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Glycoconjugates prevent *B. anthracis* toxin-induced cell death through binding while activating macrophages

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Abstract *Bacillus anthracis* toxins may be attenuated if macrophages could neutralize toxins upon contact or exposure. Glycoconjugate-bearing polymers, which have been shown to bind to *Bacillus* spores, were tested for recognition and binding of protective antigen (PA), lethal factor (LF), and edema factor (EF) toxins. We have demonstrated modulation of macrophage activity following exposure to these toxins. Without glycoconjugate (GC) activation, murine macrophages were killed by *Bacillus* toxins. GCs were shown to have a protective influence, sparing macrophages from toxin-induced cell death, as shown by increased macrophage cell viability based on trypan blue assay. Increased levels of inducible nitric oxide (NO) production by macrophages in presence of GCs suggest that GCs provide an activation signal for macrophages and stimulate their function. Results hint to GCs that promote neutralization of *Bacillus* toxins, block toxin-induced macrophage death, while increasing macrophage activation. Polymeric GCs may suggest novel approaches to improve existing or develop new vaccines as well as immunotherapeutics.

Keywords *B. anthracis* · Protective antigen (PA) · Lethal factor (LF) · Edema factor (EF) toxin · Glycoconjugate · Recognition · Macrophage · Phagocytosis · Neutralization

Abbreviations

Glyc-PAA-flu	Glycoconjugate-polyacrylamide-fluorescein polymer
GC(s)	Glycoconjugate(s)
GC1	Galα1-3 GalNAcα -PAA-flu
GC2	Galβ1-3 GalNAcβ-PAA-flu
GC3	GalNAcα1-3 GalNAcβ -PAA-flu
GC4	Galβ1-3 Galβ -PAA-flu
GC5	GlcNAcβ1-4 GlcNAcβ -PAA-flu
GC6	Fucα1-4 GlcNAcβ -PAA-flu
GC7	Galβ1-2 Galβ -PAA-flu
GC8	Fucα1-3 GlcNAcβ -PAA-flu
GC9	GlcNAcβ1-3 GlcNAcα -PAA-flu
GC10	GalNAcβ1-6 GalNAcα -PAA-flu
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Fuc	Fucose
GlcNAc	*N*-acetylglucosamine
PAA	Polyacrylamide
Flu	Fluorescein
LDH	Lactate dehydrogenase
NO	Nitric oxide
CFU	Colony forming units
M	Macrophages
PA	Protective antigen
LF	Lethal factor
EF	Edema factor
ATR	Anthrax toxin receptor

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Introduction

Bacillus anthracis, a spore-forming agent, causes anthrax [1]. *B. anthracis* spores enter the host's body through the gastrointestinal tract, the skin, or lungs [2–4]. Once *B. anthracis* spores germinate into vegetative cells, the latter produce two powerful toxins namely i) lethal toxin (LT), made up of protective antigen (PA) and lethal factor (LF), and ii) edema toxin (ET), made up of PA and edema factor (EF) [5–8]. PA binds the anthrax toxin receptor (ATR) expressed on macrophages (M) and endothelial cells [9, 10]. Soon after binding, PA supports the entry of LF and EF into host cells [9]. ET and LT cause edema and cell death, respectively [2, 3]. Toxins diminish immune cells' capacity to kill bacteria and reduce host's resistance to infection [11, 12]. Anthrax can be attenuated if macrophages [12] could recognize and neutralize any agents including toxins upon contact or exposure [5–7, 11].

PA appears to be the main target of all existing anthrax vaccines [13]. Anti-PA antibodies of anthrax vaccines recognize PA, blocking EF and LF entry into cells [14, 15]. Penicillin, ciprofloxacin, and doxycycline are the preferred antibiotics for treating anthrax infections [3]. However, antibiotics do not neutralize EF or LF toxins and will, therefore, not prevent toxemia, cellular edema or cell death [3]. In addition, antibiotic resistance has potentially life-threatening ramifications [16].

There is a need for a new vaccine candidate that will recognize and neutralize PA, EF, as well as LF. This new vaccine is anticipated to activate macrophages, and to stimulate their defense functions to control an anthrax infection. Macrophages are key players in controlling inflammation and innate immunity to microorganisms [5–7, 11, 12, 15].

Glycoscience is the field that studies natural and synthetic sugar polymers, the impact of sugars, receptor binding, and cell-cell recognition [17–19]. Synthetic sugar polymers have found applications in biomedical and pharmaceutical research due to their physicochemical properties, longer shelf-life, and low toxicity [17, 20–22].

We reported earlier that synthetic glycoconjugate-bearing polymers (Glyc-PAA-flu, or GCs) contribute to binding and recognition of *Bacillus cereus* spores [23]. Glyc-PAA-flu is a 30kD multivalent polymer ligand that consists of carbohydrate units (Glyc), a polyacrylamide (PAA) polymer backbone, and a fluorescein (flu) group [17]. Both the flu and the PAA groups are hydrophilic and generally show low binding affinity and flexibility [17]. GCs are known to inhibit *B. cereus* spores, activate macrophages and increase their viability upon *B. cereus* exposure [24–26].

In the present study, we have studied the efficacy of Glyc-PAA-flu ligands in A) binding and B) recognizing *B. anthracis* PA, LF, and EF toxins during phagocytosis using murine macrophages.

Materials and methods

Materials

Recombinant PA, EF, LF toxins and goat anti-PA primary antibody (Ab) were obtained from the List Biological Laboratories, Inc. (Campbell, CA, USA). Rabbit anti-goat HRP-conjugated secondary Ab was obtained from AbCam (Cambridge, MA, USA). GCs, namely Galβ1-3 GalNAcα-PAA-flu (1), Galβ1-3 GalNAcβ-PAA-flu (2), GalNAcα1-3 GalNAcβ -PAA-flu (3), Galβ1-3 Galβ -PAA-flu (4), GlcNAcβ1-4 GlcNAcβ -PAA-flu (5), Fucα1-4 GlcNAcβ -PAA-flu (6), Galβ1-2 Galβ -PAA-flu (7), Fucα1-3 GlcNAcβ -PAA-flu (8), GlcNAcβ1-3 GlcNAcα -PAA-flu (9), and GalNAcβ1-6 GalNAcα -PAA-flu (10) were obtained from GlycoTech, Inc. (Rockville, MD, USA). Bovine serum albumin, Griess reagent, W3500 tissue culture water, 96-wells plates, and sterile tips were purchased from Fisher Scientific (Houston, TX, USA). TMB substrate (3,3′,5,5′-tetramethylbenzidine) was obtained from Pierce Chemical Company (Rockford, IL, USA). Thioglycollate broth was obtained from Difco Microbiology, BD Bioscience (Franklin Lakes, NJ, USA). C57BL/6 mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and maintained in an AALAC-approved vivarium at the University of Arkansas for Medical Sciences (Little Rock, AR, USA). CytoTox 96® and the CellTiter 96® kits were obtained from Promega, Inc., (Madison, WI, USA).

