Single-domain antibodies pinpoint potential targets within \textit{Shigella} invasion plasmid antigen D of the needle tip complex for inhibition of type III secretion

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Numerous Gram-negative pathogens infect eukaryotes and use the type III secretion system (T3SS) to deliver effector proteins into host cells. One important T3SS feature is an extracellular needle with an associated tip complex responsible for assembly of a pore-forming translocon in the host cell membrane. \textit{Shigella} spp. cause shigellosis, also called bacillary dysentery, and invade colonic epithelial cells via the T3SS. The tip complex of \textit{Shigella flexneri} contains invasion plasmid antigen D (IpaD), which initially regulates secretion and provides a physical platform for the translocon pore. The tip complex represents a promising therapeutic target for many important T3SS-containing pathogens. Here, in an effort to further elucidate its function, we created a panel of single-VH domain antibodies (VHHs) that recognize distinct epitopes within IpaD. These VHHs recognized the \textit{in situ} tip complex and modulated the infectious properties of \textit{Shigella}. Moreover, structural elucidation of several IpaD–VHH complexes provided critical insights into tip complex formation and function. Of note, one VHH heterodimer could reduce \textit{Shigella} hemolytic activity by >80%. Our observations along with previous findings support the hypothesis that the hydrophobic translocator (IpaB in \textit{Shigella}) likely binds to a region within the tip protein that is structurally conserved across all T3SS-possessing pathogens, suggesting potential therapeutic avenues for managing infections by these pathogens.

Type III secretion systems (T3SS)$^3$ are used by many Gram-negative bacterial pathogens for the subversion of target eukaryotic cells (1). The T3SS directly injects host-modulating effector proteins in order to alter host cell activities for the benefit of the pathogen. The T3SS is a macromolecular machine comprising four key parts. A cytoplasmic sorting platform dictates the hierarchy of effector protein secretion and is powered by an imbedded ATPase. It is associated with a basal body that spans the entire bacterial envelope (inner membrane, cell wall, and outer membrane). External components of the apparatus include a hollow needle filament that bridges the pathogen and host and a needle tip complex (TC) that controls secretion status and assembles a translocon pore within the host cell membrane (2). Control of effector protein secretion by the TC occurs in response to environmental stimuli (3, 4). Despite its critical role in proper T3SS function, many questions remain about the stoichiometry and function of the nascent TC (5–7).

Components of the T3SS are well conserved with regard to their overall structure, architecture, and function despite relatively low levels of sequence identity across divergent pathogens. The TC is an example of structural conservation, and these can be clustered into seven phylogenetic families, of which three are common in human pathogens: the Ipa family (\textit{Shigella}, \textit{Salmonella}, and \textit{Burkholderia}), LcrV/Yop (\textit{Yersinia} and \textit{Pseudomonas}), and Esp (Enteropathic \textit{Escherichia coli} and \textit{Bordetella}) (8). The steps involved in TC assembly and secretion activation have been studied extensively for the enteric pathogen \textit{Shigella flexneri}, causative agent of shigellosis (bacillary dysentery). The \textit{Shigella} tip protein, IpaD, assembles at the distal end of the needle as a pentameric ring (9, 10) that prevents premature secretion of effectors until exposure to small molecules such as bile salts, which trigger a structural rearrangement and recruitment of the first hydrophobic translocator protein, IpaB, to the TC (11). By controlling recruitment and maintenance of IpaB, the TC regulates the timing of translocon pore formation, which occurs quickly upon contact with the host cell membrane. Contact with the host membrane then triggers the recruitment of a second hydrophobic translo-

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$^3$The abbreviations used are: T3SS, type III secretion system; TC, tip complex; Ipa, invasion plasmid antigen; VHH, variable domains of camelid heavy chain-only antibodies; CDR, complementarity determining region; FL, full-length.
cator protein (IpaC) to the TC with concomitant translocon pore formation and induction of effector protein secretion into the host cell to promote pathogen entry (12).

The crystal structure of IpaD has been well studied and is composed of three domains that collectively possess a dumbbell shape. A stabilizing central coiled-coil (a common motif within external T3SS components; Ref. 13) is flanked by an N-terminal \( \alpha \)-helical domain that may control oligomerization state (14) and a mixed \( \alpha/\beta \) “distal domain” that is involved in interactions with IpaB (5). These structural features are generally retained in the tip proteins across the three TC families, with a few notable exceptions including a genetically separate chaperone (in place of the N-terminal \( \alpha \)-helical domain of IpaD) for the LcrV family (14) and the absence of structural information other than a coiled-coil domain for the Esp family (15). In contrast to the abundance of high-resolution structural information on monomeric tip proteins, most information regarding the in situ multimeric TC has primarily been limited to low-resolution electron microscopy approaches. This has resulted in the generation of incongruent TC models with respect to stoichiometry, orientation, and protein composition (6, 7, 10, 16).

