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Neutralization of *B. anthracis* toxins during *ex vivo* phagocytosis

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Abstract Glycoconjugates (GCs) are recognized as stimulation and signaling agents, affecting cell adhesion, activation, and growth of living organisms. Among GC targets, macrophages are considered ideal since they play a central role in inflammation and immune responses against foreign agents. In this context, we studied the effects of highly selective GCs in neutralizing toxin factors produced by *B. anthracis* during phagocytosis using murine macrophages. The effects of GCs were studied under three conditions: A) *prior to*, B) *during*, and C) *following exposure* of macrophages to *B. anthracis* individual toxin (protective antigen [PA], edema factor [EF], lethal factor [LF] or toxin complexes (PA-EF-LF, PA-EF, and PA-LF). We employed *ex vivo* phagocytosis and post-phagocytosis analysis including direct microscopic observation of macrophage viability, and macrophage activation. Our results demonstrated that macrophages are more prone to adhere to GC-altered PA-EF-LF, PA-EF, and PA-LF toxin complexes. This adhesion results in a higher phagocytosis rate and toxin complex neutralization during phagocytosis. In addition, GCs enhance macrophage viability, activate macrophages, and stimulate nitric oxide (NO) production. The present study may be helpful in identifying GC ligands with toxin-neutralizing and/or immunomodulating properties. In addition, our study could suggest GCs as new targets for existing vaccines and the prospective development of vaccines and immunomodulators used to combat the effects of *B. anthracis*.

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	Galα1-3GalNAcα-PAA-flu glycoconjugate
GC8	Fucα1-3GlcNAcβ-PAA-flu glycoconjugate
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Fuc	Fucose
GlcNAc	*N*-acetylglucosamine
PAA	Polyacrylamide
Flu	Fluorescein
LDH	Lactate dehydrogenase
NO	Nitric oxide
M	Macrophages
ATR	Anthrax toxin receptor
PA	Protective antigen
LF	Lethal factor
EF	Edema factor
ET	Edema toxin (protective antigen-edema factor [PA-EF])
LT	Lethal toxin (protective antigen-lethal factor [PA-LF])

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Introduction

B. anthracis is considered a major bioterrorism and biological warfare agent [1–5]. *B. anthracis* causes a life-threatening infectious disease known as anthrax [1, 3, 4]. There are numerous high infectivity/mortality factors associated with *B. anthracis* such as A) the formation of dormant but metabolically active spores; B) the production of toxins and a capsule; C) a very low infectious dose, a high mortality rate, and antibiotic resistant strains that can promote the spread of anthrax [1, 2, 4, 6–10]. *B. anthracis* enters the host through cuts and abrasions in the skin (cutaneous), orally (gastrointestinal), or through the airways (inhalation) [1–4].

B. anthracis contains two plasmids: pXO1 and pXO2 [6–10]. Virulent *B. anthracis* strains express both pXO1 and pXO2 plasmids [3, 6, 9]. Attenuated non-infectious strains only express the pXO1 plasmid coding for PA, LF, and EF toxins [9]. Plasmid pXO1 is responsible for the production of toxins. The other plasmid—pXO2—is responsible for capsule synthesis [6–8]. *B. anthracis* secretes three toxin components, namely protective antigen (PA), lethal factor (LF), and edema factor (EF) [11–13]. Lethal toxin (PA-LF) is the major virulence factor causing death and cytolysis of peritoneal macrophages [14]. Edema toxin (PA-EF) prevents immune function and causes edema. Once *B. anthracis* spores enter the host, spores germinate into vegetative cells. Vegetative *B. anthracis* cells then produce PA, LF, and EF [15–17] (Fig. 1a).

PA-specific mRNA was detected within 15 min, whereas the PA protein was detected after one hour following spore germination [18]. Released PA binds the Anthrax Toxin Receptor (ATR) expressed on macrophages and endothelial cells [19–21] (Fig. 1b).

Cleaved PA forms a heptameric pore (Fig. 1c–d) and promotes entry of LF and EF into host cells [19, 22] (Fig. 1f).

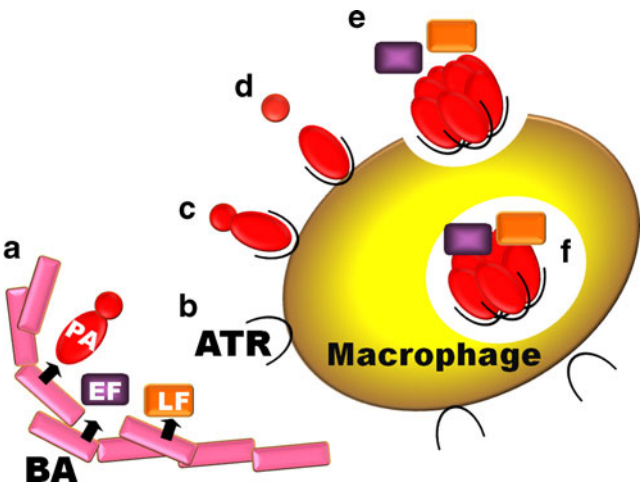

Fig. 1 *B. anthracis* pathogenesis: *B. anthracis* produces PA, EF, LF toxins (**a**); PA binds to Anthrax Toxin Receptor (ATR) (**b**, **c**); furin cleaves PA (**d**); PA forms a pore (**e**); EF/LF endocytosis (**f**) that leads to edema and death by EF/LF

Toxins simultaneously reduce macrophage capacity to kill bacteria and lower host's resistance to infection [23–25]. EF or LF toxins cause typical clinical symptoms of an anthrax infection, as well as cellular edema or cell death [1–4, 25].

An anthrax infection and its correlated toxemia may be effectively attenuated if macrophages [24] would recognize and neutralize agents and their toxins upon contact or exposure [15–17, 23]. Our group has shown that glycoconjugates (GCs) target macrophages [26–29]. GCs promote their activation and resistance to *Bacillus* spores [27–29]. In addition, we demonstrated that Galβ1-3GalNAcα-PAA-flu (GC1) and Fucα1-3GlcNAcβ-PAA-flu (GC8) exhibit binding affinity toward PA and EF toxins [30, 31].

In the present study, we have extended our research in order to determine the efficacy of GC1 and GC8 ligands in neutralizing A) single PA, LF, EF factors and B) PA-EF-LF, PA-EF (edema toxin [ET]), and PA-LF (lethal toxin [LT]) complexes or both during phagocytosis using murine macrophages. Stimulatory and neutralizing effects of GCs were studied under three conditions: A) *prior to*, B) *during*, and C) *following exposure* of macrophages to *B. anthracis* single or toxin complexes (Fig. 2).

