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# Nitric Oxide Is a Key Determinant of Group B Streptococcus–Induced Murine Macrophage Apoptosis

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Group B streptococcus (GBS; *Streptococcus agalactiae*) induces apoptosis of macrophages, and this may be an important mechanism GBS uses to suppress immune responses. The mechanisms whereby GBS induces apoptosis have not been identified. We studied GBS infection in murine macrophage–like J774A.1 cells and analyzed gene expression before apoptosis. Tumor necrosis factor (TNF)– $\alpha$ , interleukin (IL)–1, and inducible nitric oxide synthase (iNOS) gene expression coincided with apoptosis. Inhibition of iNOS gene expression by use of N<sup>G</sup>-monomethyl-L-arginine (NMMA) inhibited apoptosis, whereas inhibition of TNF– $\alpha$  and IL-1 biological activity did not. Macrophages from congenic iNOS-deficient mice were less susceptible to apoptosis than were macrophages from C57BL/6 mice. The NO donor S-nitroso-N-acetylpenicillamine (SNAP) induced apoptosis without infection, confirming its proapoptotic effect. NMMA did not impair the microbicidal activity of macrophages, however, and SNAP was not bactericidal against GBS *in vitro*. In human monocyte–derived macrophages (HMDMs), NO production was minimal, even after costimulation with IFN– $\gamma$  and lipopolysaccharide. Dose-dependent apoptosis of HMDMs occurred without a significant NO response. Thus, NO is an important mediator of GBS-induced murine macrophage apoptosis but does not contribute to antimicrobial activity or cytotoxicity in HMDMs. HMDMs and murine macrophages are killed by GBS by alternative, NO-independent mechanisms. Future studies of host-cell machinery commandeered by GBS to bring about apoptosis will be important for understanding the role played by apoptosis in defense against this important human pathogen.

Induction of apoptosis of macrophages is an important mechanism whereby bacteria modulate immune responses, and this plays an important role in shaping the dynamics of the immune response to infection [1]. Apoptosis limits acute inflammation [2], influences the chronicity of infection [3], and affects adaptive immunity [4]. Of particular relevance is the recent demonstration, in a murine model of pulmonary bacterial

infection, that apoptosis of *Streptococcus pneumoniae*–infected macrophages contributes to the immune response [5]. Understanding the interactions between macrophages and bacteria that culminate in host-cell apoptosis is, thus, important for understanding the pathogenesis of specific disease states.

Group B streptococcus (GBS; *Streptococcus agalactiae*) is a common cause of serious bacterial infection in neonates [6]. Respiratory colonization in neonates may progress to pneumonia, sepsis, and meningitis. On entry of GBS into the lung, the immediate effector cell is the alveolar macrophage. Opsonin-independent defenses are critical determinants of the ability of infants to resist GBS infection, because subprotective concentrations of anti-GBS capsular polysaccharide antibody and complement are typical of neonates [6]. After non-opsonic phagocytosis of GBS, macrophages produce cytokines such as tumor necrosis factor (TNF)– $\alpha$  [7] and nitric oxide (NO) [8, 9]. GBS also stimulates NO pro-

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duction in respiratory epithelial cells via the paracrine effects of soluble factors released by GBS-infected mononuclear cells [10]. Despite this, GBS persists inside macrophages for long periods and induce macrophage apoptosis [11]. To shed light onto how GBS might achieve prolonged survival in the host after infection, understanding the mechanisms of GBS-induced apoptosis is important.

NO exerts antimicrobial effects against many bacteria [12] and plays a role in host-cell apoptosis [13] and immunity [14]. During *Salmonella* species infection [15], NO promotes survival of infected host cells, whereas, during *Helicobacter pylori* infection, it is proapoptotic [16]. More recently, a proapoptotic role for NO during pneumococcal infection has been demonstrated [17]. The role played by NO in the macrophage apoptotic and antimicrobial response to GBS has not previously been investigated. Here, we show that NO is a mediator of murine macrophage apoptosis triggered by GBS. Surprisingly, NO was not required for macrophage killing of GBS in murine or human cells, and NO displayed no direct antimicrobial activity against GBS in vitro. Induction of apoptosis by GBS may limit host immune responses and permit bacterial persistence at sites of infection.

## MATERIALS AND METHODS

**Bacteria and reagents.** GBS 874391 is a serotype III strain [18]. *Staphylococcus aureus* 25923 and *Streptococcus pyogenes* 19615 were obtained from the American Type Culture Collection. N<sup>G</sup>-monomethyl-L-arginine (NMMA) and S-nitroso-N-acetylpenicillamine (SNAP) were purchased from Calbiochem. Murine TNF- $\alpha$ , interleukin (IL)-1 $\alpha$ , and interferon (IFN)- $\gamma$  were purchased from Endogen. Aprotinin A, leupeptin, phorbol 12-myristate 13-acetate (PMA), 5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>), and *Escherichia coli* lipopolysaccharide (LPS) were purchased from Sigma. Sulfanilamide, N-(1-Naphthyl)ethylenediamine dihydrochloride monomethanolate, and sodium nitrite were purchased from Fluka. o-phosphoric acid was purchased from Fisher Scientific.

