

# Shigella Outer Membrane Protein PSSP-1 Is Broadly Protective against Shigella Infection

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In developing countries, *Shigella* is a primary cause of diarrhea in infants and young children. Although antibiotic therapy is an effective treatment for shigellosis, therapeutic options are narrowing due to the emergence of antibiotic resistance. Thus, preventive vaccination could become the most efficacious approach for controlling shigellosis. We have identified several conserved protein antigens that are shared by multiple *Shigella* serotypes and species. Among these, one antigen induced cross-protection against experimental shigellosis, and we have named it pan-*Shigella* surface protein 1 (PSSP-1). PSSP-1-induced protection requires a mucosal administration route and coadministration of an adjuvant. When PSSP-1 was administered intranasally, it induced cross-protection against *Shigella flexneri* serotypes 2a, 5a, and 6, *Shigella boydii*, *Shigella sonnei*, and *Shigella dysenteriae* serotype 1. Intradermally administered PSSP-1 induced strong serum antibody responses but failed to induce protection in the mouse lung pneumonia model. In contrast, intranasal administration elicited efficient local and systemic antibody responses and production of interleukin 17A and gamma interferon. Interestingly, blood samples from patients with recent-onset shigellosis showed variable but significant mucosal antibody responses to other conserved *Shigella* protein antigens but not to PSSP-1. We suggest that PSSP-1 is a promising antigen for a broadly protective vaccine against *Shigella*.

*Shigella* is one of the major causes of diarrheal disease in infants and young children in developing countries (1). Approximately 1.1 million people die as a result of *Shigella* infections each year, and 60% of the deaths involve children under 5 years of age (2). In developed countries, 500,000 cases of shigellosis are reported each year by military personnel and travelers (3). The causative agents are four facultative intracellular Gram-negative *Shigella* species, i.e., *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, and *Shigella boydii*. More than 40 serotypes, as specified by the composition of the surface polysaccharide O antigen, have been identified.

Antibiotics can effectively treat shigellosis but, because of the emergence of antibiotic resistance, the World Health Organization has made the development of an effective *Shigella* vaccine a top priority. Population-based studies conducted by the International Vaccine Institute (IVI) in six Asian countries indicate that, while *S. flexneri* is the most commonly isolated species (except in Thailand), levels of different *S. flexneri* serotypes vary greatly among sites and even from year to year at a given outbreak site (4). Because a *Shigella* vaccine must include multiple serotypes and species, we tried to identify common *Shigella* antigens that could yield protection across species and serotypes. Using comparative analyses of whole-genome and virulence plasmid sequences, including publically available sequences from different species of *Shigella*, we recently identified a surface-expressed protein common to all or most species, termed pan-*Shigella* surface protein 1 (PSSP-1).

PSSP-1 is a C-terminal half-polypeptide of IcsP (5) that is expressed by more than 300 *Shigella* isolates (representing all species and serotypes) collected from field sites in Asia. It is not present among common intestinal commensal organisms. IcsP is an outer membrane protein, of the enterobacterial omptin family of proteases, that cleaves IcsA (VirG) and is involved in *Shigella* virulence (6–8). *Shigella* actin-based motility correlates with the

amount of IcsA  $\alpha$ -domain on the bacterial cell surface, which is modulated by IcsP through cleavage of IcsA between Arg758 and Arg759, removing the entire IcsA  $\alpha$ -domain from the surface of the bacterium (9).

In this study, PSSP-1, when added to cholera toxin (CT) (10) or the double mutant (R192G/L211A) of heat-labile toxin (dmlT) of *Escherichia coli* (11), showed cross-protection against multiple *Shigella* species and serotypes when tested in a mouse pneumonia model. Clinical data from shigellosis patients showed rare antibody responses to PSSP-1. The natural hierarchical level of PSSP-1 seems to be lower than that of the other *Shigella* Ipa proteins included in this study. Based on data in this study, PSSP-1 may be a relevant antigen for a *Shigella* vaccine.

## MATERIALS AND METHODS

**Bacterial strains.** *S. flexneri* serotype 2a strain 2457T (12), serotype 5a strain M90T (13), and serotype 6 (14), *S. boydii* (4), *S. dysenteriae* serotype

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1 strain 10398 (15), and *S. flexneri* serotype 2a vaccine strain SC602 (16) were used in this study. For intranasal (i.n.) immunization or challenge experiments, an aliquot of bacteria stored at  $-80^{\circ}\text{C}$  was thawed, streaked on a Bacto tryptic soy broth (BD, Sparks, MD) agar plate containing Congo red (Serva, Heidelberg, Germany), and incubated overnight at  $37^{\circ}\text{C}$ . One Congo red-binding colony was picked from the plate and cultured in Bacto tryptic soy broth at  $37^{\circ}\text{C}$ , with shaking, until the subculture reached an optical density at 600 nm ( $\text{OD}_{600}$ ) of  $\sim 0.5$  ( $\sim 2 \times 10^8$  CFU/ml). The bacteria were diluted to the optimal concentrations with phosphate-buffered saline (PBS). We confirmed the CFU values by serially diluting each bacterial challenge solution in PBS and then spreading appropriate dilutions on agar plates containing Congo red.

