Intradermal Delivery of Shigella IpaB and IpaD Type III Secretion Proteins: Kinetics of Cell Recruitment and Antigen Uptake, Mucosal and Systemic Immunity, and Protection across Serotypes


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Intradermal Delivery of *Shigella* IpaB and IpaD Type III Secretion Proteins: Kinetics of Cell Recruitment and Antigen Uptake, Mucosal and Systemic Immunity, and Protection across Serotypes

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*Shigella* is one of the leading pathogens contributing to the vast pediatric diarrheal disease burden in low-income countries. No licensed vaccine is available, and the existing candidates are only partially effective and serotype specific. *Shigella* type III secretion system proteins IpaB and IpaD, which are conserved across *Shigella* spp., are candidates for a broadly protective, subunit-based vaccine. In this study, we investigated the immunogenicity and protective efficacy of IpaB and IpaD administered intradermally (i.d.) with a double-mutant of the *Escherichia coli* heat-labile enterotoxin (dmLT) adjuvant using microneedles. Different dosage levels of IpaB and IpaD, with or without dmLT, were tested in mice. Vaccine delivery into the dermis, recruitment of neutrophils, macrophages, dendritic cells, and Langerhans cells, and colocalization of vaccine Ag within skin-activated APC were demonstrated through histology and immunofluorescence microscopy. Ag-loaded neutrophils, macrophages, dendritic cells, and Langerhans cells remained in the tissue at least 1 wk. IpaB, IpaD, and dmLT-specific serum IgG- and IgG-secreting cells were produced following i.d. immunization. The protective efficacy was 70% against *Shigella flexneri* and 50% against *Shigella sonnei*. Similar results were obtained when the vaccine was administered intranasally, with the i.d. route requiring 25–40 times lower doses. Distinctively, IgG was detected in mucosal secretions; secretory IgA, as well as mucosal and systemic IgG A-secretion cells, were seemingly absent. Vaccine-induced T cells produced IFN-γ, IL-2, TNF-α, IL-17, IL-4, IL-5, and IL-10. These results demonstrate the potential of i.d. vaccination with IpaB and IpaD to prevent *Shigella* infection and support further studies in humans. *The Journal of Immunology*, 2014, 192: 1630–1640.

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*S. flexneri* spp. are among the handful of enteric pathogens that account for most of the cases of diarrhea in children <5 y of age in sub-Saharan Africa and south Asia (1). Even if mortality is averted, the disease leads to impaired health and quality of life, particularly if acquired at an early age (2, 3). Despite the long-standing interest in control measures, no commercial vaccine is available. To be used globally, a vaccine would need to prevent infection caused by *Shigella dysenteriae* 1 (which causes epidemic dysentery), *Shigella sonnei* (affecting mostly travelers and daycare centers), and all 16 *Shigella flexneri* serotypes (mostly responsible for endemic disease) (4). Efforts to develop an effective vaccine have produced several candidates, some of which have been tested in human clinical trials with promising results (reviewed in Refs. 5–8). However, most of these vaccines are serotype specific; therefore, their protective capacity is limited to the serotype from which they were derived. In the pursuit of a broad-spectrum prophylactic intervention, we proposed the use of *Shigella* type III secretion system (TTSS) proteins IpaB and IpaD, which are highly conserved among *Shigella* serotypes, as components of a subunit-based broad protective vaccine. These proteins have an essential role in pathogenesis, because they participate in the assembly of the TTSS needle tip complex, which creates a pore in the host cell membrane and allows the translocation of bacterial effector proteins that ultimately lead to cell death (9, 10). Individuals living in endemic areas who are constantly exposed to the organism develop Ab against *Shigella* O Ag (11) and Ipa (12, 13), both of which are believed to contribute to naturally acquired protective immunity (5). An association was described between the levels of IpaB-specific serum IgG and Iga B memory cells and reduced severity of disease upon experimental challenge in human adult volunteers pre-exposed to live vaccine organisms or wild-type *Shigella* (14). In preclinical studies, mucosally delivered IpaB and IpaD were shown to protect against lethal pulmonary *Shigella* infection in mice (15, 16).

Given that *Shigella* is an enteric pathogen, oral immunization with candidate vaccines would seem the most practical approach to induce mucosal immunity that could block and prevent the organism from breaching the intestinal barrier. Disappointingly,
the success of oral vaccination has been elusive. Routine vaccines have been less immunogenic when administered orally to children living in developing countries compared with industrialized nations. This has been attributed to multiple natural barriers that interfere with immunological priming in the gut (17, 18). In animal studies, orally delivered IpaB and IpaD failed to induce substantial protection, whereas they were vigorously immunogenic and fully protective when given intranasally (i.n.).