**Collection and culture of macrophages.** Murine macrophage-like J774A.1 cells (ATCC TIB-67) were grown as described elsewhere [19]. Thioglycolate-elicited peritoneal macrophages were harvested from C57BL/6 mice and from inducible NO synthase (iNOS)-deficient B6.129P2-*Nos2*<sup>tm1Lau</sup> (iNOS<sup>-/-</sup>) mice (6–12 weeks old; Jackson Laboratories), in accordance with standard methods [20]. One-milliliter aliquots ( $1.8 \times 10^6$  cells) were seeded into 24-well plates and incubated for 24 h at 37°C in 5% CO<sub>2</sub> before infection. Human monocyte-derived macrophages (HMDMs) were obtained by treating U937 cells (ATCC CRL-1593.2;  $5 \times 10^5$  cells/well) [21] in 24-well plates with 50 ng/mL PMA for 48 h [22–25]. Monolayers were rinsed, fresh tissue-culture medium (TCM) with penicillin and streptomycin was added (24 h), and HMDMs were infected

as described below. HMDMs were supplemented with 15  $\mu$ mol/L BH<sub>4</sub> 24 h before infection, because BH<sub>4</sub> is a required cofactor for NO synthesis in human monocytes [26, 27].

**Nonopsonic phagocytosis of GBS by macrophages and antibiotic protection assay.** Monolayers were rinsed and inoculated with GBS (MOI, 100), as described elsewhere [19]. Bacterial numbers were quantified by measuring optical density at 600 nm (Spectronic Genesys 20; Milton Roy) and were confirmed by quantitative colony counts. After 2 h, monolayers were washed and TCM with 100 U/mL penicillin, streptomycin, and gentamicin was added, to kill extracellular GBS; colony counts ( $n = 3$ ) were performed as described elsewhere [19]. Duplicate wells were used to assess macrophage viability by trypan blue exclusion. NMMA (2 mmol/L), anti-TNF- $\alpha$  antibody (20  $\mu$ g/mL; Endogen), and anti-IL-1 $\alpha$  (20  $\mu$ g/mL; Sigma) antibody were added to cultures after infection as indicated below.

**Modified antibiotic protection assay to monitor escape of GBS from macrophages.** Macrophages were infected and antibiotics were added to kill extracellular GBS [19]. After 24, 48, or 72 h of incubation, 1-mL samples of the supernatants were centrifuged (10,000 g; 10 min; 22°C), to pellet bacteria; were washed twice with PBS, to remove antibiotics; and were dispensed onto Todd-Hewitt agar (THA), to confirm sterility of the extracellular milieu. Monolayers were rinsed, fresh TCM without antibiotics was added, and macrophages were reincubated. Supernatants were cultured 24 h later to identify viable GBS, which represented intracellular bacteria that were initially protected from antibiotics and then escaped or were released from macrophages.

**Detection of apoptosis.** Apoptosis was assessed by DNA fragmentation and transmission electron microscopy, as described elsewhere [19]. Reagents for apoptosis were purchased from Oncogene.

**Gene-expression microarray and Northern-blot analysis.** cDNA was prepared from J774A.1 macrophages at 4 and 24 h after infection by use of 2  $\mu$ g of RNA treated with DNA-Free (Ambion). Panorama Apoptosis Arrays (Sigma) were hybridized and exposed, in accordance with the manufacturer's instructions. Polymerase chain reaction for  $\beta$ -actin was performed on RNA samples as described elsewhere [28], to confirm the absence of genomic DNA. Array analysis was performed twice at each time point with 2 separately prepared RNA samples. Stringency limits of 4-fold or more from baseline consistent in both sets of experiments were considered to be significant. To confirm significant changes in gene expression, Northern-blot analysis was used [29].

**Measurement of NO and cytokine protein concentrations.** The Greiss reaction was used to determine NO concentrations in supernatants and cell lysates [30–32]. Triplicate samples were assayed in duplicate [27, 33]. Results are reported as means  $\pm$

**Table 1. Gene expression in group B streptococcus-infected murine macrophage-like J774A.1 cells.**

Gene	Fold up-regulated	
	4 h	24 h
Apoptosis related		
iNOS	<4.0	10 ± 6
TNFR-I	<4.0	<4.0
TNFR-II	5 ± 1	6 ± 3
TNF-α	58 ± 18	18 ± 1
IL-1ra	10 ± 1	12 ± 5
IL-1RI	<4.0	<4.0
IL-1RII	<4.0	<4.0
IL-1α	140 ± 54	221 ± 39
IL-1β	91 ± 37	640 ± 463
Control <sup>a</sup>		
β-Actin	-1.2 ± 0	0.1 ± 0.1
β2 microglobulin	1.5 ± 0.2	1.2 ± 0.2
Cyclophilin A	-1.4 ± 0.2	-0.2 ± 1.2
HPRT	-1.3 ± 0.1	-0.1 ± 1.1
L19	1.3 ± 0	-1.1 ± 0.1
Transferrin receptor	-1.6 ± 0.2	0.4 ± 1.5

**NOTE.** Data are mean ± SE fold changes for 2 independent experiments. RNA was collected from infected and noninfected (control) J774A.1 macrophages at 4 and 24 h after infection, and microarray analysis was performed by use of a targeted array of genes involved in apoptosis. HPRT, hypoxanthine phosphoribosyltransferase; IL, interleukin; IL-1R, IL-1 receptor; IL-1ra, IL-1 receptor antagonist; iNOS, inducible nitric oxide synthase; L19, ribosomal protein L19; TNF, tumor necrosis factor; TNFR, TNF receptor.

<sup>a</sup> Control genes included are from array membranes that showed no significant changes in expression in infected or control macrophages over time.

SE of at least 3 independent experiments. TNF-α, IL-1α, IL-1β, and IFN-γ concentrations were measured by ELISA (Endogen). For intracellular cytokines, lysates were prepared by sonicating macrophages in 1 mL of TCM with 10 μmol/L aprotinin A and 10 μmol/L leupeptin on ice and then were clarified. Results are reported as means ± SE of 2 independent experiments.

