Glycoconj J (2010) 27:13–25
DOI 10.1007/s10719-009-9248-7

Killing of *Bacillus* spores is mediated by nitric oxide and nitric oxide synthase during glycoconjugate–enhanced phagocytosis

Olga Tarasenko · Ashley Scott · Lee Soderberg · Usha Ponnappan · Pierre Alusta

Received: 6 March 2009 / Revised: 6 March 2009 / Accepted: 26 May 2009 / Published online: 23 June 2009

Abstract Nitric oxide (NO) is a signaling and defense molecule of major importance. NO endows macrophages with bactericidal, cytostatic as well as cytotoxic activity against various pathogens. *Bacillus* spores can produce serious diseases, which might be attenuated if macrophages were able to kill the spores on contact. Present research was carried out to study whether glycoconjugates stimulated NO and nitric oxide synthase (NOS2) production during phagocytosis killing of *Bacillus* spores. Murine macrophages exposed to glycoconjugate-treated spores induced NOS2 and NO production that was correlated with high viability of macrophages and killing rate of bacterial spores. Increased levels of inducible NOS2 and NO production by macrophages in presence of glycoconjugates suggested that the latter provide an activation signal directed to macrophages. Glycoconjugates were shown to exert a protective influence, sparing macrophages from spore-induced cell death. In presence of glycoconjugates, macrophages efficiently kill the organisms. Without glycoconjugate activation, murine macrophages were ineffective at killing *Bacillus* spores. These results suggest that glycoconjugates promote killing of *Bacillus* spores by blocking spore-induced macrophage cell death, while increasing their activation level and NO and NOS2 production. Glycoconjugates suggest novel antimicrobial approaches to prevention and treatment of infection caused by bacterial spores.

Keywords Nitric oxide · Nitric oxide synthase · Macrophages · *B. cereus* spores · Glycoconjugate · Phagocytosis

Abbreviations

Glyc-PAA-flu	Glycoconjugate-polyacrylamide-fluorescein polymer
GC1	Galα1-3 GalNAcα -PAA-flu glycoconjugate
GC3	GalNAcα1-3 GalNAcβ-PAA-flu glycoconjugate
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
PAA	Polyacrylamide
Flu	Fluorescein
LDH	Lactate dehydrogenase
NO	Nitric oxide
NOS2	Nitric oxide synthase
CFU	Colony forming units
MP	Macrophages only
OD	Optical density

Introduction

Nitric oxide (NO) is a signaling and defense molecule of major importance. NO is involved in many physiological processes in eukaryotic biology and multicellular organisms, apoptosis, differentiation, and cell proliferation [1, 2].

O. Tarasenko (✉) · A. Scott
Department of Biology, University of Arkansas at Little Rock,
2801 South University Ave.,
Little Rock, AR 72204, USA
e-mail: omtarasenko@ualr.edu

L. Soderberg · U. Ponnappan
Department of Microbiology and Immunology,
University of Arkansas for Medical Sciences,
Little Rock, AR, USA

P. Alusta
Bioinformatics Program, University of Arkansas at Little Rock,
University of Arkansas for Medical Sciences,
Little Rock, AR, USA

In addition, NO is a central component of innate immunity and an effective antimicrobial agent [2].

NO endows macrophages with bactericidal, cytostatic as well as cytotoxic activity [3–6] in host defense against a variety of viruses, bacteria, fungi, protozoa, helminthes [2, 7–14], and tumor cells [15–17]. The underlying NO mechanism of action, remains still under debate [14]. NO is lipophilic, freely diffusible radical [6, 8, 18]. It was shown that NO inhibits enzymes, damages DNA, initiates lipid peroxidation, and exacerbates peroxide–induced damage [19, 20].

Bacillus genus species cause food spoilage, and food-borne illness problems [21]. Additionally, *Bacillus anthracis* spp. are considered potential biological warfare and bio-terrorism agents [22–24]. *B. anthracis* has been placed within the *Bacillus cereus* group due to genetic similarities [25–27]. About 426 *B. anthracis* strains have been isolated worldwide [28]. Non-virulent *B. anthracis* cannot be unambiguously discerned from other *B. cereus* strains [28].

Bacillus spp. formed spores under nutrient-poor conditions [21, 22]. Spores are highly resistant to heat, dryness, radiation, and antiseptic treatments [21–28]. Spores typically exhibit exosporium, coat, inner coat, cortex, inner membrane, and a core [21–23]. *Bacillus* spores enter the body through the skin, lungs, or the gastrointestinal tract and are engulfed by macrophages [22–24, 29]. Once inside the macrophage, survival of a bacterium depends upon several mechanisms [30, 31]. Evidence suggests, *B. anthracis* survive through capsule formation and protective antigen (PA), edema factor (EF), and lethal factor (LF) toxins release that diminish the antimicrobial response [32], macrophage capacity to kill the bacteria [33–35] and reduce host resistance to infection [36]. Cellular internalization of EF and LF causes the clinical symptoms of an anthrax infection and lead to death [24, 32, 33].

Antimicrobial responses of immune cells to intracellular pathogens include but are not restricted to (i) production of superoxide (O_2^-) by NADPH oxidase and subsequent formation of hydrogen peroxide (H_2O_2) by self-dismutation of O_2^- ; (ii) generation of nitric oxide (NO), O_2^-, peroxynitrite ($ONOO^-$), and H_2O_2 by inducible nitric oxide synthase (NOS2) [37]; (iii) activation of cationic proteins [38]; and (iv) syntheses of defensins [39]. An anthrax infection might be attenuated if macrophages were able to kill the spores on contact.

Antibodies of all anthrax vaccines [40] recognize PA and block the binding and internalization of EF and LF [41, 42]. The drugs for anthrax treatment remain penicillin, ciprofloxacin, and doxycycline [24]. However, antibiotic resistance due to a high bacterial mutation rate has potential life–threatening consequences [43]. There is a need for new vaccines, immunomodulators, and drugs that decrease morbidity and mortality associated with infections caused by spores and prevent drug-resistancy development.

Bacterial and mammalian cells are decorated by complex glycoconjugate structures, including glycoproteins, glycolipids, glycosaminoglycans, and proteoglycans [44, 45]. Glycoconjugate structures are involved in cell-cell and cell-receptor/s recognition, signaling, differentiation, antigenic determinants presentations [46], and receptor activation [47–49]. Due to specific physicochemical char-

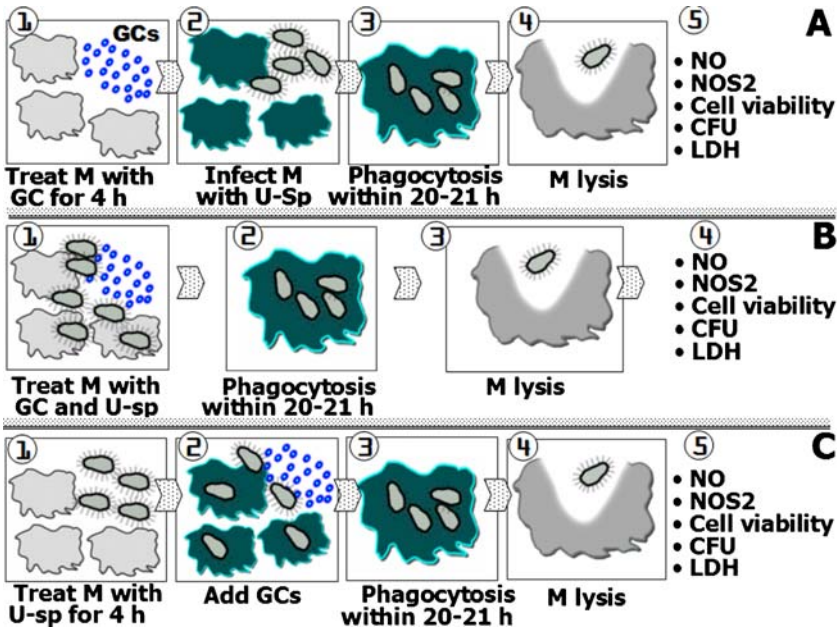

Fig. 1 Effects of glycoconjugates were studied under three conditions: A) "*prior to contact*", B) *"during contact"*, and C) *"following contact"* of macrophages with *B. cereus* spores (here and hereafter). Note: "*M*" stands for macrophage; "*GC*" for glycoconjugate; "*U-sp*" for untreated spores; "*GC-T sp*" for glycoconjugate-treated spores (here and hereafter)

acteristics, a long shelf–life, and low toxicity [50–52], glycoconjugates harbor a potential for biomedical and pharmaceutical applications [50–53]. Macrophages are considered ideal glycoconjugate targets, since they play a central role in inflammation and innate immunity to microorganisms involved with infectious diseases [22–24, 29–31, 33, 36].