To further our understanding of the function of the T3SS TC complex and to explore the possibility of generating a novel therapeutic, we generated a panel of single-chain camelid antibodies against Shigella IpaD. Camelid species produce a class of heavy chain-only antibodies that require only a single-VH domain (VHH) to recognize cognate antigens. These VHHs can be expressed as recombinant proteins in \( E. coli \) and possess high conformational stability. VHHs typically recognize conformational epitopes and can be valuable reagents to track conformational changes and probe for structure-function relationships (17). Our collection of VHHs included several that are capable of inhibiting the virulence properties of \( S. flexneri \) as well as the ability to bind directly to the surface of the pathogen. We then identified these structural epitopes by solving the co-crystal structures of IpaD in complex with four different VHHs that displayed differing degrees of pathogen neutralization. This has allowed us to identify potential functional importance of structural epitopes, especially within the IpaD distal domain, with respect to the domain structure of IpaD within the TC from \( S. flexneri \).

Results

Identification of IpaD-specific VHHs

A VHH-display phage library representing the heavy chain-only antibody repertoire of a single alpaca repeatedly immunized with purified recombinant IpaD from \( S. flexneri \) was prepared and subjected to multiple rounds of panning (see “Experimental procedures”). This allowed for the identification of 12 phagemids encoding unique VHHs with strong IpaD-binding activity when displayed by phage. After DNA sequencing of eight selected VHHs (Fig. 1A), seven were selected for expression as recombinant VHH proteins in \( E. coli \), and these were then used for further characterization (see below).

To determine the binding epitopes on IpaD for the VHHs and to identify superior binding candidates for subsequent study, two experimental methods were employed (Fig. 2 and Table 1). Initial identification of binding regions within IpaD recognized by the different VHHs was completed using IpaD deletion fragments in an immunoblot analysis (Fig. 2). The crystal structure of the IpaD monomer was previously determined (14) and found to have three predominant structural features (Fig. 2A). These include a core central coiled-coil (residues 131–177 and 273–332) flanked by an N-terminal helix-turn-helix domain previously suggested to have a self-chaperoning role (residues 1–130) and a globular distal domain comprised of mixed \( \alpha/\beta \) components (residues 178–272). Purified full-length (FL), \( \Delta1–130 \), and \( \Delta \)distal-domain IpaD proteins were used to probe for binding within each VHH cluster (Fig. 2B). As expected, all selected VHHs were capable of binding to IpaD\(^{\Delta1–130} \); however, they were also capable of binding to IpaD\(^{\Delta \)distal-domain \( \) (Fig. 2B), indicating there were no paratopes binding within the N-terminal domain for any identified VHH. Intriguingly, none of the VHHs were capable of binding to IpaD\(^{\Delta \)distal-domain \( \) (Fig. 2B), indicating that this region is required for an interaction to occur.

Competition ELISAs were then performed with recombinant VHHs differentially encoding either c-Myc or E-tag epitopes (supplemental Fig. S1). From these experiments it appeared that there were 3 unique competition groups present within the 7 selected recombinant VHHs: bin 1) JMK-E3, JMK-F11, JPS-G3, and 20ipaD; bin 2) JMK-H2; bin 3) JMK-H5 and JMK-G3 (Fig. 2C). Partial competition with JMK-H2 was observed with VHHs from bin 1, especially JMK-F11 and 20ipaD. Based on this finding, whereas JMK-H2 could be considered part of a separate bin, here it is categorized as being in bin 2 with some overlap with bin 1 (Table 1).

