

Mapping the functional B-cell epitopes of *Shigella* invasion plasmid antigen D (IpaD)

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ABSTRACT *Shigella* bacteria utilize the type III secretion system (T3SS) to invade host cells and establish local infection. Invasion plasmid antigen D (IpaD), a component of *Shigella* T3SS, has garnered extensive interest as a vaccine target, primarily due to its pivotal role in the *Shigella* invasion, immunogenic property, and a high degree of conservation across *Shigella* species and serotypes. Currently, we are developing an epitope- and structure-based multivalent vaccine against shigellosis and require functional epitope antigens of key *Shigella* virulence determinants including IpaD. However, individual IpaD B-cell epitopes, their contributions to the overall immunogenicity, and functional activities attributing to bacteria invasion have not been fully characterized. In this study, we predicted continuous B-cell epitopes *in silico* and fused each epitope to a carrier protein. Then, we immunized mice intramuscularly with each epitope fusion protein, examined the IpaD-specific antibody responses, and measured antibodies from each epitope fusion for the activity against *Shigella* invasion *in vitro*. Data showed that all epitope fusion proteins induced similar levels of anti-IpaD IgG antibodies in mice, and differences were noted for antibody inhibition activity against *Shigella* invasion. IpaD epitope 1 (SPGGNDGNSV), IpaD epitope 2 (LGGNGEVVLDNA), and IpaD epitope 5 (SPNNTNGSSTET) induced antibodies significantly better in inhibiting invasion from *Shigella flexneri* 2a, and epitopes 1 and 5 elicited antibodies more effectively at preventing invasion of *Shigella sonnei*. These results suggest that IpaD epitopes 1 and 5 can be the IpaD representative antigens for epitope-based polyvalent protein construction and protein-based cross-protective *Shigella* vaccine development.

IMPORTANCE *Shigella* is a leading cause of diarrhea in children younger than 5 years in developing countries (children's diarrhea) and continues to be a major threat to public health. No licensed vaccines are currently available against the heterogeneous *Shigella* species and serotype strains. Aiming to develop a cross-protective multivalent vaccine against shigellosis and dysentery, we applied novel multiepitope fusion antigen (MEFA) technology to construct a broadly immunogenic polyvalent protein antigen, by presenting functional epitopes of multiple *Shigella* virulence determinants on a backbone protein. The functional IpaD epitopes identified from this study will essentially allow us to construct an optimal polyvalent *Shigella* immunogen, leading to the development of a cross-protective vaccine against shigellosis (and dysentery) and the improvement of global health. In addition, identifying functional epitopes from heterogeneous virulence determinants and using them as antigenic representatives for the development of cross-protective multivalent vaccines can be applied generally in vaccine development.

KEYWORDS *Shigella* spp., epitope mapping, vaccine, invasion plasmid antigen, IpaD, type III secretion system (T3SS)

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Shigella is one of the major causes of diarrheal mortality worldwide and is estimated to lead to 212,000 deaths in 2016 (1). Shigellosis predominantly affects children under the age of 5 years, and accounts for approximately 54,900 deaths of young children annually (2). In addition, *Shigella* infections are reported to result in linear growth faltering (3, 4) and a significant impact of environmental enteric dysfunction (EED) (5), leading to poor longer-term outcomes including impaired cognitive development, academic underachievement at school, and limited economic opportunities (6–8).

Shigellosis could be treated and/or prevented theoretically. Oral rehydration therapy and antibiotic drugs are the typical treatments for severe infection but that brings heavy pressure and financial burdens to families and local healthcare systems in developing countries or regions (9). Indeed, many rural areas cannot access these facilities and resources. In addition, the increased and repeated usage of antibiotics led to the rapid acquisition of antimicrobial resistance among *Shigella* and other bacteria (10). Improved water, sanitation, and hygiene (WASH) could effectively prevent shigellosis and other enteric diseases, but financial constraints faced by developing countries, particularly countries in Southeast Asia and sub-Saharan Africa, prevent this program from being implemented for the foreseeable future. Vaccines would be a more cost-effective and practical prevention countermeasure against shigellosis. Unfortunately, there are no licensed vaccines for *Shigella* infections.

A safe, cost-effective, cross-protective, and ideally cold chain-free vaccine against heterogeneous *Shigella* species and serotypes is urgently needed. Such a vaccine would reduce morbidity and mortality in children under 5 years and also curb the threat of antibiotic resistance. However, developing vaccines against *Shigella* has been proven to be challenging. Immunological heterogeneity among *Shigella* serotypes and unique disease mechanisms are among the key difficulties in *Shigella* vaccine development. There are 4 species and over 50 serotypes in the genus of *Shigella*. These bacteria elaborate on a unique pathogenesis strategy to establish local infection, including adhesion, invasion, immune evasion, replication, and intercellular mobility (11, 12). Particularly, the type III secretion system (T3SS), a needle-like structure with an associated tip complex plays a major role in the disease mechanism (13). In T3SS, invasion plasmid antigens including invasion plasmid antigen D (IpaD) provide a physical platform and are responsible for sensing the host environment, leading to the activation of the T3SS upon contact with the host epithelial cells (11, 14). In addition, *Shigella* can impair host-adapted immunity development via IpaD-mediated B lymphocyte apoptosis (15). Targeting IpaD was indicated to be a promising approach against *Shigella* infections. Indeed, anti-IpaD antibodies were found to neutralize *Shigella* virulence properties, including invasion (16, 17) and hemolysis (18, 19). Furthermore, IpaD was identified as a broadly protective vaccine immunogen (20, 21).