We previously reported that glycoconjugates contribute to the binding and recognition [54], inhibition and killing of *Bacillus* spores [55–57]. In addition, we determined that glycoconjugates provide both defensive and simultaneous actions on spores [58].

The effects of glycoconjugates on NO and NOS2 production that contribute to antimicrobial response by macrophage and killing of *Bacillus* spores during phagocytosis were subject of this investigation. Effects of glycoconjugates were studied under three conditions, namely a) *prior to*, b) *during*, and c) *following* contact

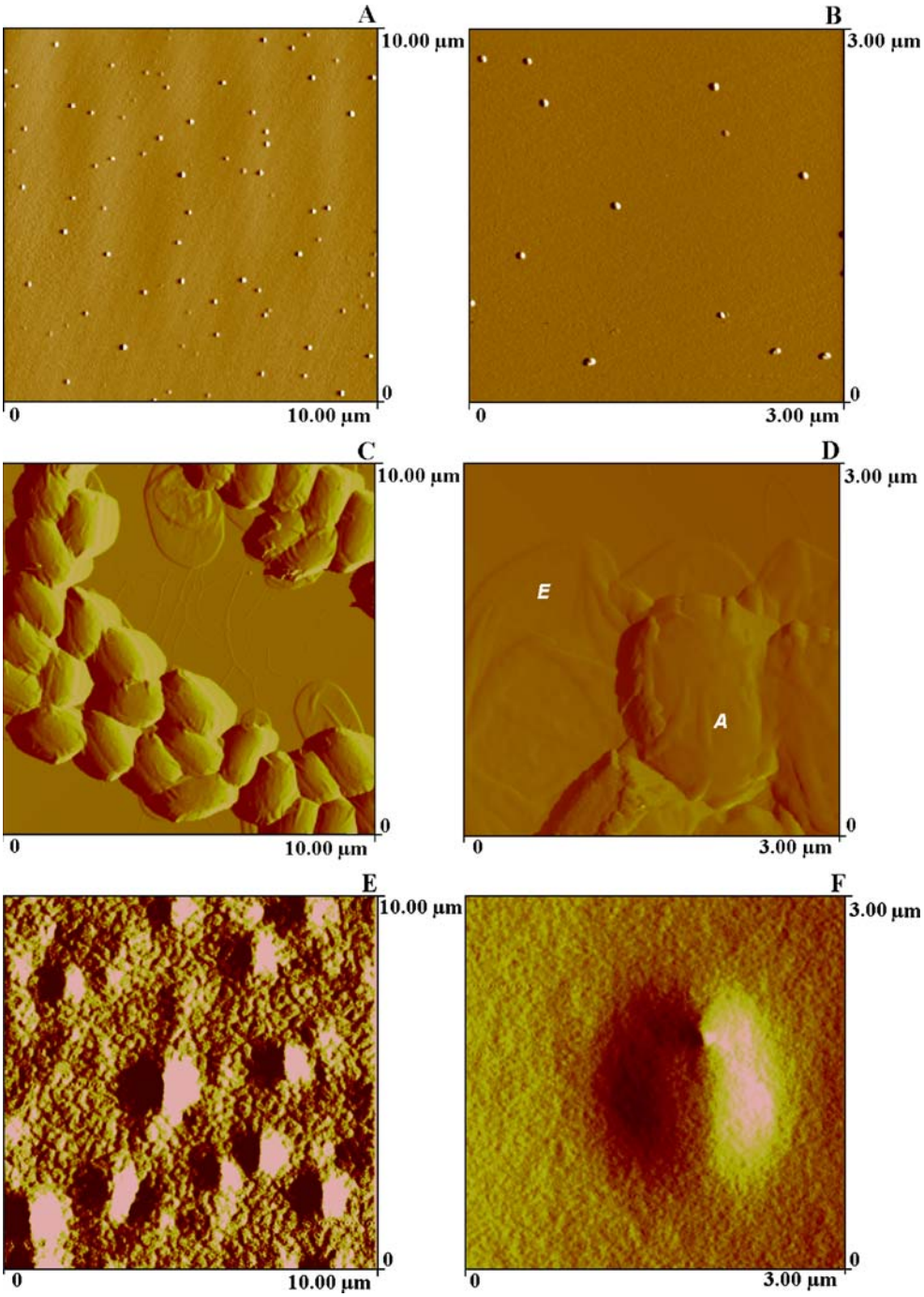

Fig. 2 AFM images of 1,000× diluted glycoconjugates (A, B), untreated (C, D) and glycoconjugate–treated (E, F) spores at both low (A, C, E) and high resolutions (B, D, F). Amplitude images are shown. White arrow *A* stands for appendages, *E* for exosporium. Amplitude images are shown (A–F)

macrophages with spores (Fig. 1). *B. cereus* was selected as a model for *B. anthracis* spores due to the close genetic relationship between both organisms [25–28].

Materials and methods

Materials

Fluoresceinated disaccharide glycoconjugates Galα1-3GalNAcα-PAA-flu (GC1) and GalNAcα1-3GalNAcβ-PAA-flu (GC3) were obtained from GlycoTech, Inc. (Rockville, MD). *B. cereus* ATCC 11778 was purchased from Raven Biological Laboratories Inc. (Omaha, NE). Trypan blue was obtained from Sigma Chemical Co. (St. Louis, MO). The CytoTox 96® kit was obtained from Promega, Inc. (Madison, WI). C57BL/6 mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained in an AALAC–approved vivarium at the University of Arkansas for Medical Sciences (Little Rock, AR). Anti-iNOS antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Gruz, CA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was purchased from Pierce Chemical Company (Rockford, IL).

Macrophage cell culture

C57BL/6 mice were intraperitoneally injected with 1.0 ml of 3% thioglycollate broth. Four days after injection, the mice were euthanized and peritoneal exudate cells were collected by lavage with RPMI 1640. Macrophages were plated in 6-well plates at 1.0–1.2×10^6/culture in RPMI 1640 containing 10% fetal calf serum, 50 nM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. In order to adhere, macrophage cultures were incubated at 37°C (95% air, 5% CO_2) for 1 h. Nonadherent cells were removed by washing. The adherent cells were maintained in RPMI 1640 only but without serum or antibiotics. Following cell washing, the macrophage concentration was of $6.0–8.0\times10^5$ macrophages per culture.

Phagocytosis studies

The effects of the glycoconjugates were studied under three conditions a) *prior to*, b) *during*, and c) *following* macrophage contact with *B. cereus* spores (Fig. 1).

For the "*prior to contact*" condition (Fig. 1A), macrophage cultures were treated with 5.0 μl of serially diluted glycoconjugates ($1–10^{-12}$ mg/ml) for 4 h. The cultures were then infected with *B. cereus* spores (2.4×10^6 CFU) at a ratio of 3–4 spores/macrophage and incubated at 37°C (95% air, 5% CO_2) for 22–24 h.

