Title page

B4GALT5-deficient CHO-Lec2 cells expressing human α1,4-galactosyltransferase: a glycoengineered cell model for studying Shiga toxin receptors

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Abstract

Human α1,4-galactosyltransferase (A4galt) is a glycosyltransferase that produces glycosphingolipid (Gb3, globotriaosylceramide, Galα1→4Galβ1→4Glc-Cer)- and glycoprotein (P1 glycotope, Galα1→4Galβ1→4GlcNAc-R)-based receptors for Shiga toxin types 1 and 2 (Stx1 and Stx2). Stx1 is bound by Gb3 and P1 glycoprotein, while Stx2 is only recognized by Gb3. To decipher the role of these receptors, CHO-Lec2 cells expressing human A4galt were modified by disrupting the hamster *B4GALT5* gene using CRISPR/Cas9 technology. The *B4GALT5* gene encodes β1,4-galactosyltransferase 5 (B4galt5), synthesizing lactosylceramide, the key substrate for Gb3 synthesis. *B4GALT5*-deficient CHO-Lec2-expressing A4galt cells lack Gb3 expression, resulting in complete resistance to Stx1 holotoxin and significantly reduced sensitivity to Stx2 holotoxin. These glycoengineered cells provide a valuable model for studying glycosphingolipid- and glycoprotein-based receptors for Stxs, enabling detailed evaluation of their roles in toxin binding and cytotoxicity.

Abbreviations

A4galt, α1,4-galactosyltransferase, Gb3/CD77 synthase; B4galt, β1,4-galactosyltransferase; Gb3, globotriaosylceramide, P^k antigen, CD77, Galα1→4Galβ1→4Glc-Cer; Genz-123346, N-[(1R,2R)-1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3pyrrolidin-1-ylpropan-2-yl]nonanamide, glucosylceramide synthase inhibitor; GP, glycoprotein; GSL, glycosphingolipid; HPTLC, thin-layer chromatography; HUS, hemolytic-uremic syndrome; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; LacCer, lactosylceramide; P1, nLc5, Galα1→4Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer; P1 glycotope, terminal Galα1→4Galβ1→4GlcNAc-R disaccharide on N-glycoproteins; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin; Stx1B, B subunit of Shiga toxin 1; Stx2B, B subunit of Shiga toxin 2.

Introduction

Human α1,4-galactosyltransferase (A4galt, P1/P^k synthase, Gb3/CD77 synthase, EC 2.4.1.228) is encoded by the *A4GALT* gene. It plays a crucial role in the synthesis of Gb3 (P^k, CD77, globotriaosylceramide, Galα1→4Galβ1→4Glc-Cer) and P1 (nLc5, Galα1→4Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer), which are glycosphingolipids (GSLs) and, to a lesser extent, N-glycoproteins with terminal Galα1→4Galβ1→4GlcNAc-R structures (called P1 glycotopes) [1, 2, 3]. A4galt catalyzes the transfer of galactose from UDP-Gal to Gal-capped oligosaccharide acceptors. Two active isoforms of human α1,4-galactosyltransferase occur in humans. The most widespread A4galt isoform (A4G) synthesizes Gb3 and P1 on GSLs (and P1 glycotope on GPs) [4, 5, 6]. A rare enzyme variant (A4Gmut) contains a p.Q211E substitution (c.631C>G mutation in *A4GALT*, rs397514502) and can transfer Gal to GalNAc-terminating glycosphingolipids, forming NOR, NOR1 and NOR2 GSLs [2, 7]. The products of human A4galt belong to the P1PK histo-blood group system (International Society of Blood Transfusion ISBT No. 003) [7, 8, 9, 10, 11].

Gb3, the main A4galt enzyme product, is considered to be the main receptor for Shiga toxins (Stxs), released by *Shigella dysenteriae* serotype 1 and Shiga-producing strains of *Eschericha coli* (STEC). These strains secrete two distinct types of Shiga toxin, Stx1, which is homologous to *S. dysenteriae*-produced Stx and more genetically distinct Stx2. Both toxins are responsible for hemorrhagic colitis and life-threatening severe hemolytic-uremic syndrome (HUS), thus pose an increasing threat to human life [12, 13, 14]. Recently, it was found that glycoprotein-based P1 glycotope, alongside Gb3 glycosphingolipid, can bind Stx1 and mediate its cytotoxicity effects. Both receptors contain the Galα1→4Galβ structure, which is essential for the interaction with the B subunit of Stxs [4, 5]. Gb3 was found to be associated with epithelial-to-mesenchymal transition, a process associated with cancerogenesis [15], while Gb3 accumulation is a hallmark of Fabry disease [16].

The dual acceptor specificity of human A4galt toward GSL- and GP-based acceptors may originate from its ability to form heterodimers with other GTs [17]. The primary substrate for human A4galt, LacCer is produced by LacCer synthase (β1,4-galactosyltransferase, B4galt), which occurs as two isoenzymes (β1,4-galactosyltransferase 5, B4galt5 and β1,4-galactosyltransferase 6, B4galt6) [18, 19]. Previous studies have shown the possibility of the existence of the A4galt-B4galt5 heterodimer by sharing by both enzymes the LXXR (X: any amino acid) sequence motifs [20, 21]. The presence of this motif in both A4galt and B4galt5 may indicate their close localization within the Golgi apparatus, thereby

facilitating heterodimer formation. Moreover, the potential interactions between human A4galt and B4galt6 were suggested by Takematsu et al. (2011) [22]. Recently, using NanoBiT technology and computational analysis of Alphafold enzyme models, it was demonstrated that A4galt can form homodimers and heterodimers with human B4galt1 and B4galt5 [17].