**Cloning and purification of PSSP-1.** A PCR-amplified fragment carrying PSSP-1 (IcsP amino acids 171 to 300; GenBank accession no. AAP78966.1) (17) was cloned between the EcoRI and XhoI sites of pET21-d and pET24-d (Novagen, Madison, WI) by using *S. flexneri* serotype 2a strain 2457T as the template. Primers used for PCR were as follows: forward, 5'-CCGGAATTCGGAGTGAACCGGGGGAGC-3'; reverse, 5'-CGGCGGCTCGAGCTAGTGGTGGTGGTGGTG AATACTGCACTATTTT-3'. The bases that are underlined are the IcsP sequences. Recombinant PSSP-1 was expressed in *E. coli* BL21(DE3) and purified from inclusion bodies as His-tagged protein. The protein was purified using nickel-nitrilotriacetic acid (NTA) His-Bind resin (Novagen), according to the manufacturer's recommendations, and was tested for endotoxin (18). Endotoxin levels of the proteins were less than 100 endotoxin units (EU)/mg. The expressed PSSP-1 polypeptide was 14.8 kDa.

**Construction of  $\Delta$ icsP *Shigella* strain.** The  $\Delta$ icsP strain was constructed by disruption of the *icsP* gene through insertion of a suicide plasmid, as described previously (19). In brief, a 600-bp internal fragment of *icsP* was amplified using PCR with the following primers: forward, 5'-GCCGGCGAGCTCACTAACTATCCACTTTTCATA-3'; reverse, 5'-GCCGGCGTCGACACCTATTGTCTTTCACCACT-3'. The resulting PCR product was inserted into the chloramphenicol resistance suicide plasmid pSW23OriT (20). The recombinant plasmid pSWicsPTr was transferred by conjugation to *S. flexneri* serotype 2a strain 2457T (19).

**Immunization and infection of mice.** We used 6-week-old female BALB/c mice purchased from Charles River Laboratories (Orient Bio, Seongnam, South Korea). Mice were kept under specific-pathogen-free conditions at the IVI, and all animal experiments were performed with approval of the IVI Institutional Animal Care and Use Committee (protocol no. 2010-011). Mice were anesthetized by intramuscular injections with ketamine hydrochloride (Yuhan Co., Ltd., Seoul, South Korea) (0.1 mg/g of body weight) combined with xylazine hydrochloride (Rompun; Bayer Korea, Seoul, South Korea) (12.5  $\mu\text{g/g}$  of body weight) and were immunized i.n. three times, at 2-week intervals, with PSSP-1 (20  $\mu\text{g}$ ) plus adjuvant, CT (2  $\mu\text{g}$ ), or dmLT (5  $\mu\text{g}$ ) (11). The total volume for intranasal immunization was 20  $\mu\text{l}$ . One week after the last immunization, the mice were inoculated i.n. with different doses of bacteria as indicated. Survival and body weights were observed daily.

**Enzyme-linked immunosorbent assay.** PSSP-1-specific antibody titers were determined by an enzyme-linked immunosorbent assay (ELISA) using serum or bronchoalveolar lavage fluid (BALF) from each mouse, as described previously (18, 21). In 96-well plates, the coating concentration of PSSP-1 in each well was 200 ng/100  $\mu\text{l}$ . The endpoint titer was expressed as the reciprocal  $\log_2$  of dilutions showing values of 0.2 at a wavelength of 450 nm.

**Enzyme-linked immunosorbent spot assay.** On day 7 after the last PSSP-1 immunization, we obtained spleens and lungs from the mice. Single-cell suspensions were prepared as described previously (18). We coated 96-well nitrocellulose microplates (Millipore, Bedford, MA) with purified recombinant PSSP-1 (30  $\mu\text{g/ml}$ ) in PBS and performed an enzyme-linked immunosorbent spot (ELISPOT) assay as described previously (18). PSSP-1-specific IgG or IgA spots were developed with the addition of 3-amino-9-ethylcarbazole (AEC)- $\text{H}_2\text{O}_2$  chromogenic sub-

strate (Sigma-Aldrich) and were counted with an ImmunoSpot analyzer (Cellular Technology, Cleveland, OH).

**Detection of cytokine production.** Single-cell suspensions from spleens or lymph nodes were cultured with PSSP-1 peptides, as described above, for 72 h at  $37^{\circ}\text{C}$  in an incubator with 5%  $\text{CO}_2$ . The culture supernatants were stored at  $-80^{\circ}\text{C}$  until cytokine analysis was performed. Cytokine levels in the supernatants were assessed by using the mouse Th1/Th2/Th17 cytokine standards of the BD cytometric bead array (CBA) kit (BD Biosciences, San Jose, CA), according to the manufacturer's recommendations.