#### Determination of antimicrobial activity of NO against GBS.

Antimicrobial activity of NO was determined by incubating  $1 \times 10^4$  cfu in 0.2 mL of M9 minimal media in microtiter plates (Corning) containing either 0, 150, or 750 μmol/L SNAP as a source of NO. M9 minimal media was used to eliminate the potential antagonism of thiol-containing groups in TCM [34]. After incubation for 20 h at 37°C in 5% CO<sub>2</sub>, 100 μL of Todd-Hewitt broth (THB) (or, for *S. pyogenes*, THB plus 2% yeast extract) was added, and plates were incubated at 37°C in 5% CO<sub>2</sub>. Bacterial growth was monitored over the course of 14 h by measuring optical density at 600 nm by use of a microtiter plate reader (Molecular Devices). *S. pyogenes* [35] and *S. aureus*, which are known to induce NO synthesis [36] and to be susceptible to NO [37], were included as controls.

**Statistics.** Macrophage mortality was compared by the non-parametric Kruskal-Wallis test (SPSS software; version 9.0; SPSS).

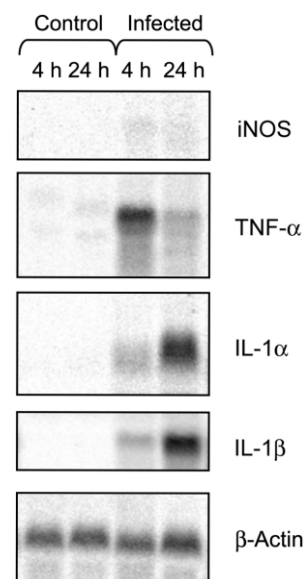
NO concentrations in infected and control macrophages were compared by analysis of variance.  $P < .05$  was considered to be significant.

## RESULTS

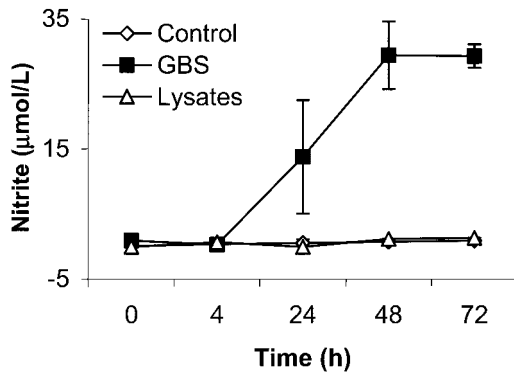
**Escape of viable GBS from infected macrophages and induction of apoptosis.** To determine whether viable GBS escape from macrophages after nonopsonic phagocytosis, we established a modified antibiotic protection assay. After 24–72 h in antibiotic-containing TCM, supernatants from infected macrophages were cultured, to confirm sterility. Fresh TCM without antibiotics was then added to the monolayers, and the cells were reincubated. The presence of viable extracellular GBS, which represented GBS that had survived intracellularly and escaped or were released after removal of antibiotics, was detected by culturing the supernatants 24 h later. In 3 experiments with a 48-h incubation, viable GBS were recovered after antibiotics had been removed. This demonstrates that, in addition to surviving intracellularly for a prolonged period and inducing apoptosis [19], viable GBS escape from infected murine macrophages after nonopsonic phagocytosis.

**Alterations in J774A.1 macrophage gene expression induced by GBS.** Genes up-regulated in J774A.1 macrophages after infection included those for iNOS, TNF-α, IL-1α, and IL-1β (table 1). Expression in Northern blots, normalized to β-actin, confirmed these findings (figure 1). Expression of housekeeping genes was not altered.

Production of NO by GBS-infected J774A.1 macrophages increased rapidly after infection (figure 2), and J774A.1 mac-



**Figure 1.** Northern-blot confirmation of the increased expression of genes shown in table 1. IL, interleukin; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor.



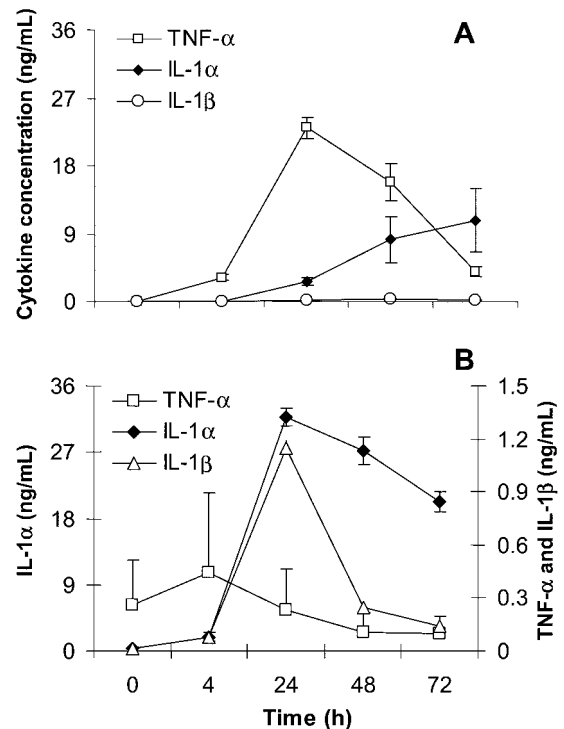
**Figure 2.** Nitric oxide (NO) production in group B streptococcus (GBS)-infected murine macrophage-like J774A.1 cells. In response to GBS, significantly higher concentrations of NO were produced by J774A.1 macrophages than by uninfected control macrophages ( $n = 3$ ;  $P < .05$ , for 24, 48, and 72 h time points [analysis of variance]).

rophages also produced TNF- $\alpha$  and IL-1 $\alpha$  (figure 3A). At 24 h, IL-1 $\beta$  was not detected in supernatants, however (figure 3A), and only low concentrations were detected in lysates (figure 3B). IFN- $\gamma$  was not detected (data not shown). Because NO, TNF- $\alpha$ , and IL-1 $\alpha$  have been implicated in apoptosis, we further investigated the roles these may play in GBS-induced apoptosis of murine macrophages.