VHH-specific inhibition of Shigella virulence traits

\( S. flexneri \) formation of translocon pores in target cell membranes requires a functional TC protein (IpaD) and functional translocator proteins (IpaB and IpaC), and this activity is routinely determined using a contact-mediated hemolysis assay. The ability of each IpaD-specific VHH to inhibit contact-mediated hemolysis was measured to determine whether any were capable of neutralizing a known virulence trait (Fig. 3 and Table 1). Four VHHs (20ipaD, JPS-G3, JMK-F11, and JMK-E3) were shown to neutralize \( S. flexneri \) contact-hemolysis activity by >75% (9). In contrast to the VHHs described herein, the anti-IpaD sera appears to exclusively recognize epitopes within the N-terminal domain (residues 1–130) (9). Therefore, it appears that both the N-terminal and distal domains contribute to productive assembly of an active T3SS translocon pore. Surprisingly, despite also requiring the presence of the IpaD distal domain, the other three IpaD-specific VHHs were essentially incapable of altering hemolysis. At this time it is unclear whether this results from structural epitopes that either are buried in the IpaD pentamer within the TC or are not important to proper T3SS function.

It was previously demonstrated that heterodimers created by covalent linkage of two inhibitory VHH monomers to create...
Figure 2. Identification of unique epitope regions within IpaD. A, crystal structure of IpaD (PDB ID 2J0O; Ref. 14) depicted in schematic ribbon format with individual domains colored as follows: N-terminal domain (H1–130, blue), central coiled-coil (green), and distal domain (red). Alpha helices (1–7) are labeled within the IpaDFL image.

B, IpaD deletion fragment immunoblots were used to identify minimal binding regions of the VHHs. The first immunoblot demonstrates that polyclonal IpaD antiserum recognizes each of the three protein constructs depicted within panel A. Immunoblot with 20ipaD is representative of all seven of the VHHs selected for further characterization, demonstrating requirement of the distal domain for reactivity.

C, visual representation of binding data derived from competition ELISAs (supplemental Fig. S1) between differentially affinity-tagged VHHs. Clustering of each VHH suggests common or overlapping epitopes are shared between each member. Blue circled numbers represent bins.
Structural analysis of IpaD–VHH complexes

Table 1
Summary of IpaD-VHH binding data and interface information

| VHH          | Neutralization of hemolysis | $k_D$ | $k_{on}$ | $k_{off}$ | Binding group | CDR  | Buried surface area |
|--------------|-----------------------------|-------|----------|-----------|---------------|------|---------------------|
| 20ipaD       | + + +                       | 1.15 ± 0.02 | 3.12 ± 0.08 | 3.52 ± 0.07 | 1             | 0/6/8 | 609                 |
| JMK-E3       | + +                         | 9.8 ± 0.19  | 4.74 ± 0.09  | 45.95 ± 0.24 | 1             | 1/5/7 | 673                 |
| JMK-F11      | + +                         | 1.70 ± 0.02 | 2.30 ± 0.01  | 3.91 ± 0.03  | 1             | –     | –                   |
| JMK-G3       | –                           | 18.7 ± 0.18 | 1.38 ± 0.01  | 25.92 ± 0.13 | 3             | –     | –                   |
| JMK-H2       | –                           | 2.02 ± 0.03 | 2.80 ± 0.03  | 5.65 ± 0.03  | 2             | 0/1/12 | 704                |
| JMK-H5       | –                           | 9.75 ± 0.10 | 1.94 ± 0.02  | 18.87 ± 0.11 | 3             | –     | –                   |
| JPS-G3       | + +                         | 4.42 ± 0.06 | 3.75 ± 0.04  | 16.56 ± 0.14 | 1             | 0/9/3 | 646                 |
| 20ipaD/JMK-G3| + + + +                     | –       | –         | –         | –             | –     | –                   |
| 20ipaD/JPS-G3| + + + +                     | –       | –         | –         | –             | –     | –                   |
| JPS-G3/JMK-G3| + + + +                     | –       | –         | –         | –             | –     | –                   |

*a* IpaD-specific VHHs were tested for the ability to inhibit contact-mediated hemolysis by *S. flexneri*. The number of plus signs indicates the degree of inhibition relative to wild-type strain. –, no reduction; +, 10–20% reduction; ++, 20–40% reduction; ++++, 40–60% reduction; ++++ +, >60% reduction.

Values indicate effective VHH concentration for half-maximal binding to IpaD as measured by bio-layer interferometry (BLI).

Number of hydrogen bonding or electrostatic interactions involving each complementarity-determining region (CDR).