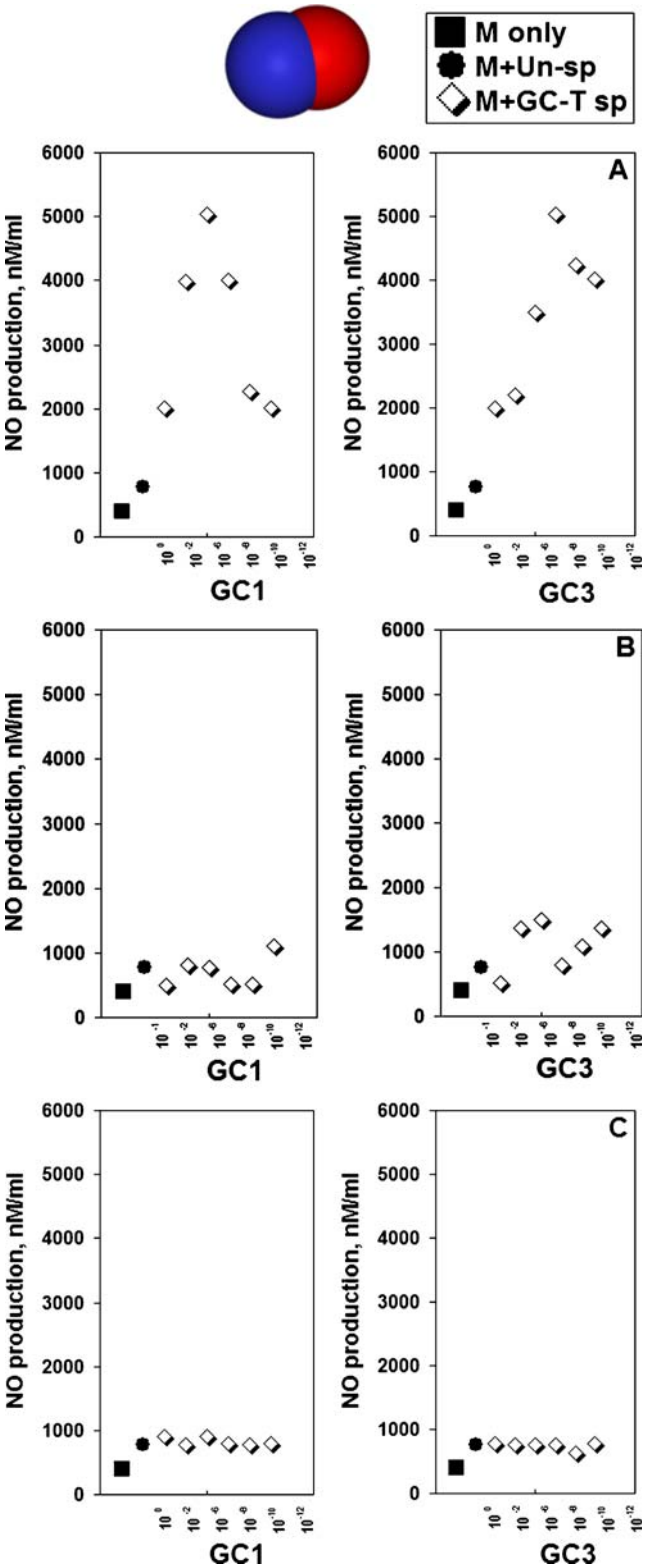

Fig. 3 Glycoconjugates GC1 and GC3 stimulated macrophage nitric oxide (NO) production, $p<0.0001$. Macrophage cultures were exposed to untreated and treated spores. Macrophages (MP) and glycoconjugate–treated macrophages (MP+GC) were used as control. After 24 h, macrophage NO production was measured using the Griess assay. GC concentrations are expressed as mg/ml (here and hereafter). These results are representative of triplicate experiments. NO molecule is shown

For the *"during contact"* (Fig. 1B), 0.5 µl of *B. cereus* spores (2.4×10^6 CFU/0.1 ml) were treated with 5.0 µl serially diluted glycoconjugates ($1–10^{-12}$ mg/ml) and incubated for 1 h at room temperature. Macrophage cultures were infected with glycoconjugate-treated spores at a ratio of 3–4 spores/ macrophage and incubated for 22–24 h.

For the "*following contact"* (Fig. 1C), macrophage cultures were infected with 0.5 µl spores (2.4×10^6 CFU) at a ratio of 3–4 spores/macrophage and incubated at 37°C for about 4 h. After their incubation, 5.0 µl serially diluted glycoconjugates were introduced to each culture and were incubated for 22–24 h.

Untreated macrophages ($6.0–8.0\times10^5$/culture) and macrophages exposed spores (2.4×10^6 CFU) in the absence of glycoconjugates were used as controls.

Glycoconjugate–spore complexes formation

Visual characterization of glycoconjugate–spore complexes formation was performed using atomic force microscopy (AFM). *B. cereus* spore suspension (0.5 µl of 2.4×10^6 CFU/0.1 mL) was treated with 5.0 µl serially diluted glycoconjugates ($1–10^{-12}$) and incubated for 1 h at room temperature. Untreated spores (2.4×10^6 CFU/ 0.1 mL) and glycoconjugates only were used as controls. Solutions of glycoconjugates, untreated, glycoconjugate–treated spore samples (10^4 CFU) were deposited on mica disks using sterile syringes, then dried in ambient air at room temperature (20–22°C) for 14 h. Prepared samples were later mounted on an AFM sample holder for imaging. All AFM observations were carried out at 20–22°C, using a Nano Scope® IIIa controller as well as a MultiMode™ microscope (Digital Instruments, Inc.) operating in tapping mode (amplitude) in conjunction with an E-scanner. A 125–µm silicon Nanoprobe (Digital Instruments, Inc.) was also employed. The calculated spring constant was 0.3 N/m. The resonance frequency remained in the range of 240–280 kHz, and the scan rate was of 1 µm/s. Flattening and high–pass filtering of the image data was performed in order to remove the substrate slope from images as well as high–frequency noise strikes, otherwise more pronounced in the high–resolution tapping mode imaging.

Post-phagocytosis studies

Infected with untreated or glycoconjugate–treated spores macrophage cultures were, subject of post-phagocytosis studies. Post-phagocytosis studies included determination of NO and NOS2 production, macrophage morphology and cell viability, lactate dehydrogenase (LDH) activity, and spore killing rate.

For NO production, macrophages were plated at 10^6 cells/culture in 6-well flat-bottomed tissue culture plates and incubated with glycoconjugates, together with treated or untreated spores. Cells were incubated at 37°C in 5% CO_2 for 24 h. Supernatants (100 µl) were then assayed for NO. The nitrite ion (NO_2-) concentration, indicative of NO, was determined using $NaNO_2$ as a standard [59]. Briefly, 100 µl of cell culture supernatant was mixed with

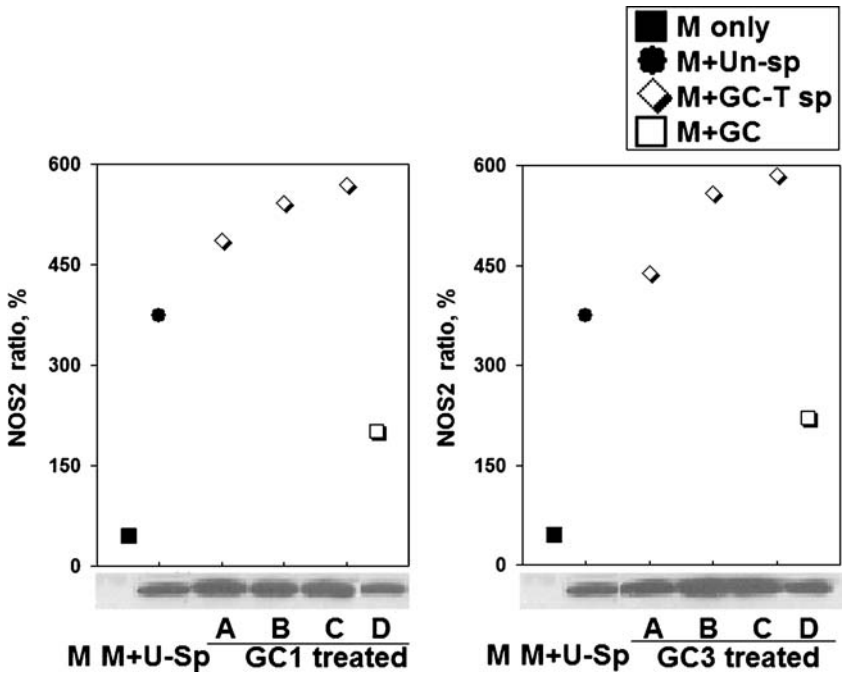

Fig. 4 Glycoconjugates stimulate NOS2 production under three conditions: A) "*prior to contact"*, B) *"during contact"*, C) *"following contact"* of macrophages with *B. cereus* spores. Macrophages treated with either GC1 or GC3 glycoconjugate (D). Data represent as a ratio % (*upper panel*) vs. measured density of resealed protein (*lower panel*). These results are representative of duplicate experiments