Recently, based on a novel vaccinology platform, multiepitope fusion antigen (MEFA) (22), we designed an epitope- and structure-based polyvalent protein antigen called *Shigella* MEFA. This *Shigella* MEFA was constructed by incorporating epitopes from several virulence factors (invasion plasmid antigen B - IpaB, IpaD, VirG, GuaB, and Shiga toxins 1 and 2) and demonstrated to induce functional antibodies against *Shigella* invasion and Shiga toxin cytotoxicity and cross-protect mice from lethal pulmonary infection by *S. sonnei* or *S. flexneri* serotype 2a, 3a, or 6 (23). However, the epitopes included in that *Shigella* MEFA protein were predicted based on antigenicity *in silico* but not functional immunity empirically. Knowing that an immunodominant epitope may not be able to induce functional immunity, this *in-silico*-based *Shigella* MEFA may not be optimal functionally. To optimize the *Shigella* MEFA protein design and maximize functional immunity, we need to first identify functional epitopes from each target virulence determinant. We have mapped the IpaB epitopes and identified the top-ranked ones for this particular virulence (24), but protective epitopes from the other key virulence factors are yet to be determined.

In this study, we mapped the functional B-cell epitopes for virulence determinant IpaD. We first *in silico* predicted the immunodominant B-cell epitopes, then genetically

fused each epitope to a carrier for an epitope fusion protein, subsequently immunized mice with each epitope fusion protein, and measured the functional activity of epitope fusion-induced antibodies against *Shigella* bacteria invasion to identify functional epitopes for IpaD. Top-ranked IpaD epitopes can then be the antigenic representatives for this key virulence determinant and used to optimize the *Shigella* MEFA protein and potentially enhance vaccine efficacy against *Shigella* infections.

RESULTS

IpaD epitopes were *in silico* predicted and genetically fused to carrier protein CsaB

Eight continuous immunodominant B-cell epitopes were identified based on *in silico* antigenicity scores, using the BepiPred 2.0 epitope prediction software (25). They were EP1 (SPGGNDGNSV), EP2 (LGGNGEVLDNA), EP3 (SAEETMKNN), EP4 (EYPINKDARE), EP5 (SPNNTNGSSTET), EP6 (TLTNSISTSS), EP7 (VQKYSNANSIFD), and EP8 (KWLTELGGTIG) (Fig. 1A). All epitopes were conserved among *S. dysenteriae* Sd197, *S. boydii*, *S. sonnei*, and *S. flexneri* 2a, and protein modeling suggested these epitopes were surface exposed on the IpaD protein (Fig. 1A and B; each epitope in a different color). Nucleotides encoding each epitope were genetically fused to carrier protein CsaB, a major subunit protein of the enterotoxigenic *Escherichia coli* (ETEC) adhesin CS4, leading to eight epitope fusion genes (Fig. 2A). Each epitope fusion gene was cloned into vector pET28a and confirmed with DNA sequencing. Expressed in *E. coli* BL21 (DE3), eight recombinant epitope fusion proteins, at the size of around 18 kDa, were generated (Fig. 2B). Recombinant epitope fusion proteins were characterized with anti-IpaBD fusion mouse antiserum in Western blot (Fig. 2C).

IpaD epitope fusion proteins induced anti-IpaD antibody response in mice

Mice intramuscularly (i.m.) immunized with each IpaD epitope fusion protein (CsaB-IpaD epitope fusion) developed robust antibody responses to IpaD protein (Fig. 3). Anti-IpaD IgG titers (\log_{10}) in serum samples of the mice immunized with EP1, EP2, EP3, EP4, EP5, EP6, EP7, or EP8 epitope fusion protein were detected at 3.68 ± 0.21 , 3.74 ± 0.22 , 3.42 ± 0.06 , 3.73 ± 0.26 , 3.81 ± 0.36 , 3.84 ± 0.18 , 3.67 ± 0.24 , 3.71 ± 0.19 , respectively. Mice i.m. immunized with recombinant protein IpaD, which was used as a positive control, had

