Selection of Nanobodies that Block the Enzymatic and Cytotoxic Activities of the Binary *Clostridium Difficile* Toxin CDT

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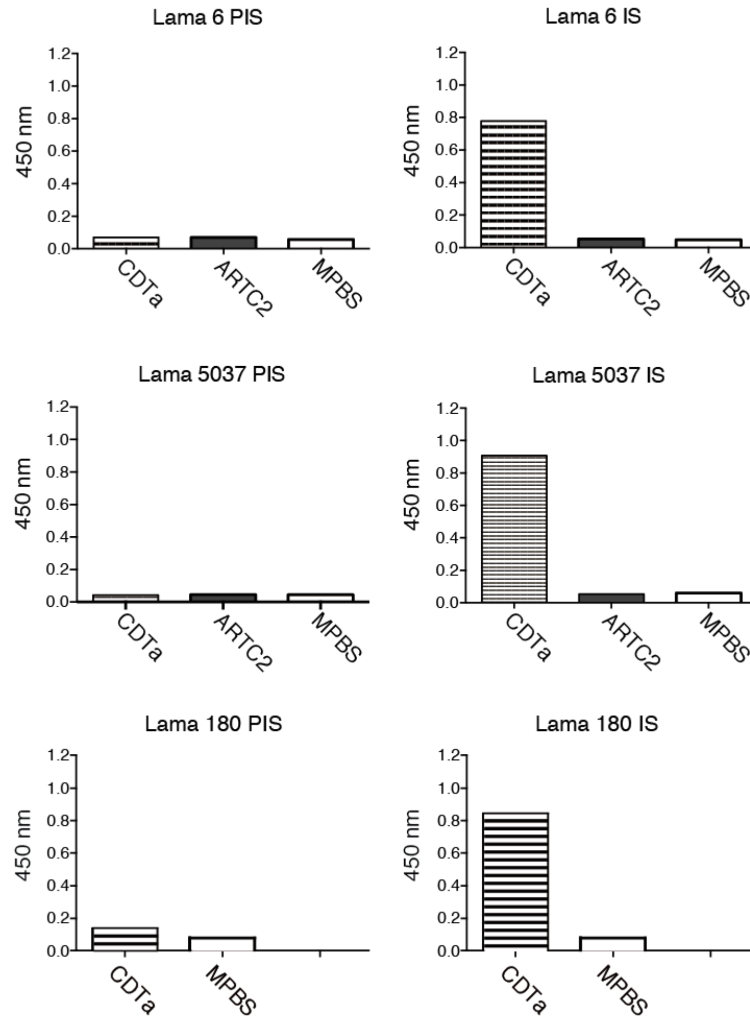
Supplementary information

Supplementary Table S1. Characterization of the phage display libraries encompassing the repertoire of VHHs in peripheral blood lymphocytes 10-20 days after the last boost immunization