In a previous study, CHO-Lec2 cells were transfected with the human *A4GALT* gene, leading to the synthesis of Gb3 and P1 glycotope. The use of CHO-Lec2 cells was motivated by the lack of a CMP-sialic acid transporter, which is essential for sialic acid-containing glycan synthesis. This feature prevents interference from sialylated glycans in Stx binding to cell surface receptors [23, 24]. Using CHO-Lec2 cells, it was found that glycotopes containing the P1 epitope (Galα1→4Galβ1→4GlcNAc-R) can be a functional receptor for Stx1, in addition to the canonical GSL-based Gb3 receptor. The goal of this study was to eliminate the Gb3 GSL receptor by disrupting the hamster *B4GALT5* gene using CRISPR/Cas9 [25]. *B4GALT5* encodes hamster B4galt5, which plays a crucial role in glycans galactosylation in CHO-Lec2 cells. Furthermore, considering the ability of human A4galt to recognize two different acceptors and A4galt-B4galt5 heterodimer formation in CHO-Lec2 cells [17], it was aimed to generate *B4GALT5*-deficient CHO-Lec2 A4G and A4Gmut cells. Therefore, these glycoengineered cells were analyzed to assess Stx binding and sensitivity to Stx1- and Stx2-mediated cytotoxicity.

Material and methods

Antibodies

Human anti-P1 (clone P3NIL100, recognizing only P1 antigen), mouse anti-P1 (clone 650, recognizing Gb3 and P1 antigens), anti-6x-His Tag antibody (clone HIS.H8), and biotinylated goat anti-mouse IgG/A/M (H/L) antibodies were purchased from Immucor Inc, Ce-Immundiagnostika, BioLegend, Thermo Fischer Scientific, and Bio-Rad Laboratories, respectively. Goat anti-human polyvalent immunoglobulin biotinylated antibody and alkaline phosphatase-ExtrAvidin were obtained from Sigma-Aldrich. The recombinant Stx1a and Stx2b His-tagged subunits (referred to as Stx1B and Stx2B), used in western blotting and flow cytometry analysis, were obtained as described in [5]. Stx1 (Verotoxin 1, from *E. coli* O157) and Stx2 (Verotoxin 2, from *E. coli* O157) holotoxins for cytotoxicity assay were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Generation *B4GALT5*-deficient CHO-Lec2 cells-expressing human A4galt using CRISPR/Cas9

CHO-Lec2 cells were transduced with *A4GALT* and *A4GALT* with c.631C>G ORFs genes, encoding A4galt (designated as A4G) and A4galt with p.Q211E substitution (A4Gmut), respectively, as described previously [5]. CHO-Lec2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). The culture of CHO-Lec2 was maintained in DMEM/F12 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified 5% CO2 atmosphere. Cells were subcultivated every 3–4 days with 0.25% trypsin/1 mM EDTA. For transduction, CHO-Lec2 cells (2×10^4) were incubated with concentrated lentiviruses and centrifuged (2460g, 150 min, 24 C) as described in [5]. After 24 h, puromycin selection was applied to obtain stable clones. For specific experiments, CHO-Lec2 A4G, CHO-Lec2 A4Gmut, and untransfected cells were cultured for 2 weeks in a complete medium containing 3 μM N-[(1R,2R)-1- (2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-pyrrolidin-1-ylpropan-2-yl]nonanamide (Genz-123346) (Merck, Darmstadt, Germany), a glucosylceramide synthase inhibitor.

Guide RNA sequences for the CRISPR/Cas9 system were designed as described in [26] with the use of CRISPOR software (http://crispor.tefor.net/crispor.py, [27]). Briefly, the top and bottom strand of 20-nt guide RNA were synthesized (Genomed, Warsaw), annealed and ligated into the pair of FastDigest BsmBI (Thermo Fisher Scientific, Waltham, MA, USA) cut plasmids, namely pSpCas9(BB)-2A-GFP (PX458) (pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang, Addgene plasmid # 48138; http://n2t.net/addgene:48138 ; RRID:Addgene_48138Addgene plasmid #48138) [26]. The ligated products were transformed into electrocompetent *E. coli* DH5α cells (Thermo Fisher Scientific, Waltham, MA, USA), and the cells were plated onto ampicillin selection plates (100 μg/mL ampicillin) and incubated at 37°C overnight. Plasmid DNA was isolated using the Gene JET Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA) and verified by Sanger sequencing. For larger-scale plasmid preparation, the Qiagen Midi kit was used (Qiagen, Hilden, Germany). The sgRNA oligonucleotide sequences are listed in Table SI.