Bi-specific antibodies typically lead to agents with increased affinity and neutralization properties (18–24). The *Shigella* T3SS TC is generally believed to be composed of five molecules of IpaD for the vast majority of the injectosomes on a given bacterium (6, 10). This suggests that there are potentially multiple VHH-binding sites available, which means that physically linking VHHs together could increase binding affinity and neutralization potency. Therefore, three VHH heterodimers were designed, each having a flexible peptide spacer (GGGGS)₃ and consisting of two monomer VHHs with varying efficacies. Two of the VHH heterodimers included a potent inhibitor VHH, 20ipaD, or JPS-G3, linked to a non-neutralizing VHH, JMK-G3, that binds a non-overlapping epitope, whereas the third heterodimer consisted of both neutralizing VHHs, 20ipaD and JPS-G3. The combination of two neutralizing VHHs was predicted to provide increased inhibition potency compared with each monomeric component.

Inhibition of *Shigella* contact-mediated hemolysis by the linked VHHs was then evaluated for each of the VHH heterodimers in a similar manner as the individual VHHs. Strikingly, the 20ipaD/JPS-G3 heterodimer consistently reduced hemolytic activity by >80% (Fig. 3 and Table 1), indicating that physical linkage of two inhibitory VHHs resulted in increased potency over either monomeric component. Heterodimers involving JMK-G3, a non-inhibitory VHH, were capable of inhibiting hemolysis by 60% (20ipaD/JMK-G3) and 50% (JPS-G3/JMK-G3). This is ~10% more than each monomeric inhibitory VHH alone ($p < 0.06$ for both heterodimers). Taken together, the data suggest that binding to IpaD at multiple sites, whether within a single polypeptide or in the context of the TC pentamer, leads to increased inhibition of *Shigella* T3SS activity. Alternatively, because a dimer consisting of a neutralizing and non-neutralizing VHH also showed enhanced inhibition of contact-hemolysis, the increased mass caused by the binding of the dimers at a single site might also contribute to increased inhibitory activity.

Characterization of VHH binding to recombinant IpaD

Binding kinetics for the IpaD-specific VHHs were analyzed using biolayer interferometry analysis to determine the relationship between functional attributes and dissociation constants ($K_D$). Intriguingly, despite clear differences in the ability to inhibit *Shigella* pathogenesis, the binding affinities of each VHH are quite similar (Table 1 and supplemental Fig. S2), with all VHHs exhibiting sub-20 nm dissociation constants. Nearly all of the VHHs had similar $k_{on}$ rates (between $1–5 \times 10^8$ M⁻¹ s⁻¹). Further insight into the relationship between binding affinity and inhibitory virulence is apparent when comparing $k_{off}$ rates across VHHs with similar $K_D$ values. For example, the VHHs with the highest inhibitory capability (20ipaD, JPS-G3, and JMK-E3) all have $K_D$ values between 1 and 10 nm and relatively similar $k_{on}$ rates ($~3 \times 10^7$ M⁻¹ s⁻¹); however, the $k_{off}$ rates between these three VHHs vary by nearly 15-fold, with 20ipaD displaying the slowest off-rate and highest inhibition of contact-hemolysis activity. It is also clear that the $K_D$ and $k_{off}$ rates are not the only mediators of inhibitory activity because the VHHs with no detectable ability to prevent *Shigella*-mediated hemolysis have binding kinetics in the same range as the neutralizing VHHs. Thus, these data indicate that differences in the ability to interact with *S. flexneri* are most likely reflective of...
unique structural epitopes that are surface-exposed within the TC rather than the affinity of the interaction.

Structural investigation of VHH binding to IpaD

To identify the regions of IpaD that are targets for antibody neutralization of virulence and that must, therefore, be exposed within the TC in situ, we determined crystal structures of IpaD in complex with four VHHs (Table 2). Three of the VHHs (JMK-E3, JPS-G3, and 20ipaD) displayed varying levels of neutralization, ranging from 30 to 50% inhibition, whereas the other VHH (JMK-H2) had essentially no effect on virulence. All four VHHs were capable of binding to recombinant IpaD with low nM affinity (Table 1). These four VHHs were capable of binding to recombinant IpaD with an inhibitory VHH, 20ipaD (J MK-E3, JPS-G3, and 20ipaD) displayed varying levels of neutralization, indicating that this region is important for T3SS activity. The most potent inhibitory VHH, 20ipaD (~50% inhibition), made contact with α3 of the coiled-coil and α4 of the distal domain (Fig. 5A) involving 14 intermolecular interactions that bury 609 Å² of available surface area. Three residues within CDR2 and five residues within CDR3 predominantly drive the 20ipaD-IpaD interaction. Of potential importance, 12/14 interactions in the complex involve IpaD residues within α4 of the distal domain, reinforcing that this region is important for T3SS activity.