Intradermal immunization using improved injection devices has gained attention as a safe, practical, and effective strategy to enhance vaccine immunogenicity (19). Because of its simplicity and efficiency, this mode of vaccination is particularly attractive for use in children. The feasibility of this approach has been demonstrated by the successful administration of the Mycobacterium bovis bacillus Calmette–Guerin (BCG) to millions of newborns and infants throughout the world. Multiple human clinical studies showed successful immunization against influenza, rabies, polio, hepatitis, and other pathogens through intradermal (i.d.) delivery of commercial vaccines (19–21). Intradermal vaccination against seasonal influenza using microneedles has been approved in Europe since 2009 (22) and in the United States since 2011 (19, 23). Vaccination via the i.d. route is simple and extremely efficient, requiring a fraction of the dosage typically given i.m. or s.c. (24). Its success has been attributed to the abundant number of specialized APC (i.e., dendritic cells [DC] and Langerhans cells [LC]) residing in the dermis and adjacent epidermal layer, which capture vaccine Ag in their proximity and process and transport them to the draining lymph nodes (DLN) for presentation and stimulation of T cells (25). Vaccine Ag that reach the DLN also activate B cells. DC activated in the skin prime CD4+ and CD8+ effector and central memory T cells. Central memory T cells recirculate, may become activated outside the DLN, and potentially migrate to effector sites (25). Particularly relevant for clearance of Shigella would be the induction of IFN-γ-secreting CD4+ T cells, as well as Ab-secreting cells (ASC) producing high-avidity Ab that could mediate microbial exclusion and phagocytic killing (5).

In this study, we investigated the early immunological events associated with i.d. immunization with Shigella IpaB and IpaD that lead to immunological priming. In particular, we examined the recruitment of immune cells to the injection site and adjacent tissues, as well as Ag uptake and activation of skin APC. We also fully characterized the immune responses induced in the systemic and mucosal compartments and the protective efficacy of this vaccine against different Shigella serotypes.

Materials and Methods

Vaccine components

The Escherichia coli double-mutant heat-labile toxin [dmLT; LT (R192G/L211A)] was produced at the Walter Reed Army Institute of Research (Silver Spring, MD), following previously described affinity-chromatography methods (26), and obtained through PATH. Recombinant IpaB complexed with the chaperone IpgC (IpaB/IpgC) and IpaD were also purified via affinity and size-exclusion chromatography (27) and quantified using 280-nm extinction coefficients (28). To obtain IpaB for immunological assays and for use as vaccine, IpaB/IpgC was treated with octyl-oIigo-oxylene to release the IpgC chaperone.

Mice, vaccination, and experimental infection with virulent organisms

Female BALB/c mice (7–8 wk old; Charles River Laboratories, Wilmington, MA) were immunized i.d. by delivering a 25-μL volume of inoculum into the upper right thigh (shaved the day before) using the NanoPass MicronJet 600 needle (NanoPass Technologies, Nes Ziona, Israel) attached to a 250-μL Hamilton 700 Series Microliter Syringe (Hamilton Company, Reno, NV). The microneedles were inserted into the bare skin at a 45° angle and locked in that position, and the inoculum was delivered slowly, monitoring the proper formation of a bubble; the needles were kept in place for 5 s before removal. Three dosage levels of IpaB/IpgC and IpaD were tested in the first experiment: 50 ng IpaB/IpgC and 100 ng IpaD (referred to as the “low” dose), 100 ng IpaB/IpgC and 250 ng IpaD (referred to as the “medium” dose), and 200 ng IpaB/IpgC and 500 ng IpaD (referred to as the “high” dose). In all instances, 100 ng dmLT was added as adjuvant. The medium and high doses, which proved the most immunogenic in the first experiment, were included in a second experiment with and without adjuvant. A group immunized i.n. with 2.5 μg IpaB/IpgC or IpaB, 10 μg IpaD, and 2.5 μg dmLT, known to generate potent responses (15, 16), was included as positive control in both experiments. Intranasal vaccination was performed by dispensing 30 μL inoculum (15 μL into each nare) with a pipette, as previously described (15, 16). Negative control groups received 100 ng dmLT or PBS i.d. All groups were vaccinated on days 0, 14, and 28. Serum samples, fecal extracts, and bronchoalveolar lavage fluid (BALF) from individual animals were prepared as previously described (15, 16). On day 56 after vaccination, mice were challenged i.n. with 5.4 × 10^9 to 5.8 × 10^7 CFU S. flexneri 2a 2457T or with 1.35 × 10^3 CFU S. sonnei 53G (15, 16); the doses used correspond to ~10–50 mouse lethal doses for each organism, and both are human virulent strains. All animal studies and procedures were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Ab, ASC, and cytokines

Ab specific for IpaB, IpaD, and dmLT were measured in serum, stool, and mucosal lavages by ELISA, as previously described (15, 16). For IgG subclass assays, HRP-labeled anti-mouse IgG1 and IgG2a (Southern Biotech, Birmingham, AL) were used as detection conjugates. The frequency of IgG and IgA ASC in the lungs, spleens, and bone marrow was measured by ELISPOT, as described before (15, 16). Cytokine levels were measured in culture supernatants of spleen cells (2 × 10^6 in 100 μl) stimulated with 1 μg/ml IpaB and IpaD, using the MSD Mouse TH1/TH2 9-Plex and IL-17 Ultra-Sensitive Kits (Meso Scale Discovery, Gaithersburg, MD) (16). Histology immunohistochemistry and confocal immunofluorescence