Sanger sequencing of mutated sites

B4GALT5 gene disruption was assessed using a PCR-based method (Laboratory of DNA sequencing and synthesis, Institute of Biochemistry and Biophysics PAS, Warsaw). Briefly, purified PCR products of *B4GALT5* were cloned into pCRScript Amp SK (Promega, Madison, WI) and transformed into *E. coli* Top10 competent cells. Using the positive colonies

as a template, a second PCR reaction with M13Forward and M13Reverse standard primers was performed. The obtained nucleotide fragments of *B4GALT5* were purified and sequenced. Mutations were identified by alignment of the sequenced alleles with the wild-type gene reference NW_003613899.1 (CriGri_1.0, scaffold2107) using GeneStudio Professional software.

Western blotting

CHO-Lec2 cells were lysed in CelLytic M (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a protease inhibitor cocktail. In total, 20 µg of cell lysate per lane was subjected to nonreducing sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% Tris-Glycine gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% cold fish gelatin in TBS for 1 h, washed three times (all washing steps were performed with TBS), and incubated overnight with primary antibody diluted 1:100 in TBS/1% BSA. After washing, the membrane was placed for 1 h in 1:1000 biotinylated secondary antibody solution, washed, and incubated with ExtrAvidin conjugated with alkaline phosphatase (1:5000, 1 h). Bands were visualized using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/ NBT, Sigma-Aldrich, St. Louis, MO, USA) reaction. The PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as the protein standard.

HPTLC orcinol staining

Orcinol staining was performed using standard procedures as previously described [1]. Briefly, dried thin-layer chromatography (HPTLC) plates were sprayed with an orcinol solution (0.2% w/v) in 3 M aqueous sulfuric acid and incubated in an oven at 110°C for 10 min.

Extraction and purification of glycosphingolipids from CHO-Lec2 cells

Isolation, fractionation, and HPTLC analysis of CHO-Lec2 neutral GSLs were performed as described [1]. Briefly, GSLs were extracted with chloroform/methanol, desalted and separated from phospholipids and gangliosides. GSLs solubilized in chloroform/methanol (2:1, v/v) were applied to HPTLC plates (Kieselgel 60, Merck, Darmstadt, Germany) and developed in chloroform/methanol/water (55:45:9, v/v/v). Dried plates were stained with orcinol or prepared for antibody assay. For this purpose, the plates were immersed in 0.05% polyisobutylmethacrylate in hexane for 1 min, dried, soaked in TBS, and blocked in 5%

bovine serum albumin in TBS for 1 h. After washing in TBS, plates were incubated overnight with human anti-P1 antibody diluted 1:50 in TBS/1% BSA, followed by incubation with anti-human biotinylated antibody (1:1000), ExtrAvidin-alkaline phosphatase conjugate (1:5000, Sigma-Aldrich, St. Louis, MO, USA), and BCIP/NBT (Sigma-Aldrich, St. Louis, MO, USA) substrate solution.

MALDI-TOF mass spectrometry analysis of GSLs extracted from CHO-Lec2 cells

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was carried out on an ultrafleXtreme MALDI TOF/TOF instrument (BrukerDaltonics, Bremen, Germany), with the use of Peptide Calibration Standard (BrukerDaltonics, Bremen, Germany) as external calibration and Norharmane (9HPyrido[3,4-b]indole, Sigma-Aldrich, St. Louis, MO) as a matrix (10 mg/ml, chloroform:methanol, 2:1, v/v). GSLs were dissolved in chloroform/ methanol (2:1, v/v). Spectra were scanned in the range of *m/z* 700–2000 in the reflectron-positive mode.

Quantitative analysis of *A4GALT* transcript in CHO-Lec2 cells

Total RNA from transfected or non-transfected CHO-Lec2 cells was prepared using Universal RNA Purification Kit (Eurx, Gdansk, Poland) and the complementary DNAs (cDNAs) were synthesized using the SuperScript III First-Strand Synthesis kit (Life Technologies, Carlsbad, CA, USA) with oligo(dT) primers. Quantitative polymerase chain reaction (qPCR) was performed with 30 ng of cDNA using the CFX Connect qPCR system (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. Human *A4GALT* and hamster *B4GALT5* transcripts were detected using the Custom TaqMan Gene Expression Assay. For endogenous control normalization, the custom assay targeting Chinese hamster *GAPDH* was used. All samples were run in triplicate. The target nucleotide sequences are shown in Table SII, and the qPCR conditions are presented in Table SIII.

Flow cytometry

CHO-Lec2 cells were scraped, washed (all washes were done with PBS), and incubated for 30 min on ice with anti-P1 (clone P3NIL100; 1:400; all dilutions were done with 1% BSA in PBS), anti-P1 (clone 650; 1:200) antibodies, washed, and incubated with secondary FITC-conjugated antibodies: anti-human IgM (1:100), anti-mouse IgM (1:100). To analyze Shiga toxin binding, cells were incubated with 1 μg/ml Stx1B or Stx2B, then washed, and incubated with anti-6x-His Tag antibody (1:1000), followed by washing and incubation with FITC-

conjugated anti-mouse IgG (1:100). After incubation with FITC-conjugates, the cells were washed, resuspended in 500 μl of cold PBS, and subjected to flow cytometry analysis using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). In total, 1×10^5 events from the gated population were analyzed by Flowing Software (Perttu Terho, University of Turku). The antibody and StxB subunits binding capacity per cell of CHO-Lec2 was determined using Quantum (Bio-Rad, Hercules, CA, USA) consisting of five populations of calibration microspheres. For quantification of cell surface antigens we used Quantum beads (Bio-Rad, Hercules, CA, USA) which carry defined quantities of FITC, and thus allow plotting calibration curves (mean fluorescence intensity versus Molecules of Equivalent Soluble Fluorochrome units). The cells were then analyzed by flow cytometry and the antibody binding capacity (ABC, the number of antibody molecules bound per cell) was calculated by interpolation from the calibration curve as described in the manufacturer's protocol and based on the fluorophore-to-protein molar ratios of the FITC-antibody conjugates. Negative control results (secondary antibody only) were subtracted from the sample results to obtain the specific antibody binding capacities.

