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# Activation of Acid Sphingomyelinase and Its Inhibition by the Nitric Oxide/Cyclic Guanosine 3',5'-Monophosphate Pathway: Key Events in *Escherichia coli*-Elicited Apoptosis of Dendritic Cells<sup>1</sup>

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Depletion of dendritic cells (DCs) via apoptosis contributes to sepsis-induced immune suppression. The mechanisms leading to DC apoptosis during sepsis are not known. In this study we report that immature DCs undergo apoptosis when treated with high numbers of *Escherichia coli*. This effect was mimicked by high concentrations of LPS. Apoptosis was accompanied by generation of ceramide through activation of acid sphingomyelinase (A-SMase), was prevented by inhibitors of this enzyme, and was restored by exogenous ceramide. Compared with immature DCs, mature DCs expressed significantly reduced levels of A-SMase, did not generate ceramide in response to *E. coli* or LPS, and were insensitive to *E. coli*- and LPS-triggered apoptosis. However, sensitivity to apoptosis was restored by addition of exogenous A-SMase or ceramide. Furthermore, inhibition of A-SMase activation and ceramide generation was found to be the mechanism through which the immune-modulating messenger NO protects immature DCs from the apoptogenic effects of *E. coli* and LPS. NO acted through formation of cGMP and stimulation of the cGMP-dependent protein kinase. The relevance of A-SMase and its inhibition by NO/cGMP were confirmed in a mouse model of LPS-induced sepsis. DC apoptosis was significantly higher in inducible NO synthase-deficient mice than in wild-type animals and was significantly reduced by treatment ex vivo with NO, cGMP, or the A-SMase inhibitor imipramine. Thus, A-SMase plays a central role in *E. coli*/LPS-induced DC apoptosis and its inhibition by NO, and it might be a target of new therapeutic approaches to sepsis. *The Journal of Immunology*, 2004, 173: 4452–4463.

Sepsis, a life-threatening disorder, is the most common cause of death in intensive care units of the industrialized world, and its incidence continues to increase (1). Despite efforts in the identification of novel therapeutic strategies, no significant improvement in the outcome of the disease has taken place in the last decades (2). Recognition of patterns expressed by microorganisms can initiate a deregulated innate response in septic patients, with exaggerated production of proinflammatory mediators, hypotension, inadequate tissue perfusion, and ensuing failure of multiple organs (2). An additional major feature of septic patients, immune suppression, prevents clearing of invading agents, promoting further activation of nonprotective, inflammatory responses, with frequent inability to eradicate primary infections and propensity to secondary nosocomial infections (3–6). Various defects in immunological functions, including depression of Th1 and Th2 cytokines, loss of CD4<sup>+</sup> T and B cells, and monocyte deac-

tivation, have been reported (4–8). Recent studies, conducted in both an animal model and tissues from affected patients, demonstrated a profound depletion of dendritic cells (DCs),<sup>3</sup> including interdigitating and follicular cells, due to death via apoptosis (9, 10). DCs play fundamental roles in the coordination of the immune responses against invading microorganisms, including sustaining the growth and viability of activated T and B cells and polarization of CD4<sup>+</sup> T cells toward a defined Th phenotype (11, 12). Depletion of DCs might thus be relevant to the pathogenesis and maintenance of immune suppression during sepsis. The mechanisms by which sepsis triggers DC apoptosis are at present largely unexplored. Elucidation of these mechanisms might ultimately be of great help in defining novel therapeutic strategies to treat this disorder.

Among sepsis-causing organisms, Gram-negative bacteria are estimated to result in 20,000 death annually in the U.S. (13). LPS, a potent inflammatory glycolipid residing in the outer membrane of these bacteria, contributes to the pathogenesis of Gram-negative septic shock (14). LPS exerts its effects through the activation of a variety of messenger molecules, including transcription factors, G proteins, protein kinases, and the sphingolipid ceramide (15–23). Ceramide derives from sphingomyelin hydrolysis, conducted by the neutral and/or the acid sphingomyelinases, or through de novo

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; A-SMase, acid sphingomyelinase; 8-Br-cGMP, 8-bromo-cGMP; CMTMR, 5-(-)-(((chloromethyl)benzoyl)amino)tetramethylrhodamine; D609, tricyclodecan-9-yl xanthate; DETA-NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazene-1-ium-1,2-diolate; G kinase, cGMP-dependent protein kinase; iNOS, inducible NO synthase; ODQ, H-(1,2,4)-oxadiazolo[4,3-*q*]quinoxalin-1-one; w.t., wild-type.

synthesis from sphinganine. Ceramide has been shown to be proapoptotic in many cell types, contributing to the effects induced by death receptor activation and environmental and chemotherapeutic agents (24–26). A role for ceramide in the apoptogenic effect of LPS has been demonstrated in endothelial cells (27); in addition, it triggers apoptosis of DCs (28).

In this study we investigated the effect of the Gram-negative bacterium, *Escherichia coli*, on the survival of DCs. We found that high numbers of *E. coli* induced apoptosis of immature DCs in vitro, and that this effect was mimicked by high concentrations of LPS. Such responses required the activation of acid sphingomyelinase (A-SMase) and the ensuing ceramide generation. We also found that A-SMase is a target for NO, a gaseous messenger that plays important roles in inflammation partly through the regulation of apoptosis in immune-competent cells (29–33). NO inhibited A-SMase activation and ceramide generation in a pathway involving formation of cGMP and stimulation of the cGMP-dependent protein kinase (G kinase). Inhibition of A-SMase by NO/cGMP resulted in protection of immature DCs from apoptosis induced by *E. coli* and LPS. Using an in vivo model of sepsis, we confirmed the biological relevance of A-SMase activation and its regulation by endogenous NO in determining the survival of DCs during sepsis. Our findings reveal for the first time the pathway regulating DC apoptosis in sepsis induced by Gram-negative bacteria.

## Materials and Methods

### Materials

The following reagents were purchased as indicated: FITC-labeled mouse mAbs anti-human or mouse CD1a, MHC class I and class II, CD80, CD86, CD40, CD14, CD3, CD11c, and PE-labeled anti-mouse CD11b from BD Pharmingen (San Diego, CA); mouse mAbs anti-inducible NO synthase (anti-iNOS) from BD Transduction Laboratories (Lexington, KY); FITC-labeled mouse-mAb anti TLR4 (HTA125) from Abcam (Cambridge, U.K.); FITC-labeled goat-anti-rabbit and Cy7-labeled goat anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA); FITC-labeled recombinant human annexin V from Bender MedSystems (Wien, Austria); recombinant human TNF- $\alpha$ , (Z)-1-[2-(2-aminoethyl)-N-(2-aminoethyl)amino]diazene-1-ium-1,2-diolate (DETA-NO), and H-(1,2,4)-oxadiazolo[4,3-*q*]quinoxalin-1-one (ODQ) from Alexis Italia (Florence, Italy); diacylglycerol kinase from BIOMOL (Hamburg, Germany); C2 ceramide, tricyclodecan-9-yl xanthate (D609), and KT5823 from Calbiochem (Bad Soden, Germany); recombinant human IL-4 from BD Pharmingen recombinant human GM-CSF from Schering Plough (Milan, Italy); recombinant mouse IL-4 and GM-CSF from R&D Systems (Minneapolis, MN); Ficoll-Paque from BioChrom (Berlin, Germany); magnetic Dynabeads M-450 goat anti-mouse IgG from Dynal Biotech (Oslo, Norway); [ $\gamma$ - $^{32}$ P]ATP, [*N*-methyl- $^{14}$ C]sphingomyelin, and Percoll from Amersham Biosciences (Little Chalfont, U.K.); the *E. coli* DH5 $\alpha$  strain from BD Clontech (San Diego, Ca); the Cell Tracker Orange 5-(–6)-((chloromethyl)benzoyl)amino tetramethyl rhodamine (CMTMR) from Molecular Probes (Eugene, OR); and LPS (from *E. coli* serotype 055:B5), imipramine, manumycin A, human placental A-SMase, and all other chemicals from Sigma-Aldrich (St. Louis, MO). Reagents for cell culture were purchased from Invitrogen Life Technologies (Gaithersburg, MD), except for FCS (clone III), which was obtained from HyClone-Celbio (Milan, Italy). The goat anti A-SMase serum (described in Refs. 34 and 35) and scyphostatin were gifts from Dr. E. Gulbins (University of Essen, Essen, Germany) and Dr. T. Ogita (Sankyo, Tokyo, Japan), respectively.

### Animals

Wild-type (w.t.) female C57BL/6 (H-2<sup>b</sup>) mice, 6–8 wk of age, were purchased from Charles River Laboratories (Calco, Italy). Female C57BL/6 iNOS<sup>–/–</sup> mice (The Jackson Laboratory, Bar Harbor, ME) were provided by Dr. S. Cuzzocrea (University of Messina, Messina, Italy). Animals were housed in the pathogen-free facility at our institution and treated in accordance with the European Community guidelines and the approval of the institutional ethical committee.

### Preparation of immature and mature DCs

Human immature DCs were obtained from PBMC by 5-day culture in the presence of human GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) exactly as

previously described (36). Depletion of residual T lymphocytes from immature DCs was routinely performed by incubating the cell preparations with the anti-CD3 mAb (1  $\mu$ g/10<sup>6</sup> cells) and goat anti-mouse IgG-coated Dynabeads M-450 as previously described (37). Residual lymphocytes accounted for <1% of the total cells. Mouse immature DCs were obtained from bone marrow precursors harvested from femurs and tibias of C57BL/6 mice by a 5-day culture with recombinant murine GM-CSF (20 ng/ml) and IL-4 (5 ng/ml) exactly as previously described (38). Human mature DCs were obtained from immature DCs by a 48-h incubation in culture medium in the presence of human TNF- $\alpha$  (50 ng/ml). Maturation was routinely assessed by measuring the exposure on the plasma membrane of specific molecules expressed by immature DCs and known to be increased in mature DCs, namely MHC class I and class II molecules, CD80, CD86, and CD40, involved in T cell costimulation. The expression of these molecules was analyzed by flow cytometry using a FACS (FACStar Plus; BD Biosciences, Sunnyvale, CA) after staining with appropriate FITC-labeled Abs as previously described (36, 37). Expression of the macrophage marker CD14 was never observed. The same protocol was used to measure the surface expression levels of TLR4 in both mature and immature human DCs. To detect intracellular A-SMase, cells were previously fixed with paraformaldehyde (4% in PBS for 10 min at room temperature), then permeabilized for 20 min at room temperature in PBS supplemented with 1% BSA and 0.1% saponin. The primary Ab was detected by flow cytometry using a FITC-labeled goat anti-rabbit secondary Ab. An isotype-matched Ab was used as a control.