Toxin, primary and secondary Ab preparations

Stable toxin solutions PA, EF, and LF were prepared according to the supplier's technical note (List Biological Laboratories, Inc., Campbell, CA). Purchased toxin samples (0.1 mg) were reconstituted using 100 μL of sterile tissue culture water. Diluted samples were immediately distributed equally into aliquots (10 μL/sterile autoclaved 1.5 mL tube) and kept at -20°C to preserve the stability of each toxin. A working solution of each studied PA, EF, and LF toxin (10 μg/mL) was prepared prior to use. Stable solutions of a goat anti-PA, anti-EF, and anti-LF primary Ab were prepared according to List Biological Laboratories, Inc. (Campbell, CA). The recommended titer of is 1:4,000 for anti-PA stable Ab solutions, and 1:1,850 for anti-EF and anti-LF primary Ab solutions. Each Ab (1.0 mg) was reconstituted in 500 μL of sterile tissue culture water (concentration: 2.0 mg/mL or 1: 4,000). Solutions were aliquoted (83.3 μL/1.5 mL sterile tube) and stored at −20°C to preserve Abs. Working solutions of anti-PA (0.789 mg/mL or 1: 10,000 titer), anti-EF(342 μg/mL or 1: 10,000 titer), anti-LF (342 μg/mL or 1: 10,000 titer) primary Ab were prepared prior to use. Rabbit anti-goat

HRP conjugated secondary Ab solutions were prepared according the supplier's manual (AbCam, Cambridge, MA) and had a final concentration of 0.1 μg/mL in the recommended 1: 10,000 titer.

Toxin binding and recognition

Binding and recognition of PA, EF, and LF toxins were assessed through our established binding procedure using GC1-GC10 [23, 29]. Briefly, each type of GC was diluted down to 0.01 mg/mL, 0.1 μg/mL, 1 ng/mL, and 1 pg/mL (or 10^{-2}, 10^{-4}, 10^{-6}, 10^{-12} dilutions) and used during binding studies. Each diluted GC (or 10 μL each) was spotted to a well of a 96-well plate. Each 96-well plate bearing spotted GC1–GC10 served as a GC sensor [23, 29] for binding and recognition of *B. anthracis* toxins. Plates were incubated at 4°C for 24 h. All plates were washed three times using 200°μL of PBST buffer (800 mL of PBS + 7.2 L ultra pure water + 8 mL Tween 20) and a Thermo Electron automatic washer (Thermo Scientific, MA, USA). All wells were blocked using 10 μL of 1% BSA blocking solution. Plates were incubated again at 4°C for 24 h. The plates were washed three times. Then, 12.0°μL of each PA, EF, and LF (10 μg/mL) was added to each well. The plates were incubated for 90 min at 37°C. Soon after, plates were washed three times using 200 μL of PBST buffer. Then, 3.2 μL of diluted anti-PA primary Ab solution (0.789 mg/mL) with either 3.2 μL of diluted anti-EF or anti-LF primary Ab (342 μg/mL) were added to each well. The plates were incubated at room temperature for 2 h. Plates were again washed three times. Subsequently, 12 μL of a secondary Ab solution (0.1 μg/mL) was added to each well. Plates were again incubated at room temperature for 2 h. After the final washing cycle, 100 μL of TMB substrate was added to all wells. The TMB solution has similar sensitivity levels to chemiluminescent detection reagents. It can detect as little as 0.15 ng of "GC ligand—Ag—primary Ab—secondary Ab" complexes within each well and consequently increase the intensity of the performed binding assay. The plates were incubated at room temperature for 20–25 min while being protected from light. Finally, 50 μL of a stopping solution (2 M H_2SO_4) was added to all wells and the optical density (OD) was measured using a Bio-TekEx800 plate reader (BioTek Instruments, MA, USA).

Molecular modeling and docking

Molecular models of toxins PA, EF, and LF were obtained from the Protein Databank (www.pdb.org) as 1ACC, 1K8T and 1JKY, respectively. Molecular models of studied toxins were rendered, optimized, and then employed in docking. GCs were modeled using ChemDraw© and optimized in Chem3D© v5.0 (by CambridgeSoft) workspace. The disaccharide moiety of GCs was declared a ligand that would dock to an active site on PA, LF and EF. The active site on PA consisted of the following residues: 182-ASP, 187-ASP, 188-LEU, 223-TYR, 229-HIS, 235-LEU and 236-TYR [45]. The active site on EF (chain A of) consisted of the following residues: 329-ARG, 346-LYS, 354-SER, 372-LYS, 491-ASP, 493-ASP, 548-THR, 577-HIS and 583-ASN [46]. The active site on LF consisted of the following residues: 148-TYR, 149-TYR, 151-ILE and 153-LYS [46]. Docking of GCs as flexible ligands onto the active sites found on PA, EF and LF was performed using BioMedCAChe software (Fujitsu Systems Inc., CA, USA) [28] and ArgusLab v4.0.1 software (www.arguslab.com by Mark Thompson and Planaria Software, LLC).

Cell cultures

C57BL/6 mice, 6 to 8 weeks old, were injected intraperitoneally with 1.0 mL of 3% thioglycollate broth. Four days after injection, mice were euthanized and peritoneal exudate cells were collected by lavage with 5.0 mL RPMI 1640. Macrophages were plated in 6-well plates at 1.2×10^6 per well in RPMI 1640 containing 10% fetal calf serum, 50 nM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubating for 1 h at 37°C (95% air, 5% CO_2), nonadherent cells were removed by washing. Adherent cells ($6.0–8.0\times10^5$ per culture well) were maintained in RPMI 1640 only.

Neutralization of toxins during phagocytosis

PA-EF-LF, PA-EF, and PA-LF toxins (7.0 μL, 3.0 μL, 3.0 μL), as well as 5.0 μL of selected GCs (GC1 and GC8) were introduced to macrophages. Phagocytosis was engendered as follows: macrophage cell cultures (10^6/mL) were infected with GC-treated and untreated toxins (controls), then incubated for 20.5 h (37°C, 95% air/5% CO_2).