To confirm proper i.d. delivery and to track tissue distribution of the inoculum, mice were injected i.d. with india ink (10% in PBS) and euthanized at 4 h, 24 h, or 1 wk after injection. Skin and muscle tissue were removed, embedded in paraffin, and sliced into 5-μm-thick sections. The slides were stained with H&E, and 20× and 40× tissue images were captured using an Olympus BH-2 microscope with a SPOT 4 Mega Pixel RT color camera and imaging software to visualize overall distribution of the dye and cellular inflammatory infiltration at the injection site. For immunohistochemistry (IHC) and immunofluorescence (IF) staining, mice were injected i.d. with IpaB, IpaD, and dmLT (medium dosage level described above) and euthanized 4 h, 24 h, or 1 wk after injection. Mice injected i.d. with PBS and euthanized 30 min after injection served as negative controls. Skin tissue, including the site of injection and surrounding areas, was removed, sectioned, embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura, The Netherlands) and flash frozen in 2-methylbutane contained in liquid nitrogen. Five-micrometer frozen tissue sections were prepared and fixed with cold acetone for staining. For IHC, slides were washed in PBS and immersed in PBS/0.3% H2O2 to exhaust endogenous peroxidase activity. The tissue sections were blocked with 10% rabbit serum and stained with anti-CD4 (clone RM4-5) and anti-CD8α (clone 53-6.7) (BD Biosciences, San Jose, CA), anti-CD20 (langerin-negative) eB0L3L3; eBioscience, San Diego, CA), and anti-macrophage–specific lectin Ab (clone ER-MP23; Abcam, Cambridge, MA). Slides were washed, and biotinylated rabbit anti-rat (Molecular Probes-Invitrogen, Grand Island, NY) was added as secondary Ab. Sections stained by IHC with anti-CD11c (BD Biosciences) were blocked with 10% goat serum before incubation with primary Ab, washed, and incubated with biotinylated goat anti-rat Ab (Sigma-Aldrich, St. Louis, MO). All sections were washed and incubated with Avidin/Biotinylated Enzyme Complex (Vector Laboratories, Burlingame, CA). A final wash was performed before the addition of 3, 3-diaminobenzidine and counterstaining in Mayer’s hematoxylin. Cells staining positive for each specific Ab were counted in the 10 representative fields at ×400 magnification. The IF staining was performed as previously described (29), with modifications. Briefly, slides were rinsed in PBS, and 1% diaminobenzine (DAB) was applied and left for 1 min. Tissues were then immersed in 10% acetic acid for 10 min, washed in PBS, and finally stained with 0.1% aqueous neutral red solution (pH 4.2) until the tissue turned red. Sections were dehydrated in a series of graded alcohols, immersed in xylene, and mounted with Permount (Fisher Biocolors, Pittsburgh, PA).
along with anti-IpaB or anti-IpaD mAb (generously provided by Dr. Edwin V. Oaks, Department of Subunit Enteric Vaccines and Immunology, Bacterial Diseases Branch, Walter Reed Army Institute of Research). The following day, sections were washed with 0.1% Triton X-100, followed by PBS. Biotin-conjugated goat anti-hamster Ab (Invitrogen) was used to detect anti-CD11c primary Ab, and goat anti-rat Ab (Inovision) was used to detect anti-r-ER-MP23, anti-Ly6G (Gr-1; clone RB6-8C5) (AbD Sero-tec, Raleigh, NC), anti-CD40 (clone: 3/23; BD Biosciences) and CD207 Ab, all prepared in the primary diluent buffer. Alexa Fluor 488 goat anti-mouse (Invitrogen) was added to detect the Ag-specific Ab. The stained slides were washed and incubated in streptavidin conjugated to Alexa Fluor 568 (Invitrogen). After another wash, DAPI was added to all of the slides to stain the nucleus, and the slides were mounted using VECTASHIELD mounting media (Vector Laboratories). IF staining was visualized using a Nikon A1 confocal laser microscope for acquisition of images, and at least six 7-μm z-stack projections were collected at 0.300-0.400-μm intervals using Nikon Elements Microscope Imaging Software (Nikon Instruments, Melville, NY). Mander’s overlap coefficient of the red channel (M red) was calculated using Velocity 3D Image Analysis Software (Improvision-PerkinElmer, Waltham, MA) to determine the extent of colocalization between cells that stained positively for a particular immune cell marker (detected with Alexa Fluor 568 in the red channel) and the Ipa (Alexa Fluor 488 under the green channel). M red represents the total sum of voxels of the overlapped red with green components divided by the total sum of red intensities. M red was reported for each treatment as an average of the four to six z-stack images where the threshold was adjusted to 5% for all images to compensate for any background noise. The confocal image acquisition and analyses were adopted from published reports (30).

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). For IHC and IF, statistically significant differences for each cell type were determined by one-way ANOVA, with Bonferroni and Tukey multiple-comparisons tests, respectively, comparing tissue from vaccine versus PBS recipients. Two-way ANOVA with Bonferroni multiple-comparisons test was used to determine statistical significance of the differences between serum Ab titers of vaccinated versus control groups. Significant differences among ASC, cytokine, and stool IgA of vaccinated versus control mice were determined by one-way ANOVA with Bonferroni multiple-comparisons test. Unpaired t test with a 95% confidence interval was used to compare IgG1 and IgG2a subclass titers in the vaccine versus PBS groups. Mean Ab titers in mucosal fluids of recipients of vaccine versus PBS were compared by one-way ANOVA with Dunnett multiple-comparisons test. Survival curves were compared using a log-rank (Mantel–Cox) test. In all analyses, differences were considered statistically significant at p values ≤ 0.05.