### Bacterial culture and preparation

*E. coli* DH5 $\alpha$  cells were cultured to late log phase in Luria broth at 37°C, centrifuged, heat-inactivated (30 min at 56°C), and resuspended (10<sup>11</sup> cells/ml) in culture medium. The bacteria were added to immature and mature DCs at various *E. coli*:DC ratios for 48 h. Some of the experiments were repeated using live bacteria, with results consistent with those obtained with the inactivated bacteria. In these experiments DCs were exposed to *E. coli* for 1 h at various *E. coli*:DC ratios; bacteria were then removed by washing, medium was supplemented with gentamicin (50  $\mu$ g/ml) and tetracycline (30  $\mu$ g/ml), and DCs were maintained in the culture medium for an additional 47 h.

### Pharmacological treatments

The conditions and concentrations of use of the various compounds interfering with the SMase/ceramide and NO signaling pathways have been described in detail previously (31, 33, 36, 37, 39). In brief, cell incubations with D609, manumycin A, scyphostatin, DETA-NO, 8 Br-cGMP, ODQ, KT5823, C2 ceramide, and exogenous A-SMase were performed for 10 min and those with imipramine for 1 h before LPS or *E. coli* administration. In the experiments in which cells were exposed to DETA-NO, the compound was dissolved in the culture medium 20 min before addition to the cells. Under these conditions the compound releases constant concentrations of NO, as measured using an NO-detecting electrode with a sensitivity of 1 nM (Mark-2 ISO NO, World Precision Instruments, Sarasota, FL) (40).

All solutions were prepared endotoxin-free. Endotoxin contamination in all drug solutions was routinely assessed by the *Limulus* amoebocyte gelification test, performed according to the manufacturer's instructions (PBI, Milan, Italy). All reagents, including the medium, scored negative.

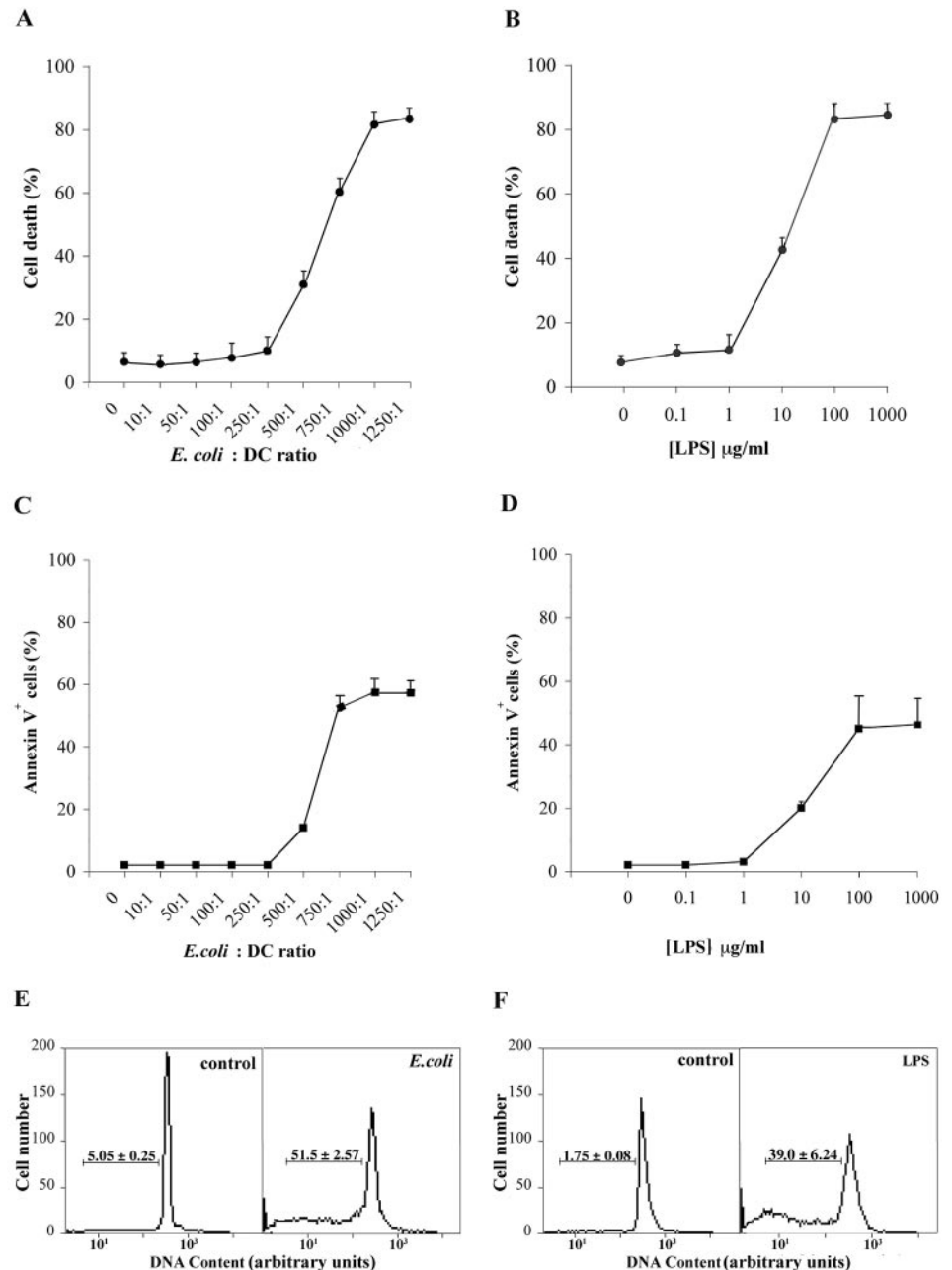
### Apoptosis detection

After pretreatment with the various compounds, DCs were treated at 37°C for 48 h in the culture medium with various concentrations of LPS or *E. coli*. Apoptosis was measured by flow cytometry, using two different protocols (29). In the first, phosphatidylserine exposure in propidium iodide-excluding cells was monitored by staining for 15 min at room temperature with FITC-labeled annexin V (2  $\mu$ g/ml). In the second approach, cells were ethanol-fixed, permeabilized with 0.01% Nonidet P-40, and stained with 50  $\mu$ g/ml propidium iodide (29). The hypodiploid DNA peak in single-parameter DNA histograms typical of apoptotic cells was then identified.

### Measurement of ceramide concentrations

DCs (10<sup>6</sup> cells/sample), preincubated at 37°C in 80  $\mu$ l of culture medium with the various compounds, were exposed to LPS (100  $\mu$ g/ml). At the time points indicated, incubation was stopped by the addition of 300  $\mu$ l of ice-cold CH<sub>3</sub>OH/CHCl<sub>3</sub> (2/1, v/v). Samples were then supplemented with 100  $\mu$ l of CHCl<sub>3</sub> and 100  $\mu$ l of NaCl (1 M). Ceramide generation was assessed by the diacylglycerol kinase assay, followed by TLC as described previously (36).

**FIGURE 1.** LPS and *E. coli* induce cell death via apoptosis of immature human DCs. *A–D*, Immature human DCs were exposed for 48 h to *E. coli* at various ratios or to increasing concentrations of LPS, as indicated in each panel. Cell samples were then collected and analyzed for various parameters describing cell death. *A* and *B*, Cell death, considered the percentage of cells that stained positively for annexin V, propidium iodide, or both ( $n = 5$ ). *C* and *D*, Apoptosis, assessed by flow cytometry measuring annexin V staining in propidium iodide-excluding cells ( $n = 4$ ). *E* and *F*, DNA content analyzed by flow cytometry measuring the binding of propidium iodide in permeabilized cells (*x*-axis, arbitrary units). DC treatment with *E. coli* (*E. coli*:DC ratio, 750:1) and LPS (100  $\mu\text{g}/\text{ml}$ ) results in the formation of a sub- $G_1$ , hypodiploid DNA peak. The panels show an experiment representative of four reproducible ones performed. Numbers in the panels indicate the percentage of cells  $\pm$  SEM, measured in the fluorescence range indicated by the bars, in the four experiments..



### Measurement of SMase activities

DCs ( $2 \times 10^6$  cells/ml) preincubated at 37°C in culture medium with the various compounds were then treated with LPS (100  $\mu\text{g}/\text{ml}$ ). At the time points indicated, the incubations were terminated by rapid immersion of reaction tubes in a methanol/dry ice bath. The suspensions were centrifuged, and the cell pellets were washed once with ice-cold PBS. Pellets were homogenized, supplemented with [*N*-methyl-<sup>14</sup>C]sphingomyelin (55 mCi/mmol; 50,000 dpm/assay), and acid and neutral SMase activities were determined by measuring the conversion of sphingomyelin to phosphorylcholine as previously described (41).