Since GCs are stimulatory, they attract macrophages and promote their phagocytic ability of either single toxins or their complexes. GCs help to clear toxicity and subsequently attenuate an anthrax infection. Recombinant toxins were used as a model of *B. anthracis*.

Materials and methods

Materials

Recombinant PA, EF, and LF toxins were obtained from the List Biological Laboratories, Inc. (Campbell, CA, USA). Galβ1-3GalNAcα-PAA-flu (GC1) and Fucα1-3 GlcNAcβ-PAA-flu (GC8) were procured from Glyco-Tech, Inc. (Rockville, MD, USA). Griess reagent, W3500 tissue culture water, 6-/96-well 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). Phosphate buffer saline (PBS), RPMI 1640 medium, fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin solution were obtained from ATCC (Manassas, VA, 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). The CytoTox 96® and the CellTiter 96® kits were obtained from Promega, Inc., (Madison, WI, USA).

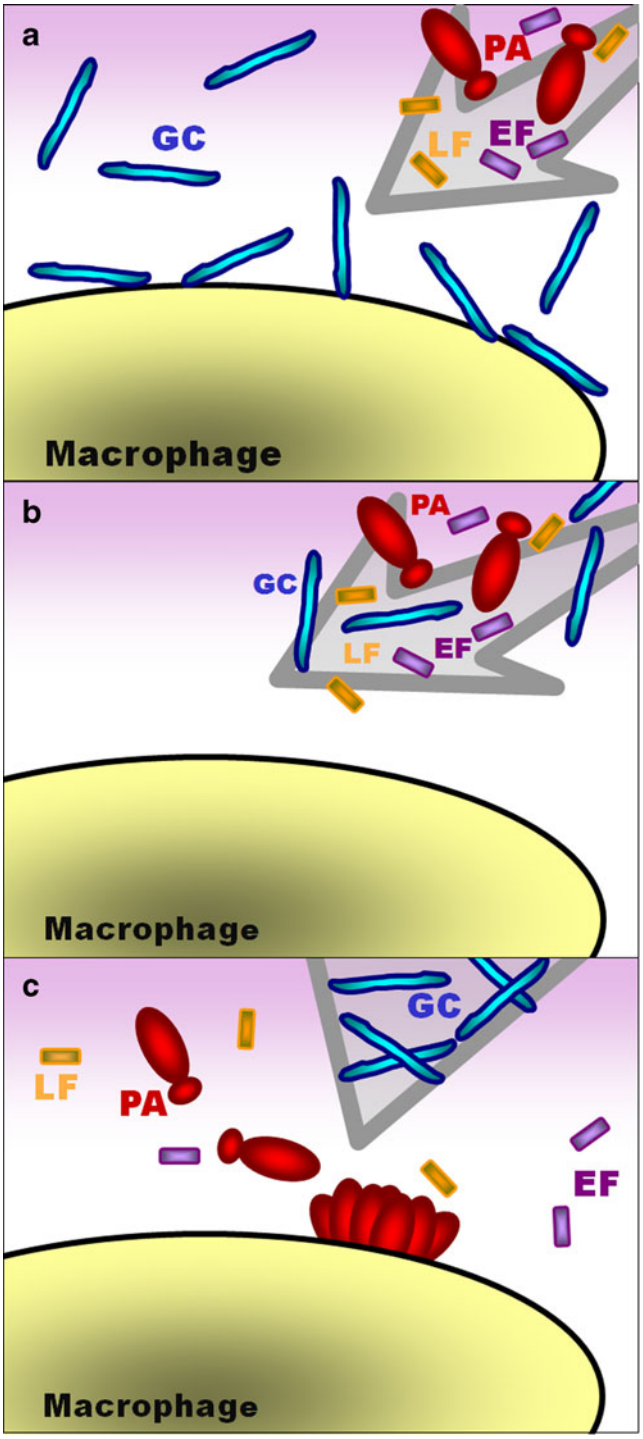

Fig. 2 Studied conditions: *prior to* (P), *during* (D), and *following* (F) *exposure* of untreated and GC-treated single toxins (PA, EF, LF) or toxin complexes (PA-EF-LF, PA-EF, PA-EF)

Cell cultures

C57BL/6 mice, 6 to 8 weeks old, were injected intraperitoneally with 1.0 mL of 3 % thioglycollate broth. Four days later, 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/culture 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) were maintained in RPMI 1640 only.

Glycoconjugates and toxins preparation

Stable GC1 and GC8 solutions were prepared according to the supplier's technical note (GlycoTech, Inc., Rockville, MD, USA). GCs (0.4 mg) were rehydrated using 400 μL of sterile 0.3 M sodium phosphate buffer (0.1 M NaH_2PO_4, 0.2 M Na_2HPO_4). 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) using sterile 0.3 M sodium phosphate buffer. Stable toxin solutions of 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* and introduced to macrophages during phagocytosis (Fig. 2).

Toxin neutralization during phagocytosis

Single toxin factors, PA (7 μL at 10 μg/mL), EF (3 μL at 10 μg/mL 3.0 μL), LF (3 μL at 10 μg/mL), or toxin complexes (PA-EF-LF, PA-EF, and PA-LF) (7 μL at 10 μg/mL, 3 μL at 10 μg/mL and 3 μL at 10 μg/mL, respectively) and GC1 or GC8 (5.0 μL at 0.01 mg/mL, 0.1 μg/mL, 1 ng/mL, and 1 pg/mL) were introduced to macrophages. Phagocytosis was performed as follows: macrophage cell cultures ($6–8\times10^5$/mL) were infected with GC-treated and untreated toxins (controls), then incubated for 20–24 h (37 °C, 95 % air/5 % CO_2). Macrophages were treated under three conditions described below (Fig. 2). In the *prior to exposure* condition (Fig. 2a), macrophages were exposed to studied GCs for 4 h prior to toxins that were added later, either as individual toxins or in combination with other toxins. Phagocytosis was then carried out at 37 °C (95 % air, 5 % CO_2) for 20 h. In the *during exposure* condition (Fig. 2b), macrophage cultures were exposed to GCs and toxins simultaneously at 37 °C (95 % air, 5 % CO_2) within 24 h. In the *following exposure* condition (Fig. 2c), macrophages were first exposed to toxins for 4 h, followed by the addition of GCs and phagocytosis at 37 °C (95 % air, 5 % CO_2) within 20 h.

Post-phagocytosis macrophage studies

Toxin-induced macrophage damage was measured by cell morphology based on trypan blue assay and lactate dehydrogenase (LDH) release. Macrophage activation was assessed using 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.

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 red color is proportional to the number of lysed cells. The optical absorbance was measured at 490 nm using a Bio-Tek Ex800 plate reader.

For the NO production, macrophages were plated at 10^6 cells/mL/culture in 6-well flat-bottom tissue culture plates, 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 [32]. 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 ANOVA. A Tukey test was performed for post-ANOVA.