Results

Inoculum distribution and recruitment of immune cells following i.d. vaccination

We investigated i.d. delivery of IpaB and IpD adjuvanted with dmLT as a novel and potentially more efficient approach (than oral delivery) for immunization against shigellosis. The vaccine was administered to mice on three occasions, 2 wk apart, using NanoPass MicronJet 600 microneedles. To establish the optimal conditions for vaccination, we first performed a series of experiments using india ink to better visualize the inoculum distribution. An inoculum volume of 25 μl was identified as optimal because it produced a visible and consistent skin bleb without leakage (Supplemental Fig. 1A). To confirm delivery into the dermis and to visualize changes in tissue morphology, the skin tissue was examined. During the first 4 h postinfection, the dye was seen as a thick dark blue layer confined to the dermal space (Supplemental Fig. 1B). At 24 h, the ink strip was tangential to the skin surface, and large numbers of phagocytic cells (mostly neutrophils and some macrophages) had been recruited. One week after infection, the dye had diffused throughout the tissue and had been almost completely ingested by phagocytic cells (Supplemental Fig. 1). We next investigated the changes in cell composition following i.d. vaccination with IpaB, IpD, and dmLT. The presence of neutrophils, macrophages, DC, LC, and CD4+ and CD8+ T cells in skin tissue was evaluated by H&E and IHC staining and confirmed by morphological analysis. Tissue from mice injected with PBS was included as control. Neutrophils rapidly infiltrated the injection site; the largest numbers were detected 4 h after injection and then gradually declined, remaining elevated for at least 1 wk (Fig. 1A). Macrophages also were recruited, albeit at lower numbers and a more gradual pace, reaching their peak at the 1-wk time point (Fig. 1A). CD11c+ DC and CD207+ (langerin+) LC were likewise detected; these cells increased significantly 24 h after vaccination and remained elevated for at least 1 wk. At either time point, the recruited DC outnumbered the LC. Interestingly, CD4+ and CD8+ T cells also were recruited to the vaccination site after 24 h, with the highest numbers detected 1 wk postinjection (Fig. 1C). Representative IHC images for each cell type in control and vaccinated mice at peak time points are shown in Fig. 1 (right panels).

FIGURE 1. Immune cells recruited into the skin following i.d. vaccination with IpaB, IpaD, and dmLT. Skin sections (bleb region) obtained following i.d. administration of IpaB (100 ng), IpaD (250 ng), and dmLT (100 ng) or PBS were stained by IHC. The number of cells stained positive with Abs recognizing mouse neutrophils (anti-Ly6G), skin macrophages (ER-MP23), DC (CD11c+), LC (CD207+), Langerin), and CD4+ and CD8+ T cells were counted at ×400 magnification in 10 representative fields of view (left panels). Data are mean cell number per field + SEM for neutrophils and macrophages (A), DC and LC (B), and CD4+ and CD8+ T cells (C). The number of neutrophils was determined based on cell morphology in H&E-stained slides. Data for PBS are mean values across time points. Right panels, H&E (top row) and IHC images representative of the highest and lowest cell counts. Asterisks indicate examples of positive staining used for counting. *p < 0.05, immunized mice versus PBS controls.
Vaccine uptake by innate immune cells

Next, we looked at the cells that might be involved in vaccine uptake by performing IF staining and confocal laser microscopy. For this analysis, we focused the z-stacks (images of planes at various depths) on tissue sections with abundant staining to investigate the presence of IpaB and IpaD (green) within specific cells (e.g., CD11c+ cells [red]). The images displayed in Fig. 2A clearly show positive staining for IpaB and IpaD colocalized with CD11c+ DC in tissue sections from vaccinated mice, as well as the absence of relevant staining in unvaccinated controls. To determine the extent of Ag sampling, we measured colocalization of IpaB and IpaD within CD11c+ DC through calculation of M_red; a value of 1.0 indicates the maximum colocalization of vaccine Ag within CD11c+ cells, and a value ~ 0.1 represents baseline (mean value for IpaB and IpaD colocalization within CD11c+ cells in the PBS controls). Skin DC containing IpaB or IpaD were detected as early as 4 h after vaccination; in fact, DC containing IpaB were still observed 1 wk postvaccination (Fig. 2B). Based on these results and the superior immunogenicity observed in parallel experiments (described below), we focused on IpaB in subsequent studies to investigate cells involved in Ag sampling as a prerequisite for induction of adaptive immunity. We hypothesized that IpaB likewise would be taken up by other immune cells in the skin, particularly LC, which are extremely efficient APC. IpaB was found within neutrophils (Ly6G+) 24 h and 1 wk postvaccination. IpaB staining also colocalized with LC and skin macrophages (ER-MP23+) at all time points examined (Fig. 3). The fluorescence intensity of IpaB overlapping that of DC, LC, and macrophages was not significantly different, suggesting a similar vaccine sampling capacity for these cell types, with colocalized Ag staining seen as early as 4 h and up to 1 wk after vaccination. We also investigated the activated phenotype (CD40+) of IpaB-containing APC and found significant overlap between IpaB and CD40+ cell fluorescent staining 24 h and 1 wk after vaccination.