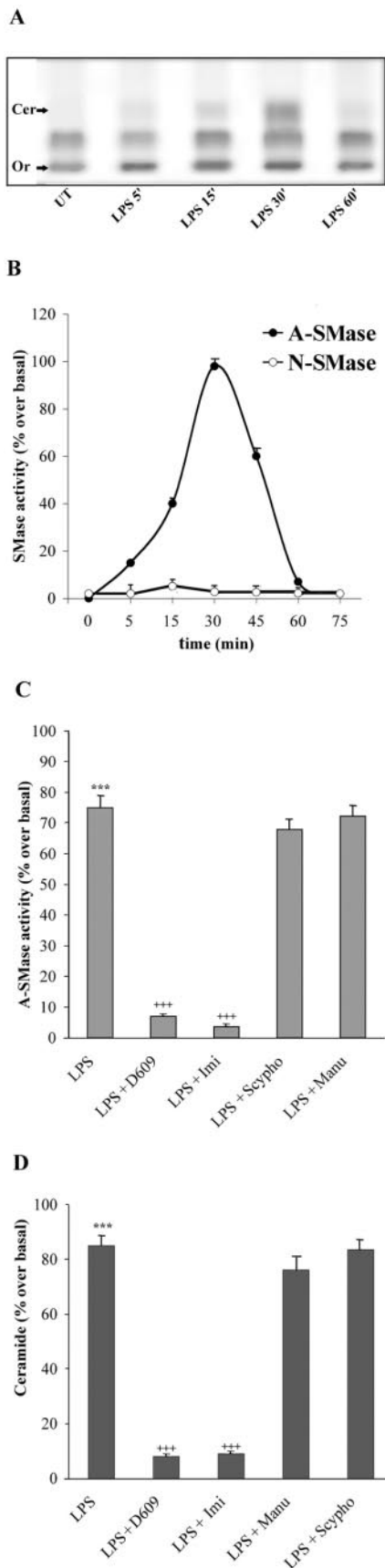
### In vivo studies

Immature murine w.t. and iNOS<sup>-/-</sup> DCs were cultured in the presence or the absence of DETA-NO (10  $\mu\text{M}$ ), 8-bromo-cGMP (8-Br-cGMP; 1 mM), or imipramine (20  $\mu\text{M}$ ) for 16 h and labeled with 10  $\mu\text{M}$  of the fluorescent dye CMTMR for 30 min (38). In vitro analysis showed that DC retained the dye for up to 72 h after loading. The various DC preparations ( $2.0 \times 10^6$  cells in 100  $\mu\text{l}$ ) were injected into the spleens of C57BL/6 female w.t. or iNOS<sup>-/-</sup> mice (groups were of 16 mice for each DC preparation). Thirty minutes later, 11 animals/group were injected i.p. with a high dose of LPS

(20 mg/kg), and five were given vehicle (control) as previously described (42). None of the vehicle-injected animals died during the experimental period. The survival of LPS-treated animals did not vary significantly among the groups treated with the various DC preparations. Animals were killed by neck dislocation at various time points as indicated in *Results*, and single spleen cell suspensions prepared. Cell numbers obtained from each spleen were determined and ranged between 98 and  $105 \times 10^6$ . No significant differences were observed in the numbers of cells recovered from control and LPS-animals. Samples of  $1 \times 10^6$  cells from each preparation were analyzed by flow cytometry, and CMTMR<sup>+</sup> cells were identified as DCs, because in control experiments they stained positively for the CD11c DC marker. DC apoptosis was measured by flow cytometry after annexin V staining. The iNOS expression in total spleen cell suspensions, macrophages, and DCs was measured by flow cytometry in permeabilized cells as described above for A-SMase.

### Statistical analysis

The results are expressed as the mean  $\pm$  SEM;  $n$  represents the number of individual experiments. Statistical analysis was performed using Student's *t* test for unpaired variables (two-tailed).



**FIGURE 2.** Exposure of immature human DCs to LPS results in activation of A-SMase and generation of ceramide. Immature human DCs were treated with LPS (100  $\mu\text{g}/\text{ml}$ ) for different periods (A and B) or for 30 min (C and D) with or without D609 (25  $\mu\text{g}/\text{ml}$ ), imipramine (Imi; 20  $\mu\text{M}$ ), scyphostatin (Scypho; 1  $\mu\text{M}$ ), or manumycin A (Manu; 5  $\mu\text{M}$ ) as indicated

## Results

### *E. coli* and LPS trigger apoptotic death of immature human DCs in a concentration-dependent way

Human monocyte-derived immature DCs were treated for 48 h in the culture medium with increasing amounts of either *E. coli* or LPS. Cell death was barely detectable up to a bacteria:DC ratio of 250:1 and an LPS concentration of 1  $\mu\text{g}/\text{ml}$ . At higher concentrations, however, both stimuli triggered DC death (Fig. 1, A and B), which was mostly due to apoptosis, because it was accompanied by phosphatidylserine exposure to the outer leaflet of the DC plasma membrane (measured by annexin V staining in propidium iodide-excluding cells; Fig. 1, C and D) and by the appearance of a hypodiploid DNA peak (Fig. 1, E and F).

### LPS activates A-SMase and generates ceramide in immature human DCs

Because LPS mimicked the effect of *E. coli* in inducing apoptosis of DCs, we decided to use the glycolipid to identify the signaling events involved in this process, concentrating on the ceramide signaling pathway. LPS (100  $\mu\text{g}/\text{ml}$ ) triggered an early wave of ceramide generation, which was detectable after 5 min, peaked at 30 min, and decreased thereafter (Fig. 2A). No further increases in ceramide were detected in the following 4 h (not shown). Ceramide can be generated either through the de novo pathway or after hydrolysis of sphingomyelin by plasma membrane SMases. These pathways, however, have different patterns of activation during apoptosis, with activation of SMases occurring as an early event, and stimulation of the de novo pathway beginning later (24–26). We therefore concentrated on SMases. As shown in Fig. 2B, LPS triggered a time-dependent activation of A-SMase, whereas it did not activate the neutral SMase. To assess the role of A-SMase in LPS-induced ceramide generation, we used a panel of structurally unrelated inhibitory compounds. To inhibit A-SMase, we used imipramine, which induces proteolysis of the enzyme (31, 33–35), and D609, which inhibits the phosphatidylcholine-specific phospholipase C, an enzyme known to be involved in A-SMase activation by LPS and other stimuli through generation of diacylglycerol (22, 23, 31, 33, 39, 43). As controls of specificity we used scyphostatin and manumycin A, which are inhibitors of the neutral SMase (39, 44, 45). When administered alone, none of the compounds had any effect on basal sphingomyelin hydrolysis (not shown). At the concentrations used, however, imipramine (20  $\mu\text{M}$ ) and D609 (25  $\mu\text{g}/\text{ml}$ ) inhibited both A-SMase activity and ceramide generation triggered by LPS, whereas scyphostatin (1  $\mu\text{M}$ ) and manumycin A (5  $\mu\text{M}$ ) did not have any effect (Fig. 2, C and D). These results indicate that activation of A-SMase accounts for the generation of ceramide triggered by LPS.

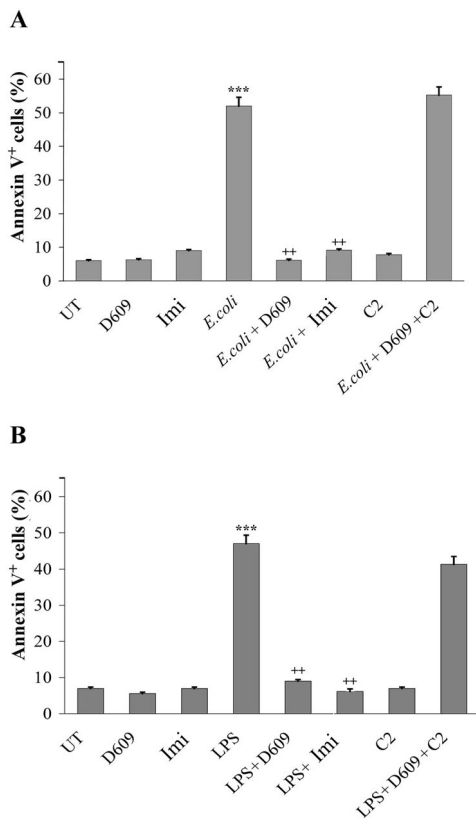
in the keys to the various panels. Ceramide levels (A and D) were quantified by TLC. Cer and Or in A indicate the positions of the standard ceramide loaded in parallel and of the origin. Values reported in D represent the percent increase over basal ceramide concentration ( $36 \pm 3.10$  pmol/mg proteins;  $n = 10$ ). The activities of the neutral SMase (N-SMase) and A-SMase (B and C) were quantified by measuring generation of phosphorylcholine from sphingomyelin. Values are expressed as the percent increase over basal SMase activity ( $1.42 \pm 0.52$  and  $0.37 \pm 0.23$  nmol/mg/h for A-SMase and N-SMase, respectively;  $n = 5$ ). Asterisks refer to the statistical significance in LPS-treated cells vs untreated controls; crosses refer to the statistical significance in cells treated with LPS plus D609 or LPS plus Imi vs cells treated with LPS alone ( $n = 5$ ; \*\*\*,  $p < 0.001$ ; + + +,  $p < 0.001$ ). The radiography shown in A is representative of five reproducible experiments.

### Activation of A-SMase accounts for the *E. coli*- and LPS-induced apoptosis of immature human DCs

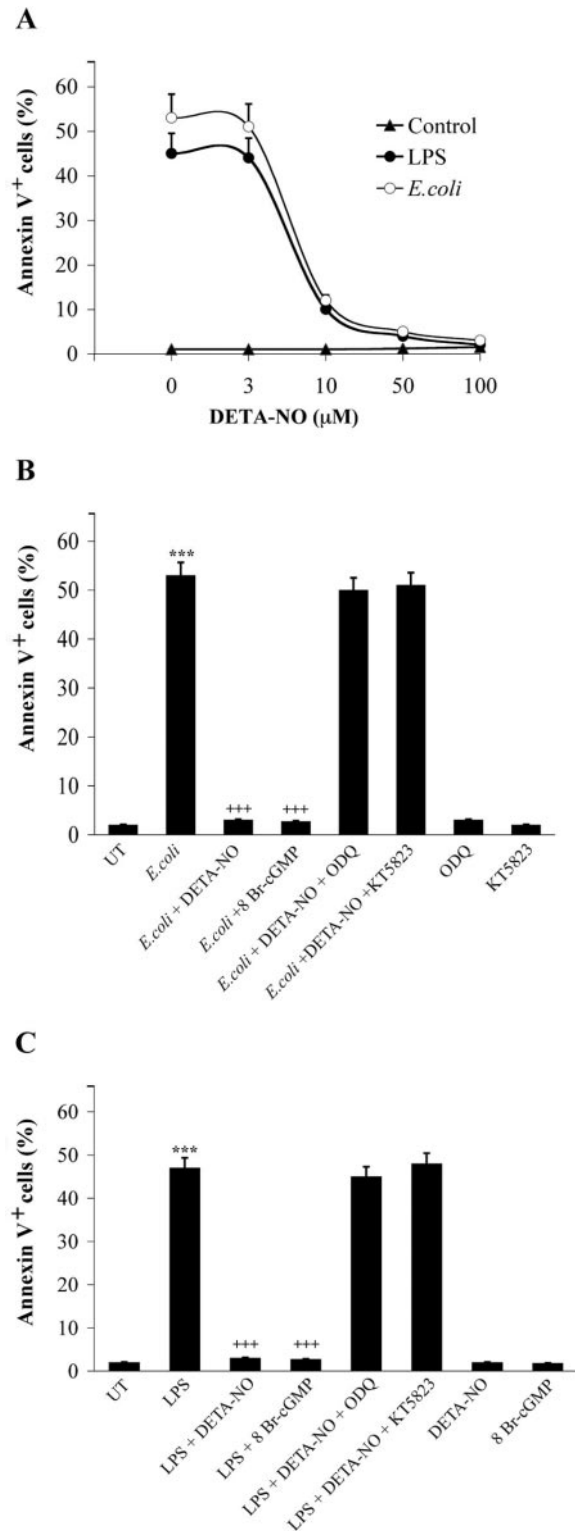
We used A-SMase inhibitors to evaluate the contribution of this enzyme to DC apoptosis triggered by *E. coli* and LPS. When administered alone, neither D609 nor imipramine had any appreciable effect on apoptosis. However, they inhibited apoptosis induction by both *E. coli* and LPS (Fig. 3). Of importance, exogenous C2 ceramide (20  $\mu$ M), although ineffective when administered alone, reversed the inhibitory effect of D609 and imipramine on *E. coli*- and LPS-induced apoptosis. The two neutral SMase inhibitors, used as controls, had no effect (not shown). These results indicate that A-SMase has a key role in the apoptotic signaling triggered by *E. coli* and LPS.