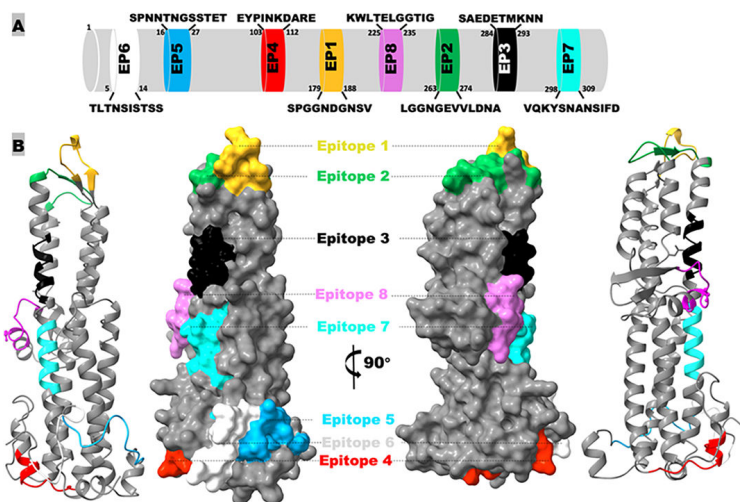


FIG 1 *In silico* identification of continuous B-cell epitopes of *Shigella* IpaD protein. (A) The epitope positions and amino acid sequences. (B) Illustration of IpaD epitope surface exposure and location on protein modeling, generated by Phyre 2 based on the *S. flexneri* 2a sequence (protein no. NP_858259) and visualized by ChimeraX-1.6.1. This model is archived in the Model archive database with the identifier "ma-rf0jy."

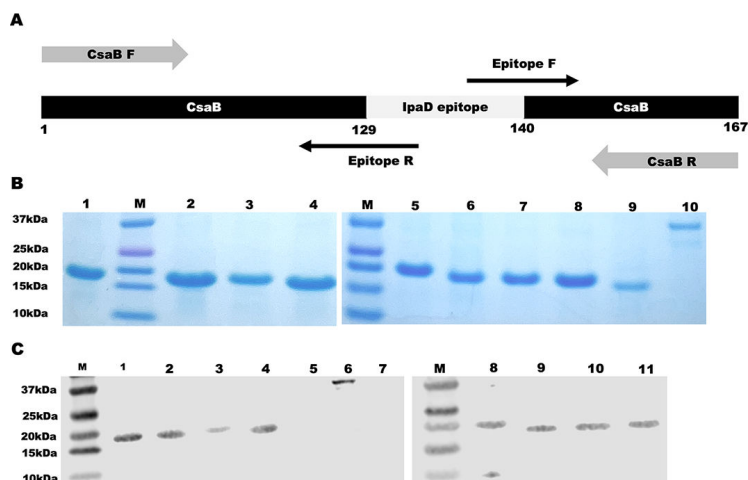


FIG 2 Construction, extraction, and characterization of IpaD epitope fusion proteins. (A) Illustration of IpaD epitope fusion construction, with two overlapping PCR amplifications using primers CsaB-F/epitope-R and epitope-F/CsaB-R to substitute a backbone CsaB epitope (amino acids 130–139) with each IpaD epitope. (B) IpaD epitope fusion protein in SDS-PAGE with Coomassie blue staining; epitope fusion protein from EP1 to EP8 in lanes 1 through 8, backbone protein CsaB in lane 9, and recombinant IpaD protein in lane 10. (C) IpaD epitope fusion protein characterization in Western blot with anti-DB fusion (IpaD and IpaB fusion protein) mouse serum (diluted 1:6,000); EP1 to EP4 in lanes 1 to 4, EP5 to EP8 in lanes 8 to 11, CsaB in lane 5, and IpaD in lane 6, and host cell *E. coli* BL21 proteins in lane 7.

anti-IpaD IgG detected at 4.63 ± 0.19 (\log_{10}). Serum samples from the groups of control mice, which were administrated with phosphate-buffered saline (PBS) or carrier protein CsaB (mouse sera from the other studies in the laboratory), showed no detectable anti-IpaD IgG response.

Antibodies derived from EP1, 2, or 5 epitope fusion protein significantly inhibited *Shigella* bacteria invasion *in vitro*

Antibody invasion inhibition assays showed that the number of *S. sonnei* bacteria (CFUs) invaded HeLa cells was reduced by $39.5\% \pm 16.62\%$, $25.2\% \pm 14.89\%$, $14.7\% \pm 21.30\%$, $17.6\% \pm 16.35\%$, $47.2\% \pm 6.73\%$, $17.5\% \pm 17.42\%$, $1\% \pm 17.24\%$, $22.2\% \pm 11.66\%$, and $64.2\% \pm 10.45\%$, respectively, after *S. sonnei* bacteria being incubated with

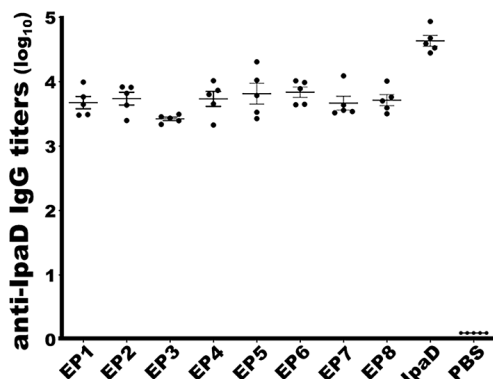


FIG 3 Mouse serum anti-IpaD IgG titers (in \log_{10}) from each immunization group or the control group. EP1 to EP8, IpaD, or PBS represents mouse sera from the group i.m. immunized with IpaD epitope fusion 1, 2, 3, 4, 5, 6, 7, or epitope fusion 8, recombinant IpaD protein, or PBS the control. $40 \mu\text{g}$ epitope fusion protein (EP1 to EP8) or the IpaD recombinant protein, or $40 \mu\text{l}$ PBS, adjuvanted with $0.2 \mu\text{g}$ of dMLT, were i.m. injected into each mouse in the group. Bars indicated IgG titer means and standard deviations.