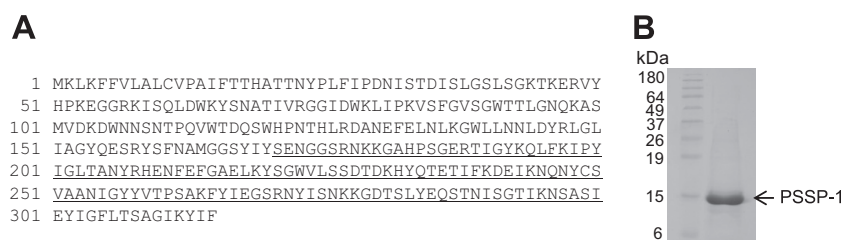
**Detection of antibody-secreting cells by ELISPOT assay in blood samples from patients with shigellosis.** The study population for this analysis included 34 adult patients with diarrhea who were admitted to the Infectious Diseases and Beliaghata General Hospital (Kolkata, India); all had  $\geq 3$  stool emissions per day, with or without blood. Informed consent was obtained according to the protocol of the Institutional Ethical Committee. Enrolled subjects were monitored for 1 month, and blood specimens were collected at different times. Stool specimens were collected immediately after hospitalization, and the first blood samples were obtained at the preliminary study examination. The second blood specimens were obtained 5 to 6 days later. History of diarrheal illness was recorded for all enrolled subjects. All subjects received oral rehydration therapy and antimicrobial treatment, in accordance with the current guidelines of the Infectious Diseases and Beliaghata General Hospital.

**Human whole-blood ELISPOT assay.** We used the ELISPOT assay with whole blood, as described previously (22), to detect the numbers of antibody-secreting cells (ASCs) (IgA or IgG) per milliliter of blood in response to PSSP-1, IpaC (23), and IpaD (24).

**Statistical analysis.** Data are expressed as mean  $\pm$  standard deviation (SD). Statistical significance between the individual groups was analyzed using the unpaired Student *t* test, with a threshold of *P* values of  $<0.05$ . A log rank (Mantel-Cox) test was used for comparison of survival rates between groups.

## RESULTS

**Selection of conserved *Shigella*-specific protein antigens based on protection against *Shigella* challenges.** To identify conserved *Shigella*-specific protein antigens that could be used in a *Shigella* vaccine to target a broad range of *Shigella* species and serotypes, we performed a comparative analysis of published genomic sequences of various *Shigella* species and virulence plasmids using the PubMed BLAST search engine. We selected 13 different genes, encoding *Shigella*-specific surface antigen, motility-associated, or secreted or secretion-related proteins (see Table S1 in the supplemental material). Each was cloned by PCR into a His-tagged fusion protein expression vector by using genomic DNA from *S. flexneri* serotype 2a strain 2457T (12) as the template. Most recombinant proteins were expressed in *E. coli* and purified. For proteins that were difficult to purify from the *E. coli* expression system, truncated forms of the proteins were cloned instead and purified. Each recombinant protein was administered intranasally (i.n.) to mice three times, at 2-week intervals, using CT as an adjuvant. A homologous challenge study with *S. flexneri* serotype 2a strain 2457T was carried out, and we found that polypeptides corresponding to a region within IcsP (GenBank accession no. AAP78966.1) or SigA (GenBank accession no. AAP18272.1) could generate homologous protection upon *S. flexneri* serotype 2a strain 2457T challenge. We also found that mice immunized with recombinant IcsP polypeptide were protected against heterologous challenge with *S. sonnei* (see Table S1 in the supplemental material). We named the IcsP polypeptide PSSP-1. The sequence homology of PSSP-1 has been confirmed for more than 300 *Shigella* isolates, representing all four species and serotypes collected



**FIG 1** Cloning and purification of PSSP-1. (A) Amino acid sequence of PSSP-1. Genomic DNA of *S. flexneri* serotype 2a strain 2457T was used as a template, and a PCR-amplified fragment carrying PSSP-1 (amino acids 171 to 300 of polypeptide IcsP [GenBank accession no. [AAP78966.1](#)]) (underlined sequence) was cloned into a protein expression vector. C-terminal His-tagged PSSP-1 was expressed in *E. coli* BL21(DE3) and purified from inclusion bodies. (B) SDS-PAGE result for purified recombinant PSSP-1. Purified PSSP-1 (10  $\mu$ g) (right lane) was subjected to SDS-PAGE before staining with Coomassie blue. Left lane, molecular mass standards.

from field sites in Asia. PSSP-1 is not present in common intestinal commensal bacteria. PSSP-1 is 14.8 kDa and includes the sequence of IcsP, an outer membrane serine protease involved in bacterial motility (25) (Fig. 1).

**PSSP-1-induced homologous and heterologous protection against *Shigella* challenges.** We tested protective efficacy conferred by PSSP-1 immunization against more species and serotypes of *Shigella* by using a mouse pneumonia model (26). While immunization with *S. flexneri* serotype 2a vaccine strain SC602 (16) generated homologous protection only against *S. flexneri* serotype 2a, i.n. PSSP-1 immunization with CT protected the animals against a number of *Shigella* species and serotypes, including *S. flexneri* serotypes 2a (12) and 5a (13), *S. dysenteriae* serotype 1 (15), and *S. boydii* (4), although the survival rates were less than 100% (Table 1). Without adjuvant, PSSP-1 alone did not protect mice (data not shown). To determine whether the cross-protection was totally due to PSSP-1, PSSP-1-immunized mice were challenged with the *icsP* gene-disrupted *S. flexneri* serotype 2a mutant strain ( $\Delta$ icsP strain). None of the PSSP-1-immunized mice survived the  $\Delta$ icsP strain challenge, while all SC602-immunized mice were completely protected (Table 1). These results suggest that PSSP-1 plays a critical role in protection against *Shigella* infection.