Cytotoxicity assay

In total, 2×10^4 CHO-Lec2 cells were seeded in 96-well plates (Wuxi NEST Biotechnology Co., Ltd, China) in complete DMEM/F12. After 24 h medium was replaced with 100 μl/well of serum-free DMEM/F12 containing 0.01, 0.05, 0.1, 0.5, and 1 ng/ml of Stx1 or Stx2 holotoxins (all concentrations were run in triplicate). After 20 h of toxin treatment, 20 μl/well of MTS tetrazolium compound (CellTiter 96 AQueous One Solution Assay, Promega, Madison, WI) was added. Plates were incubated in a humidified, 5% CO2 atmosphere for 2.5 h, and the absorbance at 490 nm was recorded on an ELISA plate reader. The background absorbance registered at zero cells/well was subtracted from the data and the absorbance of wells incubated in medium without the addition of Stx was taken as 100% of cell viability. Each experiment was performed at least three times.

Results

Generation CHO-Lec2 A4galt-expressed cells with disrupted B4GALT5 gene

Genome sequence analysis of CHO-Lec2 A4G and A4Gmut with *B4GALT5* KO showed heterozygotic complex mutations within exon 5 of the *B4GALT5* gene (Fig. S1). The mutations identified included one deletion and two insertions of nucleotides within *C. griseus*

B4GALT5 in CHO-Lec2 A4G cells (Fig. S1A), and one deletion and one insertion in CHO-Lec2 A4Gmut cells (Fig. S1B). These nucleotide aberrations led to significantly reduced *B4GALT5* gene transcript in CHO-Lec2 A4G and A4Gmut with *B4GALT5* KO (Fig. S1B, D). Therefore, the absence of B4galt5 activity was evaluated by HPTLC and MALDI-TOF mass spectrometry analysis of glycolipids isolated from the cells. These findings indicated the effective disruption of the *B4GALT5* gene in CHO-Lec2 expressing both A4G and A4Gmut, establishing a cell model deprived in globo-series GSLs.

Shiga toxins binding to CHO-Lec2 cells expressing A4G and A4Gmut with B4GALT5 KO

Shiga toxin types 1 and 2 are known to bind to the Gala1→4Gal epitope, located on the Gb3 GSL and GP-based P1 glycotope on the cell surface. It was anticipated that the generated CHO-Lec2 cells expressing A4G and A4Gmut with *B4GALT5* KO lacked globo-series GSL synthesis, including Gb3. To further confirm the depletion of Gb3 in these modified cells, the CHO-Lec2 A4G and A4Gmut cells (*B4GALT5* KO and with active gene) were treated with Genz-123346 (a glucosylceramide synthase inhibitor [29]) [5].

Quantitative flow cytometry was performed to evaluate the binding capacity of anti-P1 (clones P3NIL100 and 650) and the Stx1B and Stx2B to CHO-Lec2 A4G and A4Gmut cells. CHO-Lec2 A4G or A4Gmut cells with *B4GALT5* KO did not show Stx1B or Stx2B binding to the cell surface (Fig. 1). Treatment of CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells with the Genz-123346 inhibitor also caused no Stx1B and Stx2B binding (Fig. 1). Despite abolished Stxs binding to the cells, CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells still bound anti-P1 antibodies (cloned 650 and P3NIL100), which recognize the Gala1→4Gal epitope (Fig. 1). In summary, CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells displayed markedly reduced binding of Stx1B and no Stx2B binding to cell surface, along with retention of Galα1→4Gal epitopes recognized by anti-P1 antibody.

To evaluate the presence of Gb3 GSLs, the glycolipids isolated from CHO-Lec2 A4G and A4Gmut cells (with or without *B4GALT5* KO were analyzed by HPTLC and MALDI-TOF mass spectrometry. Orcinol staining (which recognizes glycolipids) exhibited no neutral GSLs in CHO-Lec2 A4G and A4Gmut *B4GALT5 KO* cells, similar to the cells treated with Genz-123346 (Fig. 3). Analysis of GSLs using HPTLC overlaid with anti-P1 (clone 650) antibody showed no bands corresponding to Gb3 GSL in CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells (Fig. 3). Using MALDI-TOF mass spectrometry, only HexCer isoforms were found in both CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells (Fig. 2), which corresponded to the HPTLC overlay and orcinol staining results. Taken together, CHO-Lec2

A4G and A4Gmut *B4GALT5* KO cells were Gb3 GSL-deficient, thereby preventing Stx1B and Stx2B binding.