heat-inactivated sera from the group immunized with EP1, EP2, EP3, EP4, EP5, EP6, DP7, EP8 epitope fusion protein, or the IpaD recombinant protein, with numbers of the invaded bacteria treated with the control serum were referred to as 100% (Fig. 4). Statistical analyses showed that the reduction from incubation with sera of the mice immunized with epitope fusion protein EP1 or EP5 was significantly different than the PBS control group ($P < 0.0001$).

When *S. flexneri* 2a bacteria were examined, the *in vitro* invasion was reduced by $36.9\% \pm 9.93\%$, $31.4\% \pm 8.29\%$, $16.2\% \pm 8.35\%$, $18.0\% \pm 11.99\%$, $37.2\% \pm 13.24\%$, $12.4\% \pm 12.87\%$, $-8.6\% \pm 37.52\%$, $20.1\% \pm 10.28\%$, and $62.0\% \pm 14.43\%$ respectively, after incubation with heat-inactivated sera from the group immunized with EP1, EP2, EP3, EP4, EP5, EP6, DP7, EP8 epitope fusion protein, or the IpaD recombinant protein. Statistical analyses indicated the reduction from the sera of the group immunized with epitope fusion EP1 ($P = 0.0002$), EP2 ($P = 0.0041$), or EP5 ($P = 0.0002$) was significantly different than the control (Fig. 4).

EP1 and EP5 were the top-ranked functional epitopes of *Shigella* IpaD

Serum antibodies from the mice immunized with each IpaD epitope fusion protein reduced *S. sonnei* or *S. flexneri* 2a invasion *in vitro*, except for the sera from epitope fusion EP7. However, sera from the mice immunized with EP1 epitope fusion or EP5 epitope fusion protein were shown more effective against invasion of *S. sonnei* and *S. flexneri* 2a. The antibody invasion inhibition levels from EP1 or EP5 epitope fusion protein were not significantly different compared to those from the positive control IpaD protein. For invasion inhibition against *S. sonnei*, 39.5% of EP1 was not statistically different from 64.2% of IpaD ($P = 0.08$), and 47.2% of EP5 was not significantly different than 64.2% of IpaD ($P = 0.999$). For invasion inhibition against *S. flexneri* 2a, 36.9% from EP1 compared to 62% from IpaD ($P = 0.07$), or 37.2% from EP5 than 62% from IpaD ($P = 0.08$), was not significantly different.

DISCUSSION

One of the major challenges in developing vaccines against shigellosis is the heterogeneity among *Shigella* species and serotypes. Differed from the LPS-based vaccines that target specific serotypes and thus are unable to cover different *Shigella* species

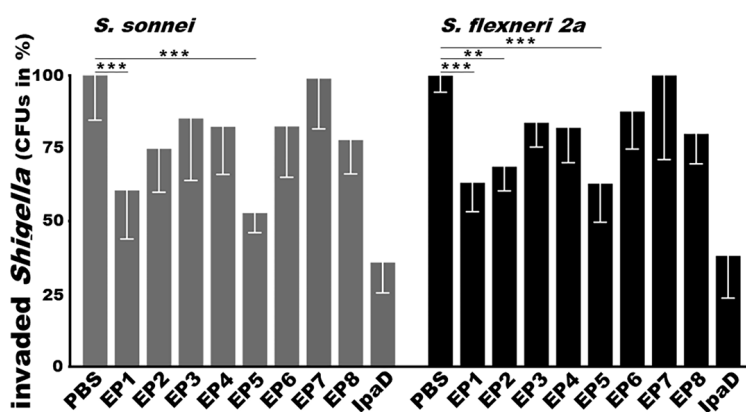


FIG 4 *In-vitro* antibody invasion inhibition against *Shigella sonnei* or *S. flexneri* 2a. Heat-inactivated mouse sera from the group i.m. immunized with PBS (control), IpaD epitope fusion 1, 2, 3, 4, 5, 6, 7, or epitope fusion 8, or IpaD recombinant protein were mixed with *S. sonnei* or *S. flexneri* 2a bacteria, and then transferred to confluent monolayered Hela cells. After eliminating extracellular *Shigella* bacteria, cells were lysed; the invaded *Shigella* bacteria were collected, cultured overnight, and counted (CFUs). CFUs were converted to percentages, referring to the CFUs from treatment with control mouse sera as 100%. Boxes and bars indicated means and standard deviations. ** and *** indicated $P < 0.01$ and $P < 0.001$, respectively.