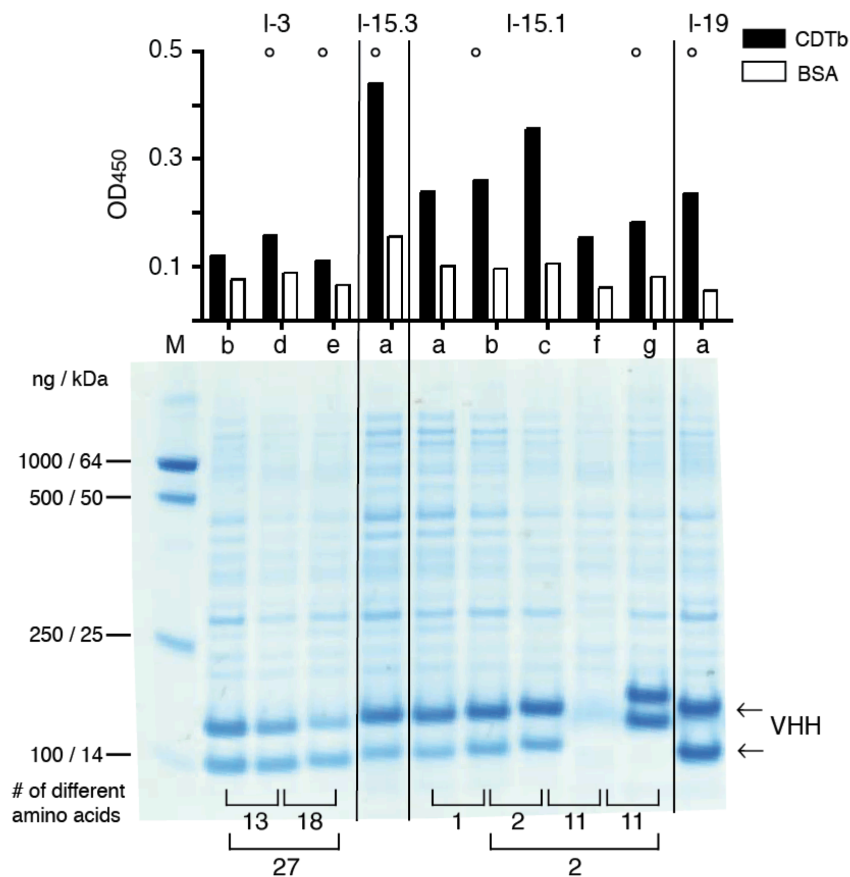
llama	size	#seq	ORFs	DUP	αCDT	CDR3
6	9.7×10^4	12	12	2	0	3-20
5037	1.2×10^5	24	17	0	0	6-19
180	7.8×10^4	24	18	0	1*	10-21

The size of the libraries is given in number of individual colonies. #seq indicates the number of clones sequenced from the primary library. ORFs indicates the number of open reading frames containg intact VHHs amongst the sequenced clones. DUP indicates the number of duplicate clones (carrying an identical amino acid sequence) among the I° clones. αCDT indicates the number of clones in the primary library containing sequences identical or similar to clones selected by panning on CDTa or CDTb. CDR3 indicates the range of CDR lengths within the sequenced I° clones. *One of 18 primary clones analyzed from llama 180, designated l-15.1k, revealed a sequence highly similar to those of other members of the CDTb-specific nanobody family l-15.1.

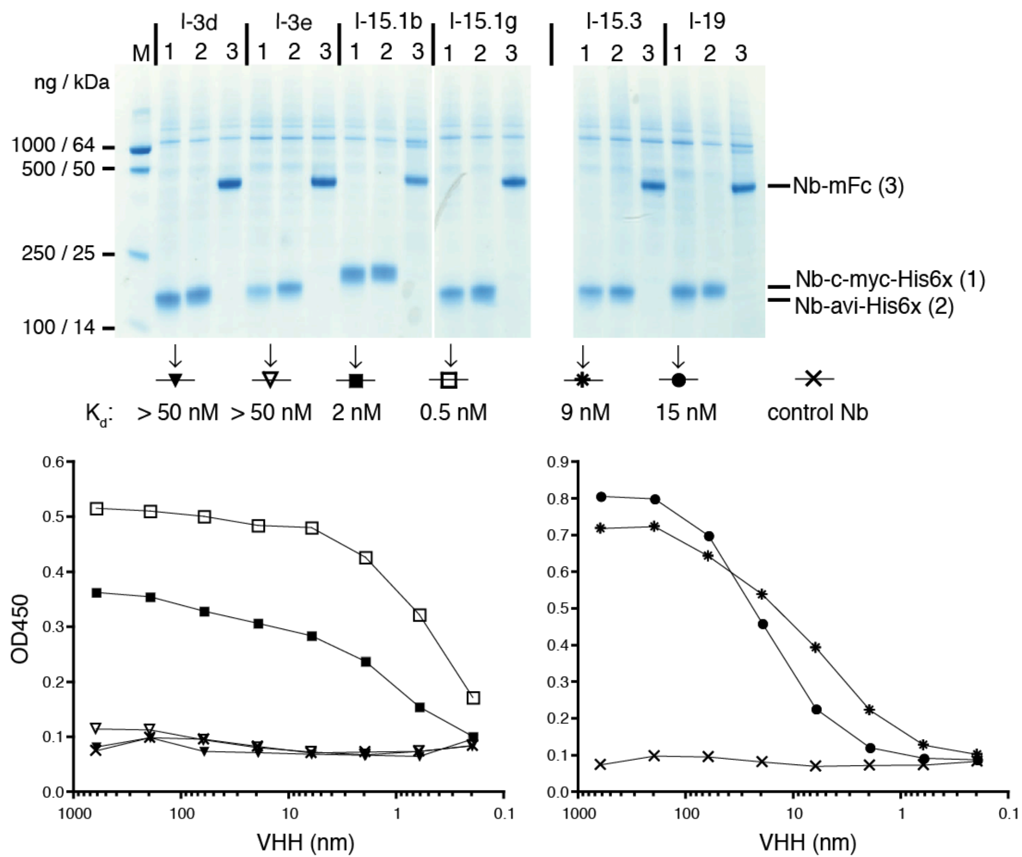
Supplementary Figure S1. Antibody response of llamas immunized with CDT. The antibody response was monitored by ELISA on microtiter plates coated with recombinant CDTa, CDTb, or ARTC2 (100 ng/ well) or with 5 % powdered milk in PBS (co). Pre-immune serum (PIS) and immune serum (IS) was obtained immediatedly before the first immunization and 9-10 d after the last immunization, respectively. Sera were diluted 1:100 in PBS for analyses. Bound llama antibodies were detected with monoclonal antibodies specific for llama heavy chain IgG2 and IgG3 antibodies[1] followed by peroxidase-conjugated anti-mouse IgG.