Post-phagocytosis macrophage studies

Toxin-induced macrophage damage was measured by cell morphology based on trypan blue assay, lactate dehydrogenase (LDH) release, and nitric oxide (NO) production. To determine macrophage cell viability, culture supernatants were replaced with 500 μL of 0.4% trypan blue solution and microscopically examined under a Nikon Eclipse E400 POL fluorescence microscope at a magnification of 400×. Digital micrographs were acquired in real time. Ten determinations were made for each culture well. Percent viability was determined by counting individual live as well as dead macrophages.

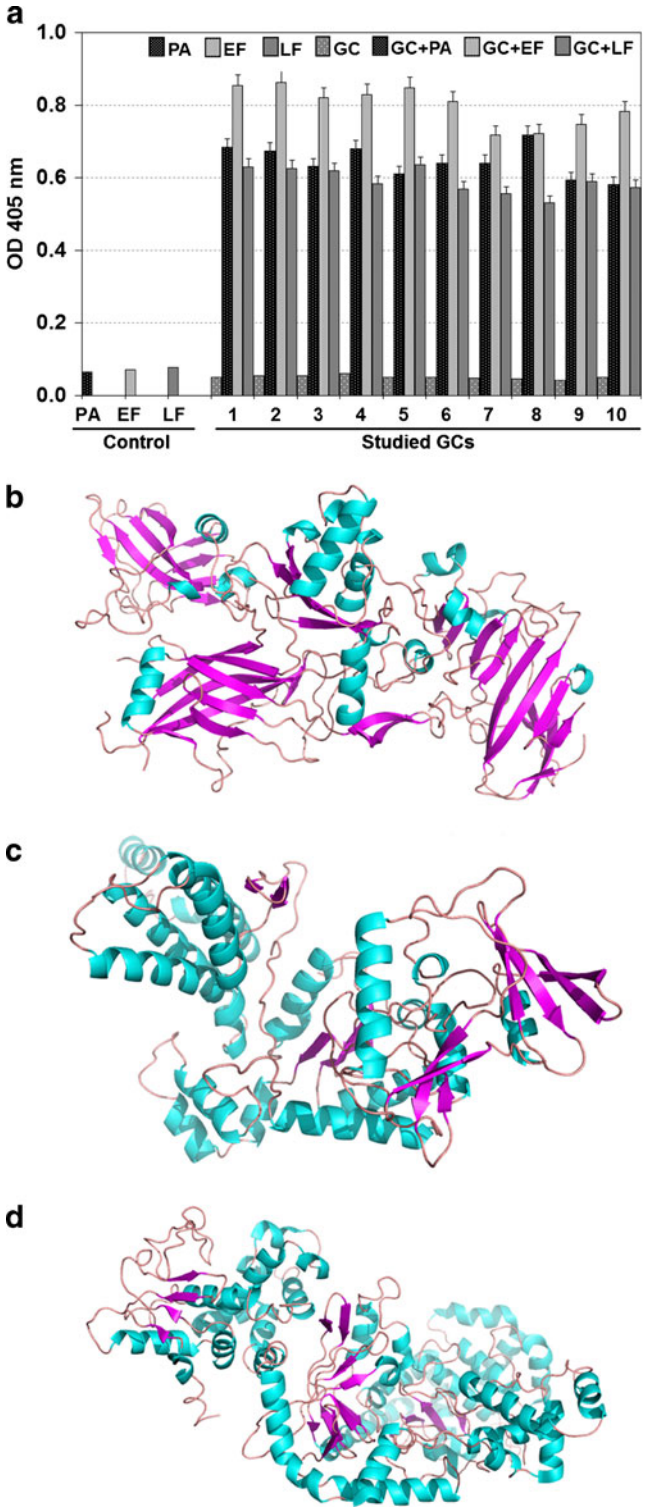

Fig. 1 GCs binding and recognition (**a**) of toxins PA (**b**); EF (**c**) and LF (**d**) based on optical density (OD) as shown (y-axis). GC1- GC10 (0.01 mg/ml) (from #1 to 10 on the x-axis) was used in binding and recognition of PA, EF, LF, $p<0.001$ vs. GCs only. PA, EF, and LF only were used as controls. FASTA sequences of PA (1ACC) (**b**), EF (1K8T) (**c**), and LF (1JKY) (**d**) toxins were obtained from the Protein Databank (www.pdb.org) and ribbon models were rendered using PyMol v0.99 (www.pymol.com)

Macrophage integrity was analyzed using a CytoTox 96® kit (Promega, Inc., Madison, WI). LDH is a stable cytosolic enzyme released upon cell lysis with conversion of tetrazolium salt to a red formazan product. The amount of color is proportional to the number of lysed cells. The optical absorbance was measured at 490 nm using a Bio-Tek Ex800 plate reader.

