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Single-domain llama antibodies as specific intracellular inhibitors of SpvB, the actin ADP-ribosylating toxin of *Salmonella typhimurium*

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ABSTRACT **ADP-ribosylation of host cell proteins is a common mode of cell intoxication by pathogenic bacterial toxins. Antibodies induced by immunization with inactivated ADP-ribosylating toxins provide efficient protection in case of some secreted toxins, *e.g.*, diphtheria and pertussis toxins. However, other ADP-ribosylating toxins, such as *Salmonella* SpvB toxin, are secreted directly from the *Salmonella*-containing vacuole into the cytosol of target cells *via* the SPI-2 encoded bacterial type III secretion system, and thus are inaccessible to conventional antibodies. Small-molecule ADP-ribosylation inhibitors are fraught with potential side effects caused by inhibition of endogenous ADP-ribosyltransferases. Here, we report the development of a single-domain antibody from an immunized llama that blocks the capacity of SpvB to ADP-ribosylate actin at a molar ratio of 1:1. The single-domain antibody, when expressed as an intrabody, effectively protected cells from the cytotoxic activity of a translocation-competent chimeric C2IN-C/SpvB toxin. Transfected cells were also protected against cytoskeletal alterations induced by wild-type SpvB-expressing strains of *Salmonella*. This proof of principle paves the way for developing new antidotes against intracellular toxins.—Alzogaray, V., Danquah, W., Aguirre, A., Urrutia, M., Berguer, P., García Véscovi, E., Haag, F., Koch-Nolte, F., Goldbaum, F. A. Single-domain llama antibodies as specific intracellular inhibitors of SpvB, the actin ADP-ribosylating toxin of *Salmonella typhimurium*. *FASEB J.* 25, 000–000 (2011). www.fasebj.org**

Key Words: intrabodies · VHH · enzyme inhibition

Salmonella are common pathogens of humans and domestic animals and are a frequent cause of enteritis. Certain strains can invade the intestinal mucosa, infect macrophages, and cause severe systemic infections (1, 2). While neutrophils can phagocytose and kill *Salmonella*, pathogenic strains survive and proliferate in macrophages (3–5). In animal models, systemic virulence of *Salmonella* is determined in large part by the SpvB gene encoded by the *Salmonella* virulence plasmid (or chromosomally in some strains) (6–9). SpvB encodes an actin ADP-ribosylating toxin that is most likely translocated from the *Salmonella*-containing vacuole to the host cell cytosol *via* the SPI-2-encoded bacterial type III secretion system (10–13). As an intracellular protein, SpvB is inaccessible to conventional antibodies. SpvB-catalyzed ADP-ribosylation of actin at R177 interferes with actin polymerization (14) and causes apoptotic cell death (9, 15). Therapeutic strategies aimed at blocking SpvB enzyme activity with small-molecule inhibitors are fraught with potential side effects of such inhibitors on endogenous mammalian ADP-ribosyltransferases (16). Here, we report the development of SpvB-specific single-domain antibodies derived from an immunized llama that specifically block SpvB-catalyzed ADP-ribosylation of actin at 1:1 M ratios. Moreover, we demonstrate that these SpvB-specific nanobodies—when expressed as intrabodies—effectively protect cells from SpvB-mediated cytotoxicity.

In camelids (camels, dromedaries, and llamas), a subset of immunoglobulins consists of heavy-chain homodimers devoid of light chains, called heavy-chain IgGs (17). Their antigen-binding site consists of one single domain, referred to as variable domain of heavy chain IgGs (VHH) (18, 19). Recombinant VHHs are also known as single-domain antibodies (sdAbs) or nanobodies because of their dimensions in the nanometer range (18, 20). These nanobodies are highly soluble and very robust and are easily converted into multivalent and/or multispecific formats (21–24). VHHs bear longer CDR3 loops than conventional antibodies (25). These long CDR3 loops protrude from the

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antigen-binding surface and can reach into and fill out molecular crevices on proteins that are usually inaccessible to conventional antibodies (26), making VHHs very useful as enzyme inhibitors (27). Nanobodies have many potential applications (20, 28) in various disease areas, including oncology (29), infection (30), inflammatory diseases (31, 32), and neurodegenerative diseases (33, 34). In most cases, nanobodies are used as soluble extracellular proteins. Nanobodies seem suited also as intrabodies because they can undergo proper folding in the reducing environment of the cytoplasm (35–37).

