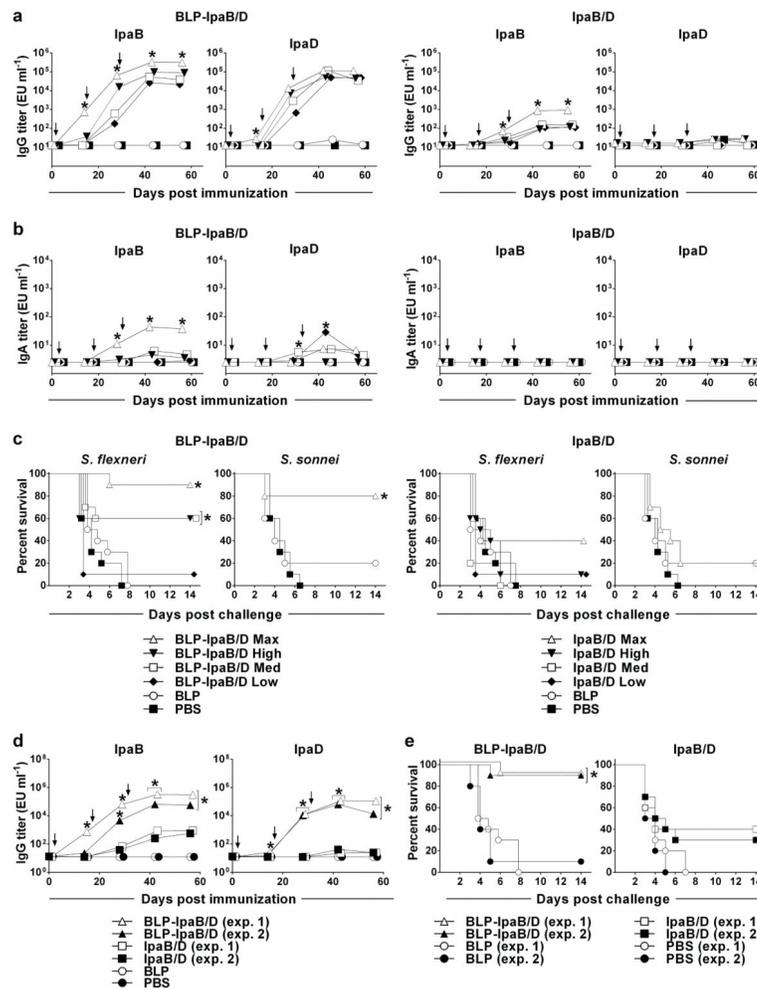
Figure 3. Serum IgG, stool IgA and protective efficacy of BLP-IpaB/D and IpaB/D in adult mice
Adult mice were immunized i.n. with increasing dosage levels (maximum, high, medium
and low) of BLP-IpaB/D or IpaB/D alone: BLP carrying 20 μg IpaB and 53 μg IpaD (BLP-
IpaB/D-Max), 10 μg IpaB and 40 μg IpaD (BLP-IpaB/D-High), 5 μg IpaB and 20 μg IpaD
(BLP-IpaB/D-Med), and 2.5 μg of IpaB and 10 μg of IpaD (BLP-IpaB/D-Low). The same
amounts of IpaB and IpaD were given alone. (**a**) Kinetics of IpaB and IpaD serum IgG (**b**)
and stool IgA titers measured by ELISA. Each data point represents the geometric mean titer
in $\log_{10}$ of at least 10 mice per group ± SEM. (**c**) Mice were challenged with wild-type *S.
flexneri* 2a 2457T or *S. sonnei* 53G 28 days after the third immunization; the curves
represent % survival post-challenge for 10 mice per group. (**d**) Serum IgG titers and (**e**)
protection following *S. flexneri* challenge in mice immunized with BLP-IpaB/D-Max or
IpaB/D alone in 2 separate experiments. Significant overall differences in $\log_{10}$ antibody
titers were determined by MANOVA. Significant differences in titers between all vaccinated
groups were analyzed by ANOVA with Tukey-Kramer multiple comparison test to compare
all groups. * *P*<0.05 for mean antibody titers significantly higher compared to all other
groups (panels a and b) and compared to Ipas alone and controls (panel d). Significant

