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Report

The Inhibitory Effect of Cholera Toxin B Subunit on *Clostridium Perfringens* Iota-Toxin-Induced Cytotoxicity

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Clostridium perfringens **iota-toxin consists of an enzymatic Ia component and binding Ib component. Ib binds to membrane receptors, forms heptamers in lipid rafts, and associates with Ia. The toxin complex is internalized into cells. In this study, we evaluated the inhibitory effects of cholera toxin B subunit (CTB), which binds to lipid rafts, on iota-toxin-mediated cytotoxicity. We examined the effect of CTB on iota-toxin-induced cell rounding activity against MDCK (Madin-Darby canine kidney) cells and Ib-induced cell death in A431 (human epithelial) cells. The presence of CTB inhibited both the cell rounding activity of iota-toxin (MDCK cells) and Ib-induced cell death (A431 cells). Moreover, CTB blocked binding of the Ib monomer to both cells at 4°C. We found by immunofluorescence microscopy that, after loading with Ib and CTB at 4°C, Ib and CTB bound to distinct regions in the plasma membranes. In MDCK cells at 37°C, Ib internalized and partially coexisted with CTB in cytoplasmic vesicles. These findings demonstrated that CTB blocks the binding of iota-toxin to target cells. CTB may provide a protective effect against infection.**

Key words *Clostridium perfringens* iota-toxin, cholera toxin B subunit, cytotoxicity

INTRODUCTION

Iota-toxin from *Clostridium perfringens* type E mediates antibiotic-associated diarrhea and enterotoxemia in rabbits.[1–5] Iota-toxin is secreted by the organism and contains two separate proteins; the enzyme component Ia and the binding/translocation component Ib. Ib interacts with the target cell-membrane receptor and accelerates Ia internalization and translocation into the cytoplasm. In cytoplasm, Ia exerts a cytotoxic effect on target cells by ADP-ribosylation of G-actin, leading to cell rounding.[1–5] Iota-toxin is a clostridial binary toxin that contains *Clostridium difficile* transferase, *Clostridium spiroforme* toxin and *Clostridium botulinum* C2 toxin.[1–5]

Iota-toxin exhibits this cytotoxic effect on host cells only when the two components act together; neither component alone shows toxic activity.[1–3] Ib docks to the target cells, forming heptamers in lipid raft microdomains.[6–8] In contrast, we previously showed that Ib causes cell necrosis to a narrow range of cell lines, including A431 and A549 cells.[9] We also demonstrated that the induced cytotoxic activity is related to functional pore formation by Ib.

It was reported that lipolysis-stimulated lipoprotein receptor (LSR) is a target cell receptor for Ib.[10,11] Iota-toxin internalizes host cells via CD44-dependent endocytosis.[12] LSR and CD44 are well-known cell-surface transmembrane proteins with roles in diverse cellular functions.[10,12] Subsequently, LSR and CD44 in lipid rafts of host cells were revealed to be functional iota-toxin receptors or co-receptors related to Ib target cell binding.[5,13] Ib forms an oligomer on cell membranes and lipid rafts. Methyl-β-cyclodextrin (MβCD), an agent that disrupts membrane rafts, inhibits the cytotoxic effect of iota-toxin.[7,8] Cholera toxin B subunit (CTB) is associated with the raft domain in various cell types.[14] CTB recognizes the cell surface ganglioside GM1.[14] It has been reported that CTB markedly blocks the internalization of *Toxoplasma gondii* through lipid rafts.[15] In the current study, we investigated the effect of CTB on the cytotoxicity of iota-toxin.

MATERIALS AND METHODS

Materials Purified recombinant Ia and Ib proteins were obtained using previously published methods.[16] Purified recombinant *C. botulinum* toxin C2I and C2II were prepared as described previously.[17] To obtain C2IIa, C2II was treated with trypsin.[17] We obtained rabbit antibody against Ib as reported previously.[7] Cholera toxin subunit B, Cell Proliferation kit I (MTT), goat anti-rabbit IgG, horse-radish peroxidase (HRP)-conjugate, sheep anti-mouse IgG, horse-radish peroxidase (HRP)-conjugate, polyvinylidene difluoride (PVDF) membranes (Immobilon P) and an ECL Western blotting kit were purchased from Merck (Tokyo, Japan). Mouse anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and Hanks' balanced salt solution (HBSS) were obtained from Nacalai Tesque (Kyoto, Japan). Alexa Fluor 488-conjugated cholera subunit B, Alexa Fluor 568-conjugated goat anti-rabbit IgG, and 4',6'-diamino-2-phenylindole (DAPI) were obtained from ThermoFisher Scientific (Tokyo, Japan).