Intrabodies are antibody-derived proteins designed to be expressed intracellularly in eukaryotic cells and to bind to specific target antigens in the cytosol, nucleus, endoplasmic reticulum, or other organelles. Intrabodies have been used as tools to interrupt, modulate, or define the functions of a wide range of target antigens at the post-translational level, *e.g.*, in the fields of cancer, microbial pathologies, autoimmune and neurodegenerative diseases, transplantation, and plant biotechnology (38–40). The efficacy of intrabodies is determined by their affinity, stability, and expression levels inside cells. The efficient cytoplasmic expression of single-chain antibody fragments (scFvs), derived by fusion of the two V domains from the light and heavy chains of conventional antibodies, is confronted with folding problems, low solubility, short protein half-life, and high tendency for aggregation. These problems are most likely caused by the reducing environment of the cell cytoplasm, which prevents formation of intrachain disulfide bridges of heavy and light chain V domains, thus resulting in unstable and/or nonfunctional intrabodies (41). VHHs are better suited than scFvs for intrabody applications because of their higher stability and expression levels. Llama intrabodies have been used, for example, to block the activity of a chloroplast enzyme and thereby modify starch composition in transgenic potato plants (42), to retain hepatitis B virions in the endoplasmic reticulum and thereby reduce HBV viremia (43), to neutralize Bax in the cytosol and prevent oxidative-stress-induced apoptosis (36), to tag and trace target antigens on different subcellular structures and intracellular compartments *via* fluorescent GFP-tagged nanobodies (chromobodies) (35), to prevent aggregation of poly-A binding protein in the nucleus of skeletal muscle cells and thereby rescue transgenic *Drosophila* from oculopharyngeal muscular dystrophy (37), to inhibit filopodia formation and migration in tumor cells (44), and to inhibit HIV replication by suppressing the Rev-dependent expression of late viral RNAs (45).

ADP-ribosylation is a common mode of action of pathogenic bacterial toxins (46). Many ADP-ribosylating toxins are secreted into the extracellular space and are subsequently translocated to the host cell cytosol with the aid of fused translocation domains (*e.g.*, diphtheria toxin) or with the aid of associated subunits (*e.g.*, binary clostridia, cholera, and pertussis toxins). These secreted toxins are susceptible to neutralization by conventional antibodies and active and passive immunization are used effectively to prevent or treat diseases mediated by some of these toxins (47, 48). Several pathogenic bacteria, including *Salmonella* and *Yersinia* inject cytotoxins into the cytosol through the plasma membrane and/or endosomal membrane *via* specialized secretion systems, thereby circumventing neutralization by antibodies. In this work we provide a proof of principle for the use of llama nanobodies as specific inhibitors of intracellular bacterial toxins, using the actin ADP-ribosylating SpvB toxin of *Salmonella enterica* serovar Typhimurium as a model. We selected several inhibitory nanobodies against SpvB from a phage display library generated from an SpvB-immunized llama. One of these nanobodies was readily expressed as an intrabody in eukaryotic cells and efficiently protected cells against the cytotoxic effects of both recombinant SpvB and SpvB-expressing *Salmonella typhimurium.*

MATERIALS AND METHODS

SpvB protein production and llama immunizations

The coding region for the catalytic domain of SpvB (aa 375–591) was amplified by PCR (**Table 1**) and cloned into pET26b (Novagen, San Diego, CA, USA), downstream of the OMP-signal peptide and upstream of a C-terminal His(6x) tag. SpvB was purified from cytoplasm lysates obtained from transformed *Escherichia coli* by HiTrap chelating HP column

TABLE 1. *Sequences of PCR primers used for cloning SpvB, the VHH repertoire, and the VHH-MBP intrabody*

Name	Sequence, 5′ > 3′
Cloning SpvB in pET26b	
SP1-NcoI	cct cct ccg acc atg gga ggt aat tca tct cga c
SP4-XhoI	gat tct tag ctc gag tga gtt gag tac cct cat gtt
Cloning VHHs in pHEN2	
Lam07-NotI	gat ggt gat gat gat gtg cgg ccg cgc tgg ggt ctt cgc tgt ggt gcg
Lam08-NotI	gat ggt gat gat gat gtg cgg ccg ctg gtt gtg gtt ttg gtg tct tgg
VH1b-SfiI	gct gga ttg tta tta ctc gcg gcc cag ccg gcc atg gcc cag gts mar ctg cag sag tcw gg
VH6b-SfiI	cgt gga ttg tta tta tct gcg gcc cag ccg gcc atg gcc gat gtg cag ctg cag gcg tct ggr gga gg
Cloning MBP-VHH5 in pCDNA6	
MBP-VHH5-Hind III	act agt gca aag ctt atg aaa atc gaa gaa ggt aaa ctg gta atc tgg
VHH5- XbaI	ggt aat cgg tct aga tca tgg ttg tgg ttt tgg tgt ctt gg

(Amersham Biosciences, Piscataway, NJ, USA). Two llamas (*Lama glama*) were immunized 3 times subcutaneously every 21 d with 180 μg of recombinant SpvB emulsified with Specol adjuvant (49). The humoral immune response was monitored in serially diluted serum by ELISA on microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) coated with 0.5 μg recombinant SpvB. The animal with the stronger response received a final boost and was bled 20 d later.