differences in survival curves were determined by log-rank test; * $P<0.05$ compared to PBS and BLP.
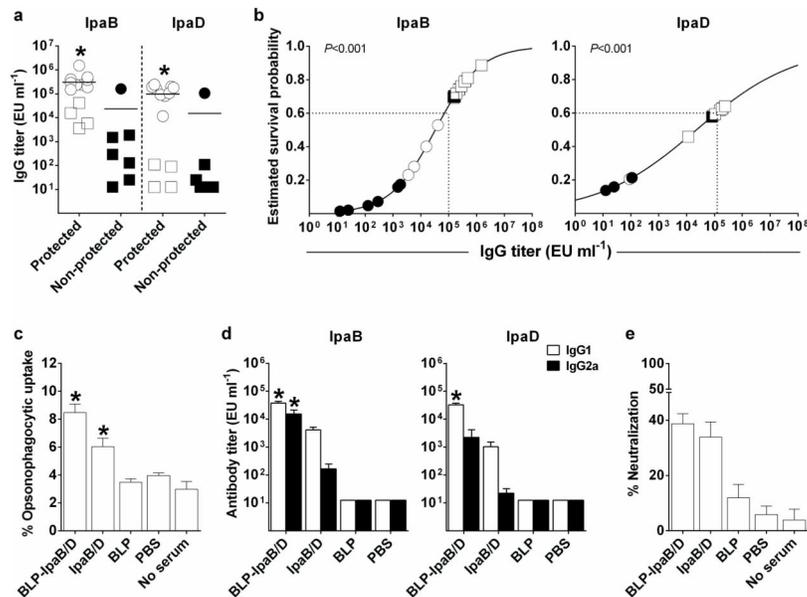
Figure 4. Antibodies and protection analysis, functional activity (opsonophagocytosis and neutralization of cytotoxicity) and IgG subclasses

Mice were immunized with BLP-IpaB/D or IpaB/D Max (20 μg IpaB and 53 μg IpaD), as described in Figure 3. (**a**) Antibody titers measured before challenge and ranked according to survival outcome; circles denote mice immunized with BLP-IpaB/D, and squares mice immunized with IpaB/D. Higher levels of IpaB or IpaD-specific serum IgG were associated with protection (survival) by logistic regression on $\log_{10}$-transformed IpaB or IpaD titers; * denotes $P < 0.001$ by likelihood ratio test in both analyses. (**b**) Estimated survival probability by logistic regression on log-transformed IpaB or IpaD IgG titers (same analysis as for panel a); mice immunized with BLP-IpaB/D-Max and IpaB/D-Max were included and depicted as circles and square, respectively. Filled and solid circles represent surviving and non-surviving mice, respectively. Dashed lines indicate level of antibodies predicting >60% survival. (**c**) Opsonophagocytic activity of serum antibodies from vaccine and control groups. Results are shown as mean % opsonophagocytic uptake of *S. flexneri* 2a 2457T in individual serum samples. (**d**) IpaB- and IpaD-specific IgG1 and IgG2a subclasses determined by ELISA; data represent mean titers from 20 mice per group. (**e**) Neutralization of macrophage cytotoxicity by serum from different treatment groups. Results are shown as mean % neutralization from replicate wells by pooled serum samples. In panels c and d, individual measurements from vaccine and control groups were compared by one-way ANOVA with Dunnett's multiple comparisons test against a common control; the tests on IpaB- and Ipa-D-specific subclasses were done using $\log_{10}$-transformed antibody titers. * denotes $P < 0.05$ compared to non-serum or PBS for OPA and to PBS for subclasses.
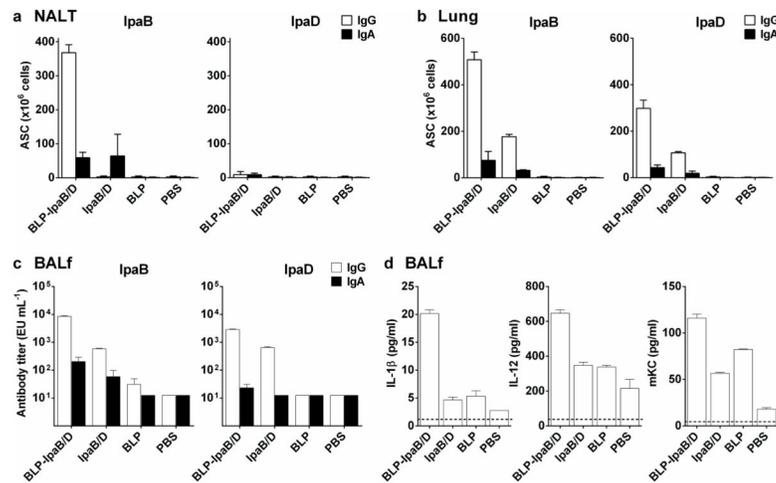
Figure 5. Mucosal IgA and IgG secreting cells, antibodies and cytokines following BLP-IpaB/D and IpaB/D vaccination