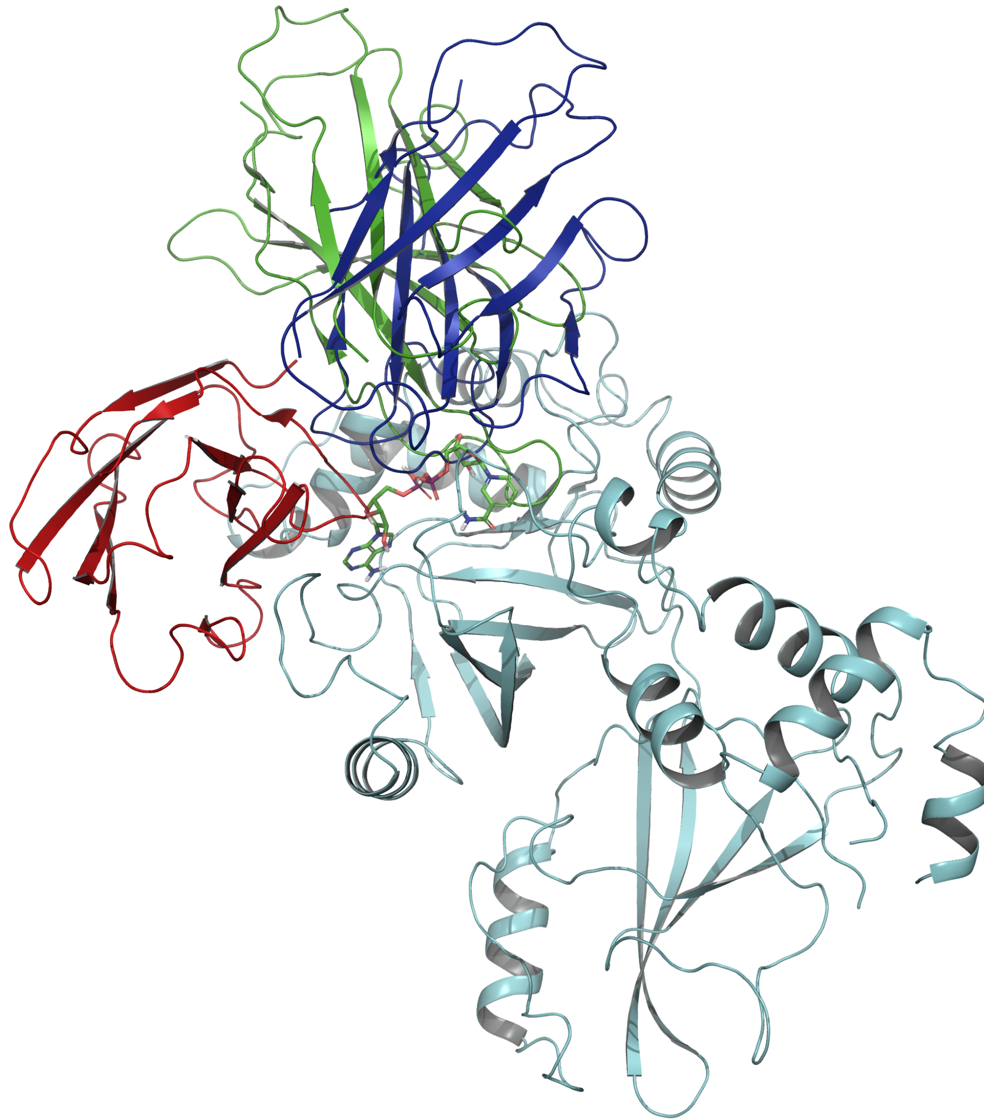
Supplementary Figure S2. Production of monovalent nanobodies in the E. coli periplasm and verification of binding specificities. *E. coli* HB2151 cells were transformed with individual VHH-encoding pHEN2 vectors and protein expression was induced with IPTG for 3 h. Periplasmic lysates were prepared by osmotic shock, clarified by centrifugation, and analyzed by SDS-PAGE and Coomassie staining (top). Binding specificity was analyzed by ELISA using plates coated with recombinant CDTb. Representative results are shown for four families of CDTb-specific nanobodies. Family names indicate the presence of a long or short hinge, the absence or presence of an additional cysteine pair in CDRs 2 and 3, and the number of amino acid residues in the CDR3 (see Table I). Family members with distinct amino acid sequences were desginated a, b, c, d etc. in order of decreasing number of isolates. Numbers on the bottom indicate the number of different amino acids between two family members. Nanobodies marked by a circle were selected for subcloning into a eucaryotic expression vector.