Cell Culture and Assay of Cytotoxicity MDCK and

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A431 cells (from RIKEN BioResource Research Center (Tsukuba, Japan)) were maintained in DMEM containing 10% fetal calf serum (FCS), streptomycin (100 μg/mL) and penicillin (100 units/mL) (FCS-DMEM) at 37°C in a 5% CO_2 concentration. For the cell rounding assay, MDCK cells (1.0×10^5 cells) were inoculated in 48-well plates. Ia and Ib were added to monolayers. The cells were observed at 4 h after incubation for morphological changes, as described previously.[7] For the cell viability assay, A431 cells were seeded in 94-well plates (5.0×10^4 cells per well) and treated with Ib for 4 h. The assay was performed with a Cell Proliferation kit I (MTT) according to the manufacturer's instructions.

Western Blotting Western blotting analysis was performed as previously described.[11] The following antibodies were used: primary antibodies, rabbit anti-Ib antibody and rabbit anti-β-actin antibody; secondary antibody, HRP-conjugated secondary antibodies.

Immunofluorescence Staining Ib and Alexa Fluor 488-conjugated cholera subunit B were incubated with cells at 4°C for 1 h in FCS-DMEM. After three washes in cold FCS-DMEM, cells were transferred to FCS-DMEM prewarmed to 37°C and incubated at the same temperature for 30 min. Immunofluorescence staining was performed as previously described.[9] Cells were incubated with the following antibodies: primary antibody, rabbit anti-Ib antibody; secondary antibody, Alexa Fluor 568-conjugated anti-rabbit IgG. Nuclei were determined by DAPI-staining.

Statistics Experimental data are presented as the mean ± standard deviation (SD). Each experiment was repeated four times. One-way analysis of variance with Bonferroni's multiple-comparison post-test was used to compare the mean values. $p<0.05$ was considered significant.

RESULTS

Inhibition of Cholera Toxin Subunit B on Cytotoxicity Induced by Iota-Toxin CTB binds to ganglioside GM1.[14] We investigated whether CTB affects the cytotoxicity of iota-toxin. Iota-toxin and C. botulinum C2 toxin, a member of the clostridial binary toxin family, enter MDCK cells and induce cytotoxicity (cell rounding).[5] Moreover, Ib induces cell necrosis in A431 cells.[9] Cells were treated with Ib and increasing concentrations of CTB for 1 h at 4°C. Washed cells were then incubated at 37°C in the absence (A431 cells) or presence (MDCK cells) of Ia. As shown in Fig. 1A, CTB treatment decreased the cell rounding activity of iota-toxin in a dose-dependent manner. Correspondingly, CTB blocked the Ib-induced cytotoxicity toward A431 cells (Fig. 1B). In contrast, the cell rounding activity of C2 toxin was not inhibited by CTB (Fig. 1A). CTB treatment (5 μg/mL) did not show cytotoxicity in MDCK and A431 cells (Fig. 1 A and B). These data indicated that CTB inhibits the cytotoxicity of iota-toxin, but not that of C2 toxin. We also examined whether ganglioside GM1 is involved in the cytotoxicity of iota-toxin. The addition of ganglioside GM1 (10 μM) had no effects on the cytotoxicities in MDCK and A431 cells caused by Ia plus Ib and Ib only, respectively (data not shown). This result indicates that the concentration of GM1 that inhibits the toxicity of cholera toxin[18] has no effect on the cytotoxicity of iota-toxin.

Effect of Cholera Toxin Subunit B on Ib Binding We compared the binding and internalization of Ib and CTB. Initially, we tested the binding and internalization of Ib using immunofluorescence staining. When MDCK cells or A431 cells were incubated with Ib and CTB at 4°C for 1 h, Ib and CTB bound distinctly and did not significantly colocalize on the cell surface (Fig. 2A and B). After incubation at 37°C for 30 min, Ib partially colocalized with CTB in cytoplasmic vesicles of MDCK cells and did not enter A431 cells (Fig. 2A and B). Cell-surface Ib and CTB that are internalized by cells are subsequently transported to early endosome.[5] In the course of intracellular trafficking, Ib partially co-localized with CTB. Binding and internalization of Ib to each cell type were similar to previously reported results.[9] To examine the inhibitory effect of CTB on Ib binding to MDCK and A431 cells, both cells were incubated with Ib in the absence or presence of CTB at 4°C for 1 h. The treated cells were lyzed in SDS-sample solution and assessed by SDS-PAGE. Ib was detected by Western blot analysis using anti-Ib antibody. As shown in Fig. 2C and D, binding of Ib monomer to both cell types was inhibited by CTB.