Mice were immunized with BLP-IpaB/D or IpaB/D Max dose as described in Figure 3. IgA and IgG ASC were measured by ELISpot in the (**a**) NALT and (**b**) lung tissue 35 days after the first immunization. Data correspond to mean ASC counts per 10^6 cells measured in pooled cell suspensions from 5 mice in each group + SEM from triplicate wells. (**c**) IpaB- and IpaD-specific IgG and IgA were measured by ELISA in bronchoalveolar lavage fluid (BALf) 35 days after immunization; data represent mean of individual titers from 5 mice per group (**d**). Cytokines measured in BALf by MSD multiarray technology; data represent mean concentrations as (pg ml−1) + SEM from triplicate wells.
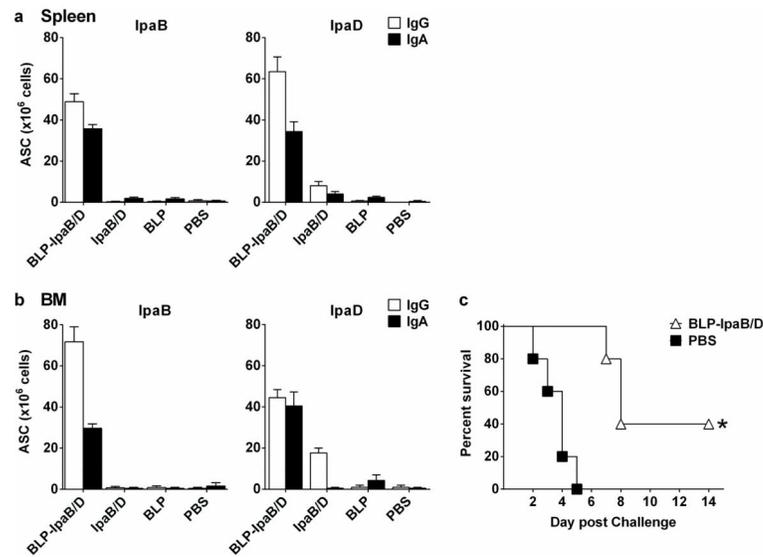
Figure 6. Systemic IgG and IgA ASC and induced by the BLP-IpaB/D and IpaB/D and protection by bone marrow adoptive transfer

Mice were immunized with BLP-IpaB/D or IpaB/D-Max as described in Figure 3. (**a**) IgA and IgG ASC measured by ELISpot in spleen and (**b**) bone marrow (BM) on day 69 after primary immunization. Data correspond to mean ASC counts per 10^6 cells from pooled cell suspensions from 5 mice in each group + SEM from triplicate wells. (**c**) Bone marrow cells from BLP-IpaB/D or PBS recipients (obtained 69 days after primary vaccination) were adoptively transferred to naïve mice that were challenged 48 h later with wild type *S. flexneri* 2a 2457T. Survival curves were compared by log-rank test; * $P<0.05$ compared to PBS.
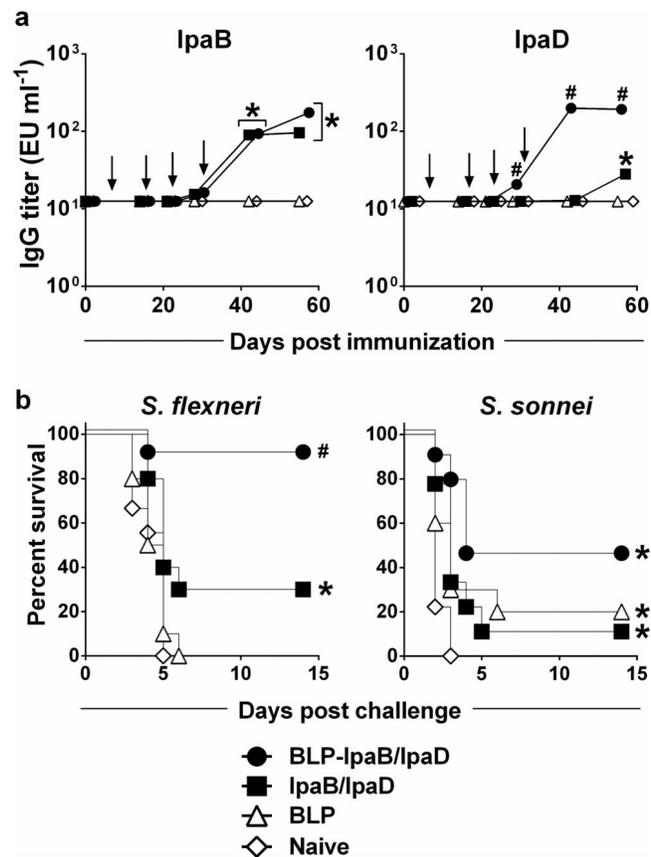
Figure 7. Serum IgG levels and protective efficacy of BLP-IpaB/D and IpaB/D in newborn mice
Mice were immunized as newborns and infants with BLP carrying 2 μg IpaB and 24 μg
IpaD or with the same amounts of IpaB and IpaD given alone. Control groups received BLP
alone or remained naïve. Vaccines were given on days 7, 14, 21 and 28 after birth (arrows).
(**a**) IpaB- and IpaD-specific IgG measured by ELISA; data represent geometric mean titers
from 12 mice per group. Significant overall differences in $\log_{10}$ antibody titers were
determined by MANOVA. Significant differences in titers between all vaccinated groups
were analyzed by ANOVA with Tukey-Kramer multiple comparison test to compare all
groups. * $P<0.05$ for mean antibody titers significantly higher compared to BLP and PBS
control groups. # $P<0.05$ for mean antibody titers significantly higher compared to all
groups. (**b**) Protection against lethal pulmonary infection with wild type *S. flexneri* 2a 2457T
or *S. sonnei* 53G (n=12). Survival curves were compared by log-rank test. * $P<0.05$
compared to naïve mice. # $P<0.05$ compared to all groups.