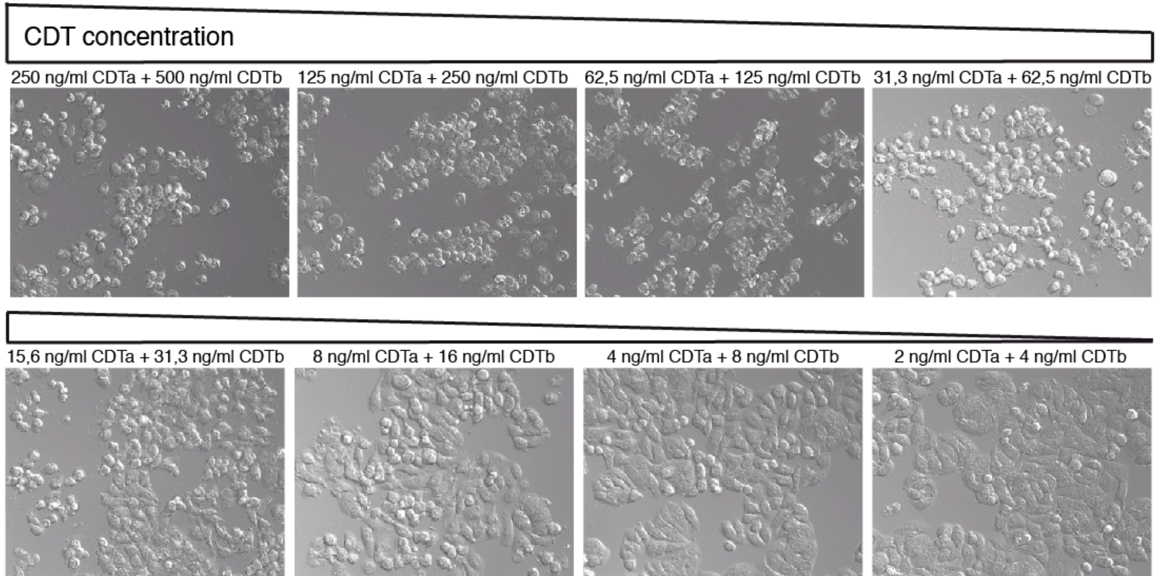
Supplementary Figure S3. Production of reformated nanobodies in transfected HEK-6E cells and comparative analyses of binding affinities. HEK-6E cells were transfected with cDNA-expression constructs encoding nanobodies fused C-terminally to chimeric tags (c-myc-His6x or avi-His6x) or to the Fc domain of mouse IgG2c (mFc). Transfected cells were cultured in serum free medium for 6 d. Cell supernatants were clarified by centrifugation and analyzed by SDS-PAGE and Coomassie staining (top). Qualitative comparison of nanobody affinities was performed by ELISA on wells coated with CDTb using serial dilutions of monovalent nanobodies. After 20 minutes of incubation, wells were washed three times to remove unbound proteins and bound nanobodies were detected with a peroxidase-conjugated monoclonal antibody directed against the c-myc tag. A c-myc tagged nanobody directed against ARTC2[2] was used as negative control. Symbols used for the respective nanobodies and the calculated dissociation constants are indicated in the middle. Representative results are shown for four families of CDTb-specific nanobodies.