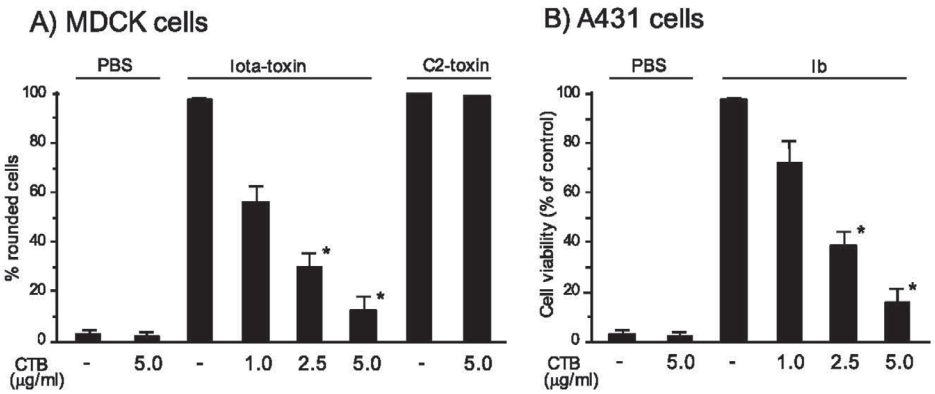

Fig. 1. Effect of Cholera Toxin Subunit B on the Cytotoxic Activity of Iota-Toxin or C2 Toxin

(A) MDCK cells were incubated with Ia (100 ng/mL) and Ib (100 ng/mL) or C2I (500 ng/mL) and C2IIa (500 ng/mL) in the presence or absence of various amounts of CTB at 37°C for 4 h. Pictures were taken. The total number of cells and number of round cells were counted from the pictures and the percentage of round cells was calculated. Values are given as the mean ± S.D. (n=4). *Significant difference (P<0.05). (B) A431 cells were incubated with Ib (1 μg/mL) in the presence or absence of various amounts of CTB at 37°C for 4 h. Cell viability was evaluated by MTT assay. Data shown are the mean ± S.D. (n=4), indicating percentage of cell viability compared with that in untreated controls. *Significant difference (P<0.05). The number of live cells is showed as a percentage of the value for controls.