**Serum Ab responses induced by i.d.-delivered IpaB, IpaD, and dmLT**

We first conducted a dose-escalation experiment in which low-, medium-, and high-dosage levels of IpaB and IpaD, alongside a constant amount of dmLT, were administered i.d. to BALB/c mice.
Groups immunized with the same Ag i.n. served as positive controls, whereas negative controls received dmLT and PBS. Intradermal immunization with IpaB and IpaD resulted in high levels of Ag-specific serum IgG Ab, regardless of the dose administered. Peak serum IgG responses to IpaB were achieved after the second vaccination, and the magnitude appeared to be similar for all groups (Fig. 4B). However, the IpaB IgG responses produced by i.d. immunization were lower than the responses induced by the protein administered i.n. For IpaD, all groups reached similar (peak) IgG levels at the time of challenge. However, in contrast to IpaB, the dosage level influenced the kinetics of IgG production. The high-dose group exhibited a faster Ab response to IpaD, attaining peak levels after the second vaccination, whereas the low-dose group required an additional immunization to reach the same level of response. It also was noticed that the IgG titers against IpaD produced by i.d. vaccination were lower than those produced by i.n. immunization. Very high IgG responses to dmLT also were elicited by the i.d.-vaccinated mice, which were very similar among groups, proving the consistency of the procedures.

Interestingly, we failed to detect Ag-specific IgA for any of the proteins (IpaB, IpaD, or dmLT) in stool supernatants following i.d. vaccination. No IgA could be detected in serum either (data not shown). However, we detected positive fecal IgA responses against all three Ag in mice immunized i.n. (Fig. 4C). No Ab responses were detected in the unvaccinated (PBS) controls.

**ASC induced by i.d. immunization with IpaB, IpaD, and dmLT**

To further investigate the induction of mucosal immune responses, we measured IpaB-, IpaD-, and dmLT-specific IgG and IgA ASC in the lungs at the time of challenge. No responses were detected in

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**FIGURE 3.** IpaB sampled by innate immune effector cells in the skin with activated phenotype. (A) Confocal IF images of skin tissue excised 24 h after vaccination show IpaB stained green and neutrophils (Ly6G⁺), macrophages (ER-MP23⁺), LC (CD207⁺, Langerin), or activated APC (CD40⁺) stained red. (B) Extent of colocalization (presented as Mₘₑₐₚ) between IpaB and the indicated cell types at different time points. Data are mean ± SD from six z-stack images. The dashed line indicates the baseline level, which corresponds to the mean Mₘₑₚ of PBS tissue images. *p < 0.05, vaccinated mice versus PBS control mice.
mice vaccinated i.d., whereas significant numbers of IgG and IgA ASC were seen in mice immunized i.n. (Fig. 5). We also measured the frequency of IpaB-, IpaD-, and dmLT-specific ASC in spleen and bone marrow (Fig. 5). Mice immunized i.d. exhibited IgG ASC responses to IpaB and IpaD in both organs, which, for the most part, increased with the vaccine dose (Fig. 5). IgG ASC specific for dmLT were also produced following i.d. vaccination. To our surprise, only IgG (not IgA) ASC were elicited in response to IpaB; dmLT alone; or PBS. IpaB-, IpaD-, and dmLT-specific serum IgG (B) and stool IgA (C). Data represent mean titers ± SEM from 10 individual mice/group. Arrows indicate immunization. *p < 0.05, vaccinated mice versus PBS control mice.

Production of cytokines upon in vitro Ag stimulation

We next examined the production of Th1, Th2, proinflammatory, and regulatory cytokines by spleen cells stimulated in vitro with IpaB or IpaD. All groups immunized i.d. showed increased production of IL-2, as well as IFN-γ and TNF-α, in response to IpaB (Fig. 6). These same cytokines, and similar magnitude of responses, were detected after i.n. vaccination (Fig. 6). IL-17 also was produced by mice immunized i.d. in response to IpaB, particularly the high- and medium-dose groups (Fig. 6). IL-2 was the only Th1-type cytokine produced at significant levels in response to IpaD by i.d.-vaccinated mice. Very high levels of Th2-type cytokines (IL-4 and IL-5) were produced by spleen lymphocytes in response to both IpaB and IpaD following i.d. vaccination (Fig. 6). The secretion of IL-4 and IL-5 by cells stimulated with IpaB was noteworthy because it greatly exceeded that of mice immunized i.n. The same was observed for IL-5 against IpaD. IL-10 was also produced by i.d.-vaccinated mice in response to IpaB and IpaD (Fig. 6, middle panels).

Ag-specific induction of IFN-γ, IL-2, and IL-5 was increased in the inguinal and popliteal DLN as a result of i.d. vaccination. These responses were remarkably high in the group that received the medium dose of vaccine and in response to IpaB (Fig. 6, bottom panels). As expected, negligible responses were seen in mice immunized i.n. No responses were detected in the dmLT and PBS controls.

Protection against lethal S. flexneri pulmonary challenge

Intradermal vaccination with IpaB, IpaD, and dmLT afforded significant protection against lethal pulmonary infection with S. flexneri. The group that received the medium dosage level had the highest survival rate (70%). Significant protection also was observed in the group that received the lowest dose (20%). Unexpectedly, all of the mice immunized with the highest dose of IpaB and IpaD succumbed to challenge (Fig. 7). The difference in protection between the medium- and high-dose groups prompted us to perform a second experiment to confirm these findings.