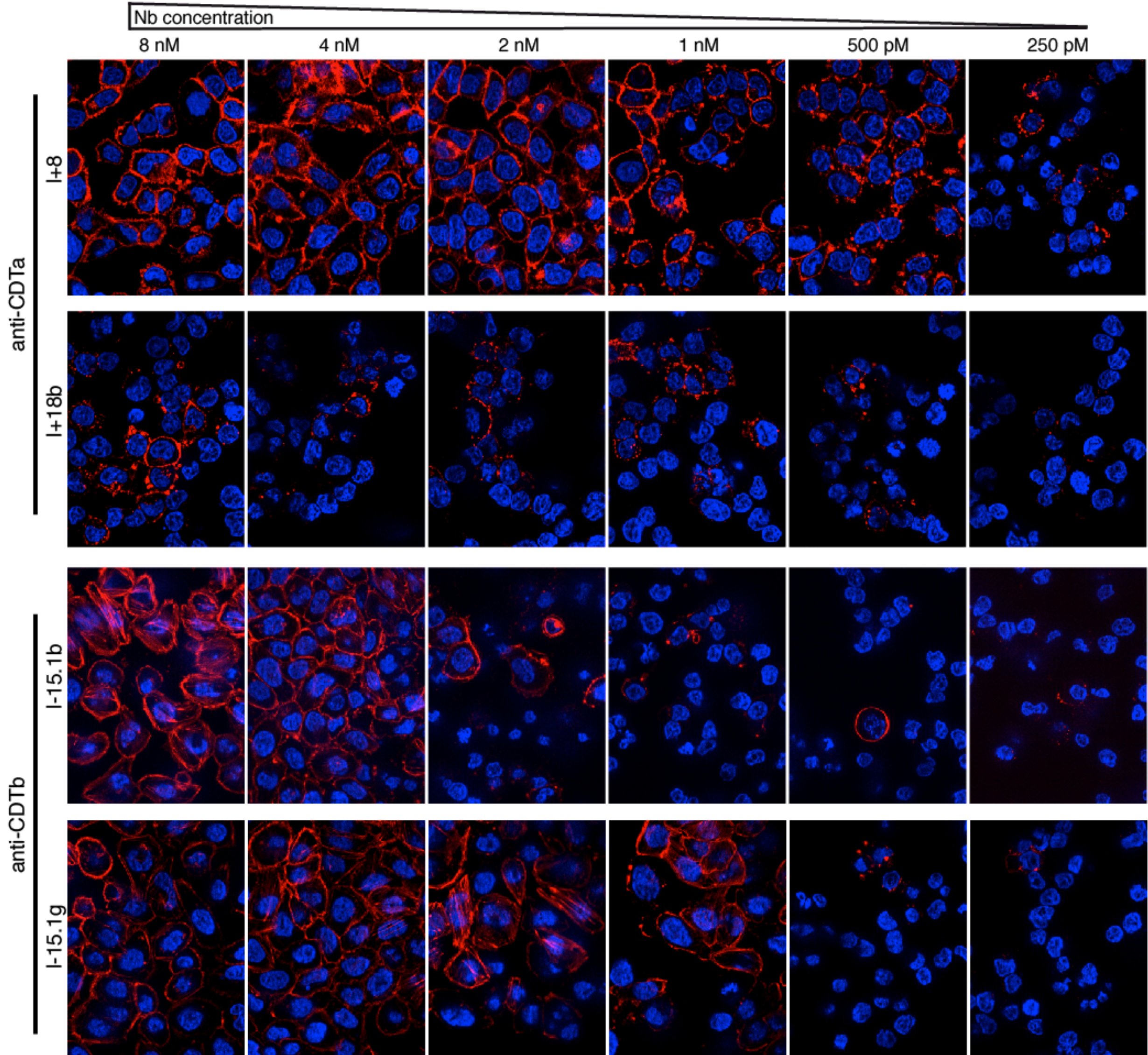
Supplementary Figure S4. Hypothetical epitopes of three CDTa-specific nanobodies based on molecular modeling. CDTa (pdb code 2WN7) is shown in cyan, nanobody l+18 in red, nanobody l-14 in green and nanobody l+18 in blue. All nanobodies are predicted to interfere with binding of NAD$^+$ (shown as a stick model) and with binding of actin (not shown). Nanobodies l-14 and l-18 are predicted to block each other but not nanobody l+8. The latter is predicted to partially occlude the binding pocket of the adenine moiety of NAD$^+$, the former that of the nicotinamide-ribose moiety of NAD$^+$.



