SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

Expression and purification of binary toxin in *E. coli.*

The nucleotide sequence of CDTa (without N-terminal 43 residue signal peptide) was codon optimized by Genscript (Piscataway, NJ) with flanking *Nde*I and *Xho*I restriction sites and cloned into the *E. coli* expression vector pET-30a (EMD Millipore, Billerica, MA). The expression vector was transformed into the *E. coli* BLR (DE3) host (Invitrogen, Carlsbad, CA). In order to decrease protein dimerization, the cysteine at position 2 was mutated to alanine using Stratagene's QuikChangeII Site-Directed Mutagenesis Kit. After sequence verification, the mutated plasmid, pET-30a-*1m_cdtA*, was transformed into *E. coli*. The recombinant proteins had a six-His tag at the C-terminus.

Expression and purification of His-tagged recombinant protein were performed according to EMD Millipore's pET System Manual. Briefly, expression was induced with the addition of IPTG to a final concentration of 1mM, and incubation continued at 37ºC for 3 hours. Bacteria were harvested by centrifugation, lysed by microfluidization at 980 bar (Microfluidics, Newton, MA) and clarified by centrifugation. Clarified lysates were loaded on a POROS 20 MC column (2.6 × 3.8 cm, Applied Biosystems, Foster City, CA) pre-loaded with Ni^{2+}, and step elution was achieved in elution buffer containing 300 mM imidazole. CDTa and 1mCDTa products were further purified using POROS 50 HS (1.6 × 14 cm, Applied Biosystems) cation exchange chromatography. CDTa and 1mCDTa

products were purified to greater than 98% purity and concentrated and buffer-exchanged into 50 mM HEPES buffer (pH 7.5, 150 mM NaCl) by tangential flow filtration (TFF) with a 5 kDa molecular weight cutoff (MWCO) Pellicon XL Biomax10 membrane (EMD Millipore) and stored at -70°C prior to use.

Expression and purification of ProCDTb and CDTb in *E. coli*. The nucleotide sequence of ProCDTb (without N-terminal 42 residue signal peptide) and CDTb (without N-terminal signal peptide and Pro domain) were codon optimized by Genscript with flanking *Bam*HI and *Xho*I sites and ligated into the expression vector pGEX-6p1 (GE Healthcare, Cleveland, OH). pGEX-6p1-*pro_cdtB* and pGEX-6p1-*cdtB* were transformed into *E. coli* BLR (DE3). The recombinant proteins had a GST tag at the N-terminus and were named GST_ProCDTb and GST_CDTb respectively. Pro refers to the Pro-domain protein sequence between amino acid 1 and amino acid 168, which is cleaved off during chymotrypsin activation.

Expression and purification of GST-tagged recombinant proteins were performed according to the GE Healthcare's instruction manual and Sundriyal et al.'s paper (1). Briefly, expression was induced with the addition of IPTG to a final concentration of 0.1mM, and incubation continued at 37ºC for 5 hours. Bacteria were harvested by centrifugation and lysed by microfluidization at 980 bar. Clarified lysates were loaded on a glutathione Sepharose 4B column (5×5 cm, GE Healthcare). Step elution was achieved in GST elution buffer containing 20 mM reduced glutathione. GST-tagged products were polished to ~95% purity and aggregates were removed by size exclusion chromatography

(SEC) with a Superdex200 column (2.6 × 60 cm, GE Healthcare). GST_ProCDTb was
48 buffer-exchanged into 50 mM HEPES buffer (pH 6.5, 150 mM NaCl) by TFF with a
10kDa MWCO membrane and the final product was stored at -70°C. GST_CDTb
50 underwent GST tag cleavage through treatment with the PreScission Protease (GE
Healthcare) according to the GE Healthcare's instruction manual. CDTb was buffer-
52 exchanged to 50 mM HEPES buffer (pH 6.5, 150 mM NaCl) by TFF with a 10 kDa
MWCO membrane and stored at -70°C prior to use.

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Expression of toxins using a Bac-to-Bac® Baculovirus Expression System
56 **(Invitrogen, Carlsbad, CA)**. Nucleotide sequence of 3mCDTa and ProCDTb were
cloned into a pFastBac™1 transfer vector and the recombinant vector was transfected into
58 Sf9 (ATCC, Manassas, VA), Sf21 (Kemp Biotechnology, Frederick, MD), or
expresSF+® (Protein Sciences Corporation, Meriden, CT) cells using Cellfectin® II
60 reagent to generate recombinant baculovirus. Insect cells were grown in suspension
culture in Sf900-III culture medium. Virus amplification was performed by infection of
62 Sf21 cells at a multiplicity of infection (MOI) of 0.1 to 1. The virus stocks were titered
using a flow cytometric baculovirus titration assay (Expression Systems, Woodland, CA).