Construction of the library and phage display selection of SpvB-specific VHHs

Mononuclear cells were isolated from 120 ml of blood by Ficoll-Paque (GE Healthcare, Little Chalfont, UK) gradient centrifugation. Total RNA was purified from these cells by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to cDNA synthesis. The VHH coding region was amplified by PCR with VHH-specific primers (Table 1). PCR products were purified from agarose gels, digested sequentially with SfiI and *Not*I (NEB), and cloned into the pHEN2 phagemid vector downstream of the PelB-leader peptide and upstream of the chimeric His6x-Myc epitope tag (50). Transformation into XL1-Blue *E. coli* yielded a library with a size of 4.0×10^7 clones. Phage particles were precipitated with polyethylene glycol from culture supernatants of transformants infected with a 10-fold excess of VCS helper phage (Promega, Madison, WI, USA). Panning of specific phage was performed using biotinylated SpvB and streptavidin-coated magnetic beads. Phage particles (1.6×10^{14}) were resuspended in PBS and incubated with 10 nM or 50 nM of biotinylated recombinant SpvB for 2 h with agitation at room temperature. Biotin-SpvB-bound phages were captured with streptavidin-coated magnetic beads. Following extensive washing, bound phages were eluted with 100 mM triethylamine and quickly neutralized with 1M Tris-HCl, pH 8. Eluted phages were titrated and subjected to two more rounds of panning, following the same procedure. Phage titers were determined at all steps by infection of XL1-Blue cells. Selected phages were purified from supernatants of individual colonies and tested for SpvB specificity by ELISA on plate bound SpvB with peroxidase-conjugated anti-M13 mAb (Sigma, St. Louis, MO, USA). Plasmid DNA were isolated from single colonies and subjected to sequence analyses using pHEN2-specific forward and reverse primers (Table 1).

Production, purification, and reformatting of VHHs

Selected anti-SpvB VHHs and a control VHH specific for *Trypanosoma cruzi* trans-sialidase (51) were expressed as soluble periplasmic proteins in *E. coli* HB2151 nonsuppressor strain. VHHs were purified by HiTrap chelating HP column (Amersham Biosciences), followed by size-exclusion chromatography using Superdex 75(Superdex 75 10/300 GL). Purity was assessed by SDS-PAGE. The coding region of VHH5 was PCR-amplified and cloned into the $pcDNA_6$ expression vector (Invitrogen) downstream of the coding region for maltose binding protein (MBP; Table 1).

Actin ADP-ribosylation and polymerization assays

ADP-ribosylation of actin in CHO cell lysates was monitored by SDS-PAGE autoradiography (11, 12). SpvB (1.2 nM) was preincubated with VHHs (2.2 μM) for 1 h at 37°C before addition to freshly prepared lysates of CHO cells in the presence of 1 mM ADP-ribose, 1 mM DTT, and 1 μCi ^{32}P-NAD$^+$ (Amersham). Samples were incubated for 30 min at 37°C, and reactions were stopped by addition of 6× SDS-PAGE sample buffer and heating for 10 min at 95°C. Proteins were size fractionated by SDS-PAGE, and ^{32}P-ADP-ribosylated actin was detected by autoradiography (Storm 840, Molecular Dynamics, Sunnyvale, CA, USA).

SpvB activity was also measured by a fluorometric assay using pyrene-labeled actin (12). SpvB (66 nM) was preincubated with VHHs at different molar ratios (1:0.15–1:5, SpvB:VHHs) for 1 h at room temperature. Pyrene actin (0.4 mg/ml, cytoskeleton) was added to these samples in ADP-ribosylation buffer (5 mM Tris-HCl, pH 8; 0.2 mM $CaCl_2$; 0.2 mM ATP; and 0.05 mM NAD) and incubated for 10 min at room temperature. Polymerization was initiated by the addition of polymerization buffer (50 mM KCl; 2 mM $MgCl_2$; and 1 mM ATP), and actin polymerization was monitored for 20 min by the increase in fluorescence resulting from the incorporation of pyrene-labeled actin into growing filaments (52). Percentages of inhibition were calculated with fluorescence signals at 800 s (100% and 0 correspond to fluorescence observed in the absence and presence of SpvB, respectively).

Macrophage infection assays with *Salmonella* strains

RAW 264.7 (American Type Culture Collection, Manassas, VA, USA) murine macrophage-like cells were grown on 12-mm coverslips in 24-well tissue culture plates in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Dartmouth, MA, USA). Wild-type and *spvB* mutant strains of *Salmonella* were grown overnight in Luria broth (LB) medium supplemented with 0.3 M NaCl at 37°C. Transfection of RAW cells with expression constructs for VHH intrabodies (1 μg plasmid/well) were performed using jetPEI transfection reagent (Polyplus Transfection Inc., New York, NY, USA) according to the manufacturer's instructions. At 24 h after transfection, cells were washed with Hank's balanced salt solution (HBSS; Gibco), and then infected with stationary-phase *Salmonella* diluted in PBS (10^7/ml). To promote contact between bacteria and cells, plates were centrifuged gently for 10 min at 200 *g* and then incubated for 30 min at 37°C. Cells were then washed and cultivated further in RPMI 1640 medium supplemented with 5% FCS and gentamicin sulfate (50 μg/ml) for 16 h. The cells were washed in HBSS, fixed in 4% paraformaldehyde for 15 min, washed twice with HBSS, and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Actin was stained with phalloidin-FITC (Sigma), bacteria were detected with anti-*Salmonella* mouse mAb (Abcam, Cambridge, MA, USA) and Alexa350-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). MBP-VHH fusion proteins were detected with rabbit anti-MBP (Abcam) and Cy^{tm}3 conjugated anti-goat anti-rabbit IgG (Amersham).