We next evaluated the protective efficacy generated by PSSP-1 immunization when PSSP-1 was administered with dmLT instead of CT, since dmLT is known to be safer (27). Intranasal immunization with 20  $\mu$ g of PSSP-1 plus 5  $\mu$ g of dmLT as adjuvant also protected mice against heterologous species and serotypes of *Shigella*, including *S. flexneri* serotype 2a strain 2457T, *S. flexneri* serotype 6, and *S. dysenteriae* serotype 1 (Fig. 2), suggesting that

dmLT, like CT, is an adjuvant that effectively promotes the induction of PSSP-1-specific immunity.

**PSSP-1-induced humoral and cellular immune responses in the presence of adjuvant (CT or dmLT).** Next, we examined the immunogenicity of PSSP-1 in mice. Each mouse was immunized i.n. three times, at 2-week intervals, with PSSP-1 plus CT or dmLT as adjuvant. Following immunization, we examined the PSSP-1-specific antibody responses in the sera. The IgG responses were comparable in the two adjuvant groups. When the levels of IgG2a and IgG1 (two subclasses of IgG, representing Th1 and Th2 CD4<sup>+</sup> T cell responses, respectively) were measured, PSSP-1-specific IgG1 levels were higher than IgG2a levels in all mice that received either CT or dmLT as adjuvant (Fig. 3A).

We next examined whether PSSP-1 immunization could elicit cytokine responses. Two weeks after the final booster immunization with CT, splenocytes harvested from immunized mice were stimulated with pooled 25-mer peptides that overlapped 14 amino acids in PSSP-1, and the levels of gamma interferon (IFN- $\gamma$ ), interleukin 17A (IL-17A), IL-2, IL-6, IL-4, and tumor necrosis factor (TNF) in cell culture supernatants were analyzed. We observed that levels of all cytokines, including IFN- $\gamma$  and IL-17A, which are widely known to be involved in generating protection against *Shigella* infections (28, 29), were increased by PSSP-1 immunization (Fig. 3B). Mice that received PSSP-1 with dmLT as adjuvant demonstrated comparable results (data not shown). Collectively, these results suggest that PSSP-1 immunization, with the use of an appropriate adjuvant, effectively induces systemic humoral immune responses as well as Th1, Th2, and Th17 cellular immune responses.

**PSSP-1-conferred protection when administered mucosally, but not systemically, in a lung pneumonia model.** We evaluated the route-dependent protective efficacy generated by PSSP-1 immunization. Mice were immunized i.n. or intradermally (i.d.) with dmLT-adjuvanted PSSP-1. Mice immunized i.n. were protected against *S. flexneri* serotype 2a strain 2457T or *S. dysenteriae* serotype 1, although the survival rates were below 100%. The i.d. route did not confer protection against either homologous or heterologous *Shigella* challenge (Fig. 4A).

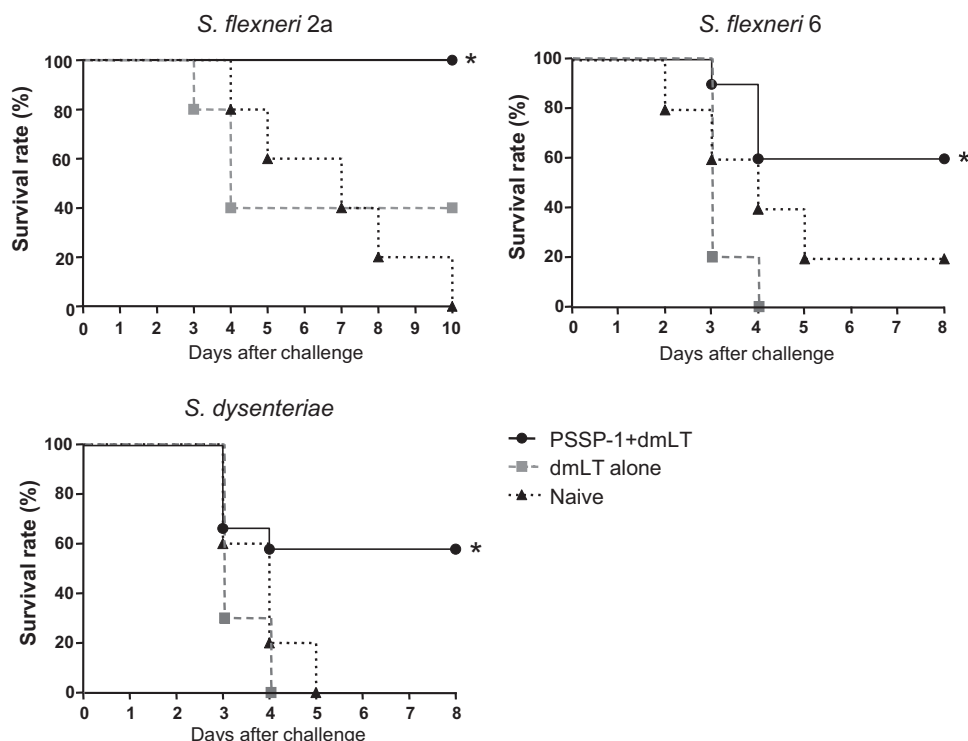
Antibody responses to PSSP-1 were also evaluated following immunization by different routes. PSSP-1-specific IgG levels in blood and bronchoalveolar lavage fluid (BALF) samples were comparable with i.n. and i.d. immunizations. However, PSSP-1-specific IgA levels were below detection levels and near zero in serum and BALF samples, respectively, after i.d. immunization. In