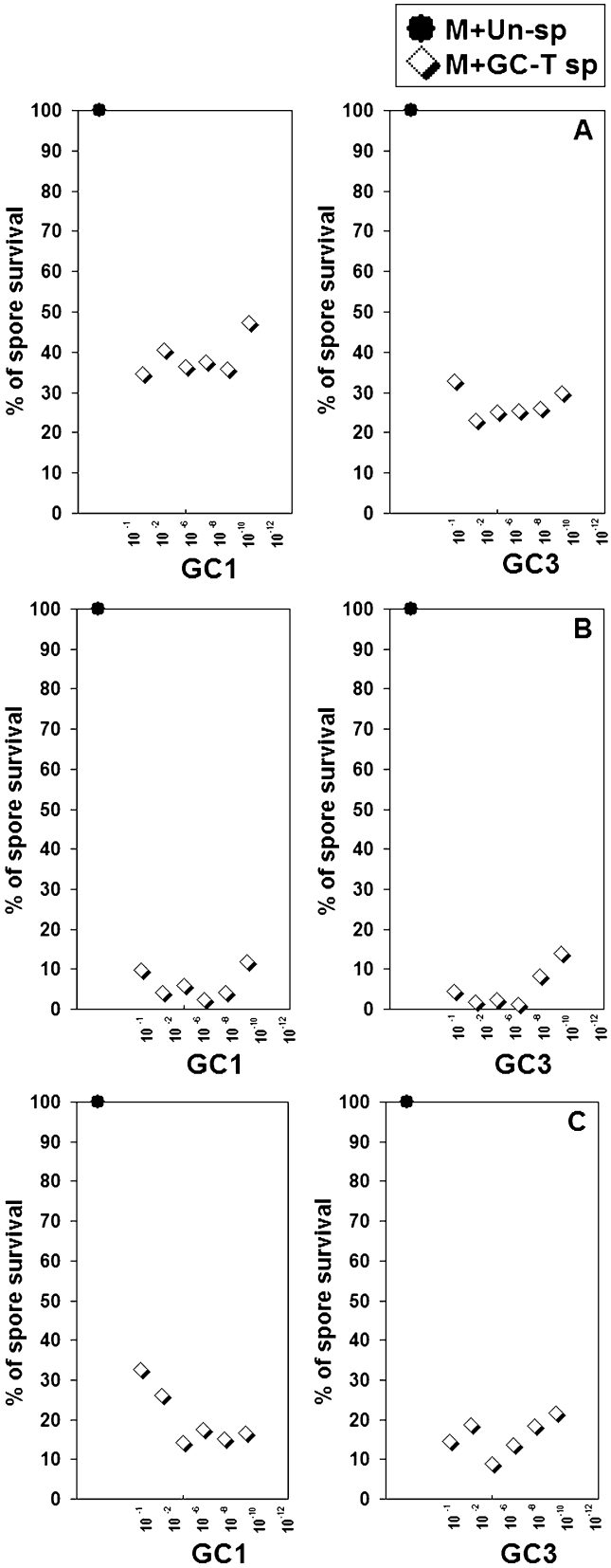

Fig. 5 Glycoconjugates facilitate spore destruction through macrophages, $p<0.01$ under three conditions: A) *"prior to contact"*, B) *"during contact"*, and C) *"following contact"* of macrophages with *B. cereus* spores. These results were expressed as mean percent bacterial killing of untreated and treated spores. These results are representative of triplicate experiments

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 absorbance was measured at 490 nm using a Bio–Tek Ex800 plate reader.

For NOS2, murine macrophages were pre-treated with either spores or GC-treated spores under *prior to*, *during* and *following* exposure conditions as describe above (Fig. 2) for 19–21 h. Cytoplasmic or nuclear extracts of the cells were resolved on SDS-acrylamide gel by electrophoresis, and transferred to polyvinylidene difluoride membranes. Membranes were incubated for 3 h at room temperature with 5% defatted milk to block non-specific sites, followed by incubation with anti-iNOS antibody (1:2000) (Santa Cruz Biotechnology Inc, CA), at 4°C for 16 h. Specific binding was detected after incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2500) (Pierce Chemical Company, IL) at room temperature for 3 h, and the addition of Supersignal (Pierce Chemical Company, IL) followed by exposure to x-ray films.

In order to analyze spore killing rates, macrophages exposed to untreated and treated spores were lysed to release spores first. The latter were then used for viability studies. Mixtures were concentrated by centrifugation using an accuSpin Micro centrifuge R (Walham, MA) for 10 min at 10,000 rpm. The resulting pellets (10.0 μl) were plated onto trypticase soy agar petri dishes. Plates were incubated overnight at 37°C and resulting colony forming units (CFU) were counted. CFU of undigested spores was a direct indication of NO and NOS2 bactericidal effect (Figs. 3, 4) and killing of *B. cereus* spores during phagocytosis (Fig. 5).

Spore-induced macrophage damage was measured by cell morphology, trypan blue, and lactate dehydrogenase (LDH) release. To determine macrophage viability, the culture supernatants were replaced with 500 μl of 0.4% trypan blue solution and examined microscopically using a Nikon Eclipse E400 POL fluorescence microscope at a magnification of 400×. Digital micrographs were acquired in real time. Percent viability was determined by counting individual live/dead macrophages. Ten determinations were made for each culture well.

Macrophage integrity was analyzed using CytoTox 96® kit (Promega, Inc., Madison, WI). LDH is a stable cytosolic

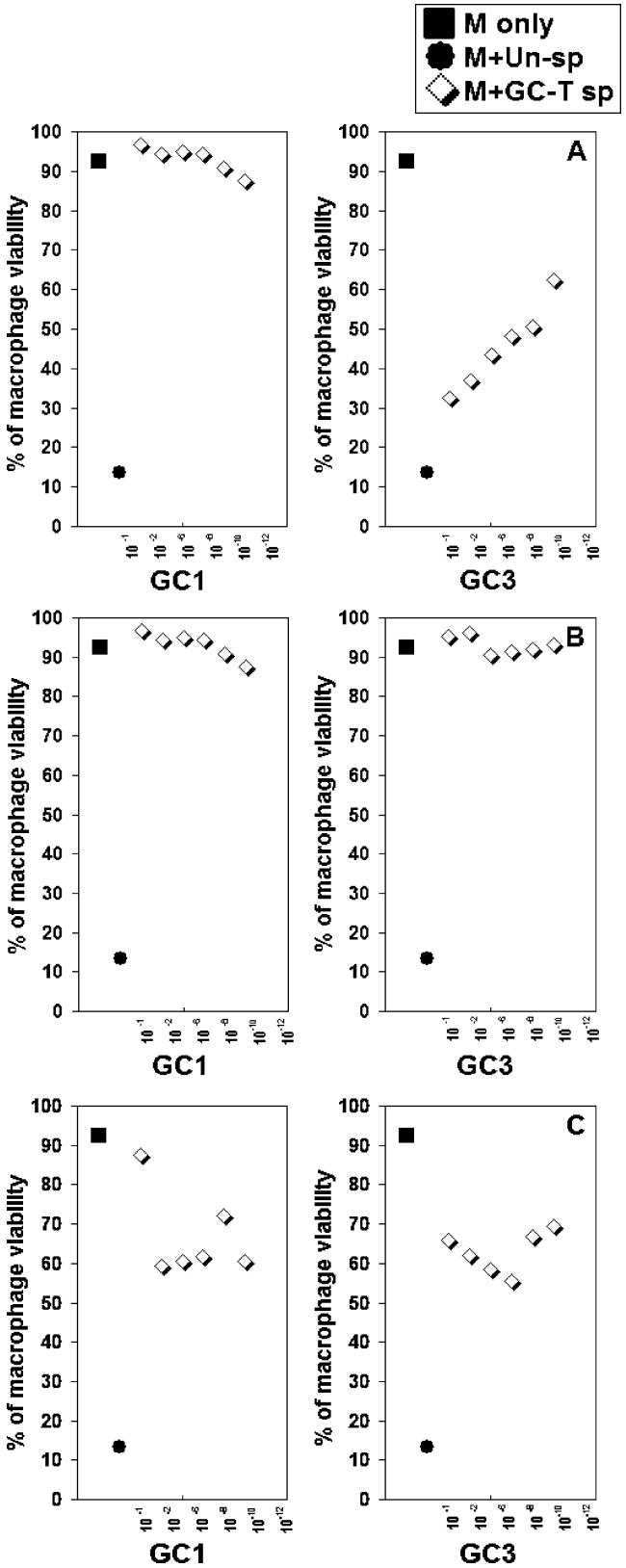

Fig. 6 Glycoconjugates protected macrophage viability after exposure to *Bacillus* spores under three conditions: A) "*prior to contact*", B) "*during contact*", and C) "*following contact*" of macrophages with *B. cereus* spore, $p<0.01$. Macrophages were exposed to untreated and GC1 or GC3 treated spores and stained for viability with trypan blue 24 h later (D). The viability for macrophages exposed to untreated spores (M+sp) (a) and glycoconjugate treated spores (b) are shown (D). These results are representative of triplicate experiments

enzyme released upon cell lysis with conversion of tetrazolium salt to a red formazan product. The amount of color was proportional to the number of lysed cells. The 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 AVONA. A Tukey test was performed for post–AVONA.