and heterogeneous serotypes, a protein-based multivalent vaccine to target protein antigens conserved across *Shigella spp.* and serotypes can potentially be cross-protective. Such a multivalent vaccine can be produced conventionally, by mixing a few protein immunogens into a cocktail product or genetically fusing two or more proteins as a fusion protein, but with the disadvantage of vaccine formulation and manufacturing complexity (26). Since not all protein segments or domains elicit host immune responses and only functional peptide domains or epitopes induce protective immunity, an alternative vaccine antigen approach has been developed. Instead of an entire protein, functional peptides or epitopes, if they are conserved, can be used as vaccine antigens to induce broadly functional immunity against heterogeneous pathogenic strains or isolates (27). To develop an epitope-based multivalent protein vaccine against heterogeneous *Shigella spp.* and serotypes, we applied an epitope- and structure-based vaccine technology, MEFA (22), and constructed a polyvalent protein antigen "*Shigella* MEFA" by presenting epitopes from IpaB, VirG, GuaB, Shiga toxin A and B subunits on backbone protein IpaD (28). We further demonstrated that the resultant polyvalent *Shigella* MEFA induced broadly functional antibodies and the antibodies inhibited invasion of all *Shigella* species and the important serotype strains and neutralized cytotoxicity of Shiga toxins; more importantly, this *Shigella* MEFA protein protected mice against pulmonary lethal challenge from *Shigella sonnei* or *Shigella flexneri* 2a, 3a, or 6 strain (28).

While the epitopes of that *Shigella* MEFA protein functioned collectively, it is unknown if each epitope was functional individually or if an individual epitope was the better one representing the target virulence factor even if it was functional. We believe that the use of top-ranked functional epitopes from each target virulence factor (IpaB, IpaD, VirG, GuaB, and Shiga toxins) will improve *Shigella* MEFA in eliciting protective immunity, thus becoming a better antigen for a more protective *Shigella* vaccine. In addition, since both IpaD and IpaB are involved in *Shigella* bacterial invasion into host cells, epitopes of these two virulence factors can function synergistically; therefore, we do not know whether the representative IpaB or IpaD epitope in the previous *Shigella* MEFA protein is functional individually. With the functional epitopes of IpaB identified empirically in a recent study (24), we focused on functional epitope mapping for IpaD in the current study. Moreover, because IpaD was designed as the backbone of the *Shigella* MEFA immunogen, identifying IpaD functional epitopes will allow us to retain the top-ranked IpaD epitope on the backbone for maximum IpaD protective immunity and improve future design of an optimal *Shigella* MEFA immunogen by substituting the less or not functional IpaD epitopes with the top-ranked functional epitopes from the other target virulence factors.

Data from this study indicated that each IpaD-epitope fusion protein elicited robust anti-IpaD antibody responses, but sera from the mice immunized with EP3, EP4, EP6, EP7, or EP8 epitope fusion were significantly less effective in inhibiting *in vitro* invasion of *S. sonnei* and *S. flexneri* 2a. By contrast, the fusion proteins with EP1 (IpaD 179–188), EP2 (IpaD 263–274), or EP5 (IpaD 16–27) were as effective as the IpaD recombinant protein in inducing functional antibodies against *S. flexneri* 2a invasion. Similarly, sera from the mice immunized with EP1 fusion, EP5 fusion, or recombinant IpaD showed similar inhibitory activities against *in vitro* invasion of *S. sonnei*. These results suggest that IpaD epitope 1 and epitope 5 can serve as surrogate antigens of IpaD to induce functional antibodies against this key virulence factor.

Early studies suggested that peptides nearby at or overlapping with EP1 or EP2 are important against *Shigella* invasion. Monoclonal antibody "IpaD-318" was demonstrated to directly bind and neutralize *S. flexneri* 2a invasion into HeLa cells (16). The epitope associated with MAb IpaD-318 was suggested to be within residues 273–278 and the area between 175 and 191 of IpaD (16). Interestingly, the EP1 (IpaD 179–188) and EP2 (IpaD 263–274) share overlapping regions with the reported binding areas of MAb IpaD-318. In addition, early site-directed mutagenesis studies showed that a deletion of IpaD peptide 161–180 (29), 171–180 (30), or 181–190 (30), or substitution of IpaD N186Y significantly impaired *Shigella* invasion (31). These peptide deletions or the substitution

occurred within the region of EP1 (179-188), supporting the important role of epitope EP1 in *Shigella* invasion and the selection of this epitope as a vaccine antigen for immunity against *Shigella* invasion.

The current study found that EP2 (IpaD 263–274) induced functional antibodies against *S. flexneri* 2a invasion. The importance of peptides in this site was also suggested by early studies. It was demonstrated that mutations, regardless they were deletions or substitutions, including deletion of IpaD peptide 241–280 (29), 261–270, or 271–280 (30), or alterations of a residue at positions N273I and Q277L (31), consistently led to an impairment in bacteria invasion. Peptide IpaD 192–267, which overlaps with EP2 (263-274), was also suggested to be the binding site for IpaB since the removal of this domain eliminated the interaction of IpaD with IpaB, a requirement for T3SS function and *Shigella* bacteria invasion (32).