Two moderately neutralizing VHHs were JPS-G3 and JMK-E3, which were capable of inhibiting Shigella hemolytic activity by ~40 and ~30%, respectively. Similar to the interaction of 20ipaD with IpaD, both JPS-G3 and JMK-E3 bind to IpaD at α3
Figure 4. Crystal structures of IpaD–VHH complexes. 

A, structures of IpaD (colored according to individual domains as in Fig. 2A) depicted in schematic ribbon format in complex with VHHs (colored gray). IpaD is oriented in a similar manner in each complex. 

B, surface representations of the equivalent IpaD–VHH complexes in panel A. IpaD is colored gray with VHH interacting residues colored green (20ipaD), orange (JMK-E3), purple (JPS-G3), and blue (JMK-H2, rotated 90° about the vertical axis). 

C, surface representation of IpaD (gray) demonstrating overlapping structural epitopes for the four VHH complexes. IpaD residues contacting VHHs (further information in supplemental Table S1) are colored according to the number of represented complexes, with 1 (yellow), 2 (orange), or 3 (red). The two IpaD residues are Glu-201 and Lys-205.

Figure 5. IpaD–VHH binding interfaces. 

Residues within hydrogen-bonding distance (2.5–3.5 Å) between each IpaD–VHH complex (A, 20ipaD; B, JPS-G3; C, JMK-E3; D, JMK-H2) are depicted as balls-and-sticks (orange, IpaD; cyan, VHH). IpaD secondary structure elements are colored as in Fig. 2A, relevant IpaD/H9251-α-helices are labeled, and each VHH is in gray. Further information on these distances can be found in supplemental Table S2.
of the coiled-coil and α4 of the distal domain (Fig. 5, B and C). The JPS–G3 interaction contributes 12 intermolecular interactions that bury 646 Å² of available surface area. CDR2 is predominantly responsible for this interaction with six residues contributing to complex formation, whereas a single residue within CDR3 (Arg-102) forms an extensive electrostatic interaction with Glu-201 of IpaD. There are 13 intermolecular interactions formed between the JMK-E3 and IpaD complex, burying 673 Å² of available surface area, the most of the 3 neutralizing VHHs (Fig. 5). Tyr-105 of JMK-E3 appears critical to the IpaD interaction as it is involved in 5/13 interactions. Additionally, JMK-E3 is the only inhibitory VHH–IpaD complex with binding interactions that involve residues within CDR1.

Intriguingly, the VHH with minimal biological effect on *Shigella* (Fig. 3), JMK-H2, interacts almost exclusively with residues from the central coiled-coil (α3 and α7) of IpaD (Fig. 5D), burying 704 Å² of available surface area, the most of all 4 VHH complexes. There are 13 intermolecular interactions within this complex, with only a single contributing hydrogen bond that is found outside of CDR3 (Thr-57). The elongated CDR3 of JMK-H2 makes extensive contacts with IpaD residues 273–287 of α7. Despite favorable binding properties in vitro ($K_D = 2 \text{ nm}$), the JMK-H2–IpaD complex suggests that this region of the coiled-coil is not important for inhibiting T3SS function, perhaps because of inaccessibility to the IpaD pentamer within the native TC.

**Discussion**

Many Gram-negative bacterial pathogens use a T3SS to inject host-modulating proteins during infection. At the distal end of the exposed portion of the *Shigella* T3SS, a TC is assembled that consists of a pentamer of IpaD that is ultimately composed of IpaD and one or both hydrophobic translocator proteins (IpaB and IpaC) depending upon its activation state. To date, the exact stoichiometry and functional topology of these proteins within the TC remains elusive, with many discordant reports suggesting either a pentamer of IpaD or a four-plus-one complex of IpaD and IpaB (6, 10). To better understand how IpaD is assembled within the *Shigella* TC, we have generated a panel of IpaD-specific VHHs. Through a variety of biochemical, molecular, and structural techniques, we have characterized these VHHs and identified structural epitopes within IpaD that are critical for proper T3SS function.