Cytotoxicity assays

Vero cells were grown in 25-cm^2 flasks in DMEM (Gibco), supplemented with 5% FCS. To assess protection of cells by anti-SpvB intrabodies, the cells were transiently transfected with MBP-VHH expression vectors (5 μg/flask) using jetPEI (Polyplus Transfection). After 6 h, cells were harvested by trypsinization (Gibco) and reseeded onto coverslips in 24-well tissue culture plates and cultivated further for 16 h. Cells were then incubated for 3 h at 37°C in the absence or presence of toxins (2.5 μg/ml C2IN-C/SpvB + 5 μg/ml C2II or 0.5 μg/ml C2I + 1 μg/ml C2II). The cells were washed in PBS, fixed in 4% paraformaldehyde for 15 min, washed twice with PBS, and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Filamentous actin and MBP-VHH fusion protein were stained as described above. To assess protection of cells by exogenous anti-SpvB nanobodies, toxins (2.5 μg/ml C2IN-C/

TABLE 2. *Sizes of input and output phages show enrichment in binders during 3 rounds of panning on biotinylated SpvB in solution*

Treatment	Round 1		Round 2		Round 3	
	Input	Output	Input	Output	Input	Output
10 nM biotinylated antigen	1.6×10^{14}	2.8×10^{7}	2.0×10^{13}	1.0×10^{9}	2.0×10^{13}	7.0×10^{10}
Negative control	1.6×10^{14}	2.2×10^{7}	2.0×10^{13}	5.2×10^{8}	2.0×10^{13}	2.0×10^{10}
50 nM biotinylated antigen	1.6×10^{14}	4.0×10^{7}	2.0×10^{13}	8.7×10^{9}	2.0×10^{13}	3.3×10^{10}
Negative control	1.6×10^{14}	2.2×10^{7}	2.0×10^{13}	2.5×10^{8}	2.0×10^{13}	7.7×10^{7}

SpvB + 5 μg/ml C2II or 0.5 μg/ml C2I + 1 μg/ml C2II) were preincubated for 15 min with VHH5 (10 μg/ml), before addition to untransfected Vero cells. After 3 h at 37°C, cells were analyzed by phase-contrast microscopy.

RESULTS

Induction and phage display selection of anti-SpvB llama antibodies

Two llamas immunized with recombinant SpvB developed high titers of antitoxin IgG. One of these llamas was selected after a final boost to construct a VHH phage display library from cDNA of peripheral blood cells (Supplemental Fig. 1). Phages displaying specific VHHs were selected by incubation with soluble biotinylated SpvB and capture of the complexes on streptavidin-coated magnetic beads (**Table 2**). The number of SpvB-specific clones increased after the first and second rounds of selection and reached a plateau after the third round, as revealed by phage ELISA (Supplemental Fig. 2). Twenty positive clones from selection rounds 2 and 3 showing the highest reactivity in phage ELISA against SpvB were selected for sequencing. The results revealed 4 distinct groups of clones based on the length and variability of the CDR3 region (**Fig. 1**). Twelve clones had a CDR3 of 16 aa (group 1). These clones evidently were clonally related and were similarly represented in the two rounds of selection. One clone had a CDR3 of 14 aa (group 2), and one clone had a CDR3 of 11 aa (group 3). Six clones had a CDR3 of 7 aa (group 4) and differed only by a single point mutation. None of these single-domain antibodies had an extra disulfide bond between the CDR3 and CDR1 as reported for ~30% of llama VHHs (53).

Selected VHHs block the enzymatic activity of SpvB *in vitro*

To analyze the inhibitory capacity of the SpvB-specific VHHs, two single-domain antibodies from group 1 (VHH5 and VHH6), one from group 2 (VHH1), one from group 3 (VHH38), and one from group 4 (VHH16) were expressed as epitope-tagged recombinant proteins in *E. coli*. Recombinant VHHs were readily purified from periplasma lysates by immobilized metal affinity chromatography followed by gel filtration. Protein yields in culture ranged from 1 to 5 mg VHH/L. The inhibitory capacity of purified VHHs against recombinant SpvB was tested by two methods: incorporation of ^{32}P-ADP-Ribose into actin (radioactivity assay), and a fluorescence assay that senses ATP-