**TABLE 1** Protective efficacy of PSSP-1 in mouse pneumonia model<sup>a</sup>

Immunization with:	Protective efficacy (%) during challenge with:				
	<i>S. flexneri</i> serotype 2a	<i>S. flexneri</i> serotype 5a	<i>S. dysenteriae</i> serotype 1	<i>S. boydii</i>	$\Delta$ icsP strain
PSSP-1	80	60	60	70	0
SC602	100	0	0	0	100

<sup>a</sup> Mice were immunized intranasally (i.n.) with PSSP-1 (20  $\mu$ g) plus CT (2  $\mu$ g) or live *S. flexneri* serotype 2a vaccine strain SC602 (10<sup>8</sup> CFU) 3 times, at 2-week intervals, and were challenged i.n. with virulent *S. flexneri* serotype 2a strain 2457T (5  $\times$  10<sup>7</sup> CFU), serotype 5a strain M90T (10<sup>7</sup> CFU), *S. dysenteriae* serotype 1 strain 10398 (10<sup>7</sup> CFU), *S. boydii* (5  $\times$  10<sup>7</sup> CFU), or  $\Delta$ icsP *S. flexneri* serotype 2a strain 2457T (5  $\times$  10<sup>7</sup> CFU). Survival was observed daily. Each experiment included 10 mice per group. *S. flexneri* serotypes 2a and 5a were tested with 5 independent experiments and *S. dysenteriae* serotype 1 and *S. boydii* with 3 independent experiments.





**FIG 2** Survival curves after PSSP-1 immunization, showing protection of mice against homologous and heterologous *Shigella* challenges. Mice were immunized intranasally (i.n.) with PSSP-1 (20  $\mu$ g) plus dmLT 3 times, at 2-week intervals, and challenged i.n. with virulent *S. flexneri* serotype 2a strain 2457T, *S. flexneri* serotype 6, or *S. dysenteriae* serotype 1 strain 10398 (all at  $10^7$  CFU). Survival was observed daily ( $n = 10$  to 12 mice per group except that  $n = 5$  for all subgroups in the *S. flexneri* serotype 2a strain 2457T challenge and the dmLT-alone and naive groups in the *S. flexneri* serotype 6 challenge). Results are representative of three independent experiments. \*, statistically significant difference for PSSP-1 plus dmLT versus naive or dmLT alone.

contrast, PSSP-1-specific IgA titers were high in both serum and BALF samples after i.n. administration (Fig. 4B).

Of note, ELISPOT assay data demonstrated that PSSP-1-specific IgG- or IgA-secreting B cells were most abundant in the lungs of mice immunized i.n. but were only minimally detected in the lungs of mice immunized i.d. In contrast, the spleens of mice immunized i.d. had numerous PSSP-1-specific IgG-secreting B cells but not IgA-secreting B cells (Fig. 4C). Our data demonstrate that mucosal immunization is more effective than systemic immunization in inducing mucosal B cell immune responses.

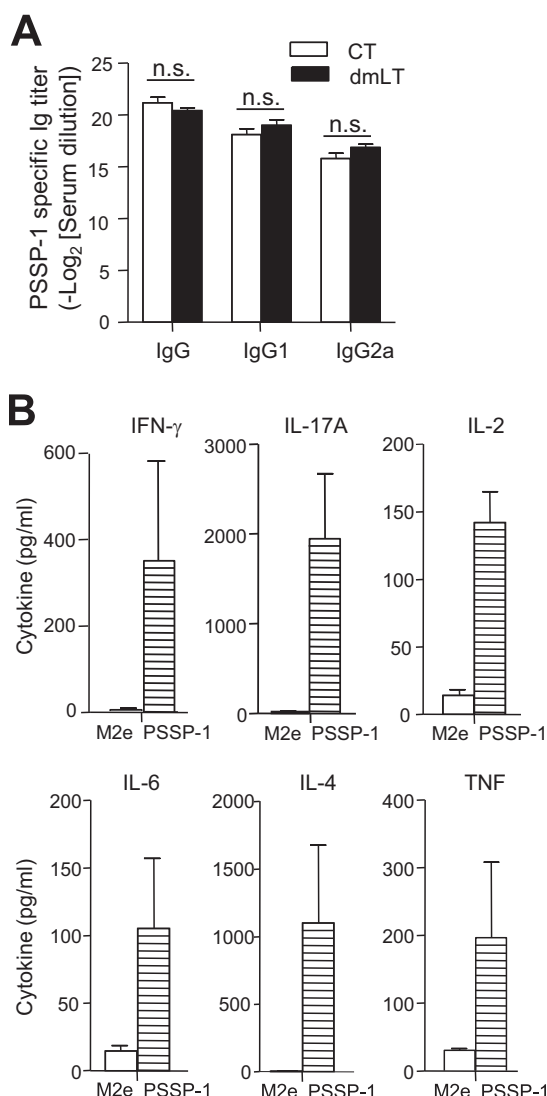
We also evaluated the IFN- $\gamma$  and IL-17A levels in response to PSSP-1 immunization via different routes. Spleens and cervical lymph nodes were isolated from mice immunized i.n. or i.d. with PSSP-1 plus dmLT as adjuvant and were stimulated with a PSSP-1 peptide pool for 72 h. Following peptide stimulation, IFN- $\gamma$  and IL-17A levels in cell culture supernatants were analyzed. We found higher levels of IFN- $\gamma$  and IL-17A in the culture supernatants of spleen cells isolated from mice immunized i.n. than in those from mice immunized i.d. High levels of IL-17A were detected from both spleens and cervical lymph nodes from mice immunized i.n. These results suggest that cellular immune responses are strongly induced only when PSSP-1 is administered i.n. with dmLT. Taken together, our data indicate that i.n. delivery of PSSP-1 produces better protective immunity in this *Shigella* pulmonary infection model.