Results

GC1 and GC8 demonstrated stimulatory effects on NO production by macrophages upon single toxin factors (PA, EF, and LF) (Fig. 3) and toxin complexes (PA-EF-LF, PA-EF, and PA-LF) exposure (Fig. 4). In order to assess the effects of GCs on NO production upon individual PA, EF and LF toxin exposure, studied toxins and GCs were introduced to macrophages during *ex vivo* phagocytosis under controlled conditions: i) *prior to* (P), ii) *during* (D), and iii) *following exposure* (F) (Fig. 3). When GC1 or GC8 (Fig. 3) was introduced to macrophages *prior to* the addition of individual toxins, NO production remained as low as the untreated control, *i.e.*, macrophages only, regardless of the

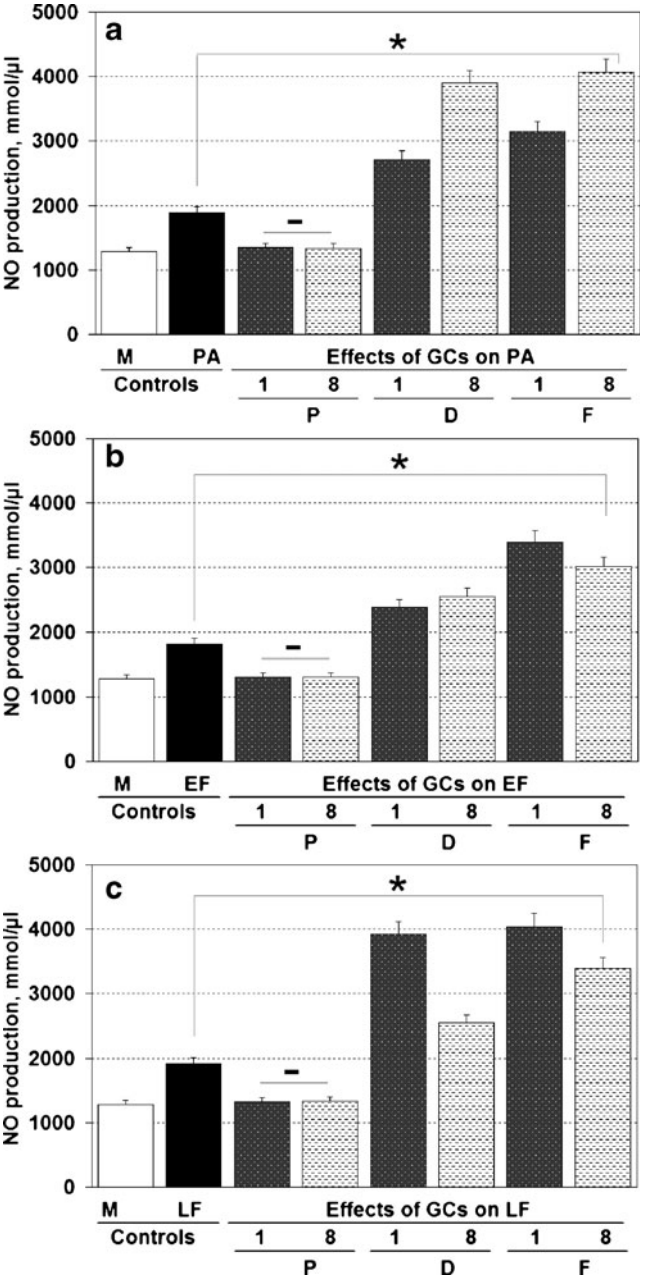

Fig. 3 Galβ1-3 GalNAcα-PAA-flu (GC1) and Fucα1-3GlcNAcβ-PAA-flu (GC8) stimulate macrophage nitric oxide (NO) production *ex vivo* during exposure to single toxins. The concentration of used GCs is expressed as pg/mL shown here and thereafter. Macrophages were treated separately with GC1 and GC8 *prior to* (P), *during* (D), and *following exposure* (F) to single toxin factors: PA (**a**), EF (**b**), and LF (**d**); *p<0.001, – non significant. After 24 h, the amount of NO produced by macrophages was measured by the Griess assay. The control consisted of macrophages (M) only and toxin factors without GCs. These results are representative of two independent experiments carried out in triplicate. The y-error bars, ranging from 1.24 % to 4.03 %, represent the standard deviations of group measurements