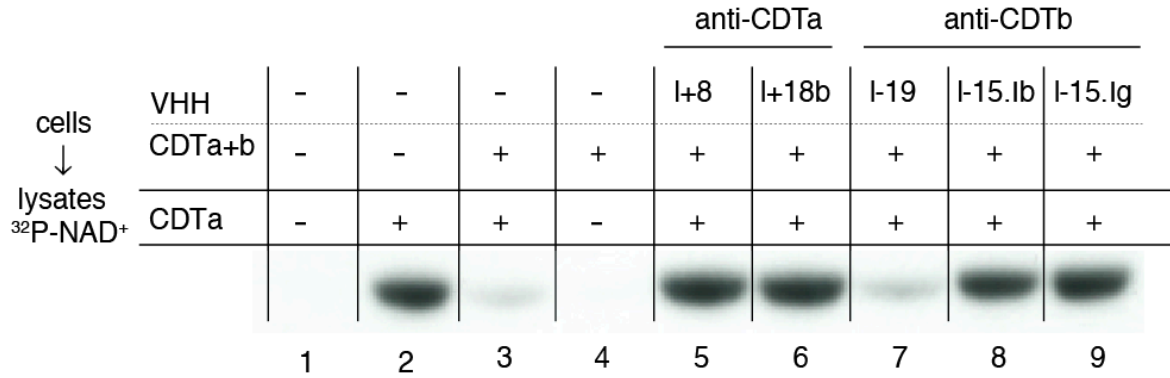
Supplementary Figure S5. Titration of CDTa+b induced cytotoxicity for HT-29. Before addition to cultures of adherent HT-29 cells, CDTa and CDTb were mixed and subjected to a 1:2 dilution series in PBS containing BSA. Four hours after addition of toxins, cells were fixed in 2% paraformaldehyde. Cellular morphology was assessed by Digital interference contrast microscopy using a Zeiss Axiovert 200M microscope.