**Low levels of blood antibody-secreting cell responses to PSSP-1 in patients with recent-onset shigellosis.** To evaluate whether natural *Shigella* infections in human patients can induce

mucosal antibody responses to PSSP-1 (which is possibly related to protective immunity), we examined the development of PSSP-1-specific gut-homing ( $\alpha 4\beta 7$ ) B cell immunity (30, 31) in human patients with shigellosis. Positive shigellosis was confirmed by culture of stool samples and by PCR findings positive for the *ipaH* gene (32). Of 34 subjects studied, 24 were microbiologically and PCR positive for shigellae. The ASC counts for *Shigella* antigens PSSP-1, IpaC (23), and IpaD (24) were determined 5 or 6 days after the first diarrheal symptoms. The cutoff value for ASC positivity was defined as  $\geq 10$  spots per milliliter of blood after subtraction of the value for the PBS control for each sample. Ten spots per milliliter represents at least 1 spot/well of the ELISPOT assay plate for each antigen. Because one person among the 24 patients with shigellosis withdrew consent, blood samples from 23 subjects were analyzed. The subjects had many fewer gut-homing PSSP-1-specific IgA (3/23 subjects) or IgG (1/23 subjects) ASCs than IpaC-specific IgA (6/23 subjects) or IgG (19/23 subjects) ASCs or IpaD-specific IgA (8/23 subjects) or IgG (16/23 subjects) ASCs (Table 2). Details of positive and negative shigellosis cases are shown in Tables S2 and S3 in the supplemental material. Since only a few subjects mounted ASC responses to the PSSP-1 protein antigen during natural infections, PSSP-1 immunization may provide cross-protective immunity in humans who previously had shigellosis.

## DISCUSSION

In this study, we found that mucosal immunization with the common *Shigella* outer membrane protein PSSP-1 could induce



**FIG 3** Systemic B cell and T cell immune responses to PSSP-1 in mice. (A) PSSP-1-specific IgG levels in serum. Mice were immunized intranasally 3 times, at 2-week intervals, with PSSP-1 protein (20  $\mu$ g) plus either CT (2  $\mu$ g) or dmLT (5  $\mu$ g). Six days after the third immunization, serum samples were collected from individual mice and levels of IgG, IgG1, and IgG2a in response to recombinant PSSP-1 were determined by ELISA ( $n = 5$  for CT and  $n = 8$  for dmLT). n.s., not significant. (B) Analysis of cytokines secreted from splenocytes of BALB/c mice immunized with PSSP-1 plus CT, 2 weeks after the third immunization with PSSP-1 plus adjuvant. After sacrifice, isolated spleen cells were stimulated with pooled whole PSSP-1 peptides (25 amino acids) for 72 h at 37°C in 5% CO<sub>2</sub>. Cytokines from culture supernatants were analyzed using the BD mouse Th1/Th2/Th17 CBA kit, according to the manufacturer's protocol. The values obtained were subtracted from unstimulated splenocyte data for individual mice ( $n = 3$  or 4). M2e, ectodomain of matrix protein 2 of human influenza virus type A (SLLETVETPIRNEWGCRCNGSSD) (used as control). Data are means  $\pm$  SD and are representative of two experiments.