Immune responses to IpaB and IpaD with or without dmLT

A second experiment was performed to ascertain the superior protection of the intermediate dosage level, investigate cross-protection against a different Shigella serotype (S. sonnei), and assess the adjuvant contribution of dmLT in the responses induced. Mice were immunized with the high and medium dose of IpaB and IpaD, as described above, and the proteins were given alone or in the presence of dmLT. A positive control group received IpaB, IpaD, and dmLT i.n., and a negative control group received PBS (Fig. 8A). The IpaB protein used in this experiment had the IpgC chaperone removed. Similar to what was observed in the first experiment, high levels of IpaB and IpaD serum IgG were produced by the proteins given i.d. admixed with dmLT. The medium-dose group produced higher IgG responses against IpaB; interestingly, these titers (as well as those measured in the high-dose group) surpassed those seen in the first experiment, suggesting a higher immunogenic capacity of IpaB compared with IpaB/IpgC when administered via the i.d. route. No differences were seen in the IpaD responses between dosage groups or experiments. The dmLT significantly enhanced the serum IgG responses to both IpaB and IpaD compared with the proteins given alone (Fig. 8B). Serum IgG responses to dmLT also were elicited, which were similar among the i.d.-vaccinated groups across experiments. A side-by-side comparison of the Ab responses obtained in both experiments is shown in Supplemental Table I.

Serum IgG2a/IgG1 subclasses and mucosal IgG and IgA Ab

In this experiment, we also examined the IgG subclass profile induced by i.d. vaccination with IpaB and IpaD, with or without dmLT. High levels of IgG1 were produced in response to IpaB and IpaD, which were similar for the medium and high dosage levels (Fig. 9A).
IgG2a was also produced, albeit at lower levels, and similarly was unaffected by the dose. The presence of dmLT allowed for increased production of IgG2a. IgG1 and IgG2a were produced in response to dmLT, and the levels were similar in all dmLT recipients.

We also investigated the presence of IgG and IgA Ab in the mucosal airways. IpaB- and IpaD-specific IgG were detected in BALF of mice immunized with the Ipa admixed with dmLT. The titers were significantly lower in the absence of dmLT, and this was

**FIGURE 5.** ASC in mucosal and systemic tissues. Mice were immunized with IpaB, IpaD, and dmLT, as described in Fig. 2A. The frequency of Ag-specific ASC was measured in lung, spleen, and bone marrow cells obtained on day 56 after vaccination (the time of challenge). The data represent mean ASC counts/10^6 cells + SEM from quadruplicate wells. *p < 0.05, vaccinated mice versus PBS controls.

**FIGURE 6.** Cytokines produced by spleen and DLN cells upon IpaD and IpaB stimulation. Mice were immunized with IpaB, IpaD, and dmLT, as described in Fig. 2A. Cytokine levels were measured in culture supernatants from spleen and DLN stimulated in vitro with IpaB and IpaD. Results show mean concentration + SEM from triplicate wells. Dashed line represents average levels produced by unstimulated cells from vaccinated animals. *p < 0.05, vaccinated mice versus PBS groups.
particular notice in the responses to IpaD (Fig. 9B). IgG Ab against dmLT were also detected; titers were similar for both i.d. groups and comparable to those measured in the i.n. control group. We were unable to detect vaccine-specific IgA in the alveolar fluid of the i.d.-vaccinated mice. In contrast, IgA specific for all three vaccine Ag was present in the BALF of mice immunized i.n. The amounts of total IgG and IgA among individual samples were not significantly different, thus confirming homogenous sampling (data not shown). However, it was noticed that the fluids of mice immunized i.n. contained larger quantities of total Ab than did mice immunized i.d. (data not shown); this was deemed consistent with nasal exposure activating (local) mucosal immunity.

Protection against *S. flexneri* and *S. sonnei* lethal pulmonary challenge

In agreement with our previous results, 70% of the mice that received the medium dose of IpaB and IpaD admixed with dmLT survived the challenge with virulent *S. flexneri* (Fig. 10A). In the absence of dmLT, the protection dropped to 50%. In contrast, the groups immunized with the highest dose of IpaB and IpaD had 30–40% protection, with dmLT providing no apparent improvement (Fig. 10A). Immunization with the medium dose of IpaB and IpaD plus dmLT also resulted in significant (50%) protection against *S. sonnei* (Fig. 10B). The percentage of survival dropped to 20% in the absence of dmLT. In both challenges, 100% protection was seen in the positive control group (Fig. 10).

Discussion

The recent Global Enteric Multicenter Study identified *Shigella* as a top priority intervention target to reduce illness and death caused by diarrheal disease in children under the age of 5 y in low-income countries (1). We have proposed the use of *Shigella* TTSS proteins IpaB and IpaD as protective Ag to formulate a broadly protective *Shigella* vaccine. The need for practical and effective ways to deliver such a vaccine prompted us to investigate the i.d. route of immunization, which, when coupled with new technologies, has emerged as a suitable alternative to i.m. injection because it is similarly effective and yet more practical and less painful. The fact that it has been the route of immunization for eradication of smallpox and continues to be successfully used to administer BCG to millions of children around the world provides a formidable precedent for its use to prevent enteric diseases in resource-poor areas. We demonstrated in this study that i.d. delivery of IpaB and IpaD admixed with dmLT is a simple and effective approach to induce protective immunity against lethal infection caused by different *Shigella* serotypes. To our knowledge, this is the first report of successful i.d. immunization with a cross-protective, subunit-based *Shigella* vaccine.