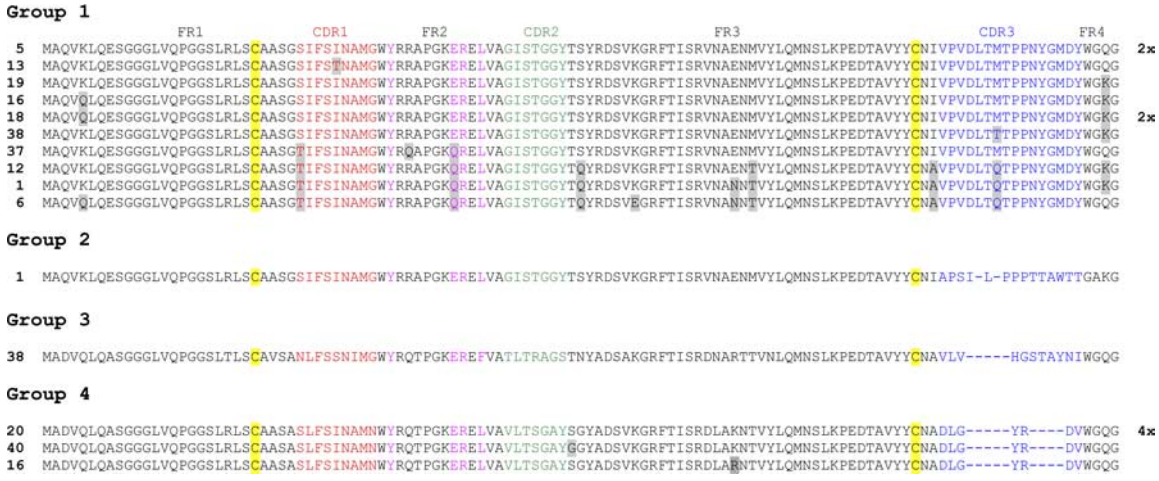

Figure 1. Amino acid sequence alignment of SpvB-specific VHHs. Nanobodies were classified into 4 groups on the basis of their CDR3 lengths: group 1, 16 aa; group 2, 14 aa; group 3, 11 aa; and group 4, 7 aa. VHH5 and VHH18 of group 1 were each found twice and VHH20 from group 4 was found 4 times in the selection rounds. FR, framework region; CDR, complementarity determining region. Color coding of amino acids is as follows: CDR1, red; CDR2, green; CDR3, blue. Hydrophilic residues in FR2 characteristic of single-domain antibodies are pink; cysteines are highlighted in yellow; variant amino acid residues within a group are highlighted in gray.

induced actin polymerization by means of pyrene-labeled actin (12). First, aliquots of a CHO cell lysate were incubated with recombinant SpvB and ^{32}P-NAD^{+} in the presence or absence of VHHs. ADP-ribosylation of actin was monitored by autoradiography (**Fig. 2*A***). In the presence of SpvB, cellular actin was strongly labeled with ^{32}P-ADP-ribose (Fig. 2*A*, lane 1). All anti-SpvB VHHs inhibited this reaction (VHH5 and VHH1 are shown as representative examples; Fig. 2*A*, lanes 2 and 3), whereas an unrelated VHH (anti-trans-sialidase) (51) did not interfere with ADP-ribosylation of actin (Fig. 2*A*, lane 4). These results indicate that all selected VHHs effectively block the SpvB enzymatic activity. Next, we performed a fluorometric assay in which an increase in fluorescence indicates polymerization of actin as monomeric pyrene-labeled actin is incorporated into growing filaments (Fig. 2*B*). When actin was preincubated with SpvB and NAD, ADP-ribosylation prevented the conversion of G-actin to F actin (visualized by the lack of increase in fluorescence; Fig. 2*B*, SpvB). When SpvB was preincubated with increasing doses of VHH5, the SpvB-mediated inhibition of actin polymerization was abrogated in a dose-dependent manner, indicating that the nanobody effectively blocked the SpvB-catalyzed ADP-ribosylation of actin. Comparative dose-response analyses revealed that VHH5 and VHH6 were both capable of fully inhibiting SpvB at a stoichiometry of 1:1 at the concentration of SpvB (66 nM) used in this assay (Fig. 2*C*). Note that the nanobodies with shorter CDR3 regions (VHH38 and VHH1) achieved complete inhibition of SpvB only at 3-fold and 5-fold molar excess, respectively (Fig. 2*C*), possibly reflecting a weaker binding to the enzyme.

When expressed as an intrabody, VHH5 can inhibit SpvB in eukaryotic cells

We selected the best inhibitor of SpvB (VHH5) for expression as an intrabody, in order to test its ability to act in the cytoplasm of eukaryotic cells exposed to the toxin or infected with *S. typhimurium*. To this end, we transiently transfected RAW macrophages with vectors encoding VHHs fused to MBP. Immunostaining with an anti-MBP antibody confirmed that both MBP-VHH5 and a control, MBP-VHHαtr (anti-trans-sialidase), were equally well expressed in the cytoplasm of transfected RAW cells (**Fig. 3*L*, *O***). The expression of these VHHs was confirmed by Western blot analysis of cell lysates. In accord with previous studies (9, 54), infection of RAW cells with *S. typhimurium* induced morphological changes characterized by rounding, partial detachment, and apoptosis, as evidenced by phalloidin staining (Fig. 3*E vs. B*). This effect can be attributed in part to SpvB, since infection with an SpvB mutant *Salmonella* strain had only mild effects on the cellular architecture (Fig. 3*H*). Transfection with MBP-VHH5 partially protected cells from the cytotoxicity mediated by wild-type *Salmonella* (Fig. 3*K*). This protective effect was specific, since cells transfected with the control MBP-VHHαtr showed rounding and disintegration of the actin cytoskeleton on transfection with wild-type *Salmonella* (Fig. 3*N*). Of the VHH5-expressing transfected cells, 94% were protected from the cytotoxic effect of *Salmonella*, thus demonstrating the significance of the qualitative analysis showed in Fig. 3.