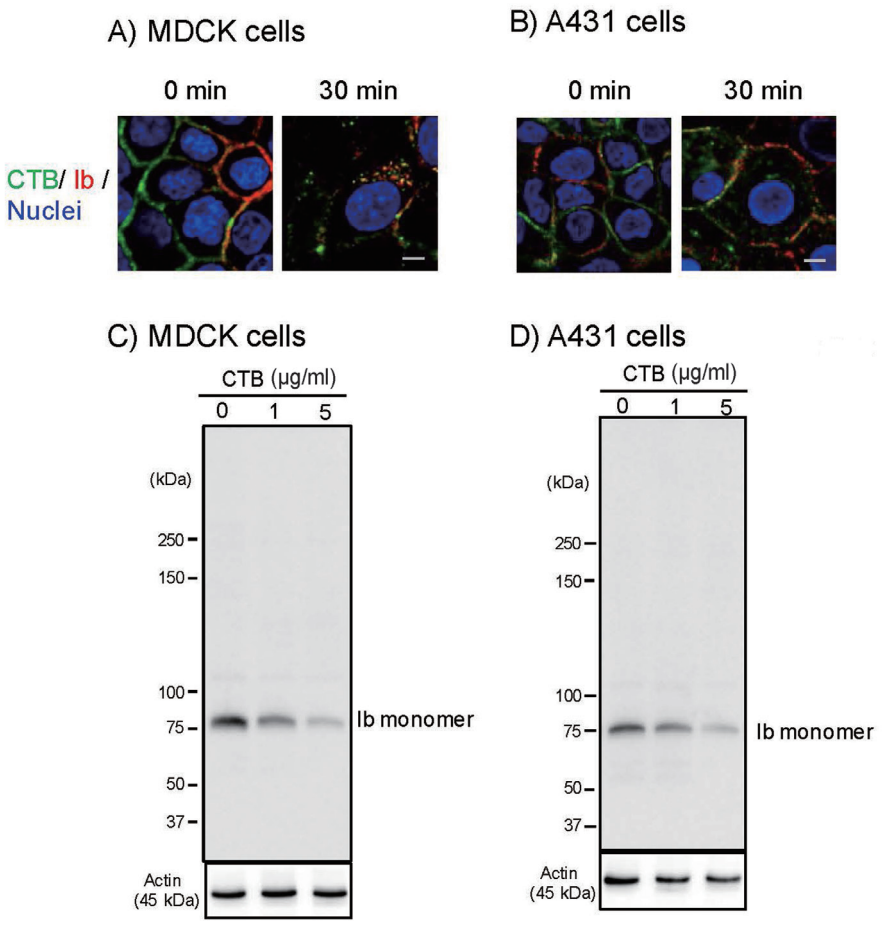

Fig. 2. Effect of Cholera Toxin Subunit B on the Binding of Iota-Toxin

Confocal microscopy study. MDCK cells (A) or A431 cells (B) were incubated with Ib (1 μg/mL) and Alexa488-labeled-CTB (1 μg/mL) at 4°C for 1 h, washed, and incubated at 37°C for 30 min. Cells were fixed, permeabilized and stained with anti-Ib antibody and DAPI. Ib (red), CTB (green) and nucleus (blue) were viewed with a confocal microscope. The experiments were repeated three times, and a representative result is shown. Bar, 5 μm. Western blot analysis. MDCK cells (C) or A431 cells (D) were incubated with Ib (1 μg/mL) in the presence or absence of CTB at 4°C for 1 h. Cells were subjected to Western bloting using anti-Ib and anti-β-actin antibodies. A typical example from three experiments is exhibited.

DISCUSSION

The current study found that the cytotoxic activity induced by iota-toxin is blocked by CTB. Since binding is a crucial step in the activities of many toxins involved in severe diseases, CTB and its derivatives could potentially be therapeutic agents for the treatment of iota-toxin-producing strains of *C. perfringens*. As *C. perfringens* type E causes enterotoxemia in young livestocks, it has been reported that associated diseases are mediated by iota-toxin.[1,3] Iota-toxin elicits fluid accumulation and histological damage in mouse ligated ileal loops.[19] In future, we will assess whether CTB decreases iota-toxin-induced fluid accumulation and histological damage in ileal loops. Thus, CTB possesses potential for therapeutic application against iota-toxin-associated disease.

CTB binds to cell surface ganglioside GM1 in lipid rafts and enters the cells.[14] Ib also binds to lipid rafts and is internalized.[7,8] In contrast, GM1 did not block the activity of iota-toxin, indicating that Ib is not associated with GM1 in lipid rafts. Previous studies have established that CTB stabilizes lipid raft microdomains, since CTB pentamer can associate with five GM1 molecules and induce GM1 clustering.[20,21] In this study, CTB inhibited the binding of Ib on the cell surface at 4°C. As a decrease in temperature leads to a decrease in membrane fluidity, clustering of GM1 by CTB is unlikely to be promoted. We therefore propose that Ib binding inhibition caused by CTB is not due to the raft stabilization by CTB.

GM1 binds to the primary binding site of CTB, but the latest reports indicate that glycosylated receptors are crucial for the secondary binding site of CTB.[22–25] The primary binding site on CTB is distinct from the secondary binding site,[23,24] with the latter being situated closer to the A subunit binding site than the former. CTB is reported to bind to histo-blood group antigens (HBGAs) at a secondary binding site. HBGAs are complex carbohydrates present on the surfaces of red blood cells and mucosal epithelial cells. CD44 is the carrier molecule of HBGAs and is co-expressed with HBGAs.[26,27] Iota-toxin binds to LSR and CD44 in lipid rafts and internalizes to target cells.[5,13] Previous works implicated LSR and CD44 as host cell receptors for Ib.[10,12] Moreover, Ib specifically binds to purified CD44.[12] In this report, CTB inhibited the binding of Ib on the cell surface at 4°C. This inhibitory mechanism seems to act through steric hindrance caused by CTB binding with CD44.

It has been reported that CTB inhibits the pathogenicities of various pathogens. CTB markedly blocks the adhesion and internalization of *Toxoplasma*,[15,28] and effectively inhibits CXCR4-tropic human immunodeficiency virus type

I (HIV-1) vector infection.[29] The adherence and the invasion of uropathogeic *Escherichia coli* to bladder epithelial cells are blocked by pretreatment with CTB.[30] In this work, CTB inhibited the cytotoxicity of iota-toxin. CTB effectively suppresses the virulence factors when pathogens enter host cells through lipid raft microdomains.