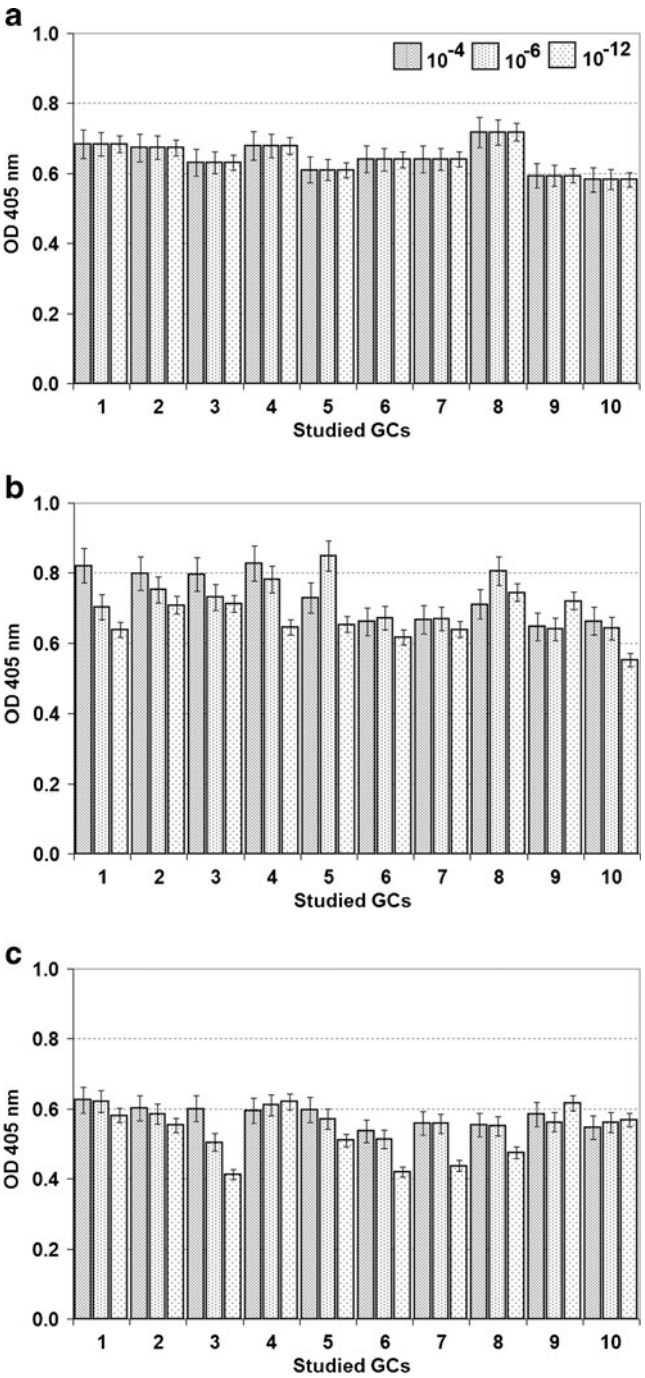

Fig. 2 Dilution effect of GCs (1–10) on binding and recognition of PA (**a**), EF (**b**), and LF (**c**) toxins. GCs concentrations 0.1 μg/mL, 1 ng/mL, and 1 pg/mL (or 10^{-4}, 10^{-6}, 10^{-12} dilutions) are used

For NO production, macrophages were plated at 10^6 cells/culture in 6-well flat-bottom tissue culture plates and incubated in parallel with GC-treated and untreated toxins. Cells were incubated at 37°C in 5% CO_2 for 24 h. Supernatants (100 μL) were then assayed for NO content. The nitrite ion (NO_2^-) concentration, indicative of NO, was determined using $NaNO_2$ as a standard [30]. Briefly, 100 μL of cell culture supernatant was mixed with an equal volume of Griess reagent [0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid]. The samples were incubated at room temperature for 20 min and optical absorbance was measured at 490 nm using a Bio-Tek Ex800 plate reader.

Statistics

Results were considered statistically significant at p-values< 0.01 using t-test or ANOVA.

Results

In this study, GC1-10 [29] was used in order to assess the affinity of GCs towards studied PA, EF, and LF toxins using our binding assay (Fig. 1).

We evaluated 10 GCs (ligands) as a mean to study anthrax toxins, namely PA, EF and LF. Figure 1 displays the optical density (OD) measured at 405 nm vs. the type of studied GC, 1 through 10. The OD is an indirect but reliable indication of GCs' affinity to bind to a specific toxin. When a GC binds to a toxin, the solution absorbs more light, hence yielding a higher OD. The higher the OD, the higher the binding affinity of GCs toward studied toxins during the binding study (Fig. 1).

The average OD signal intensity value for each GC was plotted to illustrate how strong GCs would bind to a particular toxin: PA (Fig. 1a, b), EF (Fig. 1a, c), or LF (Fig. 1a, d). It can be clearly seen that i) on the whole, GC1-10 utilized in this experiment were binding to EF with higher OD value than those bonded to PA or LF (Fig. 1a); ii) GC8 has a marked affinity for PA and EF; iii) all GCs bound to LF with low OD values compared with PA and EF.

GCs can selectively target toxins, change their spatial confirmation, and eventually prevent PA binding to ATR. By using either GC1 or GC8, one may be able to effectively prevent anthrax toxins from carrying out their deleterious actions.

Further, we studied how diluted GCs impact binding and recognition of studied PA, EF, and LF toxins (Fig. 2). Figure 2 shows the dilution effects of studied GCs (GC1 through GC10) on binding and recognition of PA, EF, LF toxins. To assess the efficacy of GCs, these were diluted 10^{-4}–10^{-12} times prior to being used.

Figures 2a-c display the OD measured at 405 nm vs. the type of studied GC, 1 through 10, taken at various dilutions. As stated earlier, the OD is an indirect but

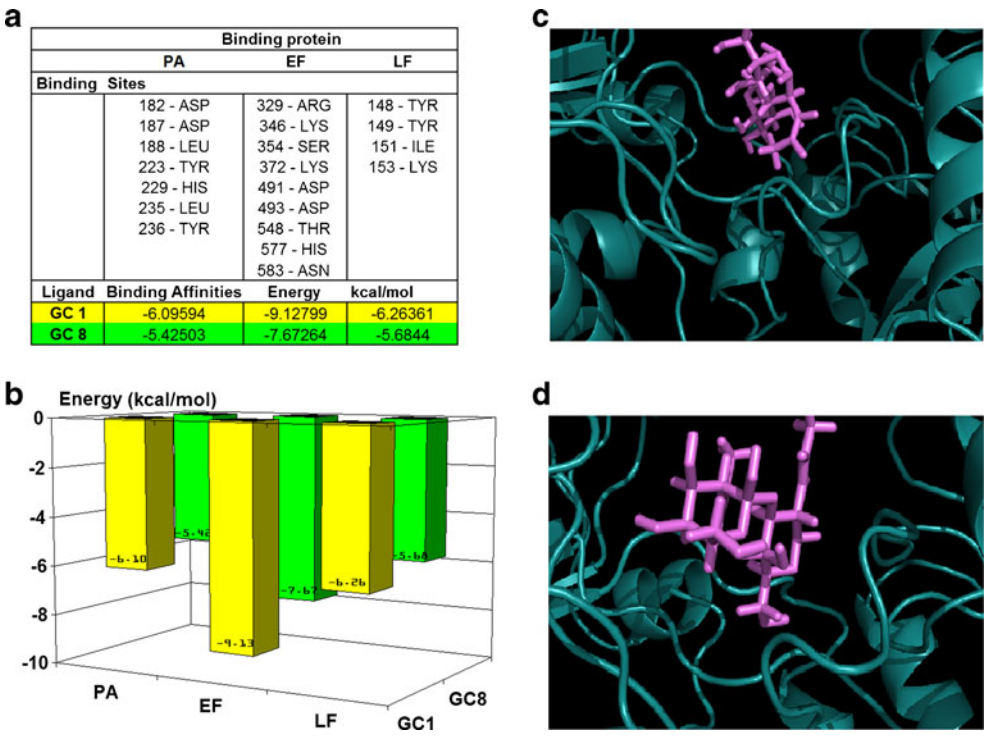

Fig. 3 Binding energies and molecular docking of GC1, GC8 and PA, EF and LF toxins. **a**, **b** The binding energy serves as a yardstick to assess binding affinity. Comparative docking representations of the disaccharide moiety (pink) of GC1 (**c**) and GC8 (**d**) with lowest binding energies are demonstrated here. Compared to GC8 (**d**), GC1 (**c**) docked to the binding site found on chain A of EF with lowest binding energy (**a**, **b**)