### NO inhibits the *E. coli*- and LPS-induced apoptosis of immature human DC through a cGMP-dependent pathway

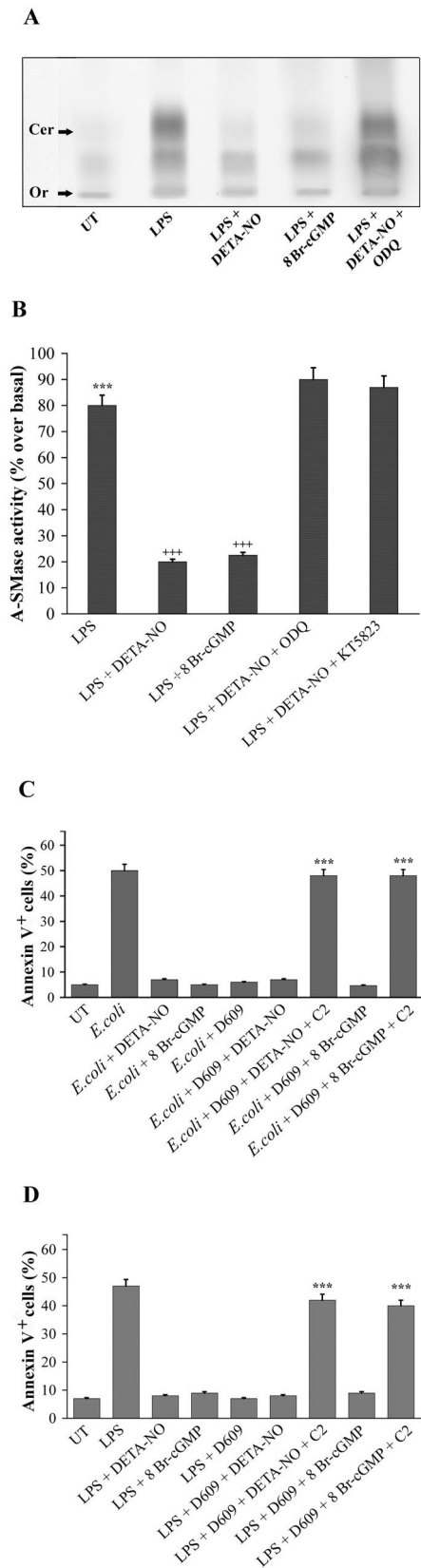
To analyze the role of NO, we used DETA-NO, a compound known to release a constant flux of NO with a period of  $\sim$ 20 h. When DETA-NO was administered at 10, 50, and 100  $\mu$ M, the steady state concentrations of NO in the medium, measured with an NO electrode (40), were  $0.028 \pm 0.001$ ,  $0.051 \pm 0.002$ , and  $0.12 \pm 0.04$   $\mu$ M ( $n = 4$ ), respectively. As shown in Fig. 4A,



**FIGURE 3.** Inhibition of A-SMase protects immature human DCs from *E. coli*- and LPS-induced apoptosis. Immature human DCs were exposed for 48 h to *E. coli* (*E. coli*:DC ratio, 750:1), LPS (100  $\mu$ g/ml), D609 (25  $\mu$ g/ml), imipramine (Imi; 20  $\mu$ M), or C2 ceramide (C2; 20  $\mu$ M) in various combinations as detailed in the keys to the panels. Phosphatidylserine exposure was quantified by annexin V staining as described in Fig. 1. Asterisks refer to the statistical significance in *E. coli*- or LPS-treated cells vs untreated controls; crosses refer to the statistical significance in cells treated with *E. coli* plus D609 or *E. coli* plus Imi vs cells treated with *E. coli* alone and in cells treated with LPS plus D609 or LPS plus Imi vs cells treated with LPS alone ( $n = 4$ ; \*\*\*,  $p < 0.001$ ; ++,  $p < 0.01$ ).



**FIGURE 4.** NO inhibits *E. coli*- and LPS-induced apoptosis of immature human DCs through a cGMP/G kinase-dependent pathway. Immature human DCs were exposed for 48 h to *E. coli* (*E. coli*:DC ratio, 750:1), LPS (100  $\mu$ g/ml), 8-Br-cGMP (1 mM), ODQ (1  $\mu$ M), KT5823 (1  $\mu$ M), or DETA-NO (100  $\mu$ M when the concentration is not specified). Phosphatidylserine exposure was quantified by annexin V staining as described in Fig. 1. Asterisks refer to the statistical probability in *E. coli*- or LPS-treated cells vs untreated controls; crosses refer to the statistical significance in cells treated with *E. coli* plus DETA-NO or *E. coli* plus 8-Br-cGMP vs cells treated with *E. coli* alone, and in cells treated with LPS plus DETA-NO or LPS plus 8-Br-cGMP vs cells treated with LPS alone ( $n = 4$ ; \*\*\*,  $p < 0.001$ ; +++,  $p < 0.001$ ).



**FIGURE 5.** The protective effect of NO/cGMP on *E. coli*- or LPS-induced human DC apoptosis is mediated through inhibition of A-SMase. Immature human DCs were exposed for 48 h to *E. coli* (*E. coli*:DC ratio, 750:1), LPS (100  $\mu\text{g}/\text{ml}$ ), 8-Br-cGMP (1 mM), ODQ (1  $\mu\text{M}$ ), KT5823 (1  $\mu\text{M}$ ), DETA-NO (100  $\mu\text{M}$ ), D609 (25  $\mu\text{g}/\text{ml}$ ), or C2 ceramide (C2; 20  $\mu\text{M}$ ) as detailed in the keys to the various panels. Ceramide generation (A), A-SMase activity (B), and phosphatidylserine exposure (C and D) were measured as described in Figs. 1 and 2. Asterisks in A refer to the statistical

significance ( $p < 0.001$ ) in LPS-treated cells vs untreated controls ( $n = 5$ ); asterisks in C and D ( $p < 0.001$ ) refer to the statistical significance in cells treated with *E. coli*, D609, C2, and DETA-NO (or 8-Br-cGMP), and with LPS, D609, C2, and 8-Br-cGMP vs cells treated in the same way but in the absence of C2 ( $n = 5$ ). Crosses in B refer to the statistical significance ( $p < 0.001$ ) in cells treated with LPS plus DETA-NO or LPS plus 8-Br-cGMP vs cells treated with LPS alone. The radiography shown in A is representative of four reproducible experiments. Cer and Or indicate the positions of the standard ceramide loaded in parallel and the origin.

DETA-NO, although ineffective when administered alone, inhibited both *E. coli*- and LPS-induced apoptosis in a concentration-dependent manner. Among the signaling pathways mediating the biological action of NO, activation of guanylate cyclase and the ensuing generation of cGMP appear relevant to human DC functions (36, 37). To analyze the dependence of the inhibitory effect of NO on cGMP generation, cells were exposed to *E. coli* or LPS in the presence of the membrane-permeant cGMP analog, 8-Br-cGMP (1 mM), or DETA-NO (100  $\mu\text{M}$ ) together with either ODQ (1  $\mu\text{M}$ ), a specific inhibitor of guanylate cyclase (46), or KT5823, an inhibitor of G kinase (47). The compounds had no effect when administered alone; however, 8-Br-cGMP mimicked, whereas ODQ and KT5823 prevented, the inhibitory effect of DETA-NO on both LPS- and *E. coli*-induced apoptosis (Fig. 4, B and C). These results indicate that NO inhibits apoptosis via a mechanism involving activation of guanylate cyclase, formation of cGMP, and activation of G kinase.