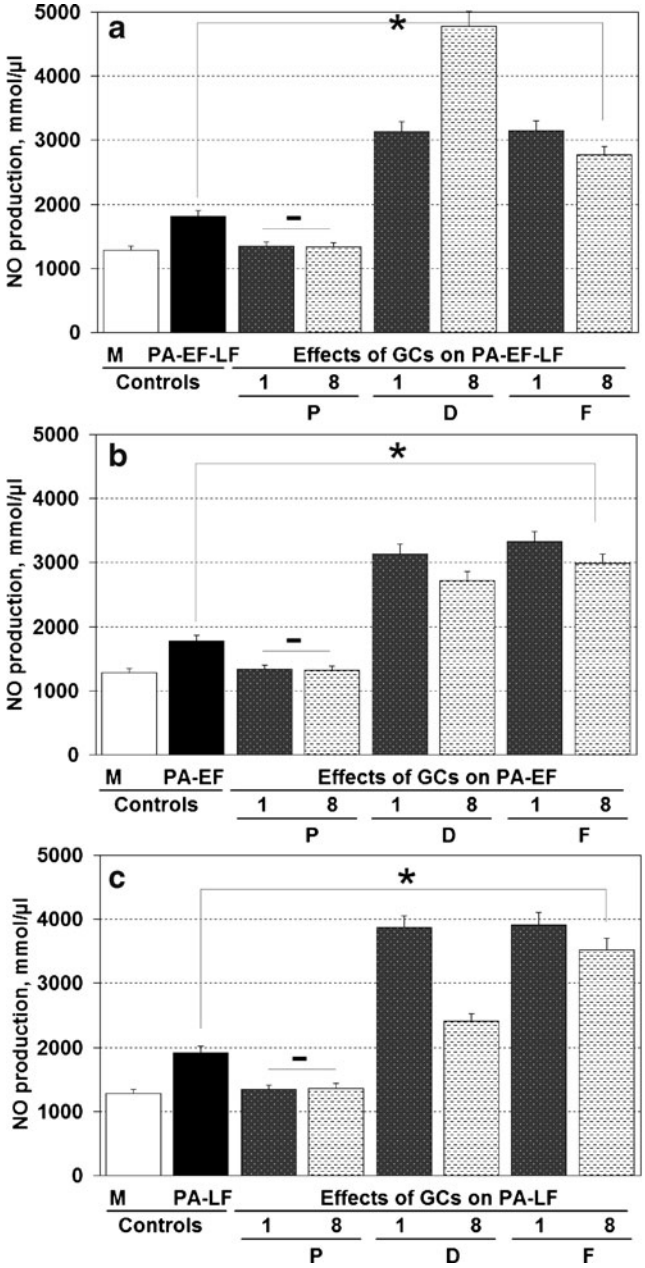

Fig. 4 GCs stimulate macrophage nitric oxide (NO) production *ex vivo* during exposure to complex toxins. Macrophages were treated separately with GC1 and GC8 *prior to* (P), *during* (D), and *following exposure* (F) of macrophages to toxin complexes PA-EF-LF (**a**), PA-EF (**b**), and PA-LF (**c**), *$p<0.001$, – non significant. After 24 h, the amount of NO produced by macrophages was measured by the Griess assay. The control consisted of macrophages (M) only and untreated toxins. These results are representative of two independent experiments carried out in triplicate. The y-error bars, ranging from 1.41 % to 3.96 %, represent the standard deviations of group measurements

GC concentration or toxins used. When introduced prophylactically, GCs protect macrophages from the deleterious effect of PA, EF, and LF toxin complexes (Fig. 3a–c).

Macrophages demonstrated a 2.5–2.7 fold increase of NO when PA (Fig. 3a) or EF (Fig. 3b) was introduced, either in the *during* or *following exposure* conditions. GC1 treatment demonstrated a 3.4 fold increased NO production by macrophages in the *during* and *followed* LF exposure condition (Fig. 3a).

On the other hand, a significantly higher NO production, namely a 3.5 fold, was noticed for GC8 when macrophages during simultaneous or followed PA exposure (Fig. 3a). NO production by GC8 was approximately same either *during* and *followed* exposure of EF (Fig. 3b) and LF (Fig. 3b).

It appears that GC8 activates macrophages against PA and EF. When GC1 was used, however, the overall NO production was slightly higher compared to GC8-treatment upon *following* EF (Fig. 3b) or LF exposure (Fig. 3c). GC1 may prove to be slightly more effective in stimulating NO production by macrophages upon LF exposure (Fig. 3c). NO secretion is regarded as an immune defense mechanism against *B. anthracis* and against one of its toxins, PA. The latter was introduced before GCs, and therefore, had ample time to react with macrophages without any competing GCs. The introduction of GCs (GC1 or GC8) consequently contributed to an increased NO production. Although added later, GCs were able to stimulate the NO production.

In this NO production experiment we used all three (Fig. 4a) or two toxin factors (Fig. 4b, c) together, as they appear *in vivo* in an actual anthrax infection. Although this experiment was performed *ex vivo*, PA-EF-LF, PA-EF, PA-LF allow the fatal endocytosis of EF and LF to occur through the circular heptameric pore consisting of seven cell-binding proteins, PA [11–13]. When GC1 or GC8 are introduced to macrophages *prior to* the addition of the PA-EF-LF toxin complex (Fig. 4a), NO production remained as low as the control, regardless of the GC concentration considered. Macrophage NO production was neither increased nor decreased when GCs (GC1 or GC8) were present *prior to* the addition of the PA-EF-LF toxin complex (Fig. 4a). This encouraging observation corroborates the assumption that GCs can protect macrophages from toxin binding and endocytosis of EF and LF into their own cytosol. There EF and LF then carry out their respective damage-inducing processes. When introduced prophylactically, GCs appear to protect macrophages from the deleterious effect of the PA-EF-LF (Fig. 4a), PA-EF (Fig. 4b), and PA-LF (Fig. 4c) toxin complexes. Although the following speculation needs to be verified, it appears that GCs block furin or ATR, making it extremely difficult for PA to form heptamer pores.

GC1 treatment in the *during* or *following* exposure of PA-EF-LF (Fig. 4b) and/or PA-LF (Fig. 4c) toxin complexes showed approximately the same NO production by activated macrophages, except PA-LF toxin and GC1 treatment (Fig. 4c). When GCs were used to counter the effect of the PA-EF-LF toxin complex, compared to GC1, GC8 yielded a higher NO production (Fig. 4a). When GC8 was used

(Fig. 4d, f), however, the overall NO production was slightly lower compared to GC1 *during* exposure to either PA-EF (Fig. 4b) or PA-LF (Fig. 4c). GC1 may prove to be slightly more effective in protecting macrophages from the exposed PA-EF or PA-LF toxin complexes (Fig. 4c, e).

Our macrophage viability (Fig. 5) study has confirmed the previously reported data that the damaging effects of either EF or LF single toxins will occur when they are combined with PA toxins [13]. Single toxins could not cause a strong effect on cells unless they will act in the complex [13].