Multiple devices have been developed to facilitate i.d. vaccine delivery that are easy to use and more reliable than the conventional needle and syringe techniques (19). The NanoPass hollow microneedles used in this study allow delivery of the inoculum (even highly viscous substances) into the dermis in a slow and controlled manner. This device has been approved by the U.S. Food and Drug Administration and was safe, effective and reliable in clinical studies of i.d. influenza vaccination (31, 32). The microneedles are barely noticeable (shorter than 1 mm) and slightly penetrate the skin, thus greatly reducing pain (and the perception of pain). Different from traditional i.d. injection with needle and syringe (i.e., Mantoux technique), which requires highly trained personnel, i.d. injection with microneedles is easier, and vaccines potentially could be self-administered at home (19, 33). To our knowledge, this is the first report of a preclinical vaccine study using the NanoPass microneedles.
Our results demonstrate consistent delivery of vaccine into the dermis and the ensuing activation of an innate immune response with immediate recruitment of large numbers of neutrophils, followed by macrophages, DC, and LC. Our finding of CD11c+ DC, LC, and macrophages containing IpaB, as well as the evidence of cell activation, imply that these cells participate in Ag sampling and shuttling to the DLN for T cell stimulation (34). In fact, the success of i.d. vaccination is predicted on the efficiency of this process. The presence of IpaB- and IpaD-specific cytokine-secreting T cells in the inguinal lymph nodes of i.d.-vaccinated mice supports this notion. In addition, the recruitment of CD4+ and CD8+ T cells and activated Ag-carrying APC in the dermis up to 1 wk after vaccination suggests the possibility of local T cell priming. The presence of CD8+ T cells is consistent with reports (34, 35) describing the involvement of skin-activated LC preferentially priming Ag-specific CD8+ T cells after i.d. vaccination.

Intradermal immunization with IpaB, IpaD, and dmLT resulted in high levels of Ag-specific serum IgG and systemic (spleen and bone marrow) IgG ASC. A distinct, unanticipated observation was the lack of serum IgA, systemic IgA ASC, and mucosal (stool and BALF) IgA when the Ag were administered i.d. Similarly unexpected was the absence of ASC in the lungs (IgG or IgA) at the time of challenge—presumably needed to secrete mucosal Ab to clear the pathogen, and yet these animals were largely protected. Considering that the timing of ASC sampling might not have been optimal (because mucosal responses usually peak soon after vaccination), we measured lung ASC and Ab in the airways 1 wk after the last immunization. Although the ASC results were mostly negative (data not shown), high levels of IgG were found in the BALF of i.d.-vaccinated mice. Still, no IgA was found. In the absence of lung ASC, it could be argued that the IgG detected in the BALF possibly derives from ASC residing in other parts of the respiratory tract or from systemic sources (i.e., circulating or spleen plasmablasts and plasma cells). These Ab may reach the mucosa by transudation or through active transport via the FcRn (36). The extent to which systemic immunity contributes to protection against Shigella is unclear. In humans, serum IgG Ab to LPS were associated with serotype-specific protection (reviewed in Ref. 6). It was hypothesized that these Ab transudate into the gut where they may neutralize and/or kill the organism, possibly through complement activation (37, 38). Serum IgG against IpaB was correlated with reduced disease severity in challenged individuals (14). Because the Ipa are expressed briefly upon bacterial–host cell contact, these Ab presumably block invasion by preventing translocation of virulent factors. Other investigators (39) proposed that if the mucosal defenses fail, the inflammatory

FIGURE 9. Serum IgG subclasses and BALF IpaB-, IpaD-, and dmLT-specific Ab. Mice were immunized with high and medium dosages of IpaB and IpaD, with or without dmLT, as described in Fig. 8A. IgG1 and IgG2 were measured on day 56. Subclass responses were also measured in mice that received dmLT alone from Experiment 1 (Fig. 5A), because this group was not included in Experiment 2. (A) Data represent mean (+ SEM) IpaB-, IpaD-, and dmLT-specific IgG1 and IgG2a titers from 10–20 mice/group. (B) Individual IpaB-, IpaD-, and dmLT-specific IgG and IgA titers measured in BALF collected on day 35 from five mice/group; horizontal lines represent mean values. *p < 0.05, vaccine groups versus PBS groups.

FIGURE 10. Protection against S. flexneri and S. sonnei lethal pulmonary challenge. Mice were immunized, as described in Fig. 8A, and challenged i.n. on day 56 with 5.4 × 10⁷ CFU of S. flexneri 2a (A) or 1.4 × 10⁸ CFU S. sonnei (B). Data represent survival curves from 10 mice/group. * p < 0.05, vaccinated mice versus PBS controls.
process that accompanies Shigella infection may also lead to transudation of serum IgG into the lamina propria and, thereby, limit tissue invasion.