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For recombinant protein generation, insect cells were grown in a 20-L Wave®
66 bioreactor. The cultures were grown to a cell concentration of 1-2 × 10^6 cells/mL and
infected at an MOI of 0.1-1.0. Cells were harvested after about 4-5 days of infection. The
68 insect cells were lysed using a non-ionic detergent and clarified by centrifugation. All
toxins were purified using a combination of chromatographic techniques yielding

70 products that were greater than 95% purity by SDS_PAGE analysis (2). The purified antigens were concentrated and buffer-exchanged exchanged to 50 mM HEPES buffer 72 (pH 7.5, 150 mM NaCl) using TFF and stored at -70ºC.

74 **SDS-PAGE and anti-CDTa and anti-CDTb western blot**. Two micrograms of purified protein was mixed with an equal volume of 2x loading buffer and resolved in a 4%-20% 76 gradient Tris-glycine gel (Invitrogen) according to the Laemmli method (3). Dithiothreitol was added to a final concentration of 50 mM for the reducing SDS-PAGE. 78 Western blotting was performed using an iBlot gel transfer device (Invitrogen) according to the manufacturer's protocol. CDTa was detected using an anti-CDTa monoclonal 80 antibody (mAb) and CDTb was detected using anti-CDTb mAb (mAbs generated at Merck Research Laboratories). HRP-goat-anti-mouse secondary antibody (Invitrogen) 82 diluted to 1-to-2000 was used as the secondary antibody and the signal was developed using the HRP-substrate kit according to the manufacturer's protocol (Bio-Rad, Hercules, 84 CA).

86 **Cell Lysate Preparation and anti-LSR western blot**. Cell lysate preparation and anti-LSR western blot were conducted similarly to methods described in the literature (4). 88 Briefly, cells were washed twice with PBS and lysed on ice using Cell Extraction Buffer (Invitrogen) supplemented with 1mM PMSF and a protease inhibitor cocktail (Sigma, St. 90 Louis, MO). Equal amounts of each cell lysate were separated by SDS-PAGE and transferred to nitrocellulose membranes for western blotting using the iBlot gel transfer 92 device. LSR was detected using polyclonal rabbit anti-LSR antibody (Santa Cruz

Biotechnology, Dallas, TX). Actin was detected using anti-actin monoclonal antibody
94 clone ACTN05 (Thermo Fisher Scientific, Waltham, MA).

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Electrospray Ionization Time-of-flight (ESI-TOF) Mass Spectrometry. Liquid
98 chromatography and MS analysis of chymotrypsin activated GST_ProCDTb was performed
with a capillary 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled
100 to a MSD-ESI-TOF VL mass spectrometer (Agilent Technologies). The samples were diluted
into 0.1% formic acid (Thermo Fisher Scientific) and injected on to a 0.5 mm x 50 mm
102 Monolithic PS-DVB column (Thermo Fisher Scientific) at 75°C and eluted with a gradient of
0.1% formic acid in water to 100% acetonitrile (Thermo Fisher Scientific) at a flow rate of
104 0.015 mL/min. The eluent was injected into the mass spectrometer. Mass reconstruction from
the raw data was accomplished using the BioConfirm software (Agilent Technologies). The
106 accurate mass of the cleaved protein (observed by TOF-MS) was compared to the protein's
primary sequence using Protein/Peptide Molecular Weight Calculation Tools (Agilent
108 Technologies) to determine the amino acid sequence of the protein.

110 **Cell lines and cell culture.** Vero (African green monkey kidney, ATCC#CCL-81),
IMR90 (human lung fibroblast, ATCC#CCL-186), T84 (human colorectal carcinoma
112 epithelial, ATCC CCL-248), HT29 (colorectal adenocarcinoma, ATCC#HTB-38),
HepG2 (human liver carcinoma, ATCC#HB-8065), and Caco-2 (human colon epithelial,
114 ATCC#HTB-37) cell lines were obtained from the American Type Culture Collection
(ATCC, Manassas, VA) and cultured as described by ATCC. Briefly, Vero and HepG2
116 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with

10% heat-inactivated fetal bovine sera (FBS, HyClone, Logan, UT) and 100 units/mL of
118 penicillin-streptomycin (Invitrogen). IMR90 cells were cultured in EMEM supplemented
with 10% FBS and 100 units/mL of penicillin-streptomycin, 2 mM L-glutamine, 1%
120 MEM non-essential amino acid (100× stock solution), 1 mM sodium pyruvate, and 1%
sodium bicarbonate (7.5%). T84 cells were cultured in a 1:1 mixture of Hank's F12
122 medium and Dulbecco's modified Eagles's medium supplemented with 5% FBS. HT29
cells were cultured in McCoy's 5a Medium supplemented with 10% FBS and 100
124 units/mL of penicillin-streptomycin. Caco-2 cells were cultured in EMEM supplemented
with 20% heat-inactivated fetal bovine sera and 100 units/mL of penicillin-streptomycin.
126 All cell culture media were purchased from ATCC. All cells were incubated in a
humidified chamber at 37ºC with 5% CO_2.