Results

The effects of GC1 and GC3 glycoconjugates on spores and macrophages were closely examined, since NO production by macrophages is important in killing phagocyted pathogens and is a well known as a marker of macrophage activation [2, 7–14, 68]. Glycoconjugates and their binding with *B. cereus* spores were examined by AFM (Fig. 2).

AFM allows visual confirmation of the glycoconjugate used, as well as untreated and glycoconjugate–treated *B. cereus* spores at a nanoscale level. Glycoconjugates diluted 1,000× consistently formed uniform clusters as seen in 2D AFM renderings (Fig. 2A, B). Section analysis (not shown) of 1,000× diluted glycoconjugate revealed the size of glycoconjugate clusters averaging 0.6 nm vertically and 46.0 nm horizontally. Furthermore, AFM allowed visual characterization of spore morphology prior to (Fig. 2C, D) and following glycoconjugate treatment (Fig. 2E, F). Untreated spores were found to be firmly affixed to the mica surface and remained sufficiently bound to be scanned with an AFM scanning probe (Fig. 2C, D). Spores appeared slightly elongated (Fig. 2C, D). The spore surface is covered with a loose layer of exosporium that spreads out and eventually attached itself to the mica surface (Fig. 2C, D). *B. cereus* spores exhibit a clearly identifiable exosporium as well as appendages (Fig. 2C, D). AFM observations of glycoconjugate–treated spores indicate that morphology of these spores are noticeably different (Fig. 2E, F) from untreated ones (Fig. 2C, D). Tiny morphological features including exosporium and appendages seem to disappear under a layer of glycoconjugate solution (Fig. 2E, F).

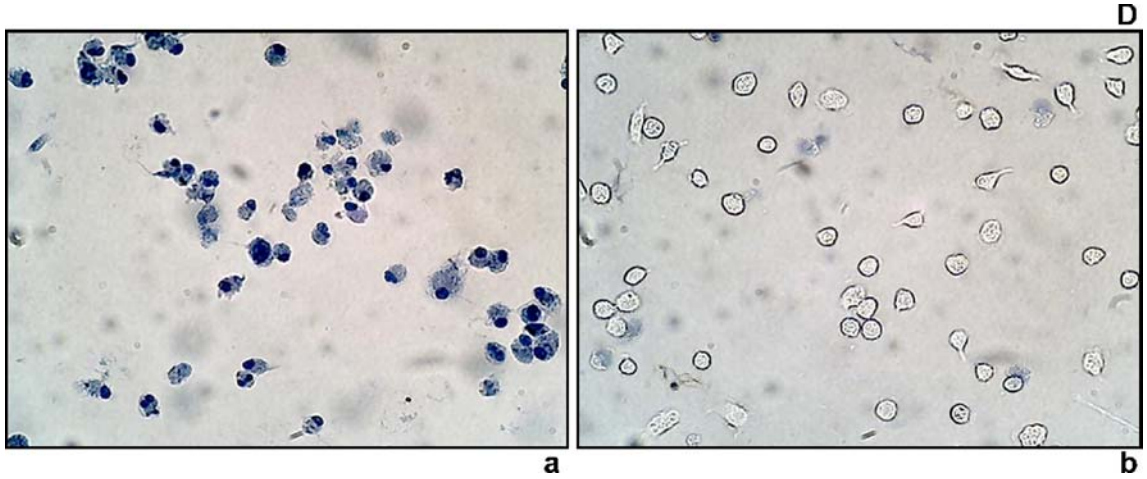

Fig. 6 (continued)

Macrophage production of NO in conjunction with glycoconjugates and spores was measured. When macrophages were exposed to untreated spores, but were not otherwise activated, the presence of either GC1 or GC3 induced macrophage NO production ($p<0.0001$) at the "*prior to contact"* condition with peak levels at dilution 10^{-6} (Fig. 3A). Glycoconjugate GC3 increased macrophage NO production, while GC1 was much less stimulatory at the *"during contact"* condition (Fig. 3B). Both glycoconjugates were least effective at the *"following"* condition.

Glycoconjugates were evaluated for the ability to modulate macrophage NOS2 synthesis (Fig. 4) resulting from exposure to glycoconjugates and/or spores.

Results show that spores stimulate macrophage NOS2 expression and that GC1 and GC3 further stimulate NOS2 (Fig. 4) under all three studied conditions. Thus, the presence of glycoconjugates induced NOS2 expression whether the macrophages regardless of the order of exposure. However, only when the cells were treated with glycoconjugates before spores were added, was NO production at a high level. The apparent dissociation between NOS2 induction and NO production likely resulted from the reduced macrophage viability reducing total NO, while NOS2 Western blotting used lysates of adherent and mostly viable cells.

Glycoconjugates were evaluated for the ability to modulate macrophage–mediated destruction of *Bacillus* spores (Fig. 5). Glycoconjugates were diluted by 10^{-1}–10^{-12} prior to addition to spores and/or macrophages.

As shown in Fig. 5A, GC1 facilitated the destruction of 60–65% ($p<0.01$) of spores, while GC3 facilitated the destruction of 70–86.4% of spores ($p<0.01$) at the *"prior to contact"* condition compared to untreated spores. Glycoconjugates stimulate the destruction of 98–99% of spores at the *"during contact"* (Fig. 5B) and 80–90% in the *"following"* condition (Fig. 5C).

As shown in Fig. 6, *B. cereus* spores reduced macrophage viability by 87%. The addition of GC1 or GC3 even at dilutions as high as a million fold protected the macrophages. The presence of GC1 resulted in macrophage viability up to 87% and 97% at the *"prior to"* and *"during"* conditions (Fig. 6A, B). GC1 and GC3 were somewhat less protective (60–87%) under the *"following"* condition (Fig. 6C).

To further characterize glycoconjugates, their effects on spore–induced damage to macrophages were examined (Fig. 7).

LDH release was determined 24 h after phagocytosis. Analysis of LDH, a cytosolic enzyme released upon toxic effects on cells, showed that macrophages exposed to untreated spores released 85% of LDH (Fig. 7). Both glycoconjugates lower LHD release ($p<0.01$) under three studied conditions vs. untreated spores.

Discussion

Following exposure to *Bacillus* spores, the latter will be rapidly taken up by phagocytes [31, 37, 42]. The capacity of phagocytes to kill pathogens including spores will determine whether the exposure will progress to a possibly serious infection. Exposure of murine macrophages to *B. cereus* spores at a ratio of 1.2 spores/macrophage resulted in macrophage death 24 h later. This was demonstrated by trypan blue (Fig. 4) and LDH (Fig. 5) assays. *Bacillus* spores do not cause cell lysis, but may induce macrophage apoptosis [30, 64]. The present study primarily focused on glycoconjugates, their stimulatory effects on NO and NOS2 by macrophages, and their protective role during