Studies on the use of monoclonal or recombinant antibodies to reduce bacterial infections are increasingly important due to several factors, including the threats of bacterial multidrug resistance and bioterrorism (26, 27). Pathogens that use a T3SS for virulence are particularly attractive due to the structural homology of their apparatus components and the global burden of the diseases they represent. Diarrheal diseases, such as those caused by *Shigella* spp. and *Salmonella enterica*, have an estimated 1.7 billion cases per year and are the second leading cause of mortality in children under the age of five (28, 29). Other pathogens with T3SS are also recognized as significant targets for new therapeutics. For example, use of such therapies could prove important for combating nosocomial infections caused by *Pseudomonas aeruginosa* or for preventing the bioterror threat of *Burkholderia pseudomallei*. Several approaches to the design and use of antibodies as therapeutics exist, with VHHs being one of the most robust options at this time (30).

The degree of inhibition by a VHH during contact-mediated hemolysis did not correlate directly with binding affinity in vitro or the extent of the association network in the described crystallized structures (e.g. number of hydrogen bonds or buried surface area). The key to inhibitory activity appeared to require binding that included interactions with the IpaD distal domain. Analysis of the VHH–IpaD crystal structures revealed that all four complexes involved structural epitopes clustered within the distal region of the protein (Fig. 4C), predominantly including residues 165–177 (coiled-coil α3) and 198–205 (distal domain α4). Furthermore, two specific IpaD residues (Glu-201 and Lys-205) were involved in all three complexes with inhibitory VHHs (Fig. 5C), suggesting that this region of the protein is critical to proper function during maturation of the TC and could reflect a potential binding site for the translocator IpaB (5). As an alternative to blocking the IpaB binding site, VHH binding to the distal domain could influence the nature of the structural changes previously proposed to promote IpaB recruitment into the TC. Future experiments will address the effects of VHH binding on the structural plasticity of IpaD and the stepwise maturation of the T3SS in order to establish a precise mechanism for VHH-mediated inhibition of *Shigella* type III secretion functions.

Multiple models of in situ TC structures have been proposed; however, no definitive structure or universal composition has been identified. A homopentameric array is the most common model proposed for TC structures including the *Shigella* TC (10, 14), the *S. enterica* TC (31) and the *Yersinia* TC (32). An alternative four-plus-one (IpaD-plus-IpaB, respectively) model has been proposed for *Shigella* (6); however, even in those studies the predominant TC composition is that of a homopentamer (>90% of injectisomes). It is possible that the VHHs prepared here will allow future studies to provide a definitive test of needle TC composition for nascent apparatuses. Regardless, some of the VHHs prepared here clearly can inhibit *Shigella* virulence activities (i.e. contact hemolysis), which are related to translocon pore formation. The epitopes recognized by these VHHs are largely localized to the mixed α/β structure distal domain of IpaD. This is consistent with previous studies suggesting that the distal domain is key for steps subsequent to nascent TC formation (5, 10). Furthermore, VHHs that fail to impair virulence functions appear to bind to epitopes that localize to the central coiled-coil of IpaD, which is proposed to be buried within the TC (6, 10).

The fact that anti-IpaD VHHs, which impair *Shigella* virulence activity, can be fused to further enhance inhibition suggests that they may be able to bind to TCS in a multivalent manner. This additive effect on inhibition may be an attractive route for the development of novel multivalent therapeutics. It cannot be ruled out, however, that this additive effect is not due to multivalency but rather to an increase in the binding affinity, and the neutralization occurs through a form of steric hindrance caused by the added bulk after a single binding event. Such a possibility is supported by the observation that a non-

**Structure of IpaD–VHH complexes**

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inhibitory VHH fused with an inhibitory one also enhances the ability of the latter to impair *Shigella* contact-mediated hemolysis; however, these do not inhibit contact-hemolysis as well as the multimers of neutralizing VHVs. Such an observation would be consistent with multivalent binding for the latter. Future studies will need to be done to determine whether increased valency (e.g. homo- and heterotrimeric, tetramers, etc.) continue to increase the inhibitory activity of neutralizing VHVs.