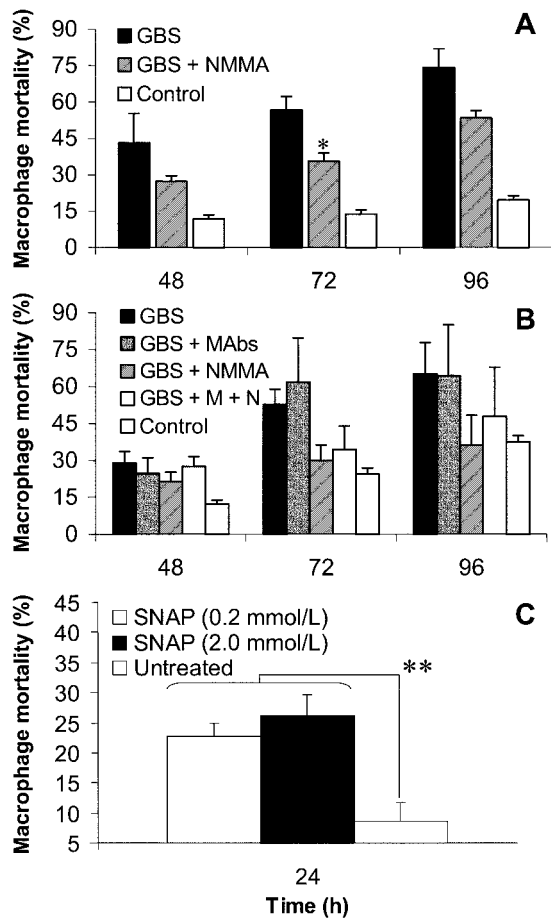
**Requirement of NO for GBS-induced apoptosis of murine macrophages.** Addition of NMMA to GBS-infected J774A.1 macrophages inhibited NO production ( $30\% \pm 5\%$  vs.  $<3 \mu\text{mol/L}$  treated) and apoptosis ( $P = .029$ ) (figure 4A). Neutralizing anti-TNF- $\alpha$  and anti-IL-1 $\alpha$  monoclonal antibodies (MAbs), in contrast, did not inhibit apoptosis (figure 4B). Treatment with NMMA, anti-TNF- $\alpha$  MAb, and anti-IL-1 $\alpha$  MAb in combination inhibited apoptosis to a degree similar to that for NMMA treatment alone (figure 4B). Incubation of noninfected macrophages with combinations of recombinant (r) TNF- $\alpha$ , rIL-1 $\alpha$ , and the NO donor SNAP was performed, to determine whether these factors are cytotoxic in the absence of infection. In preliminary experiments, a dosing schedule was determined that would maintain infection-equivalent concentrations for 48 h. At these concentrations and at 5-fold higher concentrations, neither rTNF- $\alpha$  nor rIL-1 $\alpha$  induced apoptosis (data not shown). A single dose of 0.2 mmol/L SNAP, however, triggered apoptosis ( $P < .05$ ) (figure 4C). Thus, NO, but not TNF- $\alpha$  or IL-1 $\alpha$ , triggers apoptosis of murine macrophages in the absence of GBS.

Because NO is a major macrophage antimicrobial molecule, we assessed GBS survival in NMMA-treated J774A.1 macrophages. Surprisingly, inhibition of NO did not significantly alter bacterial-clearance kinetics (figure 5). A slightly higher recovery of viable intracellular GBS from macrophages at 72 h was not significant. These results indicate that NO is not essential for killing of GBS in murine macrophages.

**GBS-induced apoptosis and responses in peritoneal macrophages.** To confirm the role played by NO in macrophage apoptosis, experiments with peritoneal macrophages from C57BL/6 and congenic iNOS $^{-/-}$  mice were performed. Peritoneal macrophages from C57BL/6 mice underwent dose-dependent apoptosis (figure 6A). An MOI of 300 induced apoptosis of peritoneal macrophages from C57BL/6 mice, and TNF- $\alpha$  secretion by peritoneal macrophages from C57BL/6 and iNOS $^{-/-}$  mice was comparable at this MOI ( $<4000 \text{ pg/mL}$ ), although higher concentrations of IL-1 $\alpha$  were secreted by peritoneal macrophages from iNOS $^{-/-}$  mice at 24 h (figure 6B). Peritoneal macrophages from C57BL/6 mice expressed higher concentrations of NO than did peritoneal macrophages from iNOS $^{-/-}$  mice ( $P < .05$ ) (figure 6C), and peritoneal macrophages from iNOS $^{-/-}$  mice exhibited comparatively less apoptosis in response to GBS ( $P < .05$ ) (figure 6D). Thus, peritoneal macrophages from C57BL/6 mice produce a high concentration of NO in response to GBS, compared with that of peritoneal macrophages from iNOS $^{-/-}$  mice, and are correspondingly more susceptible to GBS-induced apoptosis. That GBS-induced apoptosis is not completely abolished in peritoneal macrophages from iNOS $^{-/-}$  mice is consistent with the



**Figure 3.** Tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 production in group B streptococcus (GBS)-infected murine macrophage-like J774A.1 cells. Macrophages produced high levels of TNF- $\alpha$  and IL-1 $\alpha$  in supernatants (A) and in cell lysates (B). However, IL-1 $\beta$  production was comparatively minimal, despite large increases in mRNA for IL-1 $\beta$ . Data shown for cytokine assays are means  $\pm$  SE for 2 independent experiments.