Despite the difficulties to analyze the effects of whole bacteria on cell fitness, the results of the above assay revealed a partial protection when cells expressed intracellular SpvB-inhibitory nanobodies. To corroborate this result, we next studied the protective effect of cytoplasmic expressed nanobodies on cells treated with an exogenously added translocation-competent chimeric binary C2IN-C/SpvB toxin (55). In the chimera, the catalytic domain of SpvB is cloned in place of the catalytic domain of the binary *Clostridium botulinum* C2 toxin, another actin ADP-ribosylating toxin (56). Thus, the SpvB catalytic domain is fused in frame behind the N-terminal domain of component I of the C2 (C2I) toxin, generating a chimeric C2IN-C/SpvB fusion protein. C2I mediates binding to component II of the C2 toxin (C2II), which in turn mediates both host cell binding and efficient delivery of the fusion protein into

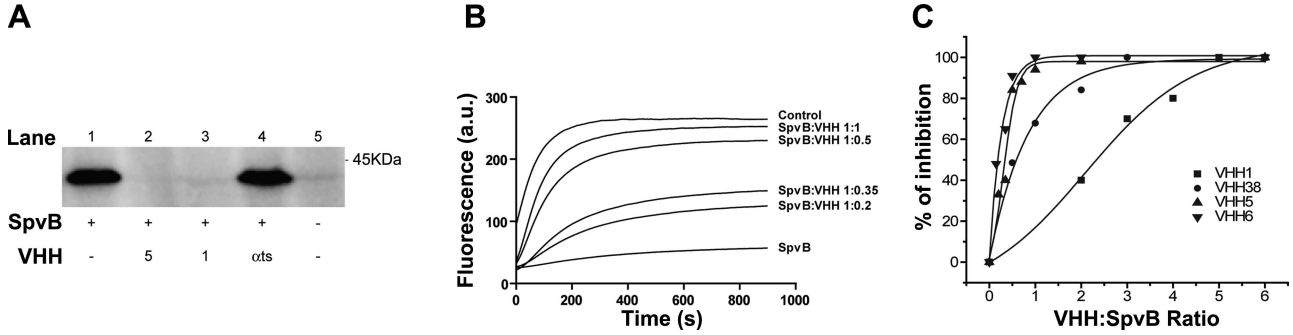

Figure 2. Specific VHHs inhibit SpvB *in vitro*, as demonstrated by radiolabeling and fluorometric assays. *A*) CHO cell lysates were incubated for 30 min with ^{32}P-NAD in the absence or presence of SpvB and SpvB-specific VHH1, VHH5, or a control VHH (anti-trans-sialidase αts). Proteins were size fractionated by SDS-PAGE, and covalent incorporation of ^{32}P-ADP-ribose into actin (40 kDa) was assessed by autoradiography. *B*) Polymerization of pyrene-labeled actin in the presence of NAD only (control) or SpvB + NAD (SpvB) was assessed by fluorometry. Addition of VHH5 at the indicated molar ratios reversed the inhibitory effect of SpvB on actin polymerization. *C*) Actin polymerization was assessed as in *B* in the absence or presence of different SpvB-specific VHHs. Results obtained for all tested nanobodies are plotted as percentages of SpvB-mediated inhibition of actin polymerization as a function of the VHH:SpvB molar ratio.

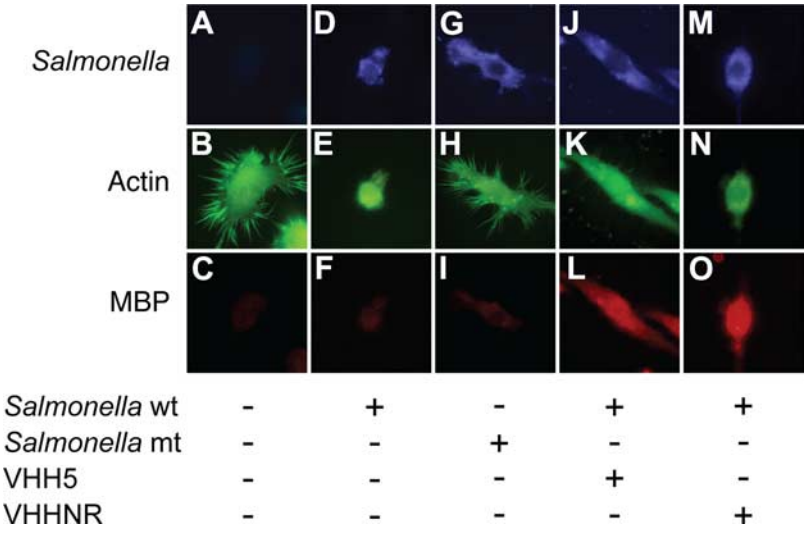

Figure 3. VHH5 intrabodies partially protect murine macrophages from the cytopathic effects of *Salmonella* infection. RAW macrophages were transiently transfected with expression constructs for MBP-VHH5 (specific anti-SpvB nanobody) or MBP-VHHαts (unrelated anti-trans-sialidase VHH as a control of specificity). At 24 h after transfection, macrophages were infected with wild-type (wt) or SpvB-mutant (mt) strains of *S. typhimurium*. Cells were fixed 16 h after infection. *Salmonella* and MBP-VHH fusion proteins were visualized by immunostaining using an anti-salmonella antibody (*A, D, G, J, M*) or an anti-MBP antiserum (*C, F, I, L, O*), respectively; actin was stained with phalloidin-FITC (*B, E, H, K, N*).