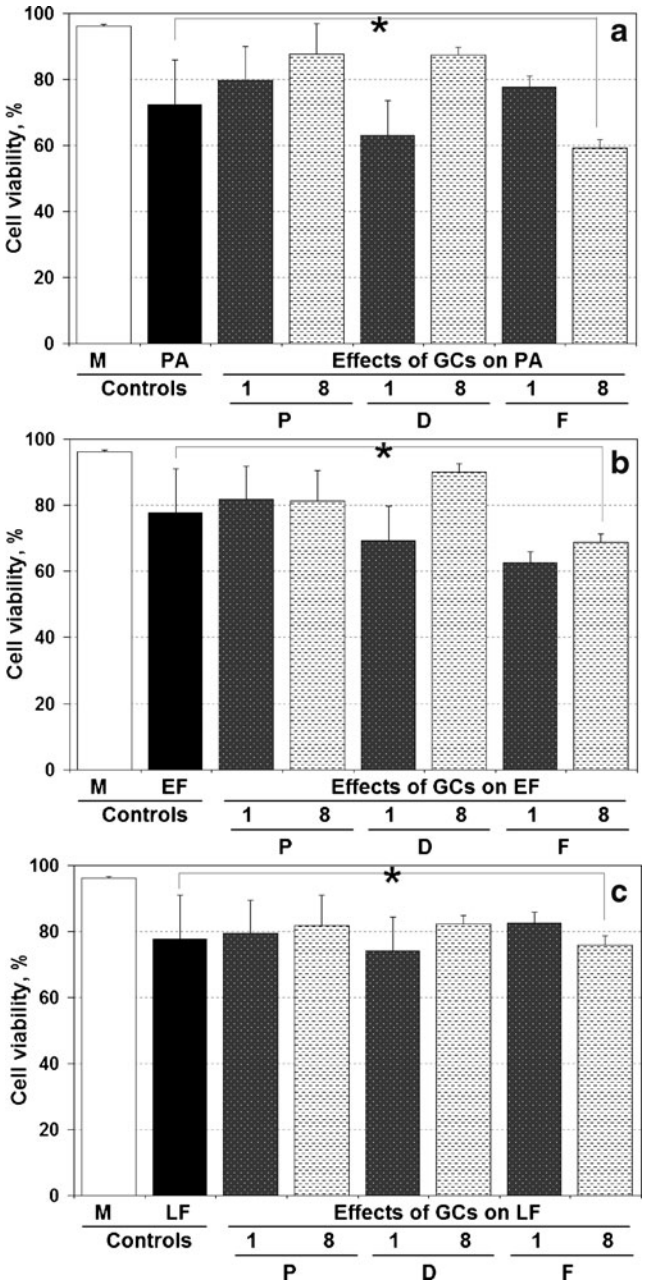

Fig. 5 Effects of GC1 and GC8 on macrophage cell viability upon single PA (**a**), EF (**b**), and LF (**c**) toxin exposure, *$p<0.001$. Macrophage (M) cultures were exposed to untreated toxins (PA, EF, and LF) *vs.* GC-treated (1 and 8) toxins within 24 h, then underwent the Trypan Blue assay. *Prior to* (P), *during* (D), and *following exposure* (F) conditions are shown. The y-error bars, ranging from 0.80 % to 3.98 %, represent the standard deviations of group measurements

Untreated macrophages (control) demonstrated 96 % cell viability (Fig. 5). Exposure to untreated controls including PA (Fig. 5a), EF (Fig. 5b), and LF (Fig. 5c) showed a cell viability of 72, 78, and 78 %, respectively.

The GC1-treatment led to an 80 % macrophage cell viability during PA, LF, or EF exposure (Fig. 5a). The effect of GC1 on PA, LF, or EF in the *during* and *following exposure* conditions yielded 62 % or 79 % cell viabilities (Fig. 5a). The GC8 treatment appeared more effective in protecting macrophages from PA as evidenced by a higher cell viability (Fig. 5a). This was particularly true in the *prior to* and *during* exposure of PA, EF, and LF where viabilities were on average as high as 80–85 %, respectively. The protective effect of GC8, however, failed in the *following exposure* of PA, EF, and LF where the cell viability dropped to a low of approximately 61 or 75 % (Fig. 5a–c).

A loss of macrophage viability was observed upon exposure to untreated PA-EF-LF toxin complexes (or 47 %), PA-EF (or 50 %), PA-LF (or 65 %), compared to macrophages only (97 %) (Fig. 6).

GC1 treatment increased cell viabilities upon exposure of PA-EF-LF (Fig. 6a) and PA-EF (Fig. 6b) up to 75 % in the *prior to exposure* condition. Exposure of PA-EF and a consecutive treatment by GC1 yields a macrophage viability as low as 61 % (Fig. 6c). GC1 deploys its full potential in the *during exposure* of PA-EF-LF (Fig. 6a), PA-EF (Fig. 6b) and PA-LF (Fig. 6c), where an overall cell viability of over 85–92 % is achieved. It is concluded that GC1 treatment is efficacious in counteracting both PA-EF and PA-LF toxin complexes.

GC8 treatment proved most effective when used selectively with the PA-EF-LF (Fig. 6a) and the PA-EF (Fig. 6b), although slight fluctuations in macrophage cell viability (80–91 %) were observed under the *prior to, during*, and *following* exposure conditions. Overall, GC8 promoted 80–91 % cell viability against PA-EF-LF, PA-EF, or PA-LF exposures under either the *prior to* or *during exposure* conditions. GC8 has the potential to neutralize PA-EF-LF and PA-EF toxin complexes, yielding in a higher cell viability (Fig. 6a, b). Incidentally, PA-EF-LF and PA-EF toxin complexes are a realistic occurrence. The GC8 treatment demonstrated 81 %, 62 %, and 70 % macrophage viabilities under the *prior to*, *during*, or *followed* of PA-LF exposures conditions (Fig. 6c).

In conclusion, the protective effect of either GC1 or GC8, were observed under the *prior to* and *during exposure* conditions of PA-EF-LF (Fig. 6a), PA-EF, and PA-LF as shown in Fig. 6.

To further characterize the protective effect of GCs, LDH levels by macrophages exposed to toxins were examined.

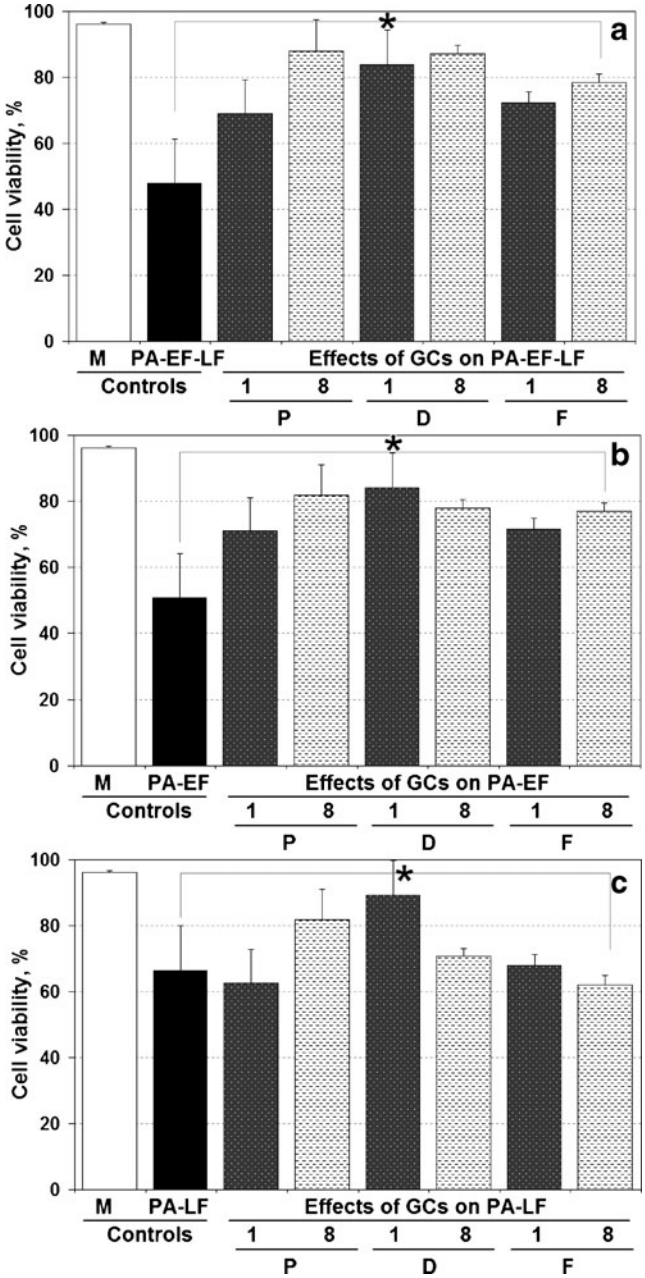

Fig. 6 GC1 and GC8 stimulate macrophage cell viability during complex PA-EF-LF (**a**), PA-EF (**b**), and PA-LF (**c**) toxins exposure, * $p<0.001$. Macrophage (M) cultures were exposed to untreated toxins (PA-EF-LF, PA-EF, and PA-LF) and GC-treated (1 and 8) toxins within 24 h, then underwent the Trypan Blue assay. *Prior to* (P), *during* (D), and *following exposure* (F) conditions are shown. The y-error bars, ranging from 1.76 % to 3.69 %, represent the standard deviations of group measurements