reliable indication of GCs' affinity to bind to a specific toxin. All diluted GCs (1–10) demonstrate a certain binding affinity for toxins, some higher than others. For PA (Fig. 2a), the dilution of individual GCs has little or no effect on binding. GC8 demonstrated an outstanding higher binding affinity toward PA (Fig. 2a). Although the optical density fluctuates for EF (Fig. 2b), in most cases the higher the dilution, the lower the optical density, with the exception of GC6 and 7, where the dilution effect is not as pronounced. Overall, the recognition of EF decreased with dilution of GC1, GC2, GC3, GC4, and GC10. Diluted GC4, GC8, and GC9 promoted the recognition of EF. The latter, however, decreased for GC4 and GC8 using a dilution of 10^{-12}. We have shown that a dilution of 10^{-6} was most efficient in *B. cereus* spore binding and killing during phagocytosis [24, 25]. In case of LF (Fig. 2c), a similar trend is observed, with the exception of GC6, GC7, and GC8, where the dilution effect is hardly noticeable. Recognition of LF decreased using diluted GC1, GC2, GC3, GC5, and GC6. Diluted GC4, GC9, and GC10 demonstrated an increased recognition of LF. Binding affinity of individual GCs to PA does not appear to be affected by dilution as shown in Fig. 2a. This characteristic is especially valuable since GCs will be binding to PA at virtually any dilution tested. Needless to say, working with diluted GCs is also more economical.

GC1 and GC8 were examined closer in molecular docking studies (Fig. 3a, b) since GC1 and 8 demonstrated high PA binding levels and noticeable binding affinity toward PA and EF during the binding study (Figs. 1 and 2). The binding energy serves as a yardstick to assess binding affinity of studied GCs.

Molecular docking (Fig. 3a) revealed that the lower the binding energy (AKA best ligand pose), the more likely an interaction (predominantly consisting of hydrogen bonds) is to occur between studied GCs and PA, EF or LF. For instance, GC1 was first docked onto the active site of PA, then EF, and finally on that of LF. GC8 was docked in the same sequence. The binding energies (kcal/mol) of all docking sessions were recorded (Fig. 3a-b). Compared to GC8 (−7.67 kcal/mol), GC1 (−9.13 kcal/mol) is more likely to bind to EF (Fig. 3b). Similarly, unlike GC8 (−5.43 kcal/mol), GC1 (−6.10 kcal/mol) is slightly more likely to bind to PA. Likewise, GC1 (−6.26 kcal/mol) appears to bind more easily to LF than GC8 (−5.68 kcal/mol) does (Fig. 3a-b). GC1 seems to bind more readily to any one of the toxins (PA, EF, and LF), compared to GC8. To prevent formation of the PA heptamer, one would consequently employ GC1 that has a greater chance of blocking any one of the aforementioned toxins. Molecular docking results demonstrated that the disaccharide moiety (pink) of GC1 (Fig. 3c) was more stable when docked onto the binding site found on chain A of EF toxin, when compared to that of GC8 (Fig. 3d).

GC1 and GC8 were further examined during phagocytosis studies to evaluate their effects on macrophages exposed to PA, EF, and/or LF. Since macrophage NO production is important in the killing of bacteria or neutralizing toxins, and is a marker of macrophage activation [7, 31–35], the effects of GCs on macrophage NO production were examined (Fig. 3).

When exposed to GC1-treated toxins, in particular the PA-LF complex, macrophages produced more NO (Fig. 4a) compared to macrophages only (M) and untreated PA-EF-LF, PA-EF, PA-LF. Similarly, GC8-treatment, especially of the PA-EF-LF complex, induced higher NO release compared to macrophages and untreated toxin complexes, namely PA-EF-LF, PA-EF, and PA-LF (Fig. 4b). Macrophages exposed to untreated PA-EF-LF, PA-EF, PA-LF toxins induced a slight increase of NO production. The addition of either GC1 or GC8 glycoconjugate induced macrophage NO production ($p<0.001$).

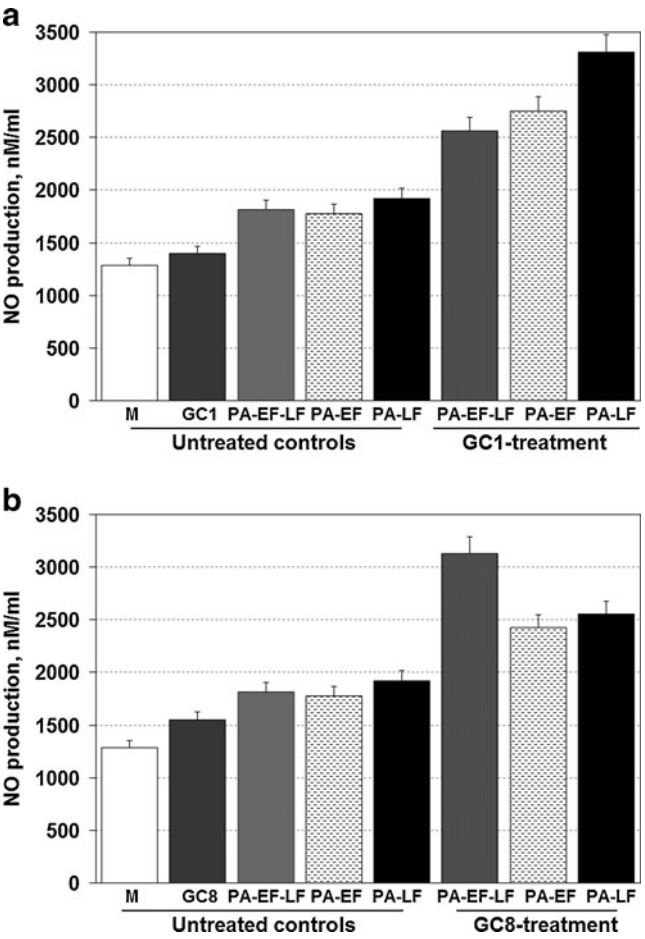

Fig. 4 GC1 and GC8 stimulate macrophage nitric oxide (NO) production. Macrophage cultures were exposed to untreated and GC1- (**a**) and GC8- (**b**) treated PA-EF-LF, PA-EF, PA-LF toxins. After 20 h, the amount of NO produced by macrophages was measured by the Griess assay. Untreated controls consisted of macrophages (M) only, GC1, GC8, and untreated toxins. These results are representative of experiments carried out in triplicate

GC1 doubled macrophage NO production (Fig. 4a), while GC8 increased NO by a 2.6-fold upon exposure to PA-EF-LF (Fig. 4b). In presence of PA-EF, both GC1 and GC8 induced approximately the same amount of NO ranging from 2,400 to 2,700 nM/mL. GC1 treatment of toxins was highly stimulatory and increased the NO production in 2.7 times, whereas GC8 treatment was less stimulatory.