the host cell cytosol (57). The advantage of this system is that—in the absence of other bacterial virulence factors—the morphological changes induced by toxin exposure can be attributed specifically to the cytotoxic effects of SpvB. Moreover, the wild-type binary C2I toxin provides a convenient specificity control. We thus tested whether nanobody VHH5, when expressed as an intracellular MBP fusion protein, was able to inhibit the cytotoxic effects of the chimeric binary C2IN-C/SpvB toxin. To this end, subconfluent Vero cells were transfected with either MBP-VHH5 or an unrelated MBP-VHHαtr (anti-trans-sialidase) and then incubated with either the binary C2 (C2I) holotoxin or with the binary C2IN-C/SpvB fusion protein plus C2II (**Fig. 4**). The results show that transfection with VHH5 specifically protected cells from the cytopathic effects caused by SpvB (Fig. 4*K, L*) but not by C2I (Fig. 4*G, H*). Control transfection with the unrelated VHHαtr did not protect cells from SpvB-mediated cytotoxicity (Fig. 4*M, N*).

As exogenous monovalent single-domain antibody, VHH5 blocks cytotoxicity of secreted binary C2IN-C/SpvB chimeric toxin

Using the same binary C2IN-C/SpvB fusion protein plus C2II system, we also investigated whether VHH5, in a soluble extracellular monovalent nanobody format, could protect cells from the cytotoxic effects of exogenously added C2IN-C/SpvB chimeric toxin. To this end, we incubated cells with either wild-type C2I binary toxin or with the binary C2IN-C/SpvB chimeric toxin in the presence or absence of SpvB-specific VHH5 and monitored cytotoxicity by microscopy (**Fig. 5**). The results confirm a specific protective effect of VHH5 on cells against the binary C2IN-C/SpvB chimeric toxin (Fig. 5*E*) but not against the binary C2I holotoxin (Fig. 5*C*). In this case, we cannot rule out the possibility that VHH5, in addition to blocking the enzymatic activity of SpvB, exerts a protective effect also by inhibiting translocation of C2IN-C/SpvB.

DISCUSSION

SpvB is the prototype of a bacterial toxin that is directly translocated to the host cell cytosol *via* the SPI-2-encoded bacterial type III secretion system, and thus, inaccessible to conventional antibodies (9, 13). Using three different complementary assays, we demonstrate that single-domain antibodies derived from a llama immunized with the catalytic domain of SpvB specifically block the enzymatic and cytotoxic activities of this actin-ADP-ribosylating toxin (Fig. 2). Remarkably, the single-domain antibody VHH5 proved functionally ac-

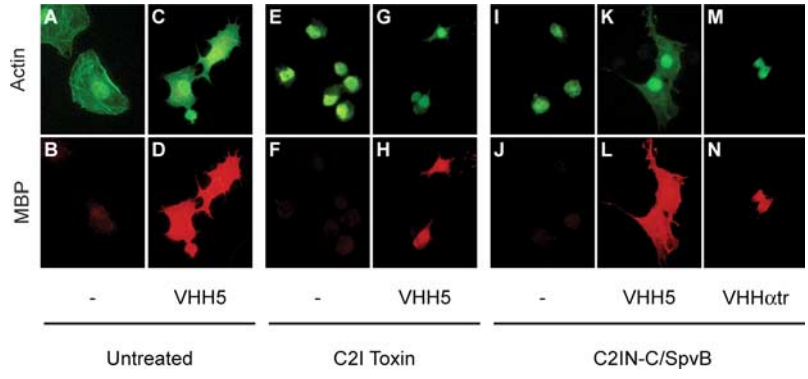

Figure 4. VHH5 intrabodies protect Vero cells from the cytopathic effects of translocation-competent C2IN-C/SpvB chimeric toxin. Vero cells were transiently transfected with expression constructs for MBP-VHH5 or MBP-VHHαts as in Fig. 3. At 24 h after transfection, cells were incubated for 3 h in the absence or presence of *C. botulinum*-based binary AB-toxins, *i.e.*, C2II and either wild-type C2I or the C2IN-C/SpvB chimeric toxin. Cells were fixed and analyzed by actin staining with phalloidin-FITC (top panels; *A, C, E, G, I, K, M*) and by immunofluorescence staining of MBP (bottom panels; *B, D, F, H, J, L, N*) as in Fig. 3.