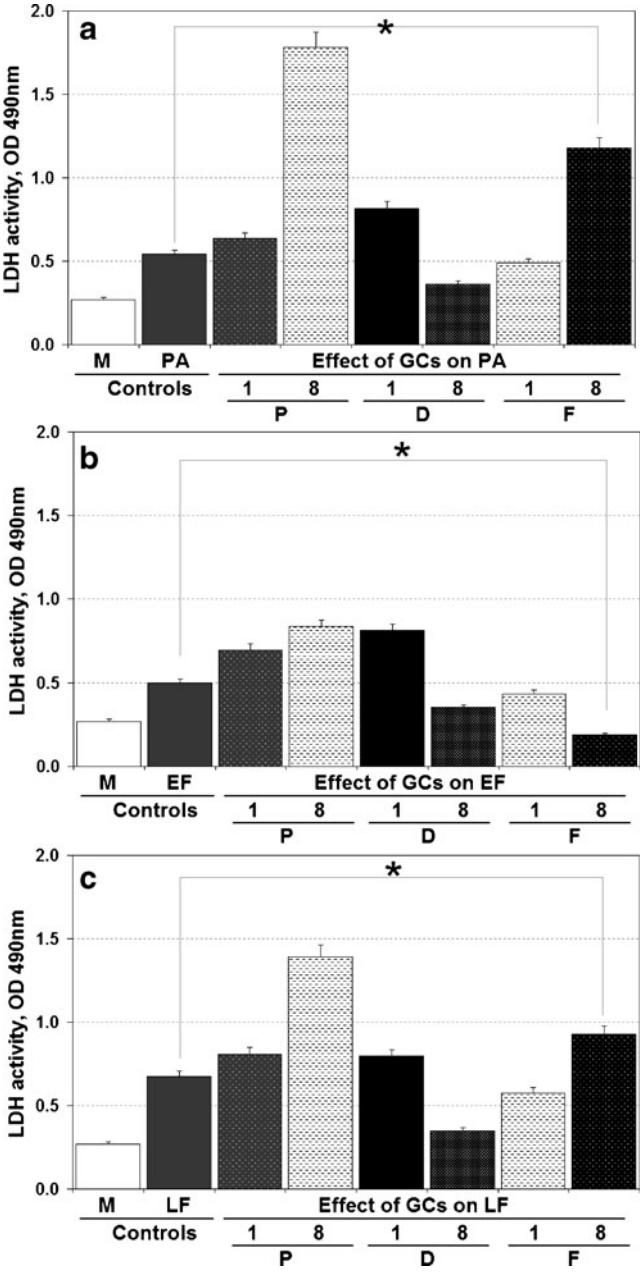

Fig. 7 LDH activity *prior to* (P), *during* (D) and f*ollowing* (F) untreated and GC-treated single PA (**a**), EF (**b**), and LF (**c**) toxins, * $p<0.01$. Macrophage (M) cultures were exposed to untreated as well as GC1- (1) and GC8- (8) treated toxins. After 24 h, LDH was assayed in macrophages (M) only and M exposed to untreated and GC-treated single toxins. These results are representative of experiments carried using out in triplicate. The y-error bars, ranging from 1.48 % to 3.99 %, represent the standard deviations of group measurements

LDH release was determined 1 day after macrophages were exposed to untreated as well as GC-treated toxins. These toxins were either single (Fig. 7) or complexes (Fig. 8).

In the *prior to exposure* condition, when GC8 was added to macrophages before the addition of PA (Fig. 7a), LDH levels soared a 7-fold compared to macrophages only. A higher LDH release was observed during the GC8 treatment during either PA or LF toxin exposure (Fig. 7). This may be due to the fact that PA binds to ATR, forms heptameric pores, promotes the entry of LF and the subsequent leakage of cell content [19, 22]. This will consequently increase the LDH level *ex vivo* because macrophages are coping with