It was reported that released NO can prevent cell death [31–34]. Our results have confirmed this statement [31–34] and have shown that the more NO is produced (Fig. 4), the more macrophages survive, and consequently more toxins will be neutralized during phagocytosis (Fig. 5).

Macrophages alone showed a 96% viability, whereas GC1 or GC8 only demonstrated 68% or 79%, respectively. A dramatic loss of macrophage viability was observed during exposure to untreated toxins (control group). Macrophages exposed to PA-EF-LF, PA-EF, PA-LF demonstrated 44%, 49%, and 62% cell viability, respectively (Fig. 5). Whether toxins were treated with GC1 (Fig. 5a) or GC8 (Fig. 5b), the observed macrophage cell viabilities rose. Macrophage cell viability remained high (90%) when the GC1 treatment was administered to the PA-EF-LF complex (Fig. 5a). Macrophage cell viability increased (82%) when GC1 was administered in conjunction with the PA-EF toxin combination and PA-LF toxin (85%). GC8 similarly increased cell viability in presence of PA-EF-LF, PA-EF, and PA-LF between 86% and 89%.

To further characterize GCs, LDH activity was examined upon toxin exposure. LDH release was determined 1 day following macrophage exposure to GC-treated or untreated PA-EF-LF, PA-EF, and PA-LF complexes (Fig. 6).

Macrophage LDH activity was higher (almost twice as high as untreated PA-EF-LF, PA-EF, PA-LF toxins) when toxins were treated with GC1 (Fig. 6a). GC8-treated PA-EF-LF, PA-EF, PA-LF complexes induced less LDH compared to controls (Fig. 6b).

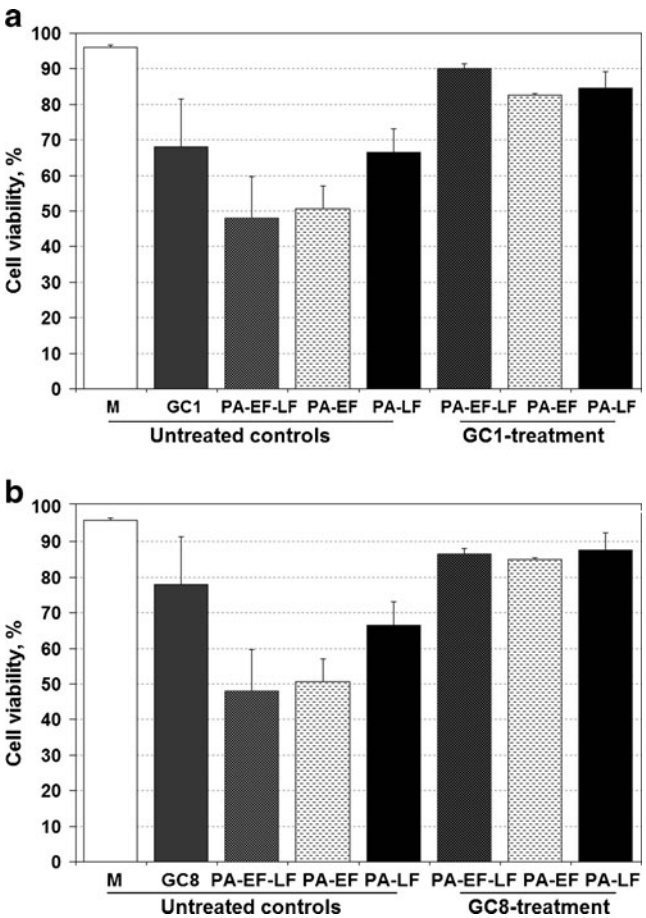

Fig. 5 GCs contribute to higher macrophage (M) cell viability upon PA-EF-LF, PA-EF, PA-LF toxins exposure. These results are representative of experiments carried out in triplicate

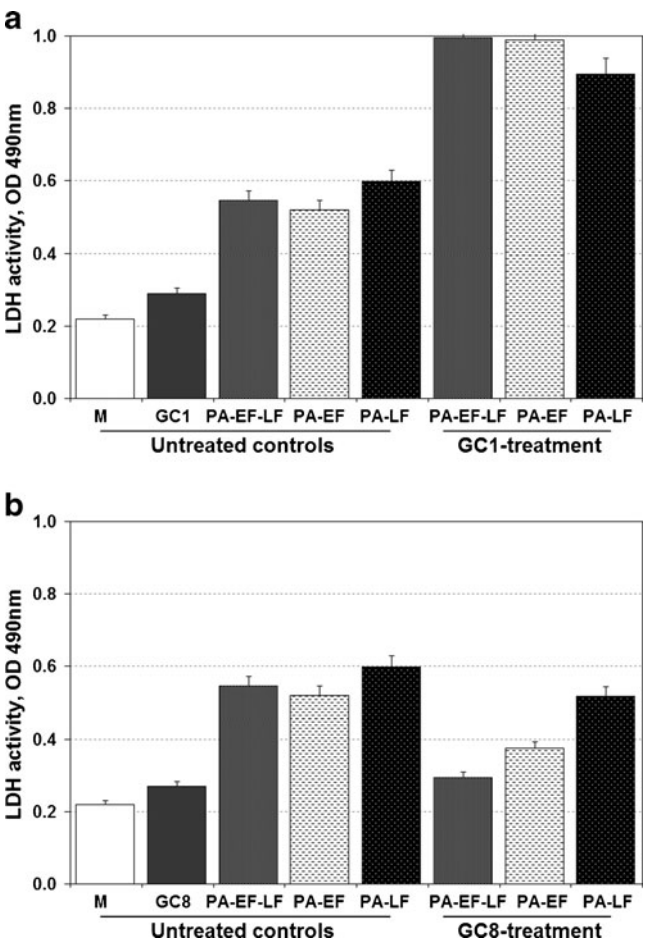

Fig. 6 LDH activity upon toxin exposure. Macrophage cultures were exposed to untreated-, as well as GC-treated toxins. After 20 h, macrophage LDH was assayed in macrophages exposed to untreated and GC1- (**a**) and GC8- (**b**) treated PA-EF-LF, PA-EF, PA-LF toxins and macrophages (M) only. These results are representative of experiments carried out in triplicate