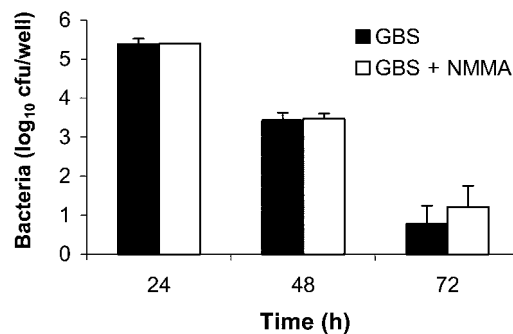
**Figure 4.** Contribution of nitric oxide (NO), but not tumor necrosis factor (TNF)- $\alpha$  or interleukin (IL)-1 $\alpha$ , to group B streptococcus (GBS)-induced apoptosis of murine macrophage-like J774A.1 cells. *A*, J774A.1 macrophages were infected with GBS and treated with 2 mmol/L N<sup>G</sup>-monomethyl-L-arginine (NMMA), and macrophage viability was determined by trypan blue staining. Significantly lower mortality in GBS-infected NMMA-treated macrophages than in infected, untreated controls is shown. *B*, Treatment of GBS-infected J774A.1 macrophages with monoclonal antibodies (MAbs) to TNF- $\alpha$  and/or IL-1 $\alpha$  did not inhibit apoptosis. Treatment of GBS-infected macrophages with NMMA, anti-TNF- $\alpha$  MAb, and anti-IL-1 $\alpha$  MAb in combination (M + N) inhibited apoptosis to a degree similar to that for NMMA treatment alone. *C*, NO induced significant apoptosis in the absence of GBS in S-nitroso-N-acetylpenicillamine (SNAP)-treated J774A.1 macrophages ( $n = 3$ ). In contrast, neither recombinant murine TNF- $\alpha$  nor IL-1 $\alpha$ , when used at infection-equivalent concentrations, induced significant apoptosis in the absence of infection (data not shown). \* $P = .029$ , Kruskal-Wallis test; \*\* $P < .05$ , Kruskal-Wallis test.

incomplete inhibition of apoptosis of NMMA-treated GBS-infected macrophages, suggesting that a second NO-independent pathway of GBS-induced apoptosis exists. Intracellular survival of GBS in peritoneal macrophages from C57BL/6 and iNOS<sup>-/-</sup> mice was not significantly different (figure 7). These results confirm that NO synthesis in murine macrophages in

response to GBS is not essential for the killing of GBS but contributes to macrophage apoptosis.

**GBS-induced apoptosis and bacterial killing in U937 HMDMs.** We next performed experiments with HMDMs derived from the U937 monocytic cell line. GBS persisted inside HMDMs for 72 h (figure 8A). Similar to observations in murine macrophages, GBS-infected HMDMs underwent dose-dependent apoptosis in response to GBS (figure 8A, inset; 48–72 h). At an MOI of  $\geq 150$ , HMDMs produced low but detectable concentrations of NO (figure 8B;  $n = 4$ ), consistent with results reported by others [38, 39]. Addition of 10 ng/mL human rIFN- $\gamma$  and/or *E. coli* LPS (20–2000 ng/mL) did not augment NO production (figure 8B). Nitrite was not observed in macrophage-free TCM (with or without GBS) [40, 41]. GBS-induced mortality in HMDMs reached 36%  $\pm$  7% at 72 h (figure 8C) and was not affected by NMMA (figure 8C). Comparison of intracellular bacterial-clearance kinetics in HMDMs demonstrated that NMMA had no effect on the intracellular survival of GBS (figure 8D). These results are consistent with our observations in murine macrophages showing a limited role for NO in intracellular killing of GBS in macrophages.

**No direct cytotoxicity of NO to GBS in vitro.** We measured the direct antimicrobial action of NO against GBS using an assay that is described elsewhere [34]. Compared with untreated GBS cultures, SNAP-treated GBS cultures showed no significant growth impairment, even when GBS was incubated with concentrations of SNAP as high as 750  $\mu$ mol/L (figure 9A). In contrast, growth of *S. pyogenes* was completely inhibited by the lowest concentration of SNAP tested (figure 9B). *S. aureus* demonstrated an intermediate phenotype (figure 9C). Hence, NO has potent, direct antimicrobial activity toward *S. pyogenes* and



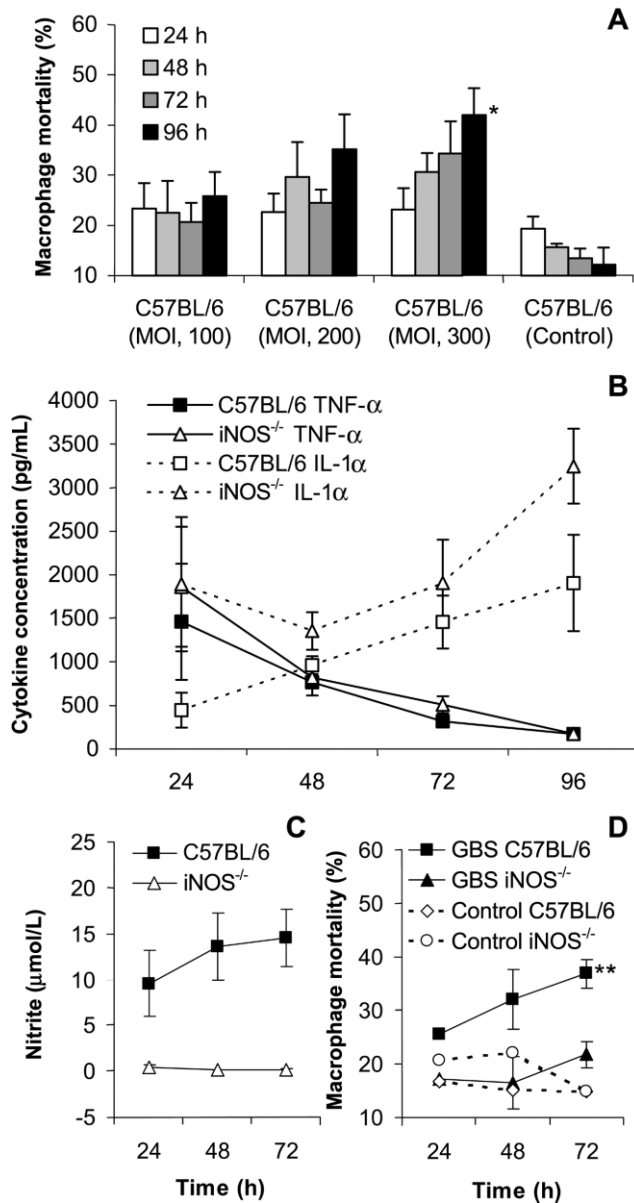
**Figure 5.** No requirement of nitric oxide (NO) for killing of intracellular group B streptococcus (GBS) in murine macrophage-like J774A.1 cells. J774A.1 macrophages were infected as described in Materials and Methods and were treated with 2 mmol/L N<sup>G</sup>-monomethyl-L-arginine (NMMA). Intracellular GBS was quantitated by disrupting infected monolayers with 0.01% Triton X-100, diluting in PBS, and dispensing lysates onto Todd-Hewitt agar for quantitative colony counts. Inhibition of NO production had no significant effect on the rate of intracellular clearance of GBS ( $n = 4$ ).