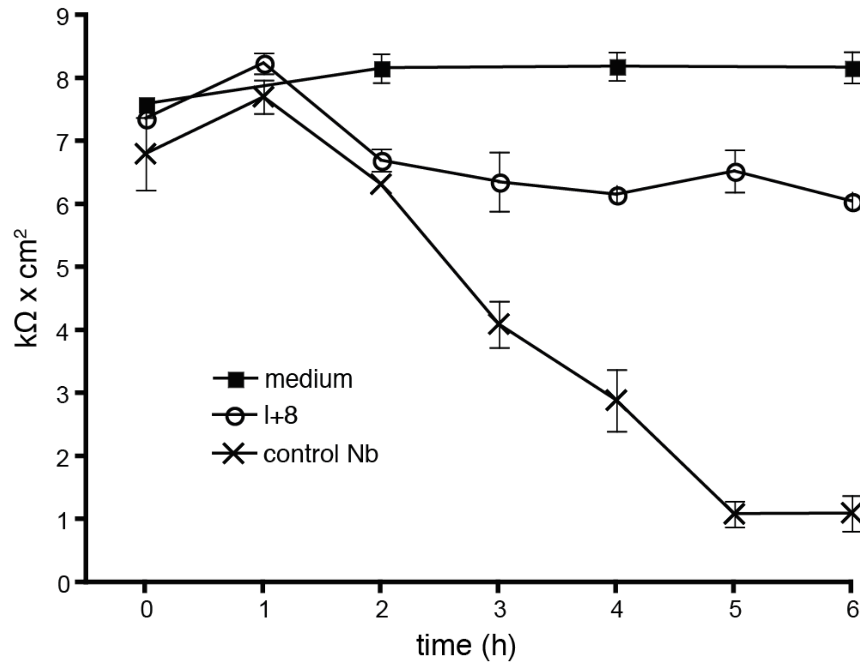
Supplementary Figure S6. Titration of nanobody-mediated neutralization of CDTa+b cytotoxicity. Before addition to a mixture of CDTa (24 ng/ml, 0.5 nM) and CDTb (30 ng/ml, 0.4 nM) nanobodies were subjected to a 1:2 dilution series in PBS containing BSA. Toxins and nanobodies were added to adherent HT-29 cells for 4 h at 37°C. Cells were fixed and stained with the actin-staining dye phalloidin-rhodamine and the DNA-staining dye Hoechst 33342. Cellular morphology and integrity of the cellular cytoskeleton were assessed by immunofluorescence microscopy using a Zeiss Axiovert 200M microscope equipped with DIC and an Apotome.



Supplementary Figure S7. Nanobodies l+8 and l+18b effectively prevent ADP-ribosylation of actin in cells treated with CDTa and CDTb. Before addition to HT29 cells, CDTa and CDTb were preincubated for 20 min with or without the indicated CDTa- or CDTb-specific nanobodies. Cells were then incubated with the toxin-nanobody mixture for 30 min to allow uptake of CDTa and CDTa-catalyzed ADP-ribosylation of actin. Cells were then washed and lysed with Triton-X-100. Cell lysates were clarified by centrifugation. In order to ADP-ribosylate un-modified actin, CDTa and ^{32}P-NAD^{+} were then added to the cell lysates and incubation was continued for 15 min before reactions were stopped by addition of SDS-PAGE sample buffer. Proteins were size fractionated by SDS-PAGE and radioactivity covalently incorporated into actin was detected by exposing the dried gel to an X-ray film. Treatment of cells with CDTa+b in the absence of nanobodies (lane 3) prevented subsequent radio-ADP-ribosylation of actin (due to prior essentially complete ADP-ribosylation of actin from non-radioactive endogenous NAD^{+}). In contrast, actin was as effectively ADP-ribosylated in cells treated with CDTa+b in the presence of nanobodies l+8, l+18b and l-15.1g (lanes 5, 6, 9) as in untreated cells (lane 2). Nanobody l-19 did not block and l-15.1b partially blocked ADP-ribosylation of actin from endogenous NAD^{+} (lanes 7, 8).



Supplementary Figure S8. CDTa-specific nanobody l+8 inhibits CDT-induced disruption of transepithelial resistance. Before addition to established monolayers of MDCK C7 cells grown on 24 well filter plates, CDTa (10 nM) and CDTb (20 nM) were preincubated for 15 min with excess CDTa-specific or control nanobodies (200 nM). The transepithelial electrical resistance (TEER) was measured every hour with a Volt Ohm meter.



References

1. Daley, L.P., Gagliardo, L.F., Duffy, M.S., Smith, M.C. & Appleton, J.A. Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids. *Clin Diagn Lab Immunol* **12**, 380-386 (2005).

2. Koch-Nolte, F. et al. Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 in vivo. *Faseb J* **21**, 3490-3498 (2007).