Western blotting was conducted to evaluate GPs-based P1 glycotope Stx receptor in CHO-Lec2 A4G and CHO-Lec2 A4G *B4GALT5* KO cell lysates using the anti-P1 (clone P3NIL100) antibody and the Stx1B and Stx2B. No significant differences were observed between the analyzed cells (regardless of the presence *B4GALT5* KO or not) (Fig. 4). Anti-P1 (clone P3NIL100) antibody detected more proteins in CHO-Lec2 A4Gmut cells, which correlated with previous results, indicating excess amounts of P1-based glycoproteins synthesized by A4galt with p.Q211E substitution, compared to the enzyme without this mutation [5]. Additionally, the binding analysis of Stx1B and Stx2B to cell lysates showed that Stx1B binds to several glycoproteins, while Stx2B did not detect any proteins, regardless of *B4GALT5* deficiency (Fig. 4). Treatment with Genz-123346 did not significantly affect the binding patterns of anti-P1 (clone P3NIL100) and Stx1B to CHO-Lec2 A4G and A4Gmut (*B4GALT5* KO and with the active gene) cell lysates (Fig. 4). Collectively, both CHO-Lec2 A4G and A4Gmut irrespective of *B4GALT5* deficiency, still produced P1 glycotope on GPs that are recognized by Stx1B.

Sensitivity of CHO-Lec2 cells expressing A4G and A4Gmut with *B4GALT5* KO for Shiga holotoxins

To assess how *B4GALT5* disruption affected the viability of CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells upon Stx holotoxin exposure, a cytotoxicity assay was performed. Untransfected CHO-Lec2 cells, which were resistant to both Stx1 and Stx2, were used as controls. CHO-Lec2 A4G *B4GALT5* KO cells showed no sensitivity to either Stx1 or Stx2 exposure (Fig. 5A). In contrast, CHO-Lec2 A4Gmut *B4GALT5* KO cells were resistant to Stx1 but exhibited slight sensitivity to Stx2 (Fig. 5A). When treated with the Genz-123346 inhibitor, both CHO-Lec2 A4G and A4Gmut *B4GALT5* KO cells showed no cytotoxic effects in response to either Stx1 or Stx2 (Fig. 5B). In summary, *B4GALT5*-deficient CHO-Lec2 A4G cells were resistant to both holotoxins, whereas CHO-Lec2 A4Gmut *B4GALT5* KO cells demonstrated a gentle sensitivity to Stx2 but remained unaffected by Stx1. These results suggested the existence of an unidentified glycolipid-based (which lacking after Genz-123346 inhibitor treatment) cell surface receptor for Stx2 holotoxins produced only in CHO-Lec2 A4Gmut cells.

Discussion

The main goal of this study was to generate *B4GALT5*-deficient CHO-Lec2 cells as a model for studying Stx-receptor interactions. In recent years, the development of glycoengineered mammalian cells using chemical, enzymatic, and genetic approaches, including CRISPR/Cas9-based gene editing, has become increasingly important in biomedicine [25]. Gene silencing has been widely used to modify cell surface receptors involved in pathogen recognition and modulation of the immune response. This is typically achieved by knocking out or silencing genes encoding glycosyltransferases. For example, decreased *ST6GAL1* gene expression in airway epithelial cells using siRNA reduced the binding of human influenza viruses to cell surface sialic acid-containing receptors [30].

Cells with modified glycosylation machinery provide insights into glycan functions and molecular interactions and serve as valuable tools for designing cells with specific glycans on their surface [31, 32]. The *B4GALT5*-deficient CHO-Lec2 cells obtained in this study could be utilized for further research on the dual acceptor specificity of human A4galt. For years, Gb3 has been considered the primary receptor for Stxs, but at present, it is stated that Stx receptors are located on GSLs and GPs, with different binding patterns for Stx1 and Stx2 [5]. Studies on pigeon A4galt homologs have revealed the existence of two enzymes with distinct acceptor specificities: one primarily utilizes GPs and the other recognizes both GSLs and GPs as substrates [6]. Similarly, human A4galt has demonstrated dual specificity toward GSLs (major substrates) and GPs (minor substrates) [5]. This phenomenon referred to as acceptor promiscuity has been well documented within GTs and has significant implications for cellular biological and pathological processes [33]. Other examples of promiscuous GTs are human A and B transferases that synthesize A and B blood group antigens on both N- and O-glycans, as well as GSLs [34]. The representatives of human α1,3-fucosyltransferases (Futs) isoenzymes depending on the enzyme isoform, can reveal varied specificity toward glycolipids and/or glycoproteins acceptors [35].

B4GALT5-deficient CHO-Lec2 cells lacking Gb3 GSLs were generated in this study and served as an excellent model for investigating N-glycoprotein Stx receptors. Previous studies have explored the relationship between Stxs resistance and GTs knock-outs (including *A4GALT* and *B4GALT5*) in various human cells, such as HeLa [36, 37], bladder cancer cells 5637 [38] and colorectal adenocarcinoma cells HT-29 cells [39]. For example, Tian et al. 2018 showed that the bladder carcinoma cell line 5637 cells with *B4GALT5* knock-out revealed > 10^5-fold increased resistance to both Stx1 and Stx2, compared to the control cells [38]. More

comprehensive studies are needed to investigate the novel glycolipid-based receptor for Stx2 holotoxin, which was identified in *B4GALT5*-deficient CHO-Lec2 A4Gmut cells (Fig. 5). It is hypothesized that the removal of the B4galt5 enzyme from CHO-Lec2 A4Gmut cells may facilitate the formation of new heterocomplexes involving human A4galt and other GSL-related GTs, which are not associated with the synthesis of globo-series GSLs.