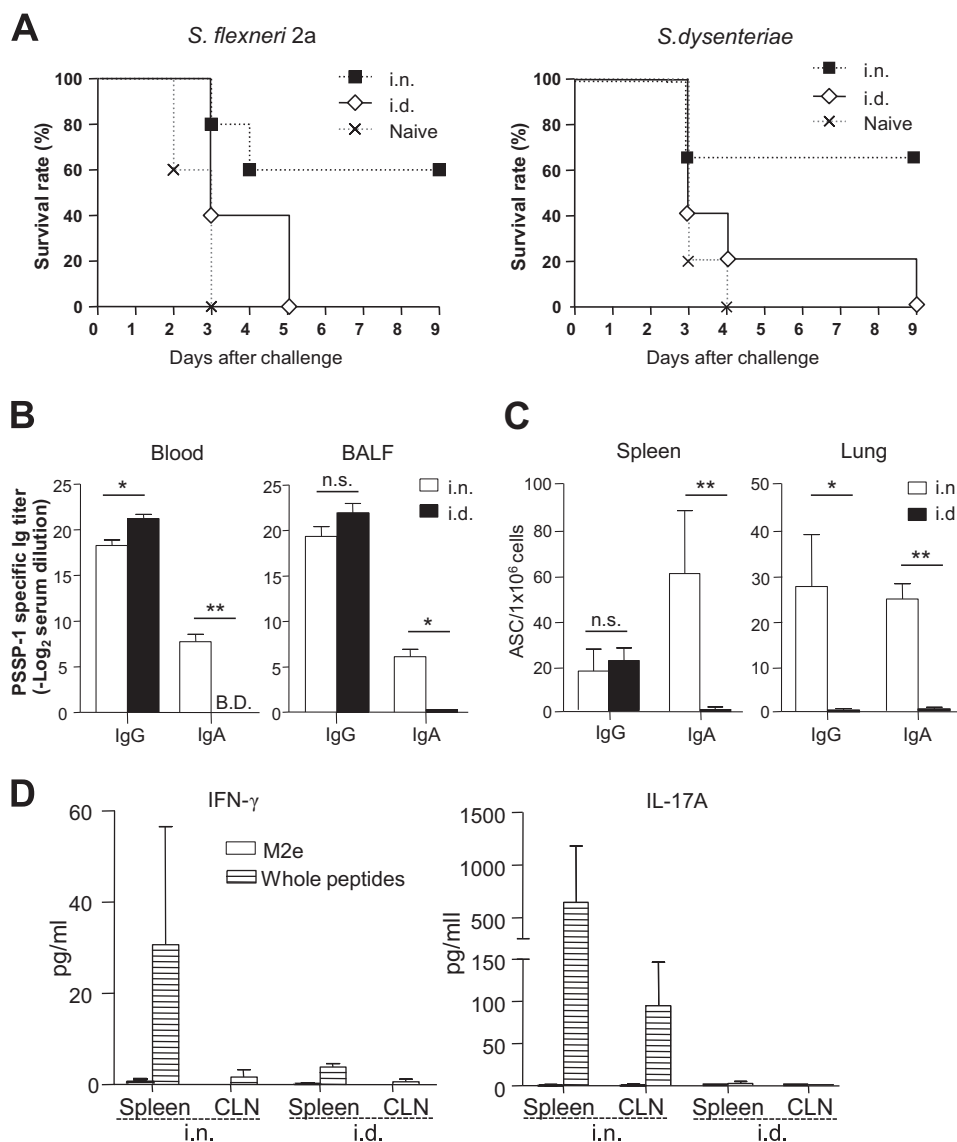
broadly protective immunity against *Shigella*. Initially, 13 genes were identified as *Shigella* specific by comparative genomic analyses. Among them, two polypeptides, encoded by partial *sigA* and *icsP* genes, showed protection against homologous *Shigella* challenges in a mouse pneumonia model (see Table S1 in the supplemental material). Of note, the partial IcsP polypeptide PSSP-1 protected the mice against heterologous *Shigella* challenges. The

failure of PSSP-1-immunized mice to survive challenge with an *icsP* gene-disrupted *Shigella* strain ( $\Delta$ *icsP* strain) confirms that PSSP-1-mediated immune responses play an important protective role against *Shigella* infections (Table 1). SC602 also has IcsP on the outer membrane, but it failed to provide cross-protection against heterologous *Shigella* challenges, probably due to the decreased magnitude of immune responses in the presence of large amounts of lipopolysaccharide (LPS). *Shigella* vaccine development targeting LPS (an important component of the outer membrane of Gram-negative bacteria) alone has not been successful (33). LPS contains three chemically linked components, i.e., lipid A, core polysaccharide, and O antigen (34). Among the three, the O antigen polysaccharide chain is known to be one of the main virulence factors for shigellae and also the major target for innate immunity (35, 36). However, the structural variability of the O antigen polysaccharide chain among serotypes makes it difficult to utilize serotype-specific LPS as a broadly protective agent in a *Shigella* vaccine. Also, polysaccharide induces a T cell-independent antibody response and has poorer immunological memory than the T cell-dependent antigens. Thus, the best way to prevent shigellosis would be to use conserved proteins that could provide broad protection against different species and serotypes.

Subunit polypeptide vaccines are considered to be safer than live attenuated vaccines, but they require adjuvant to confer protective immunity. CT produced by *Vibrio cholerae* and heat-labile toxin (LT) produced by enterotoxigenic *E. coli* are very strong mucosal adjuvants but, due to their toxicity, are not approved for human use. dmLT was designed to reduce LT toxicity and is safe for animals and humans (27). Thus, we investigated dmLT as an adjuvant for use with PSSP-1. As shown in Fig. 2 and 3, dmLT enhances systemic and mucosal antibody and T cell responses, as indicated by Th1, Th2, and Th17 cytokine production in response to PSSP-1. Also, dmLT-adjuvanted PSSP-1 protected mice against challenges with multiple *Shigella* serotypes.

To test whether the route of PSSP-1 immunization is important for the induction of protective immunity, we compared the i.n. and i.d. routes. Although i.d. immunizations with PSSP-1 could induce higher levels of PSSP-1-specific IgG in blood than i.n. immunizations, mice had no protection against either *S. dysenteriae* serotype 1 or *S. flexneri* serotype 2a strain 2457T challenge. Intranasal immunization with PSSP-1 plus dmLT as the adjuvant resulted in high levels of PSSP-1-specific IgA in lungs and protection. We found higher levels of IFN- $\gamma$  and IL-17A in response to PSSP-1 after i.n. immunizations than after i.d. immunizations. These results indicate that mucosal immunity induced by PSSP-1 plays an important role in protective immunity against *Shigella* infection. The combination of PSSP-1 and other *Shigella* proteins or novel mucosal adjuvants is under consideration for better protection against *Shigella* infection.