moderate antimicrobial activity toward *S. aureus* but limited direct cytotoxic effect on GBS.

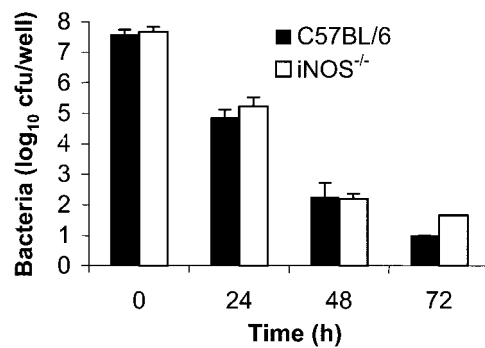
## DISCUSSION

To identify the major mediators involved in GBS-induced macrophage apoptosis, we analyzed the expression of genes for inflammatory and apoptotic mediators during the host-pathogen interaction. Phagocytosis of GBS up-regulates the expression of genes for iNOS, TNF- $\alpha$ , and IL-1 $\alpha$ . Although each of these factors has been implicated in the regulation of apoptosis in other systems, the present study has demonstrated that NO is responsible for much of the apoptosis of GBS-infected murine macrophages. As previously described [42], NO is cytotoxic to murine macrophages in the absence of infection. NO plays a similar proapoptotic role in macrophage apoptosis associated with *S. pneumoniae* infection [17]. Compared with murine macrophages, human macrophages secrete minimal amounts of NO, and NO does not appear to contribute to GBS-induced apoptosis of human macrophages. Consistent with this observation, GBS infection of peritoneal macrophages from C57BL/6 mice caused significantly more apoptosis than did GBS infection of peritoneal macrophages from iNOS<sup>-/-</sup> mice incapable of synthesizing NO from iNOS.

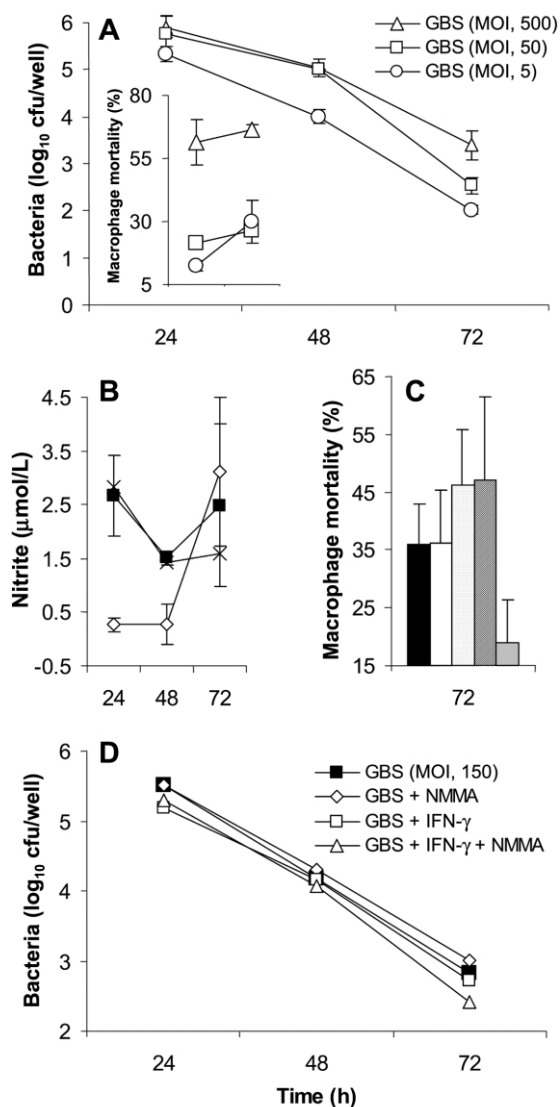
In some cell types, high concentrations of NO appear to promote mitochondrial cytochrome *c* release and caspase activation [13, 43]. At lower concentrations, NO may be cytoprotective, because of an effect associated with cyclooxygenase-2 activation [44]. Hence, NO bears the unique ability to both initiate and prevent apoptosis, depending on the cell type and nature of the insult. NO also plays dichotomous roles in models of infection. It has been reported that NO induced by *Sal-*