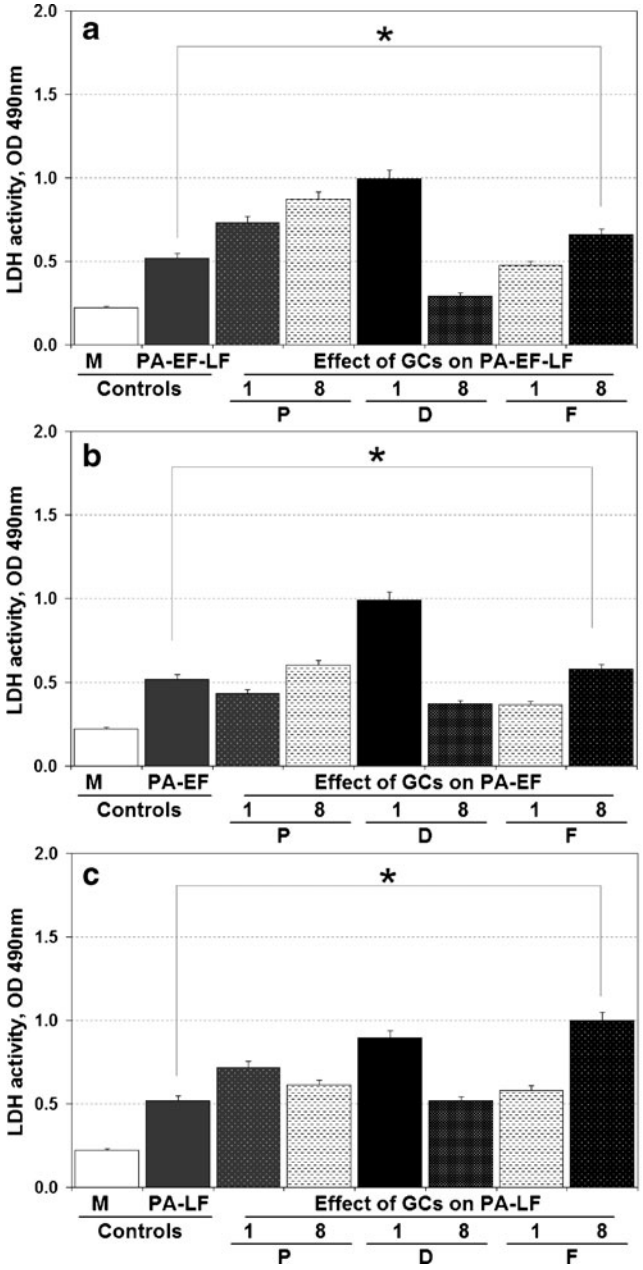

Fig. 8 LDH activity *prior to* (P), *during* (D) and *following exposure* (F) of untreated and GC-treated complex PA-EF-LF (B), PA-EF (D), and PA-LF (F) toxins, $*p<0.01$. Macrophage (M) cultures were exposed to untreated as well as GC1- (1) and GC8- (8) treated toxins. After 24 h, LDH was assayed in macrophages (M) only and M exposed to untreated and GC-treated complex toxins. These results are representative of experiments carried out in triplicate. The y-error bars, ranging from 0.93 % to 4.00 %, represent the standard deviations of group measurements

toxins and resisting toxigenic effects of toxins. Resistancy of macrophages to toxins demonstrated by increased macrophage viability (Fig. 6) and presumably the processing an antigen by activated macrophages. In return, activated macrophages will increase NO production that may lead to higher LDH. Knockout mice lacking either NO or LDH or *B. anthracis* Sterne toxingenic strain will allow us to verify the involved mechanism.

GC1 allowed less cell damage as it only moderately elevated LDH levels by a mere 2.2 fold. Similarly, GC8 was more protective under the *following exposure* condition, where LDH levels were 4.8 fold higher, compared to only a 2-fold higher level when GC1 was used (Fig. 7a). In an attempt to counter the effect of PA, GC1 proved more effective than GC8 in the *prior to* and *following* exposure conditions. When GC8 and PA were administered simultaneously (under the *during exposure* condition), GC8 yielded a noticeable low level of LDH (1.4 fold). The low LDH level may be attributable to GC8-PA interaction that could have occurred prior to being added simultaneously to macrophages.

When examining the effect of GCs on EF (Fig. 7b), the very low LDH levels (0.8 fold) were recorded when GC8 was added to macrophages under the *following exposure* condition. GC8 may be most suitable in protecting macrophages after they have been exposed to EF. GC1 was similarly effective yielding a LDH level of 1.8 fold. Administration of GC8 under the *prior to exposure* condition, and GC1 under the *during exposure* condition would yield to a high degree of cell breakdown as evidenced by 3.4 and 3.2 fold high LDH levels, respectively.

As for the single toxin PA (Fig. 7a), LDH levels followed a similar tendency as for LF. Under the *prior to exposure* condition, when LF was administered to macrophages after the addition of GC8 (Fig. 7c), LDH levels rose a 5.6 fold high (compared to that of macrophages), indicative of significant cell breakdown. GC1 allowed less cell breakdown as it only moderately elevated LDH levels a mere 3 fold. Similarly, GC8 scored higher under the following exposure condition, where LDH levels were 3.6 fold higher, compared to only a 2.4 fold higher when using GC1. In an attempt to counter the effect of EF, GC1 proves more appropriate than GC8 under the *prior to* and *following exposure* conditions. When GC8 and LF were administered simultaneously (under the *during exposure* condition), GC8 yielded a noticeable low level of LDH (1.2 fold). The low cell breakdown can be ascribed to GC8-LF interaction that could have occurred prior to being added simultaneously to macrophages.

LDH release remained fairly low (a 2.8 fold) upon exposure to a combination of all three toxins (PA-EF-LF) (Fig. 8a) after administering GC1. When administered simultaneously (under the *during exposure* condition), GC8 yielded lower LDH levels (a 1.2 fold). Finally, when used after the toxin factors, GC1 yielded lower LDH levels (a 1.96 fold), making it the better GC to be used after a confirmed anthrax infection. It would be simply unrealistic that GC8 is capable of vanquishing an anthrax infection as it happens. Practically, GC1 would, therefore, be the counter

measure that can either be used in a prophylactic (or preventative) manner (*i.e.*, when an anthrax infection is imminent or expected), or after an anthrax infection has occurred.

As for the PA-EF toxin combination (Fig. 8b), overall LDH levels, albeit a few fluctuations, were remarkably similar (Fig. 8b).

As for the PA-LF toxin (Fig. 8c), overall LDH levels, although slightly lower in intensity, were remarkably similar in pattern, as it is the case for the single toxin LF (Fig. 7c). The only exception is when GC8 was used under the *prior to exposure* condition. When the PA-LF toxin combination was administered to macrophages after the addition of GC8 (Fig. 8c), LDH levels did not rise as high (2.5 fold) as they did when LF was used alone (5.6 fold).

Discussion

It is important to intercept PA, EF, and LF toxins upon their release by *B. anthracis* (Fig. 1). PA acts as a Trojan horse, a pore or channel comprised of seven (7) PA monomers, which allows delivery of EF and LF. Toxin complexes migrate through the host cell membrane into the cytosol, where they may then catalyze reactions that disrupt normal cellular physiology. Neutralizing PA in a timely manner will subsequently prevent binding to ATR (Fig. 9) and further heptamerization, thus preventing the pore to form (Fig. 9). With no pores present, EF, or LF would have no means of being channeled into host cells (Fig. 9).

In the event PA heptamers are formed on the host cell membrane, it is then crucial—as an alternative—to prevent EF and LF from entering the pores into the cytosol where they can carry out their damage-inducing process leading to cell lysis. Blocking EF and LF from entering the cell can be achieved through steric hindrance, by having GCs bind to either EF or LF, rendering them too cumbersome for endocytosis (Fig. 9).