Discussion

The capability of macrophages to kill microorganisms and/or to survive exposure to toxins may determine whether exposure to *B. anthracis* will progress to a possibly serious infection [4–7]. The present study primarily focused on binding of toxins by GCs and their protective role on macrophages. Exposure of murine macrophages to *B. anthracis* PA-EF-LF, PA-EF and PA-LF toxin combinations resulted in a roughly 50% loss in macrophage viability 24 h later (Fig. 5). GCs have been reported to interrupt spore germination [23–27] and to bind to *B. anthracis* toxins [27]. GCs adhering to toxins could block their binding to cell surface receptors. Alteration of receptors by GCs might impair toxin binding [29]. GCs [23–27] bound to toxins might also act as opsonins, promoting endocytosis [36]. GCs may also serve as ligands that bind to macrophage receptors, thus activating macrophages.

There is some supporting evidence that carbohydrates can serve as chemoattractants or immunostimulators for macrophages [24–27, 36–38, 47–52]. Indeed, GCs increase macrophage production of inducible NO in presence of PA-EF and PA-LF toxin complexes or both (Fig. 4). Specific carbohydrate structures expressed on agents are believed to be recognized by complementary molecules expressed on the surface of interacting cells [37–42]. It was reported that natural or synthetic carbohydrate–based ligands are involved in the recognition processes, including adhesion between cells, adhesion of cells to an extracellular matrix, and specific recognition of cells by one another [37–42]. In addition, it was demonstrated that carbohydrate-carbohydrate interactions play an important role in complimentary binding of glycosphingolipids [43, 44]. Recognition and inhibition of PA-EF and PA-LF toxin complexes is based on binding affinity between disaccharide GCs acting as ligands [27, 29]. Individual GCs may exhibit different properties. This is reflected in the differences observed in inducible NO production by macrophages. Presumably, binding of GCs leads to alteration of toxins that play an essential role, either in ATR receptor binding [9], in heptamer pore formation or both [27, 29]. Even after being bound to toxins [27, 29], GCs might serve as chemoattractants for macrophages or other phagocytic cells during phagocytosis of infectious agents [24–27, 36–38, 47–52]. Macrophages may become more prone to adhere to GC-altered toxins [27, 29], resulting in increased endocytosis of toxins during phagocytosis (Fig. 5) and NO production (Fig. 4) that will consequently lead to the neutralization of toxins.

The present study could lead to an improved selection of GCs ligands exhibiting immunomodulating properties. This study could suggest new targets for improving current or developing new vaccines and immunomodulators to neutralize *B. anthracis* toxins.

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References

1. Weiss, M.M., Weiss, P.D., Weiss, J.B.: Anthrax vaccine and public health policy. Am. J. Public Health **97**(11), 1945–1951 (2007)
2. Barnaby, W.: In: Barnaby, W. (ed.) The Plague Markers: The secret World of Biological Warfare. Vision Paperbacks, London (1997)
3. Jamie, W.E.: Anthrax: diagnosis, treatment, prevention. Prim. Care Update OB/GYNS **9**, 117–121 (2002)
4. Spencer, R.C.: Bacillus anthracis. J. Clin. Pathol. **56**, 182–177 (2003)
5. Friedlander, A.M.: Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261**, 7123–7126 (1986)
6. Popov, S.G., Villasmil, R., Bernardi, J., Grene, E., Cardwell, J., Popova, T., Wu, A., Alibek, A., Bailey, C., Alibek, K.: Effect of *Bacillus anthracis* lethal toxin on human peripheral blood mononuclear cells. FEBS Lett. **527**, 211–215 (2002)
7. Singh, Y., Leppla, S.H., Bhatnagar, R., Friedlander, A.M.: Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and -resistant cells. J. Biol. Chem. **264**, 11099–11102 (1989)
8. Cote, C.K., Rossi, C.A., Kang, A.S., Morrow, P.R., Lee, J.S., Welkos, S.L.: The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb. Pathog. **38**, 209–225 (2005)
9. Bradley, K.A., Young, J.A.: Anthrax toxin receptor proteins. Biochem. Pharmacol. **65**, 309–314 (2003)
10. Bonuccelli, G., Sotgia, F., Frank, P.G., Williams, T.M., de Almeida, C.J., Tanowitz, H.B., Sherer, P.E., Hotchkiss, K.A., Terman, B.I., Rollman, B., Alileche, A., Brojatsch, J., Lisanti, M. P.: Anthrax toxin receptor (ATR/TEM8) is highly expressed in epithelial cells lining the toxin's three sites of entry (lungs, skin, and intestine). Am. J. Physiol. Cell Physiol. **288**, C1402–C1410 (2005)
11. Duesbery, N.S., Vande Woude, G.F.: Anthrax toxins. Cell. Mol. Life. Sci. **55**, 1599–1609 (1999)
12. Guidi-Rontani, C., Mock, M.: Macrophage interactions. Curr. Top. Microbiol. Immunol. **271**, 115–141 (2002)
13. Ramirez, D.M., Leppla, S.H., Schneerson, R., Shiloach, J.: Production, recovery and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis*. J. Ind. Microbiol. Biotechnol. **28**, 232–238 (2002)
14. Little, S.F., Webster, W.M., Ivins, B.E., Fellows, P.F., Norris, S.L., Andrews, G.P.: Development of an in vitro-based potency assay for anthrax vaccine. Vaccine **22**, 2843–2852 (2004)
15. Baillie, L., Read, T.D.: *Bacillus anthracis*, a bug with attitude! Curr. Opin. Microbiol. **4**, 78–81 (2001)
16. De Bolle, X., Bayliss, C.D., Field, D., van de Ven, T., Saunders, N.J., Hood, D.W., Moxon, E.R.: The length of a tetranucleotide repeat tract in Haemophilus influenzae determines the phase variation rate of a gene with homology to type III DNA methyltransferases. Mol. Microbiol. **35**, 211–222 (2000)