B4GALT5-deficient CHO-Lec2 cells could be further glycoengineered by modulating the synthesis of GP-based receptors or altering the cell surface glycome through transfection of genes encoding specific GTs. Future research should focus on elucidating the dual specificity of A4galt toward GSLs and GPs and understanding the molecular mechanisms governing the distribution of Stx receptors on different acceptor molecules. A deeper exploration of GP-based P1 glycotopes as potential decoy receptors may enhance therapeutic strategies focused on Stx sequestration and elimination from the body. It can open new avenues in Stx pathology and lead to breakthroughs in the treatment and prevention of Stx-related diseases.

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Figure legends

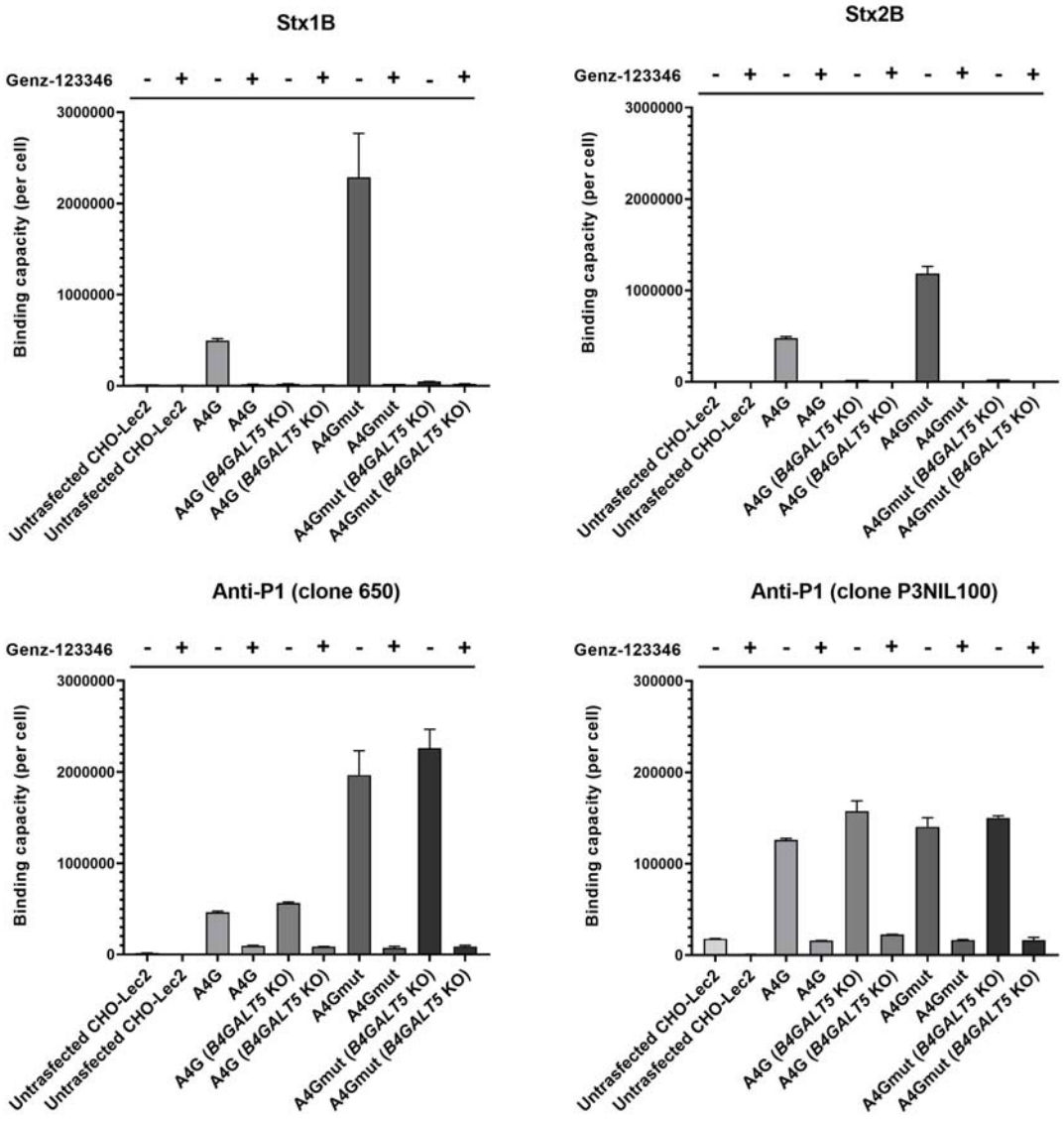

Fig. 1. Quantitative flow cytometry analysis of anti-P1 (P3NIL100), anti-P1 (650), Stx1B and Stx2B binding capacity of CHO-Lec2 cells. The cells were either untreated or treated with the Genz-123346 inhibitor.