**Figure 6.** Group B streptococcus (GBS)-induced cytotoxicity and nitric oxide (NO)/cytokine responses in peritoneal macrophages from C57BL/6 and congenic inducible NO synthase (iNOS)-deficient B6.129P2-*Nos<sup>tm1Lau</sup>* (iNOS<sup>-/-</sup>) mice. *A*, Dose-dependent cytotoxicity of GBS toward peritoneal macrophages from C57BL/6 mice. *B*, Concentrations of tumor necrosis factor (TNF)- $\alpha$  detected in cultures of peritoneal macrophages from C57BL/6 and iNOS<sup>-/-</sup> mice were similar after GBS infection, although higher concentrations of interleukin (IL)-1 $\alpha$  were detected at 24 h in cultures of iNOS<sup>-/-</sup> macrophages. Synthesis of NO in GBS-infected peritoneal macrophages from C57BL/6 mice (*C*) correlated with increasing cytotoxicity in these cells (*D*), in contrast to the significantly better survival of macrophages from iNOS<sup>-/-</sup> mice and lack of NO production in these cells ( $n = 4$ ). \* $P < .05$ , vs. MOI of 100; \*\* $P < .05$ , for GBS C57BL/6 at 72 h vs. GBS iNOS<sup>-/-</sup> at 72 h (Kruskal-Wallis test and analysis of variance).



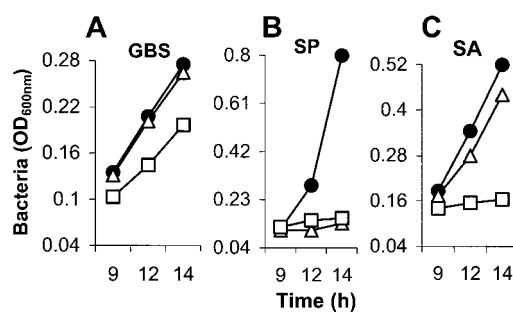
**Figure 7.** No requirement of nitric oxide (NO) for killing of intracellular group B streptococcus (GBS) in peritoneal murine macrophages. Peritoneal macrophages from C57BL/6 and congenic inducible NO synthase (iNOS)-deficient B6.129P2-*Nos<sup>tm1Lau</sup>* (iNOS<sup>-/-</sup>) mice were infected as described in Materials and Methods, and intracellular GBS was quantitated by disrupting infected monolayers with 0.01% Triton X-100, diluting in PBS, and dispensing lysates onto Todd-Hewitt agar for quantitative colony counts. Survival of GBS in peritoneal macrophages from C57BL/6 and iNOS<sup>-/-</sup> mice was not significantly different at any time point ( $n = 4$ ).



**Figure 8.** Survival of group B streptococcus (GBS) within U937 human monocyte-derived macrophages (HMDMs). GBS triggers apoptosis of HMDMs independent of nitric oxide (NO), and NO is not essential for killing of intracellular GBS. HMDMs were cultured, differentiated, and infected as described in Materials and Methods. **A**, Prolonged intracellular bacterial survival (at MOIs from 500 to 5) and dose-dependent GBS-induced HMDM apoptosis was observed to a degree and with kinetics similar to those observed for murine peritoneal macrophages (mortality is inset; 48–72 h is shown). **B**, Minimal production of NO in HMDMs in response to GBS was observed at an MOI of 150 (■), regardless of exogenous stimulation with 10 ng/mL human interferon (IFN)- $\gamma$  and 20 ng/mL lipopolysaccharide (×), compared with that in noninfected control (◇) cells. **C**, Apoptosis of HMDMs infected with GBS at an MOI of 150 (black bar) was not significantly affected by the presence of N<sup>G</sup>-monomethyl-L-arginine (NMMA) (white bar). Interestingly, IFN- $\gamma$  amplified GBS-induced macrophage cytotoxicity, both when IFN- $\gamma$  alone (dotted bar) and when IFN- $\gamma$  plus NMMA (hatched bar) were used. Noninfected control cells are shown by the gray bar. **D**, Intracellular GBS survival in HMDMs was not affected by the presence of NMMA or IFN- $\gamma$ .

*monella* species [15] and *Actinobacillus* species [45] has anti-apoptotic activity but that NO induced by *Helicobacter pylori* [46] and *Mycobacterium tuberculosis* [47] has proapoptotic activity. Our experiments demonstrated that NO, when present at sufficiently high concentrations, was proapoptotic in the murine macrophage response to GBS. Inhibition of NO, however, did not completely abolish GBS-induced apoptosis of murine macrophages, and peritoneal macrophages from iNOS<sup>-/-</sup> mice were not completely protected from bacteria-induced apoptosis. This, we believe, suggests a complex host-pathogen interaction in murine macrophages in which additional NO-independent apoptotic pathways may be triggered by GBS. These pathways appear to be critical in human macrophages—in the present study, HMDMs produced minimal concentrations of NO in response to GBS—but less significant in murine cells.

In addition to modulating pathways of apoptosis, NO plays a major role in macrophage antimicrobial defense against some pathogens [48]. Surprisingly, our experiments showed that NO has little influence on the elimination of intracellular GBS in macrophages, at least in vitro. NMMA-treated J774A.1 macrophages, peritoneal macrophages from iNOS<sup>-/-</sup> mice, and NMMA-treated HMDMs cleared intracellular GBS as efficiently as did control cells. The absence of direct antimicrobial action of NO toward GBS was confirmed by in vitro bacterial-suspension growth-inhibition assays. The resistance of GBS to NO may be related to the presence of genes that encode products associated with efflux mechanisms or resistance to oxygen/nitrogen radicals. In *E. coli* and *M. tuberculosis*, for example [49], peptide methionine sulfoxide reductase confers increased resistance to reactive nitrogen intermediates through unknown mechanisms. Similarly, *Neisseria gonorrhoeae* monomeric pilin protein PilB increases its ability to resist oxidative damage [50].