#### *The protective effect of NO is due to the cGMP-dependent inhibition of A-SMase*

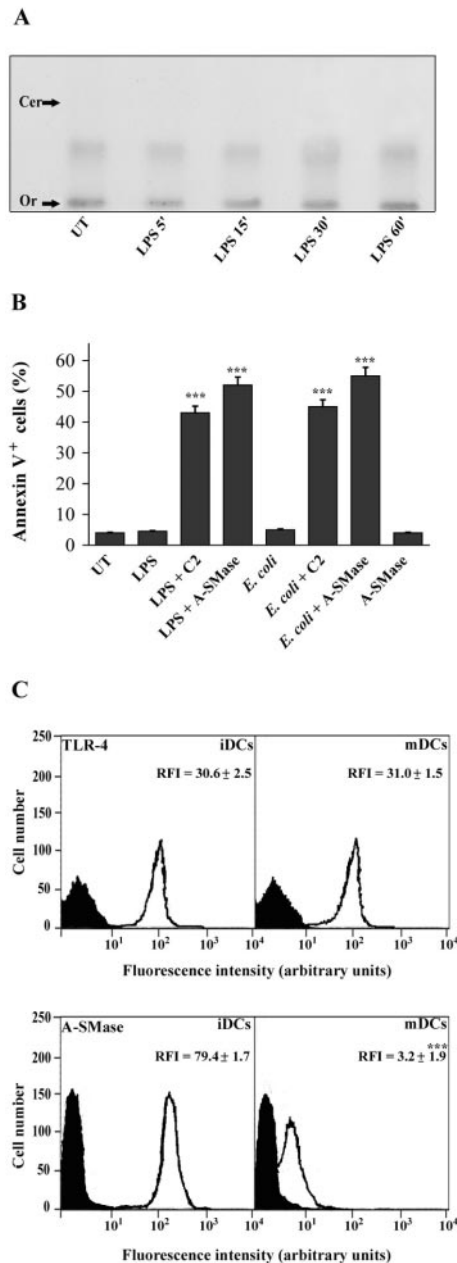
Treatment of DCs with DETA-NO inhibited both the activation of A-SMase and the generation of ceramide triggered by LPS. Moreover, this effect of NO was mimicked by 8-Br-cGMP and was prevented by ODQ or KT5823 (Fig. 5, A and B). Basal sphingomyelin hydrolysis was not modified when DETA-NO, ODQ, KT5823, or 8-Br-cGMP was administered alone (not shown). These results indicate that NO inhibits A-SMase activation by LPS in a cGMP/G kinase-dependent manner.

We then investigated whether the cGMP-dependent inhibition of A-SMase activity accounts for NO protection from *E. coli*- and LPS-induced apoptosis. Administration of either DETA-NO or 8-Br-cGMP inhibited apoptosis to an extent similar to that observed with D609 (Fig. 5, C and D). In addition, when the NO donor or the cyclic nucleotide was administered together with D609, apoptosis was inhibited no further. Finally, administration of C2 ceramide (20  $\mu\text{M}$ ) reversed the protective effect on apoptosis of DETA-NO and 8-Br-cGMP, administered alone or in combination with D609 (Fig. 5, C and D, and not shown). These results indicate that inhibition of A-SMase activity and of the ensuing ceramide generation accounts for the cGMP-dependent, inhibitory effect of NO on LPS- and *E. coli*-induced apoptosis.

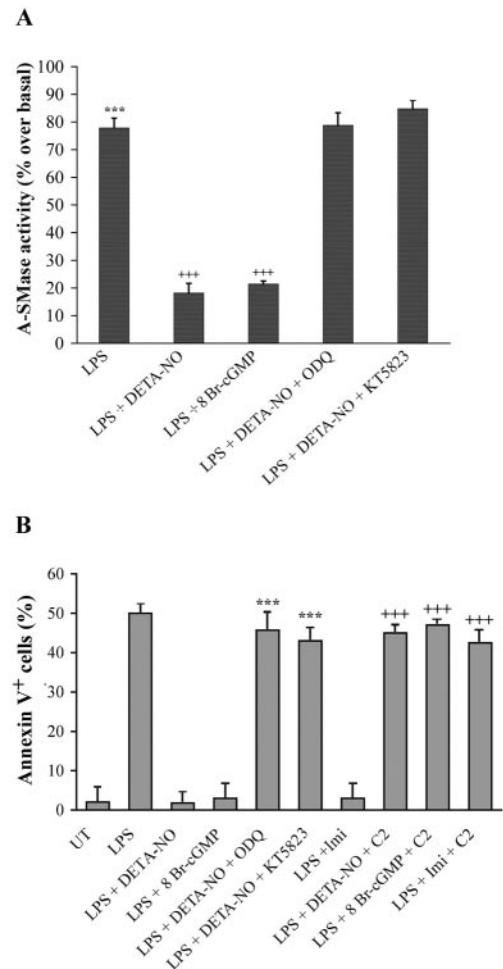
#### *Mature human DCs are resistant to E. coli- and LPS-induced apoptosis because of their reduced expression of A-SMase*

Immature DCs were treated with TNF- $\alpha$  (50 ng/ml) for 2 days. The TNF- $\alpha$ -induced differentiation of DCs toward a mature, Ag-presenting phenotype includes the coordinated up-regulation of proteins on the plasma membrane, among which are MHC classes I and II and the CD80, CD86, and CD40 costimulatory molecules (48). Maturation of DCs was routinely checked by measuring the expression of these proteins by flow cytometry as described previously (not shown) (36, 37).

significance ( $p < 0.001$ ) in LPS-treated cells vs untreated controls ( $n = 5$ ); asterisks in C and D ( $p < 0.001$ ) refer to the statistical significance in cells treated with *E. coli*, D609, C2, and DETA-NO (or 8-Br-cGMP), and with LPS, D609, C2, and 8-Br-cGMP vs cells treated in the same way but in the absence of C2 ( $n = 5$ ). Crosses in B refer to the statistical significance ( $p < 0.001$ ) in cells treated with LPS plus DETA-NO or LPS plus 8-Br-cGMP vs cells treated with LPS alone. The radiography shown in A is representative of four reproducible experiments. Cer and Or indicate the positions of the standard ceramide loaded in parallel and the origin.



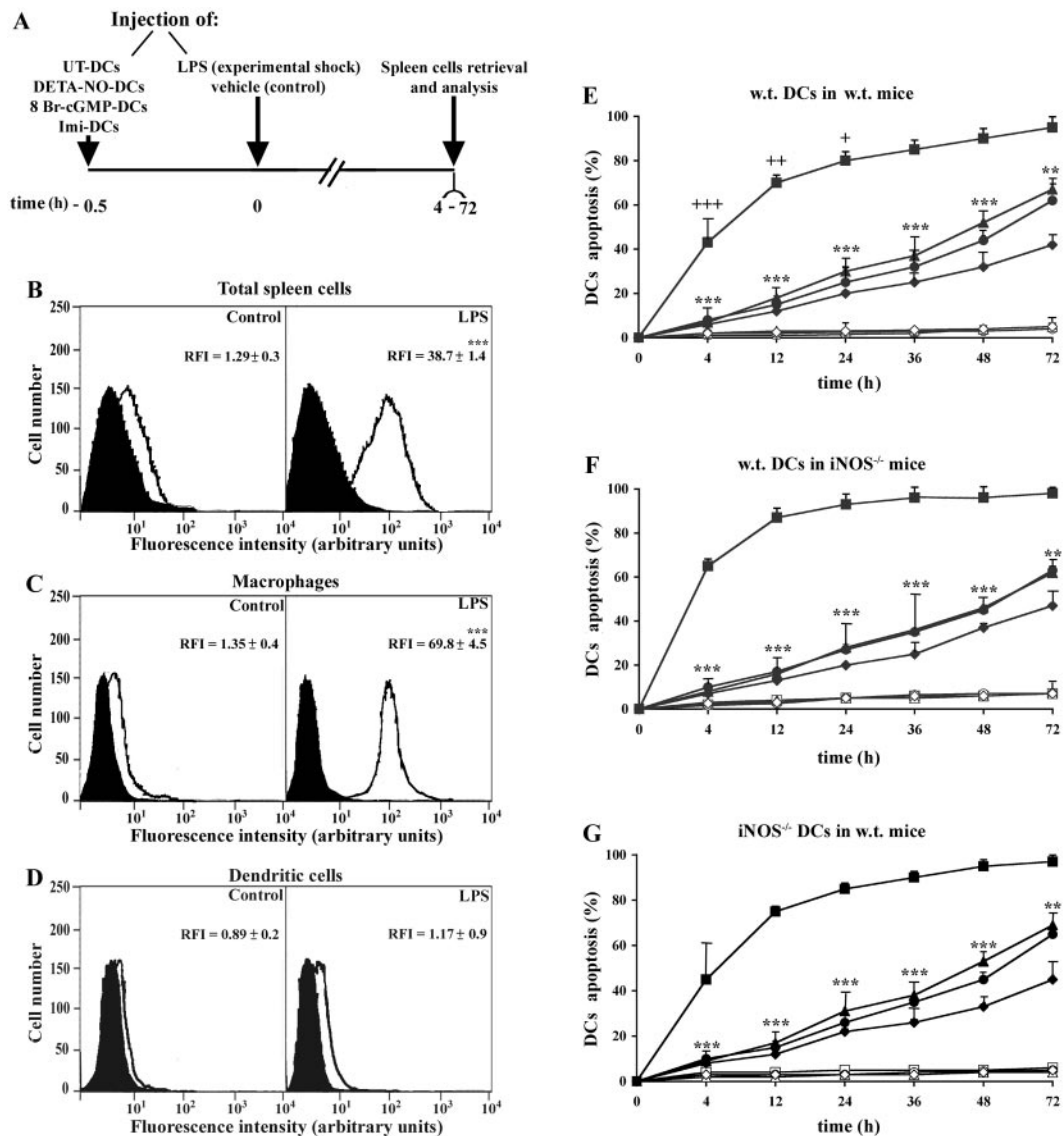
**FIGURE 6.** Resistance of mature human DCs to *E. coli*- and LPS-induced apoptosis is due to reduced A-SMase expression and impaired ceramide generation. Human immature DCs (iDCs) were treated with TNF- $\alpha$  (50 ng/ml) for 2 days. **A**, TNF- $\alpha$ -treated, mature DCs (mDCs) were exposed to LPS (100  $\mu$ g/ml), and ceramide content was measured by TLC at the indicated time points. Cer and Or indicate the positions of the standard ceramide loaded in parallel and the origin. **B**, mDCs were treated for 30 min with *E. coli* (*E. coli*:DC ratio, 750:1), LPS (100  $\mu$ g/ml), C2 ceramide (C2; 20  $\mu$ M), or human placental A-SMase (2.0 U/ml) in various combinations as detailed in the key. Phosphatidylserine exposure was quantified by annexin V staining as described in Fig. 1. Asterisks refer to the statistical significance ( $p < 0.001$ ) in cells treated with *E. coli* plus C2 or *E. coli* plus A-SMase vs cells treated with *E. coli* alone, and in cells treated with LPS plus C2 or LPS plus A-SMase vs cells treated with LPS alone ( $n = 4$ ). **C**, Comparison of the expression levels of the TLR4 and A-SMase in iDCs vs mDCs. Cell preparations were decorated with FITC-stained Abs specific for the two Ags, as detailed on the left, and were analyzed by flow cytometry ( $\square$ ). Their relative fluorescence intensity (RFI) was calculated vs negative controls ( $\blacksquare$ ). The results shown are from one experiment representative of four reproducible ones. The RFI reported in the panels are the mean  $\pm$  SEM in the four experiments. Asterisks refer to the statistical significance ( $p < 0.001$ ) in mDCs vs iDCs.