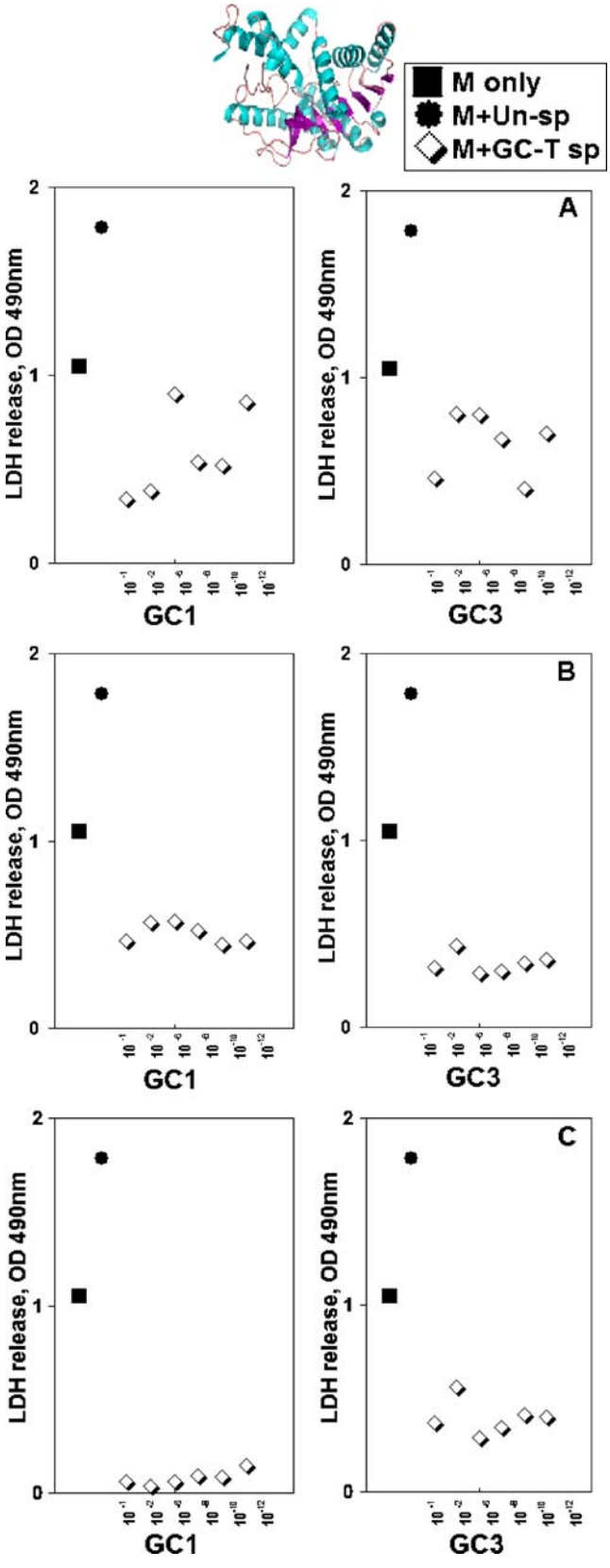

Fig. 7 Glycoconjugates protected macrophages from spore–induced damage, $p<0.01$. Macrophage cultures were exposed to untreated and treated spores. After 24 h, macrophage LDH was assayed in macrophages exposed to MP, MP+GC, untreated and treated spores. These results are representative of triplicate experiments. LDH molecule is shown

phagocytosis of spores. The presence of glycoconjugates stimulated NO and NOS2 production and prevented the widespread death of macrophages exposed to spores. Moreover, glycoconjugates promoted macrophage killing of the spores. Glycoconjugates have been reported to interrupt spore germination [54–58], which could contribute to macrophage phagocytosis [55, 56]. Carbohydrates found on the spore coat likely serve as potential receptors [54, 60–64] for interactions with monosaccharide units of the glycoconjugates leading to adhesion [54]. Binding of the receptors by glycoconjugates might impair spore germination [54–58] and also act as opsonins, promoting phagocytosis [65]. Even when highly diluted, glycoconjugates were very effective at stimulating NO (Fig. 3) and NOS2 production (Fig. 4). NOS2 induction was less effective when macrophages were first exposed to spores. This was likely due to interruption of macrophage metabolism by the spores. NOS2 induction with GC exposure indicates activation of the macrophages, which were, thus, better able to kill the spores and protect the macrophages from spore-induced cell death. There is evidence that glycoconjugates can serve as chemoattractants and/or immunostimulators for macrophages [55, 56]. Thus, glycoconjugates contribute to an increased macrophage production of inducible NO, important in intracel-

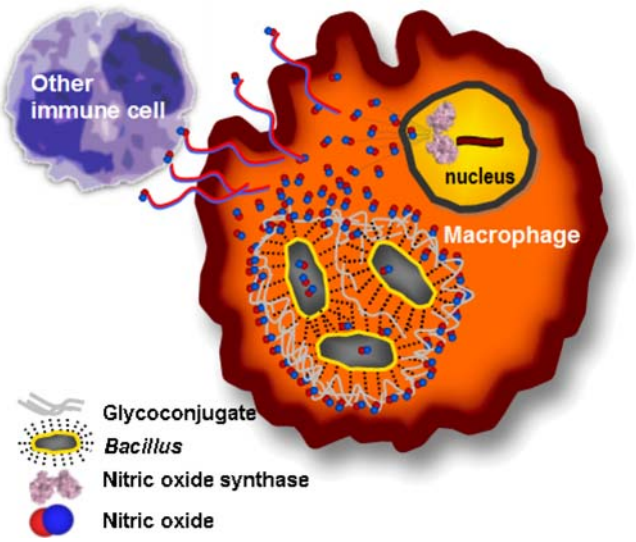

Fig. 8 Proposed antimicrobial mechanism achieved by glycoconjugates against spores and macrophages

lular [2, 7–14, 66, 69] killing of *B. cereus* spores (Fig. 4) [55–57]. Released NO by macrophages stimulates killing of spores and/or germinating bacteria, and recruit other immune cells [65]. Macrophages of the mammalian immune system generate inducible NO as part of their arsenal employed against a variety of pathogens [2, 7–14, 68]. In addition, it was postulated that NO inhibits aerobic respiration, depresses ATP levels, triggers the σ^B general stress regulon [70, 71] via the energy stress branch [72, 73], and suppresses several bacterial enzymes [70, 71].

Specific carbohydrate structures expressed on pathogens are believed to be recognized by complementary molecules expressed on the surface of interacting cells [45–49]. Previous studies show that the external spore layers are, in fact, glycosylated [54, 60–64]. Receptors on *B. cereus* spore exterior [54, 64] are likely to be involved in interactions with glycoconjugates [53–57]. Differences in carbohydrates [54, 60–64] on bacterial spores could lead to differences in glycoconjugate effectiveness [55, 56].

Previous reports suggest that complex carbohydrates are involved in recognition processes, including adhesion between cells, adhesion of cells to the extracellular matrix, and specific recognition of cells by one another [45, 74, 75]. Carbohydrate–carbohydrate interactions play an important role in the complimentary binding of glycosphingolipids [76–78]. We have postulated that recognition [54] and inhibition [55–57] of bacterial spores is based on multivalent carbohydrate–carbohydrate interactions [45, 74–78] between disaccharide glycoconjugates, acting as ligands [54–58], and carbohydrates expressed on spores [55, 56, 60–64]. Different glycoconjugates may exhibit diverse activities and this may be reflected by the differences observed in inducible NO production by macrophages (Figs. 3 and 4). Furthermore, it was shown that a high level of NO can promote apoptosis in some cells, whereas lower NO levels inhibit apoptosis in others [66, 67]. Our results show that lower dilution of glycoconjugates promotes viability of macrophages (Fig. 6), lower LDH release (Fig. 7), and NO and NOS2 production (Figs. 3 and 4).

Taken together, the results presented in this report lead us to propose a novel antimicrobial defense mechanism by glycoconjugates against bacterial spores and/or bacteria (Fig. 8).

Binding of glycoconjugates leads to alteration of receptors that play an essential role in spore germination [54–58]. This binding prevented spore germination into bacteria and delayed bacterial defensive [77–79] mechanism (Fig. 8). In addition, we postulated that glycoconjugates bond or deactivate released NO by *Bacillus* (Fig. 8). Prospective bioinformatics studies on NO and glycoconjugate/s interaction studies will elucidate this issue. It was shown that freely diffusible radical NO [6, 8, 18] causes enzymes and DNA damages, initiates lipid peroxidation, and exacerbates peroxide–induced damage of pathogens [19, 20]. The ability of pathogens, like *B. anthracis,* to survive in macrophages depends critically on the state of their oxidative stress defense system [79–81], which is apparently interrupted by glycoconjugates treatment. Glycoconjugates activate macrophages, inducing NOS2 synthesis and NO production and increasing their ability to kill engulfed spores and reduce spore-induced cell damage. From other hand, glycoconjugates and/or NO probably recruit other immune cells. Prospective studies with other immune cells will clarify this issue.

The present studies could lead to an improved screening and selection of glycoconjugate ligands having bactericidal, possibly, immunomodulating properties and could suggest new targets for vaccine and/or immunomodulator and/or therapeutics development against *B. cereus* or *B. anthracis* spores.

Acknowledgements The present study was supported in part by start–up funds and a National Science Foundation EPSCoR RII Grant "Arkansas AASSET Initiatives" (#EPS-0701890) and the Arkansas Science & Technology Authority; Kathleen Thomsen Hall Charitable Trust Grants; American Society for Microbiology URF and UTF programs. We would like to acknowledge Professor Kalle Levon, Polytechnic Institute of New York University, for allowing us to use the atomic force microscope. The authors acknowledge Dr. Dina Kudasheva for the assistance during atomic force microscopy, and Kristin Hester, Michelle Park Kim, Deirdre McManus, undergraduate students for the assistance during NO and LDH studies.