**Figure 9.** Direct antimicrobial activity of nitric oxide (NO) toward group B streptococcus (GBS). Bacteria were cultured in M9 minimal media in the presence of 0 (●), 150 (△), or 750 (□) μmol/L S-nitroso-N-acetylpenicillamine (SNAP), as described in Materials and Methods. Twenty hours later, fresh growth media was added and bacterial growth was monitored by measurement of optical density at 600 nm over a 14-h period. Growth of GBS was not significantly affected by the presence of SNAP. However, SNAP was potently bacteriostatic to *Streptococcus pyogenes* (SP) even at low concentrations and inhibited growth of *Staphylococcus aureus* (SA) at high concentrations.

We searched the National Center for Biotechnology Information database for homologues of *msrA* in *S. agalactiae* 2603V/R (serotype V) and NEM316 (serotype III) but found no significant similarities. It is possible that other mechanisms are in place in GBS that confer increased resistance to reactive nitrogen intermediates. NO may, however, exert antimicrobial activity toward GBS in vivo via interactions not readily modeled under the experimental conditions used here [51]. A recent study [52] demonstrated that GBS carotenoid pigment is protective against oxidative damage from reactive oxygen species produced by macrophages.

Another study, by Hoehn et al. [53], showed that gaseous NO has a bacteriostatic effect on GBS. In that study, bacterial cultures on agar were exposed to gaseous NO, and growth was measured after a 24-h incubation. Three key differences may explain the discrepant results with regard to the effects of NO on growth of GBS. First, different forms of NO were used (gaseous vs. SNAP derived) under different growth conditions (agar vs. suspension culture), limiting comparison of the 2 studies. The concentrations and physiological availability of NO from the different sources may differ. THB provides a less restrictive growth environment than does agar, which may improve recovery of GBS. Moreover, potential differences in NO-induced pH changes in agar versus suspension cultures may affect bacterial viability [54]. It is also important to note that, in the study by Hoehn et al., gaseous NO was not bacteriostatic to GBS at lower, clinically relevant NO concentrations [54]. Second, the bacterial inoculum used to assess the antimicrobial effect of NO in these systems may influence interpretation of NO susceptibility testing results. In the study by Hoehn et al., inhibition of GBS growth by high concentrations of gaseous NO was only significant at exceedingly low GBS inocula. In the present study, we used  $\geq 1 \times 10^4$  bacteria, an inoculum more reflective of the typical clinical situation, in which the neonatal lung is infected with substantial numbers of GBS [55]. In a different study that assessed NO susceptibility in *Salmonella typhimurium*, similarly high bacterial inocula were used [34]. Finally, 2 other organisms (*S. aureus* and *Pseudomonas aeruginosa*) tested for susceptibility to gaseous NO in the study by Hoehn et al. were unaffected by the presence of high concentrations of gaseous NO. These findings contradict reports that indicate that NO synthesized from iNOS exerts antimicrobial activity against these organisms [56, 57].

Because macrophages kill GBS inefficiently, persistence within macrophages might allow GBS to evade opsonization and recognition by neutrophils. GBS infections are typically treated with antibiotics that penetrate cells poorly, and this may exacerbate this effect [58]. Although phagocytosis might temporarily benefit GBS, these bacteria are poorly adapted to the intracellular environment and would presumably benefit from macrophage death. Consistent with this hypothesis, we have

shown that viable GBS escape from macrophages after phagocytosis. The ability of GBS to manipulate macrophages in this manner may contribute to the characteristic persistence of GBS colonization and infection [58].

We used the J774A.1 cell line and primary murine peritoneal macrophages to model GBS infection. Numerous studies have employed J774A.1 macrophages as an in vitro model of pathogenesis [59–63]. In some cases, however, murine macrophages display increased susceptibility to pathogen-induced cytotoxicity or other dissimilar responses, compared with human cells [64, 65]. This may be related to the efficiency of phagocytosis or killing, or this may reflect inherent differences between the responses of murine and human cells. Specifically, murine macrophages may elaborate more NO than human macrophages (reviewed in [66–69]). Indeed, studies have shown that bacterial infection of human macrophages often does not result in significant NO production [68].

We used HMDMs to compare the results of GBS infection in murine macrophages. PMA-differentiated U937 HMDMs display characteristics of fully mature human macrophages [22]. GBS infection of HMDMs demonstrated that, compared with murine macrophages, NO production in response to GBS is low. NMMA treatment of GBS-infected HMDMs had no effect on intracellular bacterial clearance, confirming our hypothesis that NO is not an antimicrobial effector molecule against GBS in macrophages. Production of reactive oxygen species by GBS-infected macrophages may play a more prominent role in antimicrobial effector activity [52]. It is also important to highlight several recent studies of bacteria-induced NO in human macrophages in the context of the present experiments. In contrast to the findings of the present study, it has been reported that pneumococcal infection of human macrophages [17] results in NO synthesis and contributes to the apoptotic response. These observations demonstrate that NO acts as an important regulatory molecule in the response of human macrophages to some bacteria [70]. Human macrophages possess the machinery to generate NO [14, 68, 71], and it may be that the in vitro conditions used here limit responses that occur in vivo. To further define the host apoptotic pathways important in human immune responses to GBS infection, extensions of the observations presented here will be significant.

The present study has demonstrated that NO is a critical, but not an exclusive, determinant of GBS-induced apoptosis of murine macrophages but that NO is not necessary for the killing of GBS. Production of NO during infection may impair immune responses by causing elimination of macrophages. Additional studies are needed to understand the complex interactions between GBS and macrophages. Study of the death pathways that are activated to bring about apoptosis may lead to the identification of novel interventional strategies that can improve the outcome of these serious infections.

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