The best characterized T3SS-tip proteins from animal pathogens can be subdivided into three main families: 1) IpaD-SipD-BipD from *Shigella, Salmonella*, and *Burkholderia* species; 2) LcrV-PcrV-AcrV from *Yersinia, Pseudomonas*, and *Aeromonas* species; 3) EspA-Bsp22 from enteropathogenic *E. coli* and *Bordetella* species (8). Despite low levels of amino acid conservation, available structural information from tip proteins within each family indicates the presence of a conserved antiparallel coiled-coil with variability in the globular domains at each end of this coiled-coil (14, 33). Furthermore, tip-translocator interactions have been described within each of these three families, suggesting that translocon assembly and presentation occurs upon a stable tip platform (5, 34, 35). Visualization of TCS from the LcrV-PcrV-AcrV family also indicated that the TC was composed of a pentamer in a similar orientation described for IpaD (32). Furthermore, protective epitopes derived from both LcrV and PcrV localize to the region equivalent to α4 within the IpaD distal domain (36, 37). Although structural data are relatively lacking for the EspA-Bsp22 family, both proteins appear to form pilus-like assemblies and are capable of eliciting protective antibody responses in mice (34, 38). Altogether, these observations in tandem with the data presented herein support the hypothesis that hydrophobic translocators within the *Shigella/Salmonella/Burkholderia* family bind to a conserved region within the tip protein. The successful development of a VHH heterodimer capable of reducing *Shigella* hemolytic activity >80% suggests this approach could be applied toward other T3SS-possessing human pathogens, specifically targeting this putative hydrophobic translocator-binding site.

**Experimental procedures**

**Alpaca immunizations and VHH display library preparation**

One alpaca (*Vicugna pacos*) was used in this study. Immunizations and VHH display library construction were performed as previously described (19, 40). Specifically, the alpaca received five successive subcutaneous injections at 3-week intervals using an immunogen consisting of purified recombinant IpaD (14). Peripheral blood lymphocytes were obtained from blood 5 days after the final immunization of the alpaca. RNA and then cDNA were produced from the peripheral blood lymphocytes, and PCR was used to amplify the VHH-coding repertoire from cDNA. Using high efficiency *E. coli* transformation methods, >10<sup>6</sup> independent clones were obtained and pooled to generate the VHH-display phage library.

**Anti-IpaD VHH identification, expression, and purification**

Panning, phage recovery, and clone fingerprinting were performed as previously described (18, 19, 24). Two rounds of panning were performed with purified IpaD coated onto Nunc Immunotubes. A single low stringency panning approach was employed using 10 μg/ml target antigen. After phages were eluted, they were amplified and subjected to a second round of panning at high stringency with 1 μg/ml target antigen employing 10-fold reduced input phage, shorter binding times, and longer washes. After the second high stringency round of panning, individual *E. coli* colonies were picked and grown overnight at 37 °C in 96-well plates. A replica plate was then prepared. The bacteria were then grown, and protein expression was induced with isopropyl 1-thio-β-D-galactopyranoside so that the culture supernatants could be assayed for IpaD binding as determined by ELISA.

From each of the two-cycle panning regimens, >50% of VHH clones bound to IpaD as shown by ELISA reactivity values that were >2-fold greater than negative controls. The strongest positive binding clones for IpaD were characterized by DNA fingerprinting (18). Multiple clone groups with unique fingerprints were identified among the VHHs selected for binding to IpaD. DNA sequences of the VHH coding regions were obtained for representatives from each identified fingerprint group, and sequences were compared for homologies. Based on this sequence analysis, IpaD VHVs identified as being unlikely to have common B cell clonal origins were selected for protein expression.

Selected VHH coding DNAs were cloned into a pET32b expression vector (Novagen) for cytosolic expression in *E. coli* Rosetta-gami 2 (DE3)pLacI (Novagen) as a fusion to thioredoxin. All VHVs were expressed with hexahistidine (His<sub>6</sub>) to facilitate subsequent purification using standard nickel-immobilized metal ion affinity chromatography (Ni-IMAC) chromatography methods and a C-terminal epitope tag for detection, either E tag (GAPVPYPDPLEPR) or myc tag (MEQKLISEEDL).

**ELISA**

Competition ELISAs were performed as follows. Purified VHVs expressed with a c-myc tag were tested for binding in the presence of a 40-fold excess of purified VHVs having an E tag or vice versa. Nunc MaxiSorb plates were coated overnight at 4 °C with 1.0 μg/ml IpaD antigen. Plates were blocked with 4% non-fat dried milk in PBS-Tween 0.1% for 2 h at 37 °C. The competitor VHVs, *i.e.* myc-VHVs, were diluted in blocking solution to 10 μg/ml and added to each well of the plate going down the columns and allowed to incubate for 30 min at 23 °C on a rocker platform. The plate was then thoroughly washed with PBS-T followed by PBS. E-tag VHVs were added to the washed wells at 0.25 μg/ml in blocking solution going across the rows of the plate and allowed to incubate for 1 h at 23 °C on a rocker platform. The plate was then thoroughly washed with PBS-T followed by PBS. To the clean wells, HRP goat anti-E-tag mAb diluted 1:10,000 in blocking solution was added to each well. After incubation, wells were washed with PBS-T followed by PBS. Bound HRP was detected using the SureBlue Peroxidase Substrate (KPL, Gaithersburg, MD). The reaction was quenched with 1 μl phosphoric acid, and absorbance was read at 450 nm.
**Contact-mediated hemolysis**

To determine the effect of VHHs on a known *Shigella* virulence activity, a modification of a standard contact-mediated hemolysis assay was used as previously described (41). In the modified assay the bacteria were incubated with VHHs (0.1 mg/ml) for 30 min before the addition of sheep red blood cells.