**FIGURE 7.** Apoptosis of immature murine DCs depends on A-SMase activation and is inhibited by NO through cGMP/G kinase-dependent inhibition of A-SMase activity. Immature murine DCs were treated for 30 min (**A**) or 48 h (**B**) with LPS (100  $\mu$ g/ml), DETA-NO (100  $\mu$ M), 8-Br-cGMP (1 mM), ODQ (1  $\mu$ M), KT5823 (1  $\mu$ M), imipramine (Imi; 20  $\mu$ M), or C2 ceramide (C2; 20  $\mu$ M) in various combinations as detailed in the keys to the panels. A-SMase activity (**A**) and phosphatidylserine exposure (**B**) were measured as described in Figs. 1 and 2. Asterisks in **A** refer to the statistical significance in LPS-treated cells vs untreated controls; asterisks in **B** refer to the statistical significance ( $p < 0.001$ ) in cells treated with LPS, DETA-NO, and ODQ (or KT5823) vs cells treated with LPS plus DETA-NO. Crosses in **A** refer to the statistical significance ( $p < 0.001$ ) in cells treated with LPS plus DETA-NO or LPS plus 8-Br-cGMP vs cells treated with LPS alone; crosses in **B** refer to the statistical significance ( $p < 0.001$ ) in cells treated with LPS, DETA-NO, and C2; LPS, 8-Br-cGMP, and C2; or LPS, Imi, and C2 vs cells treated in the same way but in the absence of C2 ( $n = 4$ ).

At variance with immature DCs, exposure of mature DCs to LPS resulted in neither generation of ceramide (Fig. 6A) nor activation of SMases (not shown). In addition, mature DCs did not undergo apoptosis when exposed to either *E. coli* or LPS. Sensitivity to apoptosis, however, was restored by administration, together with LPS or *E. coli*, of exogenous C2 ceramide or A-SMase (2.0 U/ml; Fig. 6B). We then investigated the molecular basis of the difference in sensitivity to LPS and *E. coli* between mature and immature DCs. In particular, we measured the expression levels of both A-SMase and the LPS TLR4 (49). In agreement with previous results (50) we did not find any difference in the levels of the TLR4 (Fig. 6C). By contrast, the levels of A-SMase were significantly lower in mature DC with respect to those observed in immature





**FIGURE 8.** Treatment of immature DCs with NO, cGMP, or the A-SMase inhibitor imipramine confers resistance to LPS-induced apoptosis in an *in vivo* model of sepsis. **A**, Diagrammatic representation of the experimental protocol. Immature DCs obtained from w.t. and *iNOS*<sup>-/-</sup> mice were treated for 16 h in the absence (UT-DCs) or the presence of DETA-NO (100  $\mu$ M; DETA-NO-DCs), 8-Br-cGMP (1 mM; 8 Br-cGMP-DCs), or imipramine (Imi; 20  $\mu$ M; Imi-DCs), stained with the red fluorescent dye CMTMR (10  $\mu$ M), and injected into the spleens of w.t. or *iNOS*<sup>-/-</sup> C57BL/6 mice ( $2.0 \times 10^6$  cells/animal; -0.5 h). Thirty minutes later (time zero), mice were injected i.p. with either LPS (20 mg/kg) or vehicle. Spleens were removed after 4, 12, 24, 36, 48, and 72 h, and spleen suspensions were prepared. **B--D**, Comparison of iNOS expression in spleen suspensions from vehicle-injected and LPS-injected w.t. mice at the 12 h point, measured by flow cytometry as described in Fig. 6. Shown are the levels of expression measured in the total cell suspension (**B**), in CD11b<sup>+</sup>/CD11c<sup>-</sup> macrophages (**C**), and in CMTMR<sup>+</sup> DCs (**D**). The relative fluorescence intensity (RFI;  $\square$ ) was calculated vs negative controls ( $\blacksquare$ ). The results shown are from one experiment representative of three reproducible ones. The RFI reported in the panels are the mean  $\pm$  SEM in the three experiments. Asterisks refer to the statistical significance (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ) in iNOS expression of LPS-injected vs vehicle-injected mice ( $n = 3$ ). **E--G**, Phosphatidylserine exposure by CMTMR<sup>+</sup> DCs was measured after annexin V staining of the various cell preparations as described in Fig. 1. **E**, W.t. DCs injected into w.t. mice; **F**, w.t. DCs injected into *iNOS*<sup>-/-</sup> mice; **G**, *iNOS*<sup>-/-</sup> DCs injected into w.t. mice.  $\blacksquare$ ,  $\bullet$ ,  $\blacktriangle$ , and  $\blacklozenge$ , UT-DCs, DETA-NO-DCs, 8-Br-cGMP-DCs, and Imi-DCs, respectively. Filled and open symbols refer to DCs retrieved from LPS-injected and vehicle-injected mice, respectively. Asterisks refer to the statistical significance in DETA-NO-DCs vs UT-DCs (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ); crosses to the statistical significance in w.t. UT-DCs, vs *iNOS*<sup>-/-</sup> UT-DCs (+,  $p < 0.05$ ; ++,  $p < 0.01$ ; +++,  $p < 0.001$ ), analyzed after injection in w.t. mice treated with LPS ( $n = 3$ ).

DCs. Taken together, these results indicate that down-regulation of A-SMase is responsible for the insensitivity of mature DCs to LPS/*E. coli*-induced apoptosis.

#### Activation of A-SMase and its inhibition by NO/cGMP regulate sensitivity to LPS-induced apoptosis in immature murine DCs

Immature murine DCs were obtained from mouse bone marrow progenitors as previously described (38). We first characterized the

LPS-induced SMase activities and apoptosis as well as the effects of NO/cGMP on these parameters. Exposure of DCs to LPS (100  $\mu$ g/ml) for 30 min did not activate neutral SMase (not shown), but gave rise to a significant activation of A-SMase ( $1.13 \pm 0.03$  and  $2.07 \pm 0.11$  nmol/mg/h before and after LPS administration, respectively;  $p < 0.01$ ;  $n = 4$ ), which was inhibited by  $91 \pm 5.2\%$  ( $n = 4$ ) in the presence of imipramine. A-SMase activation was also inhibited when DCs were exposed to LPS in the presence of

DETA-NO. The effect of the NO donor was mimicked by 8-Br-cGMP and was prevented by either ODQ or KT5823 (Fig. 7A). LPS also triggered DC apoptosis that was inhibited to similar extents by imipramine, 8-Br-cGMP, and DETA-NO, the latter in an ODQ- and KT5823-sensitive fashion (Fig. 7B). Furthermore, sensitivity to apoptosis was in all cases restored by administration of C2 ceramide (Fig. 7B). Neither apoptosis nor changes in sphingomyelin hydrolysis were observed when DETA-NO, ODQ, KT5823, 8-Br-cGMP, imipramine, or C2 ceramide was administered alone (not shown). Taken together these results indicate that LPS triggers apoptosis of murine DCs in an A-SMase-dependent way, and that protection by NO occurs through inhibition of the activity of the enzyme in a cGMP-dependent way. Thus, murine immature DCs behave similarly to their human counterparts.

#### Generation of NO by iNOS and inhibition of A-SMase protect immature murine DCs from LPS-induced apoptosis in an in vivo model of sepsis

A model of sepsis was used to investigate in vivo the relevance of A-SMase activation and its inhibition by NO/cGMP in murine DC apoptosis. Because iNOS plays fundamental roles in the regulation of immune responses, and its expression may be triggered by LPS (32), we evaluated the role of endogenous NO generated by this enzyme. Fig. 8A shows a diagram of the protocol used in these experiments. DCs, derived from w.t. or iNOS<sup>-/-</sup> mice, were treated ex vivo for 16 h in the absence or the presence of DETA-NO, 8-Br-cGMP, or imipramine, stained with the fluorescent dye CMTMR, which is retained by the cells for long periods, and then injected into the spleen of w.t. or iNOS<sup>-/-</sup> C57BL/6 mice. Thirty minutes later, mice were injected i.p. with either LPS (20 mg/kg) or vehicle, and spleens were removed 4, 12, 36, 48, and 72 h thereafter. Two types of studies were conducted using this protocol. We first measured iNOS expressed by spleen cells derived

from w.t. mice injected with untreated w.t. DCs. Although cells from vehicle-injected mice did not express iNOS, those recovered from LPS-injected mice expressed the enzyme from the 12 h point throughout the 72-h observation period (Fig. 8B and not shown). Most iNOS-expressing cells were CD11b<sup>+</sup>/CD11c<sup>-</sup> macrophages (Fig. 8C). In contrast, DCs did not express the enzyme at any time point (Fig. 8D).

We then analyzed apoptosis of the injected, CMTMR<sup>+</sup> DCs. The numbers of CMTMR<sup>+</sup> DCs in spleen cell suspensions in each experimental condition are specified in Table I. Fig. 8, E and F, reports the results obtained using w.t. DCs, untreated or treated ex vivo with DETA-NO or 8-Br-cGMP before their injection into spleen. Treatment of the mice with LPS induced apoptosis of untreated DCs. Apoptosis increased with the time of exposure to LPS in vivo and was significantly higher in DCs recovered from iNOS<sup>-/-</sup> mice than in DCs recovered from w.t. mice, indicating that generation of endogenous NO played a protective role (compare E and F in Fig. 8). This was confirmed by the observation that DCs treated ex vivo with DETA-NO or 8-Br-cGMP before their injection into LPS-treated w.t. and iNOS<sup>-/-</sup> mice were significantly protected from apoptosis (Fig. 8, E and F). Of importance, protection from apoptosis under these conditions was similar to that observed in DCs treated with imipramine (Fig. 8, E and F). Finally, we analyzed apoptosis of iNOS<sup>-/-</sup> DCs treated ex vivo in the presence or the absence of DETA-NO, 8-Br-cGMP, or imipramine and then injected into w.t. animals. Sensitivity to LPS-induced apoptosis in these cells was similar to that found in their w.t. counterparts (compare E and G in Fig. 8), providing a functional correlate to the observation, reported in Fig. 8D, that DCs do not express iNOS in LPS-treated mice in vivo. In control experiments using vehicle-treated mice, DC apoptosis was always negligible (Fig. 8, E-G).