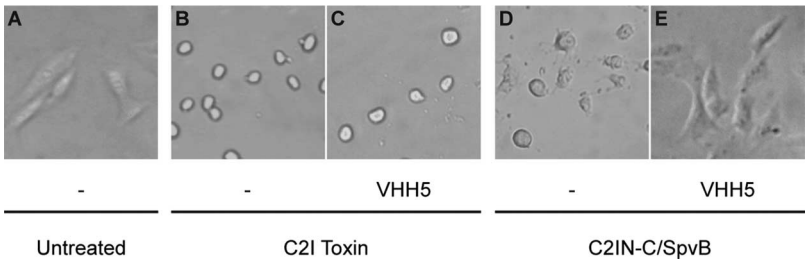

Figure 5. Extracellular VHH5 nanobody protects Vero cells from exogenously added C2IN-C/SpvB fusion toxin. *C. botulinum*-based binary AB-toxins, *i.e.*, C2II and either wild-type C2I or the C2IN-C/SpvB chimeric toxin, were preincubated with VHH5 for 15 min prior to addition to Vero cells. After 3 h at 37°C, cells were analyzed by phase-contrast microscopy. *A*) Untreated cells. *B, C*) Cells treated with C2I toxin. *D, E*) Cells treated with chimeric toxin.

tive in both the extracellular milieu (Fig. 5) and in the reducing environment of the cytoplasm (Figs. 3 and 4). These results provide a proof of principle for the concept that single-domain antibodies can serve as specific inhibitors of intracellular bacterial toxins. The SpvB-blocking nanobodies not only constitute useful experimental research tools, they might also provide the basis for new therapeutic reagents, *e.g.*, *via* transfection and/or transgenic expression of intrabodies, fusion to translocation domains, or leads for CDR3 peptide mimetics.

First, transgenic introduction and tissue-specific expression of foreign genes is well established for several domestic animals. Transgenic expression of SpvB-neutralizing VHHs as intrabodies in the cytosol of susceptible cells, *e.g.*, epithelial cells of the gut and/or macrophages, could serve as an experimental strategy for enhancing the resistance of domestic animals to pathogenic *Salmonella* strains. In this context, it is of interest to note that transgenic expression of llama-derived single-domain antibodies has already been used successfully in an insect (*Drosophila*) to block aggregation of a nuclear protein (37) and in a plant (potato) to block the activity of a starch-processing chloroplast enzyme (42). While transgenic tools are not applicable for use in humans, improved cell-transfection technologies may, in the future, provide means to express toxin-neutralizing intrabodies also in human cells, *e.g.*, on transfection with cDNA expression constructs for toxin-blocking nanobodies *ex vivo* (*e.g.*, macrophages) or *via* the gastrointestinal route (epithelial cells) (40).

Second, genetic fusion to translocation domains can serve as a mechanism to translocate proteins across the cell membrane into the cytosol (58, 59). Such an approach has recently been used to introduce a single-chain variable fragment (scFv; *i.e.*, the variable domains of the light and heavy chains of a conventional monoclonal antibody genetically fused *via* a linker peptide into a single polypeptide) directed against the *myc* oncogene into the cytosol of a human cell line (59). Similarly, fusion of a nanobody to a suitable protein or peptide-based translocation domain could provide a means of delivery for SpvB-neutralizing single-domain antibodies to the cytosol of epithelial cells. Owing to their high solubility and refolding capacity, single-domain antibodies from llamas may be even more suitable for such translocation strategies than conventional antibodies. With respect to macrophages and other antigen-presenting cells, targeted delivery of SpvB-neutralizing VHHs to the endosomal compartment of macrophages might also be achieved by genetic fusion of the nanobody to an antibody specific for an endocytosis receptor such as CD209/DCSIGN (60). Nanobodies are especially amenable for conversion into bispecific formats (24).

Third, the extended CDR3 regions of llama nanobodies can serve as lead for the synthesis of peptide mimetics. The CDR3 of llama single-domain antibodies typically is much longer than that of conventional antibodies and often constitutes a large portion of the antigen-binding paratope. Thus, synthetic peptide-based mimetics of the CDR3 (peptibodies) that retain the specificity and functionality of the original single-domain antibodies can be designed. This principle has recently been demonstrated for cAbLys3, a lysozyme-blocking single-domain antibody derived from an immunized dromedary (61). Peptides are easier to produce in large quantities and can more readily translocate across the plasma membrane than protein domains.

The proof of principle provided here for SpvB-neutralizing nanobodies could provide a model for developing specific neutralizing nanobodies against related ADP-ribosylating toxins and other type III effector proteins. Like SpvB, exoenzyme S of *Pseudomonas aeruginosa* and HopU1 of *Pseudomonas syringae* are ADP-ribosylating cytotoxins that are translocated to the cytosol of animal and plant cells, respectively, *via* a type III secretion system (62, 63). Moreover, SpvB is one of several cytotoxic effector proteins delivered *via* the SPI-2-encoded bacterial type III secretion system to the host cell cytosol (5, 64–66). Considering their high stability in various environments and in multivalent formats, nanobodies seem particularly suited also for "cocktail" strategies, *i.e.*, coexpression of two or more neutralizing reagents against different effectors.

In sum, our results underscore the suitability of camelid nanobodies for blocking biologically interesting enzymes in extra- and intracellular compartments and illustrate the advantages of these single-domain antibodies *vs.* the combined variable domains derived from conventional antibodies. FJ

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