According to a recent publication by Tran et al. (37), IcsP is preferentially concentrated at the new pole of nonseptating cells and at the septum of dividing cells and is masked by LPS O antigens. This is consistent with our data showing that PSSP-1-specific antibodies could not bind IcsP on the bacterial surface of wild-type *S. flexneri* serotype 2a strain 2457T, while the same polyclonal antibodies could bind the LPS mutant *Shigella* strain, which has only one unit of O antigen (38) (data not shown). This suggests that PSSP-1-specific antibodies may react to or neutralize shigellae during certain stages of the dividing cycle, when less LPS is expressed on the surface of the bacteria at the gut site. Here, we



**FIG 4** Data showing better protection against *Shigella* infections with mucosal administration of PSSP-1 than with systemic administration in a mouse lung model. (A) Mice immunized with PSSP-1 (20  $\mu$ g i.n. or 40  $\mu$ g i.d.) plus dmLT (5  $\mu$ g i.n. or 0.5  $\mu$ g i.d.) 3 times, at 2-week intervals, were challenged with lethal doses of *S. dysenteriae* serotype 1 strain 101398 ( $5 \times 10^6$  CFU) or *S. flexneri* serotype 2a strain 2457T ( $5 \times 10^7$  CFU). Survival was observed daily. Results are representative of two independent experiments ( $n = 5$  or 6 mice per group). (B) At 7 days after the last immunization with PSSP-1 plus dmLT, serum and BALF samples were obtained and PSSP-1-specific immunoglobulin titers were determined by ELISA ( $n = 4$  per group). B.D., below detection level. (C) At 7 days after the second immunization with PSSP-1 plus dmLT, spleen and cervical lymph node cells were prepared, and anti-PSSP-1 IgG- or IgA-producing B cells were detected with an ELISPOT assay. The numbers of antibody-secreting cells (ASC) per  $10^6$  cells are shown ( $n = 4$ ). Results are representative of three independent experiments. (D) At 14 days after the third immunization with PSSP-1 plus dmLT, single cells were prepared from spleens and cervical lymph nodes (CLN) from the mice. Cells were stimulated for 72 h with a PSSP-1 whole-peptide pool or influenza virus matrix protein 2 ectodomain (M2e), and culture supernatants were analyzed for secretion of IFN- $\gamma$  and IL-17A ( $n = 5$  per group). Data are means  $\pm$  SD and are representative of two independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; n.s., not significant.

found that mucosal immunity to PSSP-1 is important for protection against *Shigella* infections. Detailed mechanisms that are involved in PSSP-1-induced protection need to be further elucidated. When the ASCs in blood samples from patients with recent-onset shigellosis were analyzed, the hierarchical level of mucosal antibody responses to PSSP-1 seemed to be lower than those of other *Shigella* Ipa proteins (Table 2). Due to our inability to obtain blood samples from patients after discharge from the hospital, we could not measure PSSP-1-specific antibody levels later. However, early antibody responses to PSSP-1 were rare in

shigellosis cases, and thus it is assumed that people with naturally acquired shigellosis have much less B cell memory for PSSP-1. Therefore, vaccination with PSSP-1 can provide protective immunity to vaccinees against repeated *Shigella* infections.

Because there is no murine gut model of shigellosis, the mouse pneumonia model has been used to test *Shigella* vaccine candidates (28). Invasions of lung epithelial cells occur with i.n. *Shigella* infections but do not mimic the gut environment in which *Shigella* infections evoke pathology in humans. We have also evaluated the guinea pig rectocolitis model of shigellosis, using intrarectal ad-

TABLE 2 Blood antibody-secreting cell responses ( $\alpha 4\beta 7$ , IgA, and IgG) to *Shigella* antigens in patients with recent-onset shigellosis<sup>a</sup>

Antigen and isotype	No. of responders/ total no.	ASC count (range) (cells/ml blood)
PSSP-1		
IgA	3/23	0–30
IgG	1/23	0–107.5
IpaC		
IgA	6/23	0–297.5
IgG	19/23	0–297.5
IpaD		
IgA	8/23	0–102.5
IgG	16/23	0–445

<sup>a</sup> ASC counts in response to *Shigella* antigen PSSP-1, IpaC, or IpaD were determined on day 7 of natural infections. For mucosal responses, IgA- or IgG-secreting cells in blood were selected on the basis of expression of the gut-homing phenotype  $\alpha 4\beta 7$ .

ministration of shigellae (39). As guinea pigs aged (weights of >300 g), however, they ceased to respond to intrarectal inoculation of virulent *Shigella*, which made it difficult to test the protective efficacy of *Shigella* vaccine candidates with long-term immunization. Further improvements of the protocols will be required to evaluate novel *Shigella* subunit vaccine candidates using this model. In conclusion, our study provides evidence that mucosal immunization with PSSP-1 induces cross-protective immunity against *Shigella* infections and therefore PSSP-1 can be utilized as a promising universal *Shigella* vaccine candidate.

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