Table I. Numbers ( $\times 10^6$ ) of CMTMR<sup>+</sup> DCs recovered from vehicle- and LPS-treated mice

| Mouse | DCs                 | Mouse Treatment     | DC Treatment | Time (h) of Recovery |              |              |              |              |              |              |              |
|-------|---------------------|---------------------|--------------|----------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
|       |                     |                     |              | 4                    | 12           | 24           | 36           | 48           | 72           |              |              |
| w.t.  | w.t.                | Vehicle             | UT           | 1.99 ± 0.025         | 1.65 ± 0.017 | 1.37 ± 0.032 | 1.28 ± 0.026 | 1.20 ± 0.035 | 0.98 ± 0.046 |              |              |
|       |                     |                     | DETA-NO      | 1.99 ± 0.008         | 1.61 ± 0.020 | 1.36 ± 0.019 | 1.29 ± 0.030 | 1.20 ± 0.023 | 0.97 ± 0.033 |              |              |
|       |                     |                     | 8-Br-cGMP    | 1.99 ± 0.015         | 1.63 ± 0.009 | 1.37 ± 0.027 | 1.28 ± 0.039 | 1.18 ± 0.028 | 0.98 ± 0.040 |              |              |
|       |                     |                     | Imipramine   | 1.98 ± 0.017         | 1.60 ± 0.018 | 1.38 ± 0.023 | 1.28 ± 0.019 | 1.21 ± 0.036 | 0.96 ± 0.037 |              |              |
|       |                     | LPS                 | UT           | 1.95 ± 0.035         | 1.59 ± 0.020 | 1.28 ± 0.022 | 1.10 ± 0.030 | 0.95 ± 0.028 | 0.80 ± 0.045 |              |              |
|       |                     |                     | DETA-NO      | 1.94 ± 0.012         | 1.57 ± 0.032 | 1.26 ± 0.033 | 1.08 ± 0.027 | 0.95 ± 0.033 | 0.80 ± 0.042 |              |              |
|       |                     |                     | 8-Br-cGMP    | 1.95 ± 0.015         | 1.59 ± 0.037 | 1.26 ± 0.030 | 1.05 ± 0.019 | 0.94 ± 0.039 | 0.79 ± 0.051 |              |              |
|       |                     |                     | Imipramine   | 1.96 ± 0.023         | 1.58 ± 0.028 | 1.27 ± 0.029 | 1.08 ± 0.034 | 0.95 ± 0.027 | 0.80 ± 0.049 |              |              |
|       |                     | iNOS <sup>-/-</sup> | w.t.         | Vehicle              | UT           | 1.99 ± 0.025 | 1.65 ± 0.017 | 1.37 ± 0.032 | 1.28 ± 0.026 | 1.20 ± 0.035 | 0.97 ± 0.052 |
|       |                     |                     |              |                      | DETA-NO      | 1.98 ± 0.008 | 1.61 ± 0.020 | 1.36 ± 0.019 | 1.29 ± 0.030 | 1.21 ± 0.040 | 0.96 ± 0.050 |
|       |                     |                     |              |                      | 8-Br-cGMP    | 1.98 ± 0.015 | 1.63 ± 0.009 | 1.37 ± 0.027 | 1.28 ± 0.039 | 1.18 ± 0.028 | 0.96 ± 0.038 |
|       |                     |                     |              |                      | Imipramine   | 1.99 ± 0.017 | 1.60 ± 0.018 | 1.38 ± 0.023 | 1.28 ± 0.019 | 1.21 ± 0.036 | 0.96 ± 0.047 |
| LPS   | UT                  |                     |              | 1.94 ± 0.042         | 1.58 ± 0.031 | 1.27 ± 0.037 | 1.09 ± 0.017 | 0.95 ± 0.019 | 0.79 ± 0.035 |              |              |
|       | DETA-NO             |                     |              | 1.93 ± 0.033         | 1.57 ± 0.027 | 1.25 ± 0.042 | 1.09 ± 0.039 | 0.95 ± 0.009 | 0.78 ± 0.038 |              |              |
|       | 8-Br-c-GMP          |                     |              | 1.93 ± 0.025         | 1.57 ± 0.040 | 1.26 ± 0.015 | 1.07 ± 0.024 | 0.95 ± 0.024 | 0.78 ± 0.042 |              |              |
|       | Imipramine          |                     |              | 1.93 ± 0.030         | 1.56 ± 0.023 | 1.26 ± 0.035 | 1.08 ± 0.014 | 0.96 ± 0.036 | 0.79 ± 0.030 |              |              |
| w.t.  | iNOS <sup>-/-</sup> |                     |              | Vehicle              | UT           | 1.99 ± 0.040 | 1.67 ± 0.022 | 1.40 ± 0.032 | 1.30 ± 0.009 | 1.25 ± 0.035 | 0.98 ± 0.029 |
|       |                     |                     |              |                      | DETA-NO      | 1.98 ± 0.020 | 1.65 ± 0.027 | 1.39 ± 0.021 | 1.29 ± 0.025 | 1.21 ± 0.021 | 0.98 ± 0.034 |
|       |                     |                     |              |                      | 8-Br-c-GMP   | 1.98 ± 0.025 | 1.65 ± 0.031 | 1.37 ± 0.030 | 1.30 ± 0.040 | 1.21 ± 0.042 | 0.98 ± 0.037 |
|       |                     |                     |              |                      | Imipramine   | 1.98 ± 0.032 | 1.67 ± 0.037 | 1.38 ± 0.018 | 1.30 ± 0.027 | 1.23 ± 0.006 | 0.97 ± 0.054 |
|       |                     | LPS                 | UT           | 1.93 ± 0.037         | 1.57 ± 0.020 | 1.26 ± 0.034 | 1.12 ± 0.050 | 0.95 ± 0.054 | 0.79 ± 0.024 |              |              |
|       |                     |                     | DETA-NO      | 1.92 ± 0.044         | 1.56 ± 0.032 | 1.26 ± 0.047 | 1.10 ± 0.031 | 0.94 ± 0.049 | 0.80 ± 0.036 |              |              |
|       |                     |                     | 8-Br-c-GMP   | 1.93 ± 0.049         | 1.56 ± 0.037 | 1.25 ± 0.051 | 1.09 ± 0.029 | 0.93 ± 0.047 | 0.79 ± 0.051 |              |              |
|       |                     |                     | Imipramine   | 1.93 ± 0.037         | 1.56 ± 0.028 | 1.27 ± 0.037 | 1.10 ± 0.043 | 0.93 ± 0.041 | 0.79 ± 0.032 |              |              |

Immature murine w.t. and iNOS<sup>-/-</sup> DCs were cultured in the absence (UT) or presence of DETA-NO (10  $\mu$ M), 8-Br-cGMP (1 mM), or imipramine (20  $\mu$ M) for 16 h and labeled with 10  $\mu$ M of the fluorescent dye CMTMR for 30 min (38). The various DC preparations ( $2.0 \times 10^6$  cells in 100  $\mu$ l) were injected into the spleens of C57BL/6 female w.t. or iNOS<sup>-/-</sup> mice. LPS or vehicle was injected i.p. 30 min later (42). Animals were killed at the indicated time points, spleens were recovered, and CMTMR<sup>+</sup> CD11c<sup>+</sup> DCs were identified by flow cytometry. The number of CMTMR<sup>+</sup> DCs was determined in  $1 \times 10^6$  cell samples from each spleen preparation and normalized to the total number of cells (both CMTMR<sup>+</sup> and CMTMR<sup>-</sup>) recovered from the same spleen ( $n = 3$ ).

Taken together our results demonstrate that activation of A-SMase in DCs and their exposure to NO generated by iNOS-competent cells play crucial roles during LPS-induced DC apoptosis in vivo and strongly suggest that protection by NO/cGMP in vivo occurs through inhibition of A-SMase activation.

## Discussion

In this study we examined, by both an in vitro approach and an in vivo model of sepsis, the relevance of A-SMase and its regulation by NO in the *E. coli*/LPS-induced apoptosis of DCs. Using human and murine DCs, we show that the in vitro interaction with *E. coli* can trigger apoptotic cell death of immature DCs and that a single pathogen-associated molecular pattern, LPS, is sufficient to activate the process. We found that the mechanism of death induction by LPS requires activation of the sphingomyelin-hydrolyzing enzyme, A-SMase, and generation of ceramide. The obligatory role of this pathway is demonstrated by two observations. Firstly, pharmacological inhibition of A-SMase prevents ceramide generation and apoptosis induction by LPS. Secondly, reduced expression of A-SMase, with no detectable ceramide generation, occurs in mature DCs, which are resistant to LPS-induced apoptosis; sensitivity to apoptosis could be restored in these cells by administration of exogenous ceramide or A-SMase. The in vivo relevance of this observation was validated using a model of LPS-induced sepsis. In this model we found that DCs injected in septic mice were committed to die unless they were previously exposed to the A-SMase inhibitor imipramine.

The role of A-SMase in apoptosis has been established for various cell types, especially after the development of a knockout mouse model for the enzyme (51). Resistance to apoptosis induced by various stimuli was observed, among others, in lymphocytes, hepatocytes, macrophages, and endothelial cells from A-SMase knockout mice (27, 52–56), confirming and expanding the data obtained through pharmacological inhibition of the enzyme (29, 30, 57–59). All these cells participate in sepsis together with DCs (60) and undergo apoptotic death (6–8, 61–63). The involvement of A-SMase in apoptosis, however, appears to depend on the stimulus and the cell type involved (53, 64). With the notable exception of endothelial cells (27) in no other cell type has it been established whether the sepsis-induced apoptosis is mediated through the same A-SMase-dependent pathway we found activated in DCs. Elucidation, in the A-SMase<sup>-/-</sup> mouse model, of which other cells are protected from apoptosis would be important to define precisely the biological role of A-SMase in sepsis.