Results from the current study, while they agree with some early studies, also challenge other observations from previous studies. Specifically, earlier studies showed that deletions of IpaD peptide 1–20 (29), 221–230 (30), 231–240 (30), 281–290 (30), or 281–320 (29) significantly altered *Shigella* invasion. In this study, we found that antibodies derived from the fusion with EP3 (IpaD 284–293, overlaps with 281–290 and 281–320), EP6 (IpaD5-14, overlaps with 1–20), EP7 (IpaD298-309, overlaps with 281–320), or EP8 (IpaD 225–235, overlaps with 221–230 and 231–240) did not significantly affect *in vitro* invasion of either *Shigella* strain tested (*S. sonnei*, *S. flexneri* 2a). On the other hand, our data support other early studies, which indicated residue substitution at the N292Y (within EP3) (31), Y301A and N305A (within EP7) (33), W226A (within EP8) (33) not significant in affecting *Shigella* invasion. We found that EP3 (IpaD 284–293, including N292), EP7 (IpaD 298–309, including Y301 and N305), and EP8 (IpaD225-235, including W226) play a less important role in *Shigella sonnei* or *Shigella flexneri* 2a invasion. While the exact causes of these discrepancies among these early studies remain unclear to us, one possible reason could be the structural or antigenic alteration resulting from residue deletion in the region, since a peptide deletion could more drastically alter the antigenicity when compared to a substitution mutation (34).

Data from this study showed that EP5 (IpaD16-27) plays a role in inducing functional antibodies against *Shigella* bacteria invasion. EP5 is located in the N-terminal which is poorly understood. An early study showed that, unlike deletions in the C-terminus, truncation of the N terminus resulted in a less severe attenuation in *Shigella* invasion ability (29). However, other studies demonstrated that IpaD N-terminus induced protective immunity against *Shigella* infection in the Sereny test and suggested that antibodies derived from IpaD N terminus could potentially block *Shigella* invasion (35–37).

In conclusion, data from this study indicated that antibodies derived from fusion proteins with epitope EP1 (179-188), EP2 (263-274), or EP5 (IpaD16-27) inhibited *Shigella sonnei* or *flexneri* 2a invasion *in vitro*. We need to point out that the binding structure of IpaD to the functional antibodies is yet to be determined, future monoclonal antibody production and mapping studies, or better *in vivo* studies such as immunizing mice with the epitope fusion antigen and then challenging mice with *Shigella* bacteria in a pulmonary model, should help us to further validate IpaD functional or protective epitopes. Also, we focused on B-cell epitopes in the current study, T-cell epitopes which play a role in lasting immunity will need to be examined in future studies. In addition, we tested the two most important species in this study, whether antibodies from these epitopes also protect against invasion from other *Shigella spp.* or other serotypes is yet to be determined. Nevertheless, results from this study could be significant for understanding the antigenicity of IpaD, and the functional epitopes identified from this study will guide us to optimize the *Shigella* MEFA immunogen and develop a better protective vaccine against shigellosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers

Bacteria and plasmids used in this study are listed in Table 1. *E. coli* DH5 α (Promega; Madison, WI) was used to host the plasmid carrying each IpaD epitope fusion gene, and BL21(DE3) (Agilent Technologies, Santa Clara, CA) was used for epitope fusion protein expression. *S. sonnei* 53g (9904) and *S. flexneri* 2a 2457T (9905) provided by Dr. David A. Sack at the Johns Hopkins University and Dr. Eileen Barry at the University of Maryland School of Medicine, respectively, were used in antibody invasion inhibition assays. Plasmid 9765, pET28 α vector with ETEC adhesin CS4 major subunit *csaB* gene (38), was used as the template to construct IpaD epitope fusion genes. All IpaD epitope fusion genes were cloned into vector pET28 α .

In silico prediction of IpaD B-cell immunodominant epitopes and construction of epitope fusion genes

IpaD protein continuous B-cell immunodominant epitopes were *in silico* predicted first based on IpaD amino acid sequence (accession: [NP_858259](#)), using BepiPred-2.0 software (25). As previously described (22, 24), amino acid sequences of the predicted epitopes were examined and confirmed to be conserved among *S. dysenteriae* Sd197, *S. boydii*, *S. sonnei*, and *S. flexneri* 2a. Epitopes were ranked based on antigenicity scores and the top eight epitopes were selected and designed as epitopes 1 through 8 (EP1 to EP8). IpaD protein structure was visualized with UCSF ChimeraX-1.6.1 (39), and each epitope was verified for surface exposure and localized on the IpaD protein model (ID ma-rf0iy) using the Protein Homology/AnalogY Recognition Engine V 2.0 (Phyre 2) (40).