17. Gabius, H.J., Gabius, S.: Glycosciences: Status and Perspectives. Chapman & Hall, London (1997)
18. Crocker, P.R., Feizi, T.: Carbohydrate recognition systems: functional trials in cell- cell interaction. Curr. Opin. Struct. Biol. **6**, 679–691 (1996)
19. Feizi, T.: Carbohydrate-mediated recognition systems in innate immunity. Immunol. Rev. **173**, 79–88 (2000)
20. Karlsson, K.A., Angstrom, J., Bergstrom, J., Lanne, B.: Microbial interaction with animal cell surface carbohydrates. APMIS Suppl. **27**, 71–83 (1992)
21. Axford, J.: The impact of glycobiology on medicine. Trends Immunol. **22**, 237–239 (2001)
22. Borman, S.: Carbohydrate vaccines. Chem. Eng. News. **9**, 31–35 (2004)
23. Tarasenko, O., Islam, Sh, Paquiot, D., Levon, K.: Glycoconjugates for recognition of *Bacillus* spores. Carb. Res. **339**, 2859–2870 (2004)
24. Tarasenko, O., Burton, E., Soderberg, L., Alusta, P.: Polymeric glycoconjugates protect and activate macrophages to promote killing of *Bacillus* cereus spores during phagocytosis. Glycoconj. J. **25**, 473–480 (2008)
25. Tarasenko, O., Scott, A., Soderberg, S., Ponnappan, U., Alusta, P.: Killing of *Bacillus* spores is mediated by nitric oxide and nitric oxide synthase during glycoconjugate—enhanced phagocytosis. Glycoconj. J. **27**(1), 13–25 (2010)
26. Tarasenko, O., Soderberg, L., Hester, K., Park Kim, M., McManus, D., Alusta, P.: Glycoconjugates enhanced the intracellular killing of *Bacillus* spores, increasing macrophage viability and activation. Arch. Microbiol. **189**, 579–587 (2008)
27. Castleberry, J., Alusta, P., Soderberg, L., Tarasenko, O.: Binding and neutralization of *Bacillus anthracis* protective antigen toxin and its complexes using glycoconjugates. PMSE **98**, 841–842 (2008)
28. Tarasenko O., Alusta P.: Docking approach to study receptor-ligand interactions. In: MidSouth Computational Biology and Bioinformatics Society Fourth Annual Conference. New Orleans, Louisiana, February 1st–3 rd, (abstract/poster) (2007)
29. Levon K., Tarasenko O., Bin Yu.: Glycoconjugate sensors. U.S. Patent Application Serial No.: 20040161861 (2004)
30. Keller, R., Geiges, M., Keist, R.: L-arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. Cancer Res. **50**, 1421–1425 (1990)
31. Beauvais, F., Michel, L., Dubertret, L.: The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. FEBS Lett. **361**, 229–232 (1995)
32. Choi, B.M., Pae, H.O., Jang II, S., Kim, Y.M., Chung, H.T.: Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J. Biochem. Mol. Biol. **35**, 116–126 (2002)
33. Dimmelder, S., Haendeler, J., Nehls, M., Zeiher, A.M.: Suppression of apoptosis by nitric oxide via inhibition of interleukin-1β-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J. Exp. Med. **185**, 601–607 (1997)
34. Kwon, Y.G., Min, J.K., Kim, K.M., Lee, D.J., Billiar, T.R., Kim, Y.M.: Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. J. Biol. Chem. **276**, 10627–10633 (2001)
35. Vespa, G.N.R., Cunha, F.Q., Silva, J.S.: Nitric oxide is involved in control of *Trypanosoma cruzi*-induced parasitemia and directly kills the parasite *in vitro*. Infect. Immun. **62**, 5177–5182 (1994)
36. Black, J.G.: In: Microbiology: Black J.G. (ed.) Principles and Explorations, pp. 463–484. John Wiley & Sons, Inc. Publishing 2005
37. Crocker, P.R., Feizi, T.: Carbohydrate recognition systems: functional trials in cell- cell interaction. Curr. Opin. Struct. Biol. **6**, 679–691 (1996)
38. Feizi, T.: Carbohydrate-mediated recognition systems in innate immunity. Immunol. Rev. **173**, 79–88 (2000)
39. Bertozzi, C.R., Kiessling, L.L.: Chemical glycobiology. Science **291**, 2357–2364 (2001)
40. Feizi, T.: Progress in deciphering the information content of the 'glycome' - a crescendo in the closing years of the millennium. Glycoconj. J. **17**, 553–565 (2000)
41. Kiessling, L.L., Gestwicki, J.E., Strong, L.E.: Synthetic multivalent ligands in the exploration of cell surface interactions. Curr. Opin. Chem. Biol. **6**, 696–703 (2000)
42. Kiessling, L.L., Pohl, N.L.: Strength in numbers: non-natural polyvalent carbohydrate derivatives. Chem. Biol. **3**, 71–77 (1996)
43. Kojima, N., Hakomori, S.: Sialyllactose-mediated cell Interaction during granulosa cell differentiation. J. Biol. Chem. **264**, 20159–20162 (1989)
44. Kojima, N., Hakomori, S.: Carbohydrate-carbohydrate interaction of glycosphingolipids. J. Biol. Chem. **266**, 17552–17558 (1991)
45. Cunningham, K., Lacy, D.B., Mogridge, J.: Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. PNAS **99**(10), 7049–7053 (2002)
46. Lacy, D.B., Mourez, M., Fouassier, A., Collier, R.J.: Mapping the anthrax protective antigen binding site on the lethal and edema factors. J. Biol. Chem. **277**(4), 3006–3010 (2002)
47. Taylor, M.E.: Recognition of complex carbohydrates by the macrophage mannose receptor. Biochem. Soc. Trans. **21**, 468–473 (1993)
48. Stein, M., Keshav, S., Harris, N., Gordon, S.: Interleukin-4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J. Exp. Med. **176**, 287–292 (1992)
49. Stahl, P.D.: The mannose receptor and other macrophage lectins. Curr. Opin. Immunol. **4**, 49–52 (1982)
50. Aderem, A., Underhill, D.M.: Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. **17**, 593–623 (1999)
51. Rosenberger, C.M., Finlay, B.B.: Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. Mol. Cell. Biol. **4**, 385–396 (2003)
52. DeFife, K.M., Jenney, C.R., Colton, E., Anderson, J.M.: Disruption of filamentous actin inhibits human macrophage fusion. FASEB **13**, 823–832 (1999)