References

1. Moncada, S., Higgs, E., Bagetta, G.: In: Nitric Oxide and the Cell: Proliferation, Differentiation and Death. Portland, London (1998)
2. Nathan, C., Shiloh, M.U.: Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl Acad. Sci. USA **97**, 8841–8848 (2000). doi:10.1073/pnas.97.16.8841
3. Zhu, L., Gunn, C., Beckman, J.S.: Bactericidal activity of peroxynitrite. Arch. Biochem. Biophys. **298**, 452–457 (1992). doi:10.1016/0003-9861(92)90434-X
4. Fang, F.C.: Mechanisms of nitric oxide–related antimicrobial activity. J. Clin. Invest. **99**, 2818–2825 (1997). doi:10.1172/JCI119473
5. Beckman, J.S.: Oxidative damage and tyrosine nitration from peroxynitrite. Chem. Res. Toxicol. **9**, 836–844 (1996). doi:10.1021/tx9501445
6. Hibbs Jr., J.B., Taintor, R.R., Vavrin, Z., Rachlin, E.M.: Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. **157**, 87–94 (1988). doi:10.1016/S0006-291X(88)80015-9
7. Akaike, T., Suga, M., Maeda, H.: Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. Proc. Soc. Exp. Biol. Med. **217**, 64–73 (1998)
8. Doi, T., Ando, M., Akaike, T., Suga, M., Sato, K., Maeda, H.: Resistance to nitric oxide in *Mycobacterium avium* complex and its implication in pathogenesis. Infect. Immun. **61**, 1980–1989 (1993)

9. Vouldoukis, I., Riveros-Moreno, V., Dugas, B., Ouaaz, F., Becherel, P., Debre, P., Moncada, S., Mossalayi, M.D.: The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the Fc epsilon RII/CD23 surface antigen. Proc. Natl. Acad. Sci. USA **92**, 7804–7808 (1995). doi:10.1073/pnas.92.17.7804
10. Gobert, A.P., Daulouede, S., Lepoivre, M., Boucher, J.L., Bouteille, B., Buguet, A., Cespuglio, R., Veyret, B., Vincendeau, P.: L–Arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. Infect. Immun. **68**, 4653–4657 (2000). doi:10.1128/IAI.68.8.4653-4657.2000
11. Pacelli, R., Wink, D.A., Cook, J.A., Krishna, M.C., DeGraff, W., Friedman, N., Tsokos, M., Samuni, A., Mitchell, J.B.: Nitric oxide potentiates hydrogen peroxide–induced killing of *Escherichia coli*. J. Exp. Med. **182**, 1469–1479 (1995). doi:10.1084/jem.182.5.1469
12. Dykhuizen, R.S., Fraser, A., McKenzie, H., Golden, M., Leifert, C., Benjamin, N.: *Helicobacter pylori* is killed by nitrite under acidic conditions. Gut **42**, 334–337 (1998)
13. Kosaka, H., Oda, Y., Uozumi, M.: Induction of umuC gene expression by nitrogen dioxide in *Salmonella typhimurium*. Mutat. Res. **142**, 99–102 (1985). doi:10.1016/0165-7992(85)90047-8
14. Levitz, S.M., DiBenedetto, D.J.: Differential stimulation of murine resident peritoneal cells by selectively opsonized encapsulated and acapsular *Cryptococcus neofonnans*. Infect. Immun. **56**, 2544–2551 (1988)
15. Lepoivre, M., Flaman, J.M., Henry, Y.: Early loss of the tyrosyl radical in ribonucleotide reductase of adenocarcinoma cells producing nitric oxide. J. Biol. Chem. **267**(32), 22994–23000 (1992)
16. Stuehr, D.J., Nathan, C.F.: A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169**, 1543–1555 (1989). doi:10.1084/jem.169.5.1543
17. Challis, B.C.: The chemistry of formation of N–nitrosocompounds. In: Gibson, G.G., Ioannides, C. (eds.) Safety Evaluation of Nitrosatable Drugs and Chemicals, pp. 16–55. Taylor and Francis, Ltd., London (1981)
18. Ischiropoulos, H.: Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. **356**, 1–11 (1998). doi:10.1006/abbi.1998.0755
19. Umezawa, K., Akaike, T., Fujii, S., Suga, M., Setoguchi, K., Ozawa, A., Maeda, H.: Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against *Salmonella typhimurium* infection in mice. Infect. Immun. **65**, 2932–2940 (1997)
20. Woodmansee, A.N., Imlay, J.A.: A mechanism by which nitric oxide accelerates the rate of oxidative DNA damage in *Escherichia coli*. Mol. Microbiol. **49**, 11–22 (2003). doi:10.1046/j.1365-2958.2003.03530.x
21. Barnaby, W.: In: Barnaby, W. (ed.) The Plague Markers: The Secret World of Biological Warfare. Vision Paperbacks, London (1997)
22. Drobniewski, F.A.: *Bacillus* and related species. Clin. Microbiol. Rev. **6**, 324–338 (1993)
23. Hanna, P.: Anthrax pathogenesis and host response. Curr. Top. Microbiol. Immunol. **225**, 13–35 (1998)
24. Jamie, W.E.: Anthrax: diagnosis, treatment, prevention. Prim. Care Update Ob Gyns **9**, 117–121 (2002). doi:10.1016/S1068-607X(02)00100-2
25. Ash, C., Farrow, J.A., Dorsch, M., Stackebrandt, E., Collins, M.D.: Comparative analysis of *Bacillus anthracis, Bacillus cereus* 2 and related species on the basis of reverse transcriptase sequencing of the 16S rRNA. Int. J. Syst. Bacteriol. **41**, 343–346 (1991)
26. Ash, C., Collins, M.D.: Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. FEMS Microbiol. Lett. **73**, 75–80 (1992). doi:10.1111/j.1574-6968.1992.tb05292.x
27. Patra, G., Sylvestre, P., Ramisse, V., Thérasse, J., Guesdon, J.L.: Isolation of a specific chromosomic DNA sequence of *Bacillus anthracis* and its possible use in diagnosis. FEMS Immunol. Med. Microbiol. **15**, 223–231 (1996). doi:10.1111/j.1574-695X.1996.tb00088.x
28. Keim, P., Price, L.B., Klevytska, A.M., Smith, K.L., Schupp, J. M., Okinaka, R., Jaackson, P.J., Hugh-Jones, M.E.: Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. **182**, 2928–2936 (2000). doi:10.1128/JB.182.10.2928-2936.2000
29. Spencer, R.C.: Bacillus anthracis. J. Clin. Pathol. **56**, 182–187 (2003). doi:10.1136/jcp.56.3.182
30. Ireland, A.W.J., Hanna, P.C.: Macrophage-enhanced germination of *Bacillus anthracis* endospores requires *gerS*. Infect. Immun. **70**, 5870–5872 (2002). doi:10.1128/IAI.70.10.5870-5872.2002
31. Weiner, M.A., Hanna, P.C.: Macrophage-mediated germination of *Bacillus anthracis* endospores requires the *gerH* operon. Infect. Immun. **71**, 3954–3959 (2003). doi:10.1128/IAI.71.7.3954-3959.2003
32. Guidi-Rontani, C., Weber-Levy, M., Labruyere, E., Mock, M.: Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. **31**, 9–17 (1999). doi:10.1046/j.1365-2958.1999.01137.x
33. Friedlander, A.M.: Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261**, 7123–7126 (1986)
34. 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). doi:10.1016/S0014-5793(02)03228-3
35. Duesbery, N.S., Vande Woude, G.F.: Anthrax toxins. Cell. Mol. Life Sci. **55**, 1599–1609 (1999). doi:10.1007/s000180050399
36. Guidi-Rontani, C., Mock, M.: Macrophage interactions. Curr. Top. Microbiol. Immunol. **271**, 115–141 (2002)
37. Weaver, J., Porasuphatana, S., Tsai, P., Pou, S., Roman, L.J., Rosen, G.M.: A comparative study of neuronal and inducible nitric oxide synthases: generation of nitric oxide, superoxide, and hydrogen peroxide. Biochim. Biophys. Acta **1726**, 302–308 (2005)
38. Patterson-Delafield, J., Martinez, R.J., Lehrer, R.I.: Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host defense mechanism. Infect. Immun. **30**, 180–192 (1980)
39. Ganz, T., Lehrer, R.I.: Defensins. Pharmacol. Ther. **66**, 191–205 (1995). doi:10.1016/0163-7258(94)00076-F
40. 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). doi:10.1038/sj.jim.7000239
41. 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). doi:10.1016/j.vaccine.2003.12.027
42. Baillie, L., Read, T.D.: *Bacillus anthracis*, a bug with attitude! Curr. Opin. Microbiol. **4**, 78–81 (2001). doi:10.1016/S1369-5274(00)00168-5