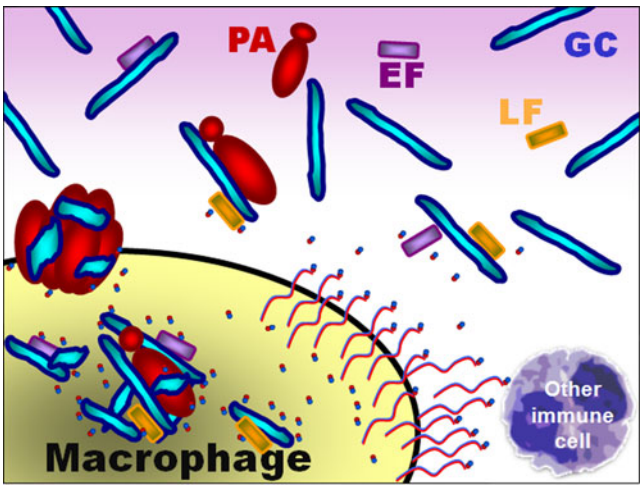

Fig. 9 Role of GCs on neutralization of either single or toxin complexes

Neutralizing single PA, EF, or LF (Fig. 9) may prevent toxemia caused by*B. anthracis* toxins [23–25]. Blockage of either PA-EF or PA-LF will prevent symptoms of an anthrax infection, in particular edema and cell death [23–25]. The GCs that most effectively bind to PA and EF are GC1 and GC8 [30, 31]. GCs can selectively target foreign agents and counteract them through binding [25–30]. It appears that GCs protect macrophages from the deleterious effects of LT (or PA-LF) and ET (or PA-EF) as evidenced by the higher macrophage viability, NO secretion and moderate LDH release. It was shown that NO has multiple important physiologic and stimulatory functions and participates in antimicrobial defense during the exposure of infectious agents and/or inflammation [32–38]. It is speculated that GCs either bind to macrophages, to furin or to ATR found on macrophage cell surface, making it difficult for PA to form heptameric pores, membrane channels for EF and LF (Figs. 1d–f, 9).

Based on an increased NO production (Figs. 3, 4) and macrophage viability (Fig. 6), it appears that macrophages exhibit an obvious defense in presence of toxins. As a result of this "exposure", highly reactive NO is secreted by macrophages as an immune response in elevated amounts. Macrophages offer an increased level of resistance towards intruding PA or toxin complexes as demonstrated by an increased NO production by macrophages (Figs. 3, 4).

Since single toxin (PA) or toxin complexes and GCs (GC1or GC8) were simultaneously introduced to macrophages, it is thought that GCs and PA toxin were competing for the same receptors found on macrophage cell membranes. By being introduced simultaneously with PA to macrophages, GCs (GC1 or GC8) may not have had sufficient time to bind to receptors found on macrophage cell membranes, thus offering protection from PA. This explains the higher NO production (Figs. 3, 4). Higher macrophage viability (Fig. 6) was consistent with NO production.

The ingestions and an antigen processing (bacteria, virus, and toxins) by macrophages [33] may further yield higher level of LDH release as evidenced by our results using either single toxins (Fig. 7) or complex toxin (Fig. 8). LDH level will decrease upon antigen processing due to blood flow increase, cells recruitment, gas exchange, perspiration, activity of liver and spleen, and blood filtration by kidneys *in vivo*. Regarding the LDH metabolism, no conclusions can be drawn since the experiments were carried out *ex vivo* using tissue culture plates. NO is a cardinal signal of macrophage activation that lead to increase activity of macrophages [32–38], processing and neutralization toxins [14–16], and consequently may lead to increase LDH *ex vivo*. It is possible that NO increased LDH toxicity [39]. It was shown that low levels of NO inhibit apoptosis of B

cells, albeit the mechanisms involved have not been elucidated [40, 41] (27, 28). By contrast, high levels of NO induce apoptosis of macrophages [42]. It was earlier proposed that simultaneous generation of NO and reactive oxygen species cause a formation of peroxynitrite, which initiates the cellular damage [43].

Prospective *in vivo* studies using a *B. anthracis* Sterne toxingenic strain will ultimately verify the involvement and LHD/NO impact on macrophages and other tissues/systems within organism upon exposure and neutralization of toxins and consecutive GC-treatment.

Alteration of receptors on macrophages or other cells by GCs will impair toxin binding. GCs bound to toxins might also act as opsonins, promoting their endocytosis and neutralization during phagocytosis achieved by macrophages [33]. Phagocytosis plays an important role in a variety of cell functions ranging from nutrition in ameba to innate, adaptive immunity, tissue repair, morphogenetic remodeling, and homeostasis in mammals [44].

GCs are prone to interrupt toxemia by blocking either individual toxin factors or toxin complexes (Fig. 9). Blocking of either single toxins or toxin complexes was correlated with low NO production as demonstrated by the *prior* to exposure conditions (Figs. 3, 4). The defense level of macrophages offered in presence of GCs remains evidently unchanged upon exposure of individual toxins (Fig. 3) or toxin complexes (Fig. 4). Since no threat is perceived by macrophages, the latter do not need to secrete any more NO compared to that of the background NO level [32–38].

Carbohydrates located on either macrophages or toxins serve as a potential multivalent receptor [45–47]. Such carbohydrates and carbohydrate-based structures are recognized by other carbohydrate moieties found on GCs [26–29]. It was shown that specific carbohydrate structures expressed on each cell type are believed to be recognized by complementary molecule(s) expressed on the external surface of counteracting cells [48–51]. It was further demonstrated that complex carbohydrates are directly involved in the recognition processes, including adhesion between cells, adhesion of cells to extracellular matrices, and specific recognition of cells by one another [26, 45–47, 50, 52]. Recognition and inhibition of toxin complexes is based on binding affinity between disaccharide GCs acting as ligands [30, 31].

NO is one of activation responses in macrophage defense during the exposure of infectious agents and/or inflammation [32–38]. Activated macrophages are prone to adhere to GC-altered toxins, leading to increased resistance and higher macrophage viability (Fig. 6). Individual GCs may exhibit different neutralizing or stimulatory properties. This is reflected in the differences observed in NO production induced by macrophages, and macrophage viability.

Presumably, binding of GCs leads to changes of toxins that play an essential role in *B. anthracis* pathogenesis, either in ATR receptor binding, in pore formation or both [3, 11–13] (Fig. 9). Even after being bound to toxins, GCs attract macrophages or other phagocytic cells during phagocytosis of foreign agents [33, 44]. GCs further stimulated the neutralization of foreign agents by means of activated macrophages. All together, GCs promote the neutralizing endocytosis of toxins and trigger a higher NO production (Figs. 3, 4). As a result, macrophage viability rises (Fig. 6).

Significance of the current work demonstrated that macrophages are more prone to adhere to GC-altered PA-EF-LF, PA-EF, and PA-LF toxin complexes. This adhesion results in a higher phagocytosis rate and neutralization of toxin complexes during phagocytosis. In addition, GCs enhance macrophage viability, activate macrophages, and stimulate NO production. When administered prophylactically, GCs manage to protect macrophages from the deleterious effect of single toxins (PA, EF, or LF) and toxin complexes (PA-EF-LF, PA-EF, or PA-LF) under the *prior to exposure* condition. Studies using *B. anthracis* Sterne toxin-producing strain will allow us to study the protective and stimulatory GCs effects in *ex vivo* and *in vivo*.

The present study may be helpful in identifying GC ligands with antitoxic, neutralizing, and/or immunomodulating properties. Our study suggests that GCs may be useful for development of prospective vaccines and immunomodulators to combat *B. anthracis* infection.

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