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RESULTS

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Cell-line screening in the cytotoxicity assay. HT29, T84, Caco-2, HepG2, and IMR-90
132 cells were compared to Vero cells to determine whether these cell lines were sensitive to
binary toxin utilizing the cytotoxicity assay conditions identified above. The geometric
134 mean TC_{50}s of 1mCDTa in binary toxin were 62 pM, 13 pM, 13 pM, 15 pM, and 29 pM
for Vero, HT29, T84, Caco-2, and HepG2, respectively (FIG S1A). IMR-90 cells were
136 found to be completely insensitive to binary toxin. The sensitivity of cell lines to binary
toxin was consistent with the amount of LSR receptor expressed in each cell line (FIG
138 S1B). T84, Caco-2, and HepG2 cell lines were not selected for the high throughput assay
because they grew much slower than Vero cells under culture conditions described in the

140 method. HT-29 cells grew only slightly slower than Vero cells and were good cell substrates in the high throughput assay; however, we observed that incubation of HT29

142 cells with a_CDTb alone resulted in cell cytotoxicity at concentrations greater than 750 pM through a mechanism that was independent of F-actin depolymerization. Therefore,

144 we did not select HT-29 cells for the binary toxin NAb assay.

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REFERENCES

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164 **TABLE S1**. Experimental design of binary toxin NAb assay DOE

Analyst	Day	Block	Cell Density (cells/well)	Toxin Conc. (pM)	Pre-Incubation Time (minutes)
1	1	Block 1	1000	968	30
1	1	Block 1	1000	242	120
1	1	Block 1	1500	484	60
1	1	Block 1	2000	242	30
1	1	Block 1	2000	968	120
1	1	Block 1	1000	242	30
2	1	Block 2	1000	968	120
2	1	Block 2	1500	484	60
2	1	Block 2	2000	968	30
2	1	Block 2	2000	242	120
2	1	Block 2	1000	484	60
2	1	Block 2	1500	484	30
1	2	Block 3	1500	484	60
1	2	Block 3	2000	484	60
1	2	Block 3	1500	242	60
1	2	Block 3	1500	484	60
2	2	Block 4	1500	968	60
2	2	Block 4	1500	484	120
2	2	Block 4	1000	968	30
2	2	Block 4	1000	242	120

166 Note: The 18 runs were divided between 2 analysts and 2 days of testing, with the four combinations of analyst and day defining the four blocks. The 18 runs consisted of the
168 eight combinations of the three factors at their low and high settings, the six face-centered axial points, and the center condition tested once within each block (total of 4
170 times).

172 **FIGURES AND FIGURE LEGENDS**

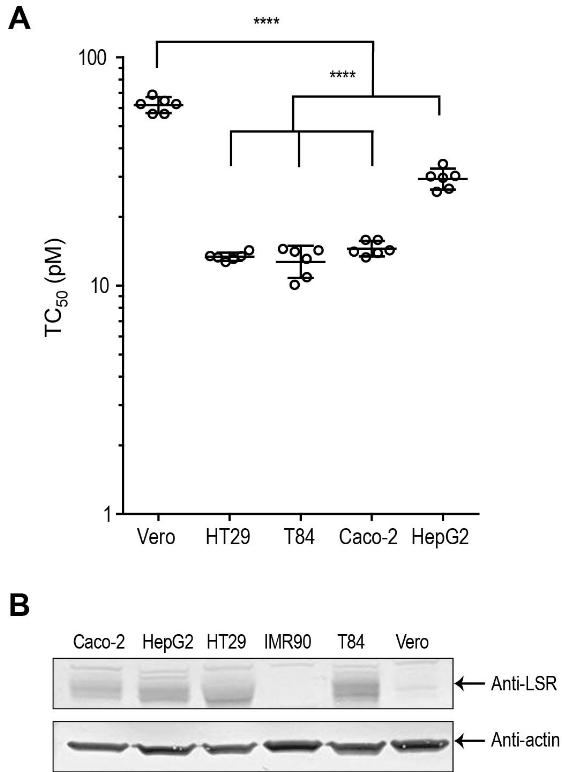

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FIG S1 Cell-line screening in the binary toxin cytotoxicity assay. (A) TC_{50} of 1mCDTa in

176 binary toxin with Vero, HT29, T84, Caco-2 and HepG2 cells substrates. 1mCDTa was mixed with a_CDTb at 1-to-7 molar ratio. Midpoint lines indicate the geometric mean

178 value. Error bars indicate the 95% confidence interval of the geometric mean (n=6). Statistical comparisons were performed using the Bonferroni's Multiple Comparison

180 Test. ***, P≤0.001. (B) Anti-LSR western blot of cell lysates. There is no visible band in the IMR-90 cell lysate and a weak band in the Vero cell lysate. However, the anti-LSR

182 bands were much darker in the HT29, T84, Caco-2, and HepG2 cell lysates. An anti-actin monoclonal antibody was used to assess equal protein loading of the cell lysates.