43. 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). doi:10.1046/j.1365-2958.2000.01701.x
44. Varki, A.: Biological roles of oligosaccharides: all of the theories are correct. Glycobiol. **3**, 97–130 (1993). doi:10.1093/glycob/3.2.97
45. Gabius, H.J., Gabius, S.: In: Gabius, H.J., Gabius, S. (eds.) Glycosciences: Status and Perspectives. Chapman & Hall, London (1997)
46. Wang, D., Kabat, E.A.: Carbohydrates antigens (polysaccharides). In: Regenmortal, M.H.V.V. (ed.) Structure of Antigens, Vol. 3, pp. 247–276. CRC, Boca Raton (1996)
47. Crocker, P.R., Feizi, T.: Carbohydrate recognition systems: functional trials in cell- cell interaction. Curr. Opin. Struct. Biol. **6**, 679–691 (1996). doi:10.1016/S0959-440X(96)80036-4
48. Feizi, T.: Carbohydrate-mediated recognition systems in innate immunity. Immunol. Rev. **173**, 79–88 (2000). doi:10.1034/j.1600-065X.2000.917310.x
49. Karlsson, K.A., Ängstr m, J., Bergstr m, J., Lanne, B.: Microbial interaction with animal cell surface carbohydrates. APMIS **27** (Suppl.), 71–83 (1992)
50. Axford, J.: The impact of glycobiology on medicine. Trends Immunol. **22**, 237–239 (2001). doi:10.1016/S1471-4906(01)01890-7
51. Borman, S.: Carbohydrate vaccines. Chem. Eng. News **82**(32), 31–35 (2004)
52. Anderson, P.W., Pichichero, M.E., Stein, E.C., Porcalli, S., Betts, R.F., Connuck, D.M., Lorones, D., Insel, R.A., Zahradnik, J.M., Eby, R.: Effect of oligosaccharide chain length, exposed terminal group, and hapten loading on the antibody response of human adults and infants to vaccines consisting of Haemophilus influenzae type b capsular antigen unterminally coupled to the diphtheria protein CRM197. J. Immunol. **142**, 2464–2468 (1989)
53. Kasper, D., Paoletti, L.C., Wessels, M.R., Guttormsen, H., Carey, V.J., Jennings, H.J., Baker, C.J.: Immune response to type III group B streptococcal polysaccharide-tetanus toxoid conjugate vaccine. J. Clin. Invest. **98**, 2308–2314 (1996). doi:10.1172/JCI119042
54. Tarasenko, O., Islam, S., Paquiot, D., Levon, K.: Glycoconjugates for recognition of *Bacillus* spores. Carb. Res. **339**, 2859–2870 (2004). doi:10.1016/j.carres.2004.10.008
55. 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). doi:10.1007/s10719-007-9097-1
56. 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). doi:10.1007/s00203-008-0352-z
57. Tarasenko, O., Burton, E., Soderberg, L., Alusta, P.: Glycoconjugates and their role in phagocytosis and destruction of *B. cereus* spores. PMSE **96**, 946–947 (2007)
58. Tarasenko, O., Lone, S., Alusta, P.: Defensive and simultaneous actions of glycoconjugates during spore decontamination. Carb. Res. **343**, 2243–2250 (2008). doi:10.1016/j.carres.2008.03.038
59. 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)
60. Fox, A., Black, G.E., Fox, K., Rostovtseva, S.: Determination of carbohydrate profiles of *Bacillus anthracis* and *Bacillus cereus* including identification of O-methyl methylpentoses by using gas chromatography-mass spectrometry. J. Clin. Microbiol. **31**, 887–894 (1993)
61. Steichen, C., Chen, P., Kearney, J.F., Turnbough Jr., C.L.: Immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. J. Bacteriol. **185**, 1903–1910 (2003). doi:10.1128/JB.185.6.1903-1910.2003
62. Sylvestre, P., Couture-Tosi, E., Mock, M.: Polymorphism in the collagen-like region of the *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. J. Bacteriol. **185**, 1555–1563 (2003). doi:10.1128/JB.185.5.1555-1563.2003
63. Waller, L.N., Stump, M.J., Fox, K.F., Harley, W.M., Fox, A., Stewart, G.C., Shahgholi, M.: Identification of a second collagen–like glycoprotein produced by *Bacillus anthracis* and demonstration of associated spore–specific sugars. J. Bacteriol. **187**, 4592–4597 (2005). doi:10.1128/JB.187.13.4592-4597.2005
64. Tarasenko, O., Islam, S., Levon, K.: Exo–and endoglycosidases as tool for study of assembly of macromolecules bacteria and spores> In 228th National Meeting of American Chemical Society, Philadelphia, PA, August 2004; AGFD-189
65. Black, J.G.: In Microbiology: Principles and Explorations, pp. 463–484. John Wiley and Sons, Inc. Publishing (2005)
66. 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). doi:10.1016/0014-5793(95)00188-F
67. Choi, B.M., Pae, H.O., Jang, S.I., 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)
68. Dimmelder, S., Haendeler, J., Nehls, M., Zeiher, A.M.: Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J. Exp. Med. **185**, 601–607 (1997). doi:10.1084/jem.185.4.601
69. Gazzinelli, R.T., Oswald, I.P., Hieny, S., James, S., Sher, A.: The microbicidal activity of interferon–gamma–treated macrophages against *Trypanosoma cruzi* involves an L–arginine–dependent, nitrogen oxide–mediated mechanism inhabitable by interleukin–10 and transforming growth factor–beta. Eur. J. Immunol. **22**, 2501–2506 (1992). doi:10.1002/eji.1830221006
70. Moore, C.M., Nakano, M.M., Wang, T., Ye, R.W., Helmann, J.D.: Response of *Bacillus subtilis* to nitric oxide and the nitrosating agent sodium nitroprusside. J. Bacteriol. **186**(14), 4655–4664 (2004). doi:10.1128/JB.186.14.4655-4664.2004
71. Nakano, M.M.: Induction of ResDE–dependent gene expression in *Bacillus subtilis* in response to nitric oxide and nitrosative stress. J. Bacteriol. **184**, 1783–1787 (2002). doi:10.1128/JB.184.6.1783-1787.2002
72. Butler, C.S., Seward, H.E., Greenwood, C., Thomson, A.J.: Fast cytochrome *bo* from *Escherichia coli* binds two molecules of nitric oxide at CuB. Biochem. **36**, 16259–16266 (1997). doi:10.1021/bi971481a
73. Hori, H., Tsubaki, M., Mogi, T., Anraku, Y.: EPR study of NO complex of *bd*–type ubiquinol oxidase from *Escherichia coli*. J. Biol. Chem. **271**, 9254–9258 (1996). doi:10.1074/jbc.271.21.12451
74. 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). doi:10.1016/S1367-5931(00)00153-8
75. Bertozzi, C.R., Kiessling, L.L.: Chemical glycobiology. Science **291**, 2357–2364 (2001). doi:10.1126/science.1059820
76. Hakomori, S.: Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu. Rev. Biochem. **50**, 733–764 (1981). doi:10.1146/annurev.bi.50.070181.003505
77. Kojima, N., Hakomori, S.: Sialyllactose-mediated cell Interaction during granulosa cell differentiation. J. Biol. Chem. **264**, 20159–20162 (1989)

78. Kojima, N., Hakomori, S.: Carbohydrate–carbohydrate interaction of glycosphingolipids. J. Biol. Chem. **266**, 17552–17558 (1991)
79. Adak, S., Aulak, K.S., Stuehr, D.J.: Direct evidence for nitric oxide production by a nitric–oxide synthase–like protein from *Bacillus subtilis*. J. Biol. Chem. **277**, 16167–16171 (2002). doi:10.1074/jbc.M201136200
80. Raines, K.W., Kang, T.J., Hibbs, S., Cao, G.-L., Weaver, J., Tsai, P., Baillie, L.A., Cross, S., Rosen, G.M.: Importance of nitric oxide synthase in the control of infection by *Bacillus anthracis*. Infect. Immun. **74**, 2268–2276 (2006). doi:10.1128/IAI.74.4.2268-2276.2006
81. Adak, S., Bilwes, A.M., Panda, K., Hosfield, D., Aulak, K.S., McDonald, J.F., Tainer, J.A., Getzoff, E.D., Crane, B.R., Stuehr, D.J.: Cloning, expression, and characterization of a nitric oxide synthase protein from *Deinococcus radiodurans*. Proc. Natl Acad. Sci. USA **99**, 107–112 (2002). doi:10.1073/pnas.012470099