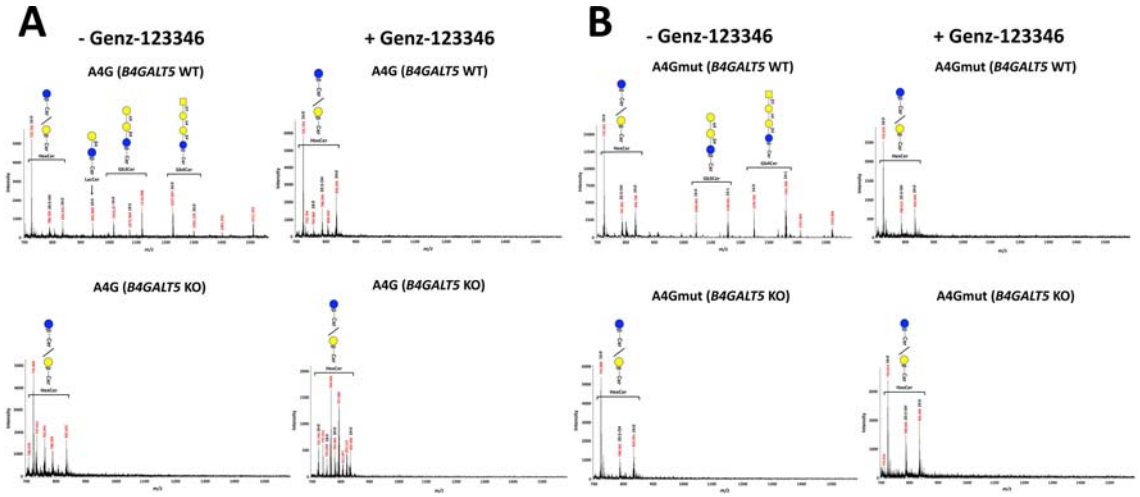

Fig. 2. MALDI-TOF analysis of neutral glycosphingolipids isolated from CHO-Lec2 cells expressing human A4galt (A4G, A) and its mutein form (A4Gmut, B), cultured in the presence or absence of the Genz-123346 inhibitor.

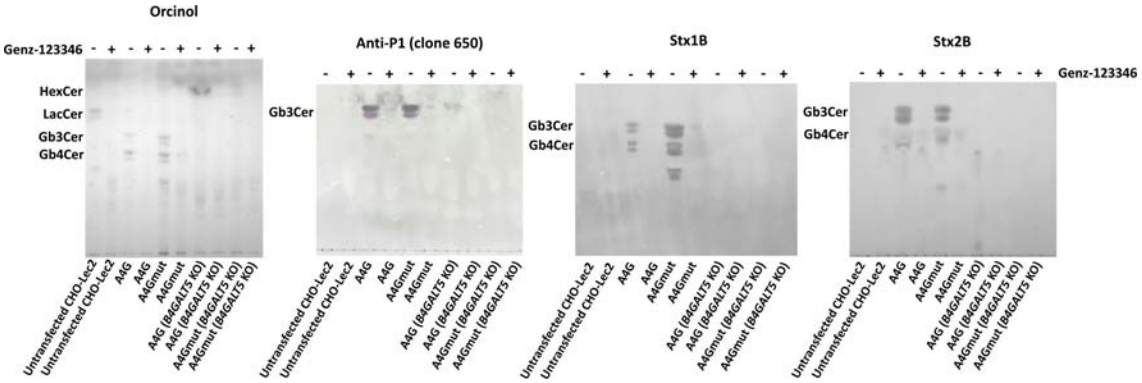

Fig. 3. HPTLC analysis of neutral glycosphingolipids purified from CHO-Lec2 cells expressing human A4galt (A4G) and its mutein form (A4Gmut), with or without hamster *B4GALT5* KO. The samples were stained with orcinol, anti-P1 (clone 650), Stx1B and Stx2B.