Plasmid 9765 with the *csaB* gene cloned in pET28 α was used as the DNA template for IpaD epitope fusion gene construction. Nucleotides coding each of the eight selected IpaD epitopes were inserted into the *csaB* gene using splicing overlap extension (SOE) PCR as previously described (24), with specific PCR primers (Table 2). Each CsaB-IpaD epitope fusion gene was cloned into pET28 α , at NheI and EagI restriction enzyme sites

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source/reference
<i>E. coli</i> strains		
DH5 α	<i>fhuA2</i> Δ (<i>argF-lacZ</i>) U169 <i>phoA glnV44</i> ϕ 80 Δ (<i>lacZ</i>)M15 <i>gyrA96</i>	Promega
BL21(DE3)	<i>recA1 relA1 endA1 thi-1 hsdR17</i> <i>huA2</i> Δ (<i>argF-lacZ</i>) U169 <i>phoA glnV44</i> ϕ 80 Δ (<i>lacZ</i>)M15 <i>gyrA96</i>	Agilent
9765	CS4 major subunit (CsaB) in pET28 α / DH5 α , Kan ⁺	(38)
9736	IpaD in pET28 α /BL21, Kan ⁺	This study
9933	IpaD-epitope-1-CsaB in pET28 α /BL21	This study
9934	IpaD-epitope-2-CsaB in pET28 α /BL21	This study
9935	IpaD-epitope-3-CsaB in pET28 α /BL21	This study
9936	IpaD-epitope-4-CsaB in pET28 α /BL21	This study
9939	IpaD-epitope-5-CsaB in pET28 α /BL21	This study
9940	IpaD-epitope-6-CsaB in pET28 α /BL21	This study
9941	IpaD-epitope-7-CsaB in pET28 α /BL21	This study
9942	IpaD-epitope-8-CsaB in pET28 α /BL21	This study
<i>Shigella</i> strains		
9904	<i>Shigella sonnei</i> 53G	Johns Hopkins University
9905	<i>Shigella flexneri</i> 2457T	University of Maryland
Plasmid		
pET28 α	Protein expression vector	Novagen

TABLE 2 PCR primers used to insert each IpaD epitope into *E. coli* CS4 adhesin major subunit gene *csaB* in the study

Primer	Sequence (5'–3')	Amplified region
IpaD-F	CGGGCTAGCATGAATATAACAACCTGACTAA	Upstream of ipaD, with NheI site
IpaD-R	CGGCCGTCAGAAATGGAGAAAAAGTT	Downstream of ipaD, with EagI site
Ep1F	CGGAGGTAACGACGGAAACTCCGTGttagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep1 into CsaB
Ep1R	AGTTTCCGTCGTTACCTCCGGGAGAtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep1 into CsaB
Ep2F	AAATGGCGAGGTTGTGCTAGATAATGCAttagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep2 into CsaB
Ep2R	CTAGCACAACTCGCCATTTCCACCTAGtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep2 into CsaB
Ep3F	CGAAGATGAAACAATGAAAAATAATtagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep3 into CsaB
Ep3R	TTTTATTGTTTCATCTTCGGCAGAtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep3 into CsaB
Ep4F	TCCAATTAATAAGACGCAAGAGAAAttagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep4 into CsaB
Ep4R	TTGCGCTTTTATAATTGGATATTCtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep4 into CsaB
Ep5F	CAATACCAACGGTTCATCAACCGAAACAttagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep5 into CsaB
Ep5R	TTGATGAACCGTTGGTATTGTTGGACTtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep5 into CsaB
Ep6F	GACTAATAGTATTTCCACCTCATCAttagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep6 into CsaB
Ep6R	AGGTGGAAATACTATTAGTCAGAGTgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep6 into CsaB
Ep7F	ATACAGTAATGCCAATAGTATTTTTGATtagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep7 into CsaB
Ep7R	TACTATTGGCATTACTGTATTTTTGAACtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep7 into CsaB
Ep8F	CTTACAGAATTAGGTGGAACAATCGGCttagtgattggtgcgactac	Paired with CsaB-R to insert IpaD-ep8 into CsaB
Ep8R	TGTTCCACCTAATTCTGTAAAGCCATTTtgacgttccaaaattaatt	Paired with CsaB-F to insert IpaD-ep8 into CsaB
CsaB-F	CTAGCTAGCtagagaaaaatcactgtaa	Forward primer to amplify <i>csaB</i> gene
CsaB-R	TCACGGCCGTTAttatgatgctaaggctcattaa	Reverse primer to amplify <i>csaB</i> gene

for tag-less recombinant proteins, and the resultant plasmid was hosted by *E. coli* DH5a or BL21 (DE3) strain.