Conclusions We demonstrated that CTB inhibits the cytotoxicity of iota-toxin. Ib is bound and internalized via lipid raft microdomains. CTB effectively blocked Ib binding to host cells. Moreover, CTB is commercially available and avirulent. Our results represent a novel strategy for the development of Ib inhibitors.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Sakurai J, Nagahama M, Oda M, Tsuge H, Kobayashi K. *Clostridium perfringens* iota-toxins: structure and function. *Toxins (Basel)*, **1**, 208–228 (2009).
2) Aktories K, Lang AE, Schwan C, Mannherz HG. Actin as target for modification by bacterial protein toxins. *FEBS J.*, **278**, 4526–4543 (2011).
3) Stiles BG, Pradhan K, Fleming JM, Samy RP, Barth H, Popoff MR. *Clostridium* and *bacillus* binary enterotoxins: bad for the bowels, and eukaryotic being. *Toxins (Basel)*, **6**, 2626–2656 (2014).
4) Knapp O, Benz R, Popoff MR. Pore-forming activity of clostridial binary toxins. *Biochim. Biophys. Acta*, **1858**, 512–525 (2015).
5) Takehara M, Takagishi T, Seike S, Oda M, Sakaguchi Y, Hisatsune J, Ochi S, Kobayashi K, Nagahama M. Cellular Entry of *Clostridium perfringens* iota-toxin and *Clostridium botulinum* C2 toxin. *Toxins (Basel)*, **9**, 247 (2017).
6) Nagahama M, Nagayasu K, Kobayashi K, Sakurai J. Binding component of *Clostridium perfringens* iota-toxin induces endocytosis in Vero cells. *Infect. Immun.*, **70**, 1909–1914 (2002).
7) Nagahama M, Yamaguchi A, Hagiyama T, Ohkubo N, Kobayashi K, Sakurai J. Binding and internalization of *Clostridium perfringens* iota-toxin in lipid rafts. *Infect. Immun.*, **72**, 3267–3275 (2004).
8) Hale ML, Marvaud JC, Popoff MR, Stiles BG. Detergent-resistant membrane microdomains facilitate Ib oligomer formation and biological activity of *Clostridium perfringens* iota-toxin. *Infect. Immun.*, **72**, 2186–2193 (2004).
9) Nagahama M, Umezaki M, Oda M, Kobayashi K, Tone S, Suda T, Ishidoh K, Sakurai J. *Clostridium perfringens* iota-toxin b induces rapid cell necrosis. *Infect. Immun.*, **79**, 4353–4360 (2011).
10) Papatheodorou P, Carette JE, Bell GW, Schwan C, Guttenberg G, Brummelkamp TR, Aktories K. Lipolysis-stimulated lipoprotein receptor (LSR) is the host receptor for the binary toxin *Clostridium difficile* transferase (CDT). *Proc. Natl. Acad. Sci. USA*, **108**, 16422–16427 (2011).
11) Nagahama M, Takehara M, Kobayashi K. Interaction of *Clostridium perfringens* iota toxin and lipolysis-stimulated lipoprotein receptor (LSR). *Toxins (Basel)*, **10**, 405 (2018).
12) Wigelsworth DJ, Ruthel G, Schnell L, Herrlich P, Blonder J, Veenstra TD, Carman RJ, Wilkins TD, Van Nhieu GT, Pauillac S, Gibert M, Sauvonnet N, Stiles BG, Popoff MR, Barth H. CD44 promotes intoxication by the clostridial iota-family toxins. *PLoS One*, **7**, e51356 (2012).
13) Fagan-Solis KD, Reaves DK, Rangel MC, Popoff MR, Stiles BG, Fleming JM. Challenging the roles of CD44 and lipolysis stimulated lipoprotein receptor in conveying *Clostridium perfringens* iota toxin cytotoxicity in breast cancer. *Mol. Cancer*, **13**, 163 (2014).
14) Pang H, Le PU, Nabi IR. Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent endocytosis of cholera toxin to the Golgi apparatus. *J. Cell Sci.*, **117**, 1421–1430 (2004).
15) Cruz KD, Cruz TA, Veras de Moraes G, Paredes-Santos TC, Attias M, de Souza W. Disruption of lipid rafts interferes with the interaction of *Toxoplasma gondii* with macrophages and epithelial cells. *BioMed Res. Int.*, **2014**, 687835 (2014).