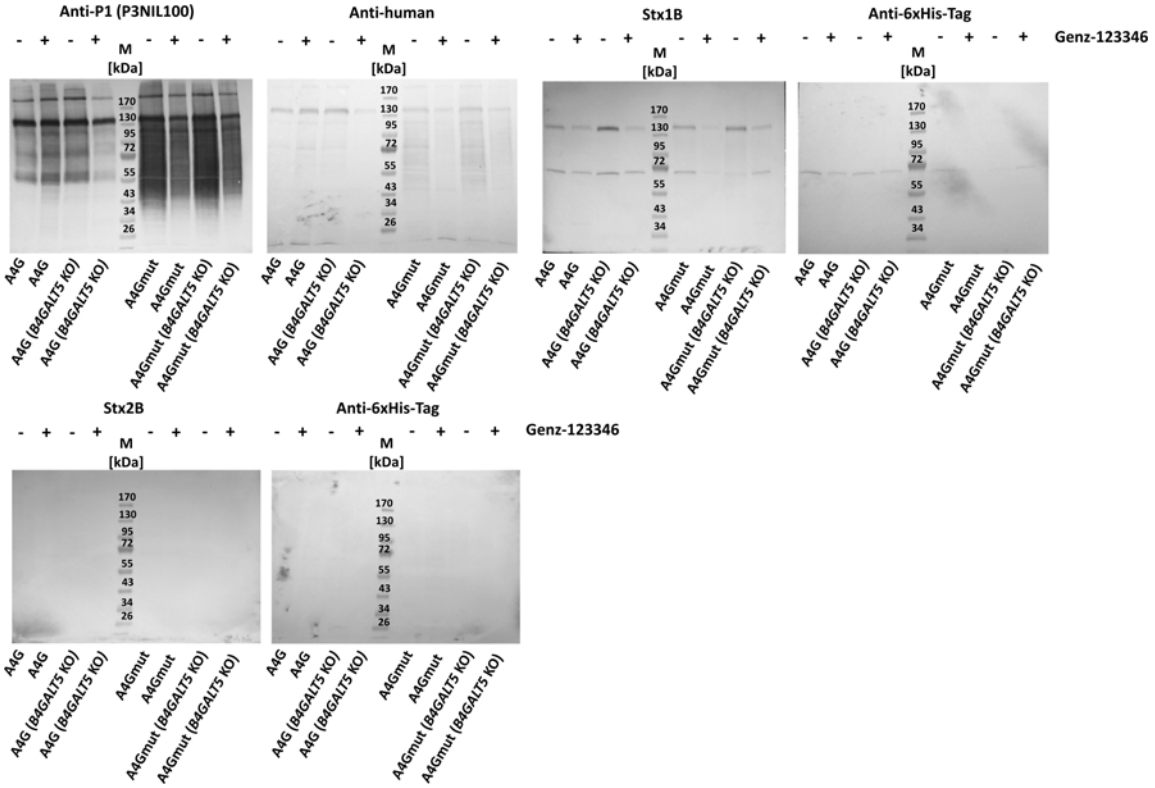

Fig. 4. Western blotting analysis of cell lysates from CHO-Lec2 expressing human A4galt (A4G) and its mutein form (A4Gmut), with or without hamster *B4GALT5* KO, stained with anti-P1 (P3NIL100) antibody. The cells were supplemented with or without the Genz-123346 inhibitor.

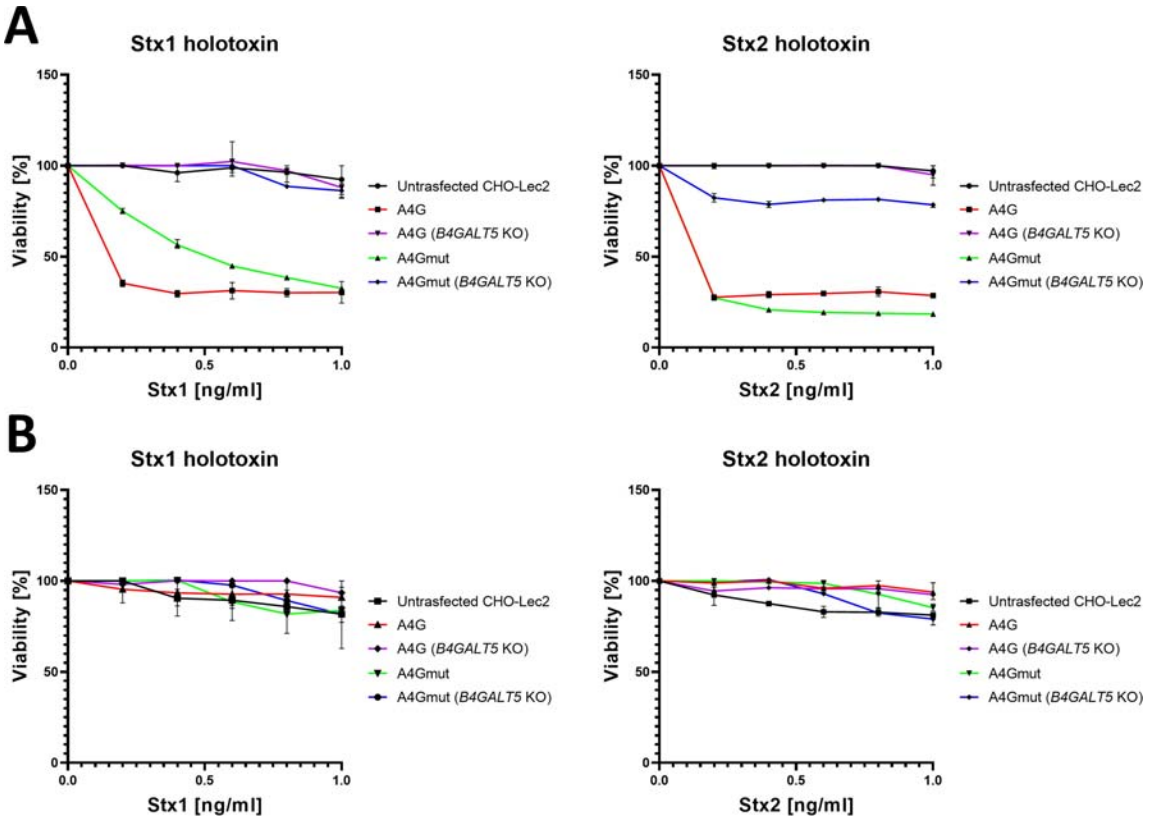

Fig. 5. Shiga toxins cytotoxicity assay. Viability of CHO-Lec2 cells expressing human A4galt (A4G) and its mutein form (A4Gmut) with or without hamster *B4GALT5* KO was analyzed after exposure to Stx1 (A) or Stx2 (B) in the presence (A) or absence (B) of Genz-123346 inhibitor.