IpaD epitope fusion protein expression, purification, and characterization

Tag-less epitope fusion proteins were expressed, extracted, and refolded as we described previously (23, 24, 41). Briefly, a single overnight-grown colony of IpaD epitope fusion strain (in *E. coli* BL21 DE3) was cultured overnight in Luria-Bertani (LB) broth supplemented with kanamycin (30 µg/mL), then sub-cultured in fresh 2xYT broth (kanamycin 30 µg/mL) and grown to the log phase (OD₆₀₀ ranging from 0.5 to 0.7) in a shaking incubator (37°C, 200 rpm). Bacteria were induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma, MO) for approximately 4 h, then harvested with centrifugation (13,000 × *g*, for 15 min at 4°C), and stored in a –80°C freezer overnight. Bacteria pellets were thawed on ice, suspended with 10 mL bacterial protein extraction reagent (B-PER, in phosphate buffer, pH 7.5; Thermo Fisher Scientific, Rochester, NY), further lysed with lysozyme (200 µg/mL), and sonication (130W, 20 kHz for 15 min by Ultrasonic processor GEX130; Cole Parmer, Vernon Hills, IL). Lysates were collected for extraction of inclusion body proteins by following the manufacturer's protocol (Thermo Fisher Scientific).

Inclusion body proteins were solubilized in 50 mM CAPS [3-(cyclohexylamine) propane sulfonic acid, pH11.0; Sigma] supplemented with 0.3% N-lauroylsarcosine and 1 mM dithiothreitol (DTT). Solubilized proteins in Spectrum membrane tubing (6–8 kDa; Fisher Scientific) were refolded with dialysis buffer 1M Tris-HCl (pH 8.5) in a cold room, with 2x exchanges of the buffer (8 h per exchange). Refolded proteins were collected, aliquoted, examined in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Coomassie blue staining, and characterized in Western blotting using anti-IpaBD fusion antibodies (42).

Mouse intramuscular immunization with IpaD epitope fusion proteins

A total of 10 groups of 8-week-old BALB/c female mice (Charles River Laboratories, Wilmington, MA), 5 mice per group, were included in the immunization study. Eight groups were administrated intramuscularly the eight IpaD epitope fusion proteins respectively, 40 µg fusion protein per mouse. One group was immunized with 40 µg IpaD

recombinant IpaD as the positive control, and another group with 1× PBS as the control. Double mutant heat-labile toxin (dmLT, LT_{R192G/L211A}; a gift from Dr. Jacob Bitoun at Tulane University), at a dose of 0.2 µg, was included in all groups. One primary was followed by two boosters, at an interval of 2 weeks. Two weeks after the final booster, all mice were sacrificed; blood samples were collected from each mouse, and sera were stored at −80°C until use. The mouse study (protocol #22088) was approved by the IACUC at the University of Illinois at Urbana-Champaign.

Mouse serum anti-IpaD IgG titration

Serum samples from each mouse were examined for antibody response to IpaD in enzyme-linked immunosorbent assay (ELISA) as described previously (43). Briefly, 100 ng IpaD recombinant protein, in 100 µL bicarbonate/carbonate antigen coating buffer (50 mM), was coated to each well of 96-well Immulon 2-HB plates (Thermo Fisher Scientific). After incubation at 37°C for 1 h and 4°C overnight, wells were blocked with 10% skim milk (in PBST), and then incubated with twofold serum dilutions (1:400 to 1:25,600), in triplicates. Wells were washed with PBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Sigma, St. Louis, MO) at 37°C for 1 h. After washes (3×) with PBST, wells were added with 3,3',5'-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD) and incubated at room temperature for 20 min. OD was read at a wavelength of 650 nm with a SPECTROstar^{Nano} spectrometer (BMG LABTECH, Germany). OD₆₅₀ readings at the highest serum dilution (>0.3 after subtraction of background readings) were converted to antibody titers and expressed in log₁₀ as previously described (43).

Mouse serum antibody invasion inhibition assay to measure antibody function against *Shigella* bacteria invasion

Shigella bacteria invade HeLa cells; functional antibodies derived from IpaD or IpaB epitopes block *Shigella* invasion *in vitro*. As described previously (24), mouse sera from each group, after inactivation at 56°C for 30 min, were mixed with *S. sonnei* or *S. flexneri* 2a (~ 2×10⁷ CFUs, colony forming units). After 25 min at room temperature, each serum and bacteria mixture was transferred to 95%–100% confluent monolayered HeLa cells, a gift from Dr. Dongwan Yoo at the University of Illinois Urbana-Champaign and cultured in wells of a 24-well tissue culture plate. After 2 h of incubation, cells were rinsed with PBS and then treated with DMEM containing gentamicin (300 µg/mL, Sigma) to eliminate extracellular bacteria. Cells were then rinsed and lysed with 0.5% Triton X-100 solution to release the invaded intracellular *Shigella* bacteria. Lysates were suspended with PBS, serially diluted, and plated on LB agar plates. After overnight culture at 37°C, CFUs were counted and converted to a percentage by referring the CFUs from bacteria treated with the control mouse sera as 100%.

Statistical analyses

Statistical analyses were performed using the GraphPad Prism version 7.0.0 (GraphPad Software, San Diego, California, USA). Two-way analysis of variance (ANOVA) was performed, and Bonferroni's multiple comparisons test was used to examine the statistical difference in antibody invasion inhibition across treatment groups. A $P < 0.05$ indicated a difference statistically significant. Error bars represent standard deviations from the means.

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