16) Nagahama M, Sakaguchi Y, Kobayashi K, Ochi S, Sakurai J. Characterization of the enzymatic component of *Clostridium perfringens* iota-toxin. *J. Bacteriol.*, **182**, 2096–2103 (2000).
17) Nagahama M, Takehara M, Takagishi T, Seike S, Miyamoto K, Kobayashi K. Cellular uptake of *Clostridium botulinum* C2 toxin requires acid sphingomyelinase activity. *Infect. Immun.*, **85**, e00966–e16 (2017).
18) Cervin J, Wands AM, Casselbrant A, Wu H, Krishnamurthy S, Cvjetkovic A, Estelius J, Dedic B, Sethi A, Wallom KL, Riise R, Bäckström M, Wallenius V, Platt FM, Lebens M, Teneberg S, Fändriks L, Kohler JJ, Yrlid U. GM1 ganglioside-independent intoxication by Cholera toxin. *PLoS Pathog.*, **14**, e1006862 (2018).
19) Redondo LM, Redondo EA, Dailoff GC, Leiva CL, Díaz-Carrasco JM, Bruzzone OA, Cangelosi A, Geoghegan P, Fernandez-Miyakawa ME. Effects of *Clostridium perfringens* iota toxin in the small intestine of mice. *Anaerobe*, **48**, 83–88 (2017).
20) Day CA, Kenworthy AK. Functions of cholera toxin B-subunit as a raft cross-linker. *Essays Biochem.*, **57**, 135–145 (2015).
21) Raghunathan K, Wong TH, Chinnapen DJ, Lencer WI, Jobling MG, Kenworthy AK. Glycolipid crosslinking is required for cholera toxin to partition into and stabilize ordered domains. *Biophys. J.*, **111**, 2547–2550 (2016).
22) Vasile F, Reina JJ, Potenza D, Heggelund JE, Mackenzie A, Krengel U, Bernardi A. Comprehensive analysis of blood group antigen binding to classical and El Tor cholera toxin B-pentamers by NMR. *Glycobiology*, **24**, 766–778 (2014).
23) Heggelund JE, Burschowsky D, Bjørnestad VA, Hodnik V, Anderluh G, Krengel U. High-resolution crystal structures elucidate the molecular basis of cholera blood group dependence. *PLoS Pathog.*, **12**, e1005567 (2016).
24) Heim JB, Hodnik V, Heggelund JE, Anderluh G, Krengel U. Crystal structures of cholera toxin in complex with fucosylated receptors point to importance of secondary binding site. *Sci. Rep.*, **9**, 12243 (2019).
25) Cervin J, Boucher A, Youn G, Björklund P, Wallenius V, Mottram L, Sampson NS, Yrlid U. Fucose-galactose polymers inhibit cholera toxin binding to fucosylated structures and galactose-dependent intoxication of human enteroids. *ACS Infect. Dis.*, **6**, 1192–1203 (2020).
26) Lin W, Karsten U, Goletz S, Cheng R, Cao Y. Co-expression of CD173 (H2) and CD174 (Lewis Y) with CD44 suggests that fucosylated histo-blood group antigens are markers of breast cancer-initiating cells. *Virchows Arch.*, **456**, 403–409 (2010).
27) Blanas A, Sahasrabudhe NM, Rodríguez E, van Kooyk Y, van Vliet SJ. Fucosylated antigens in cancer: an alliance toward tumor progression, metastasis, and resistance to chemotherapy. *Front. Oncol.*, **8**, 39 (2018). (Correction: Front. Oncol. 2018, 8,150)
28) Barrias ES, Dutra JM, De Souza W, Carvalho TM. Participation of macrophage membrane rafts in *Trypanosoma cruzi* invasion process. *Biochem. Biophys. Res. Commun.*, **363**, 828–834 (2007).
29) Kamiyama H, Kakoki K, Shigematsu S, Izumida M, Yashima Y, Tanaka Y, Hayashi H, Matsuyama T, Sato H, Yamamoto N, Sano T, Shidoji Y, Kubo Y. CXCR4-tropic, but not CCR5-tropic, human immunodeficiency virus infection is inhibited by the lipid raft-associated factors, acyclic retinoid analogs, and cholera toxin B subunit. *AIDS Res. Hum. Retroviruses*, **29**, 279–288 (2013).
30) Duncan MJ, Li G, Shin JS, Carson JL, Abraham SN. Bacterial penetration of bladder epithelium through lipid rafts. *J. Biol. Chem.*, **279**, 18944–18951 (2004).