**VHH affinity determinations**

The binding affinity of purified His-tagged IpaD (residues 39–322) with VHHs was monitored by biolayer interferometry using an Octet RED96 instrument (Pall ForteBio). IpaD was loaded onto nickel-nitrilotriacetic acid biosensors (ForteBio) at a concentration of 6.25 μg/ml for 5 min in 1 × kinetics buffer (1 × PBS, pH 7.4, 0.01% BSA, 0.002% Tween 20). All reactions were performed at 25 °C. Real-time data were analyzed use Octet Software version 8.2 (ForteBio). Binding kinetics (association and dissociation) as well as steady-state equilibrium concentrations were fitted using a 1:1 Langmuir-binding model.

**Crystallization**

IpaD (residues 39–322) was purified as previously described (25). IpaD-specific VHHs were purified as described above, with one modification. Before expression, the thrombin cleavage sequence within pET32b was modified to a tobacco etch virus cleavage sequence for ease of purification. Before crystallization, IpaD and individual VHHs were mixed in a 1:1 molar ratio and injected onto a HiLoad 26/60 Superdex 200 column. The primary peak, containing both IpaD and VHH, was collected and concentrated by ultrafiltration to 10 mg/ml in 10 mM Tris-HCl, pH 8.5, 30% (w/v) PEG 4000. Crystals were flash-cooled in a cryoprotectant solution consisting of mother liquor supplemented with 20% (w/v) PEG 200.

**Diffraction data collection, structure determination, refinement, and analysis**

X-ray diffraction data were collected on all IpaD–VHH crystals at 1.0000 Å at 100 K using a Dectris Pilatus 6M pixel array detector at IMCA-CAT beamline 17ID at the APS (Table 2). After data collection, individual reflections from each dataset were integrated with XDS (42) and scaled with Aimless (43). Experimental phase information was obtained for each IpaD–VHH structure using molecular replacement (PHASER) within the Phenix suite (44, 45). Search models for each VHH were generated using PHYRE (46), whereas the IpaD model was obtained from PDB entry 2J0O (14). Search models for each VHH were integrated with XDS (42) and scaled with Aimless (43).

**Structure refinement for each IpaD–VHH structures was carried out using Phenix (44, 45). One round of individual coordinates and isotropic atomic displacement factor refinement was conducted, and the refined model was used to calculate both 2Fo – Fc and Fo – Fc difference maps. These maps were used to iteratively improve the model by manual building with Coot (47, 48) followed by subsequent refinement cycles. TLS refinement (49) was incorporated in the final stages to model anisotropic atomic displacement parameters. Hydrogen atoms were included during all rounds of refinement. Ordered solvent molecules were added according to the default criteria of Phenix and inspected manually within Coot before model completion. The following residues within each complex were not modeled as a result of poor map quality: IpaD/JMK-H2: IpaD, residues 39–40; JMK-H2, residues 1, 127–128; IpaD/JMK-E3: IpaD, residues 39–41, 127–128 (chain A), residues 39–42 and 321–322 (chain C), residues 39–41 and 124–126 (chain E), residues 39–41 and 321–322 (chain G), residues 39–41, 182–185, 240–241, and 322 (chain I); JMK-E3: residue 128 (chain D), residue 128 (chain F), and residues 127–128 (chain J); IpaD/JPS-G3: IpaD: residues 39, 124–127, and 182–185; JPS-G3: residue 128; IpaD/20ipaD: IpaD, residues 39–41, 97–109, 116–118, 122–136, and 321–322; 20ipaD, residue 1.

**Multiple sequence alignments and figure modeling**

Multiple sequence alignments were carried out using ClustalW (50) and aligned with secondary structure elements using ESPRIT (51). Three-dimensional structures were superimposed using the local-global alignment method (LGA) (52). Representations of all structures were generated using PyMOL (53).

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