Precedent exists for routine parenteral vaccines that preferentially induce systemic immunity to prevent mucosal infections (e.g., pneumococcal and Haemophilus influenzae type b conjugates, Salmonella Typhi Vi and pertussis vaccines). The same principle may operate in our model of i.d. immunization with IpaB and IpaD and lung exposure to virulent Shigella. Meanwhile, robust mucosal and systemic IgA and IgG ASC and Ab directed to all vaccine Ag were seen in mice immunized i.n. These differences likely reflect distinct pathways of immunological priming, with i.d. immunization favoring the induction of systemic IgG, and i.n. immunization favoring both systemic and mucosal IgA and IgG.

The high (and seemingly exclusive) IgG levels produced by i.d. vaccination could be explained, in part, by the fact that Ag delivered i.d. have access to dermal DC, which induce differentiation of naive T cells into T follicular helper cells, a subset that contributes to class switch and proliferation of B cells within germinal centers (34). Strikingly high levels of IL-4 and IL-5 were produced by Ag-stimulated T cells from i.d.-vaccinated mice. Skin LC are known to induce CD4+ Th2 cell differentiation (40). Thus, the demonstration of CD207+ DC carrying vaccine Ag in our study is consistent with the unusually high levels of Th2-type cytokines observed, which largely surpassed those of the i.n. group. Vaccine-induced T cells also produced IL-2 and IFN-γ, as well as IL-10, indicating that Th1-type and T regulatory responses were likewise induced.

IpaB was the more immunogenic of the two proteins (both for Ab and T cell responses), in agreement with results from previous studies (15, 16). The dmLT adjuvant increased Ab production and particularly the level of IgG2a, promoting a Th1-type response. To our knowledge, this is the first preclinical demonstration of tolerability and robust adjuvanticity of dmLT when administered i.d. alongside a protein subunit vaccine. A phase 1 clinical study to evaluate the safety and immunogenicity of dmLT administered i.d. to humans is being planned by the Division of Microbiology and Infectious Diseases at the National Institute of Allergy and Infectious Disease of the National Institutes of Health, in collaboration with PATH.

Importantly, mice immunized i.d. with IpaB, IpaD, and dmLT were protected from S. flexneri were protected from Infectious Diseases at the National Institute of Allergy and Immunology and robust adjuvanticity of dmLT when administered i.d.

In conclusion, to our knowledge, our results provide the first preclinical evidence of cross-protective immunity of i.d.-delivered IpaB and IpaD against Shigella infection. The efficiency, ease of delivery, improved safety (avoiding the use of needles), and reduced cost (due to dose sparing) of i.d. vaccination are appealing features for implementing this approach to immunize against shigellosis children who live in high-risk areas, using an IpaB and IpaD-broad spectrum vaccine. These results and considerations warrant further investigation of i.d.-delivered IpaB and IpaD in humans.

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Supplemental Figure 1. Intradermal injection and uptake of injected dye. 25 μl of India Ink dye was administered i.d. into the shaved thigh of Balb/c mice using the NanoPass MicronJet 600 needle. (A) Macroscopic picture of the injection site during the procedure (top panel), immediately after injection, at 4 and 24 h and 1 wk after injection (bottom panel). (B) Histological images by light microscopy of H&E stained thigh tissue sections following i.d. India ink injection obtained with higher magnification (40x) to focus on the cellular infiltrate surrounding the injected fluid (top panel) and lower magnification (2x) to display the entire tissue section (bottom panel). Arrows indicate the location of hair follicles in the epidermal layer. Tissue from untreated mice served as controls. The low magnification images of the entire section (bottom row) show: 1) the inoculum delivered into the dermis and above the muscle (4 h after injection); 2) its progressive diffusion to nearby areas and influx of phagocytic cells with dense black cytoplasm (24 h); and 3) its dispersion into the subcutaneous layer (1 wk) (Supplemental Figure 1B, bottom). These observations were consistent from histological analysis of five individual mice.
### Supplemental Table I. Comparison of serum IgG antibody responses against vaccine antigens

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Route</th>
<th>Vaccine</th>
<th>Serum IgG titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IpaB*</td>
<td>IpaD*</td>
</tr>
<tr>
<td>1</td>
<td>i.d.</td>
<td>IpaB/IpgC+IpaD+dmLT (High)</td>
<td>239,452</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>IpaB+IpaD+dmLT (High)</td>
<td>388,638</td>
</tr>
<tr>
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<td>i.d.</td>
<td>IpaB/IpgC+IpaD+dmLT (Medium)</td>
<td>225,112</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>IpaB+IpaD+dmLT (Medium)</td>
<td>696,761*</td>
</tr>
<tr>
<td>1</td>
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<td>846,368</td>
</tr>
<tr>
<td>2</td>
<td>i.n.</td>
<td>IpaB+IpaD+dmLT</td>
<td>1,259,924</td>
</tr>
<tr>
<td>1</td>
<td>i.d.</td>
<td>PBS</td>
<td>28.15</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>PBS</td>
<td>20.33</td>
</tr>
</tbody>
</table>

*aMean EU/ml values in serum samples from day 55 (n=10 for experiment 1 and n=20 for experiment 2). Asterisks denote significant differences (p<0.05) between Ab titers against individual antigens from all samples in Experiment 1 compared to the same group in Experiment 2, determined by one-Way ANOVA with Tukey’s multiple comparisons test.*