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Table 1

Association between vaccine-induced antibodies and survival rates post-challenge

	No. mice	Survival vs. antibody responses[a]			Survival and ELISA titers[a]		
		Survival rates among responders	Survival rates among non-responders	p[b]	Odds ratio[c]	95% CI[d]	p[e]
IpaB total IgG	80	27/65	1/15	0.016	3.46	1.89–6.33	<0.0001
IpaD total IgG	80	24/44	4/36	<0.0001	1.76	1.30–2.38	<0.0001
IpaB IgG$_1$	20	12/19	0/1	0.80	2.74	0.93–8.08	0.038
IpaB IgG$_{2a}$	20	10/12	2/8	0.031	3.00	1.17–7.73	0.007
IpaD IgG$_1$	20	10/13	2/7	0.10	2.31	1.12–4.78	0.011
IpaD IgG$_{2a}$	20	6/6	6/14	0.048	–[f]	–[f]	0.0002
IpaB stool IgA	80	10/11	18/69	0.0002	19.76	2.54–153.87	<0.0001
IpaD stool IgA	80	6/9	22/71	0.087	4.65	1.08–20.07	0.024

[a] Mice were classified as responders or non-responders according to the total serum IgG, IgG$_1$, IgG$_{2a}$ or stool IgA ELISA titers at day 56 after immunization. A titer of at least 50 ELISA units ml^{-1}, corresponding to 4 times the lower limit of detection (12.5 EU ml^{-1}) was considered a positive response in serum and a titer of at least 10 ELISA units ml^{-1}, corresponding to 4 times the lower limit of detection (2.5 EU ml^{-1}) was considered a positive response in stool. Survivors were mice that remained alive 14 days after pulmonary challenge with *S. flexneri* 2a. Total IgG and IgA titers were measured in mice immunized with max, high, med and low BLP-IpaB/D and IpaB/D dosage levels; subclasses were measured in mice that received the max dose.

[b] p-value from two-sided Fisher's exact test.

[c] Odds ratio for survival from logistic regression for an increase of 1 in log10(titer).

[d] Confidence interval for odds ratio, based on Wald test.

[e] p-value from likelihood ratio test.

[f] Calculation did not converge; all mice that died were non-responders (i.e., had titers <50 ELISA units ml^{-1}).

Table 2

Antibody titers measured in serum and mucosal secretions and protective efficacy

	Serum IgG[a]		Stool IgA[a]		BALf IgG[b]		BALf IgA[b]		Survival[c]	
	IpaB	IpaD	IpaB	IpaD	IpaB	IpaD	IpaB	IpaD	S. flexneri	S. sonnei
BLP-Ipa Max	310,021	109,915	37.9	6.6	2,616	1,095	145.9	19.2	90	80
IpaB/D Max	918.1	25.2	2.5	2.5	315.5	57.2	29.2	12.5	40	20
BLP	12.5	13.4	2.5	2.5	18.4	12.5	12.5	12.5	0	20
PBS	12.5	12.5	2.5	2.5	12.5	12.5	12.5	12.5	0	0

[a] Serum and stool values are provided as geometric mean titers (EU ml^{-1}) from 10 mice per group measured on Day 55 (time of challenge).

[b] BALf was collected from 5 mice per group on day 35 following the initial vaccination. Geometric mean titers are provided in EU ml^{-1}.

[c] Survival is listed as the percentage of 10 mice per group surviving lethal pulmonary challenge with the indicated *Shigella* organism.