Although obligatory, A-SMase activation might not be the only signaling event involved in DC apoptosis induced by LPS. The biological effects of this glycolipid are known to be mediated by synergic cooperation of various signaling pathways, including the transcription factor NF- $\kappa$ B, G proteins, and several protein kinases belonging to the Akt, JNK, mitogen-activated, stress-activated, and Src-related families (15–18, 20, 21, 23, 65, 66), some of which activated as a consequence of A-SMase stimulation (22, 23, 65). Whether and how activation of one or more of the above signaling pathways contributes to apoptosis induced by high concentrations of LPS remain to be investigated.

The second important finding of our study is that NO protects DCs from apoptosis in sepsis and that the mechanism of its action is the inhibition of A-SMase. The importance to DC protection of NO generated by iNOS during sepsis was clearly demonstrated in the in vivo model by two lines of evidence. First, DCs died from apoptosis faster and at higher rates in iNOS<sup>-/-</sup> mice than in w.t. mice; second, exposure to exogenous NO/cGMP or imipramine increased DC survival significantly.

We did not find significant differences in resistance to apoptosis between w.t. and iNOS<sup>-/-</sup> DCs. A likely explanation comes from the observation that in vivo, LPS did not trigger iNOS expression in w.t. DCs. Similarly to iNOS<sup>-/-</sup> DCs, therefore, w.t. DCs were exposed only to the NO generated by neighboring, iNOS-competent cells. Interestingly, human DCs do not express iNOS when exposed to cytokines and bacterial products (36, 37). Our in vivo results, therefore, may also be a valid model for human DC during sepsis. At variance with the situation in vivo, some in vitro studies showed that murine DCs express iNOS after stimulation with LPS and cytokines (32), confirming the reported variability in the expression of the enzyme among in vivo and in vitro models (67, 68).

The observation that NO protects DCs against apoptosis is relevant to the overall understanding of the role of this messenger in sepsis. NO is known to induce both detrimental effects, such as hypotension, vasoplegia, lactic acidosis, and impaired thermoregulation, and beneficial actions, such as maintenance of renal and hepatoportal blood flow, inhibition of platelet aggregation, and maintenance of the cardiac function (68–70). The survival to sepsis of iNOS<sup>-/-</sup> mice was found to be less than, more than, or equal to that of their w.t. counterparts depending on the model of endotoxemia, thus reflecting the complexity of the actions of NO described above (71–75). Despite this complexity, however, iNOS-generated NO has been shown to contribute substantially to protection from apoptosis during sepsis in various organs, including liver, heart, kidney, and thymus (74, 76–79). To date, the intracellular signal transduction events responsible for such protective action had been studied to only a limited extent. Our in vitro results show that inhibition of A-SMase is central to the protective action of NO in DCs, and that this inhibition occurs through stimulation of guanylate cyclase, generation of cGMP, and activation of protein kinase G. These findings provide a target and a mechanism of action for NO that may open new vistas to our understanding of apoptosis in sepsis and its prevention.

In particular, relevant to apoptosis in sepsis are activation of caspases, changes in the expression levels of members of the Bcl-2 family of proteins, and reduction in the mitochondrial membrane potential (63). In various models of apoptosis, all these signaling events have been reported to be inhibited by NO, acting through cGMP (80). Likewise, inhibition of these events also takes place as a consequence of inhibition of A-SMase, including that by NO (24–26, 29–31, 33, 57). It is conceivable, therefore, that the inhibition of A-SMase by NO shown in this study switches off the signaling pathways leading to sepsis-induced apoptosis, thus acting as a key mechanism of protection.

Protection of immune-competent cells, including DCs, from apoptosis has been recently envisaged as a means to ameliorate the multiple organ failure/dysfunction syndrome and thus the final outcome of the disease (63). The obligatory role of A-SMase in *E. coli*/LPS-induced apoptosis of DCs and its regulation by NO/cGMP revealed by our studies may therefore be relevant to the development of sepsis. The experimental protocol we used for our in vivo experiments was designed to specifically study the mechanism of protection of DCs from sepsis induced-apoptosis and not the possible beneficial role of this protection as a therapeutic strategy. However, our results suggest that DCs obtained from A-SMase<sup>-/-</sup> mice (51) may have a potential role in sepsis, ameliorating the immune suppression associated with this condition. Validation of the protective effect of A-SMase<sup>-/-</sup> DCs by appropriate models (48) may be the first step in developing new therapeutic approaches to this disease.

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## References

- Angus, D. C., W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky. 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* 29:1303.
- Marshall, J. C. 2003. Such stuff as dreams are made on: mediator-directed therapy in sepsis. *Nat. Rev. Drug Discov.* 2:391.
- Meakins, J. L., J. B. Pietsch, O. Bubenick, R. Kelly, H. Rode, J. Gordon, and L. D. MacLean. 1977. Delayed hypersensitivity: indicator of acquired failure of host defenses in sepsis and trauma. *Ann. Surg.* 186:241.
- Docke, W. D., F. Randow, U. Syrbe, D. Krausch, K. Asadullah, P. Reinke, H. D. Volk, and W. Kox. 1997. Monocyte deactivation in septic patients: restoration by IFN- $\gamma$  treatment. *Nat. Med.* 3:678.
- Puyana, J. C., J. D. Pellegrini, A. K. De, K. Kodys, W. E. Silva, and C. L. Miller. 1998. Both T-helper-1- and T-helper-2-type lymphokines are depressed in post-trauma anergy. *J. Trauma* 44:1037.
- Hotchkiss, R. S., K. W. Tinsley, P. E. Swanson, R. E. Schmiege, Jr., J. J. Hui, K. C. Chang, D. F. Osborne, B. D. Freeman, J. P. Cobb, T. G. Buchman, et al. 2001. Sepsis-induced apoptosis causes progressive profound depletion of B and CD4<sup>+</sup> T lymphocytes in humans. *J. Immunol.* 166:6952.
- Ayala, A., G. Y. Song, C. S. Chung, K. M. Redmond, and I. H. Chaudry. 2000. Immune depression in polymicrobial sepsis: the role of necrotic (injured) tissue and endotoxin. *Crit. Care Med.* 28:2949.
- Mannick, J. A., M. L. Rodrick, and J. A. Lederer. 2001. The immunologic response to injury. *J. Am. Coll. Surg.* 193:237.
- Hotchkiss, R. S., K. W. Tinsley, P. E. Swanson, M. H. Grayson, D. F. Osborne, T. H. Wagner, J. P. Cobb, C. Coopersmith, and I. E. Karl. 2002. Depletion of dendritic cells, but not macrophages, in patients with sepsis. *J. Immunol.* 168:2493.
- Tinsley, K. W., M. H. Grayson, P. E. Swanson, A. M. Drewry, K. C. Chang, I. E. Karl, and R. S. Hotchkiss. 2003. Sepsis induces apoptosis and profound depletion of splenic interdigitating and follicular dendritic cells. *J. Immunol.* 171:909.
- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
- Moser, M., and K. M. Murphy. 2000. Dendritic cell regulation of TH1-TH2 development. *Nat. Immunol.* 1:199.
- Pinner, R. W., S. M. Teutsch, L. Simonsen, L. A. Klug, J. M. Graber, M. J. Clarke, and R. L. Berkelman. 1996. Trends in infectious diseases mortality in the United States. *JAMA* 275:189.
- Danner, R. L., R. J. Elin, J. M. Hosseini, R. A. Wesley, J. M. Reilly, and J. E. Parrillo. 1991. Endotoxemia in human septic shock. *Chest* 99:169.
- Narumi, S., J. M. Tebo, J. H. Finke, and T. A. Hamilton. 1992. IFN- $\gamma$  and IL-2 cooperatively activate NF $\kappa$ B in murine peritoneal macrophages. *J. Immunol.* 149:529.
- Hambleton, J., S. L. Weinstein, L. Lem, and A. L. DeFranco. 1996. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc. Natl. Acad. Sci. USA* 93:2774.
- Jakway, J. P., and A. L. DeFranco. 1986. Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide. *Science* 234:743.
- Herrera-Velitz, P., and N. E. Reiner. 1996. Bacterial lipopolysaccharide induces the association and coordinate activation of p53/56lyn and phosphatidylinositol 3-kinase in human monocytes. *J. Immunol.* 156:1157.
- Barber, S. A., G. Detore, R. McNally, and S. N. Vogel. 1996. Stimulation of the ceramide pathway partially mimics lipopolysaccharide-induced responses in murine peritoneal macrophages. *Infect. Immun.* 64:3397.
- Herrera-Velitz, P., K. L. Knutson, and N. E. Reiner. 1997. Phosphatidylinositol 3-kinase-dependent activation of protein kinase C- $\zeta$  in bacterial lipopolysaccharide-treated human monocytes. *J. Biol. Chem.* 272:16445.
- MacKichan, M. L., and A. L. DeFranco. 1999. Role of ceramide in lipopolysaccharide (LPS)-induced signaling. LPS increases ceramide rather than acting as a structural homolog. *J. Biol. Chem.* 274:1767.
- Monick, M. M., A. B. Carter, G. Gudmundsson, R. Mallampalli, L. S. Powers, and G. W. Hunninghake. 1999. A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide-stimulated human alveolar macrophages. *J. Immunol.* 162:3005.
- Procyk, K. J., M. R. Rippe, R. Testi, F. Hofmann, P. J. Parker, and M. Baccarini. 2000. Lipopolysaccharide induces jun N-terminal kinase activation in macrophages by a novel Cdc42/Rac-independent pathway involving sequential activation of protein kinase C  $\zeta$  and phosphatidylcholine-dependent phospholipase C. *Blood* 96:2592.
- Kolesnick, R. N., and M. Krönke. 1998. Regulation of ceramide production and apoptosis. *Annu. Rev. Physiol.* 60:643.
- Levade, T., and J.-P. Jaffrézou. 1999. Signalling sphingomyelinases: which, where, how and why? *Biochim. Biophys. Acta* 1438:1.
- Pettus, B. J., C. E. Chalfant, and Y. A. Hannun. 2002. Ceramide in apoptosis: an overview and current perspectives. *Biochim. Biophys. Acta* 1585:114.
- Haimovitz-Friedman, A., C. Cordon-Cardo, S. Bayoumy, M. Garzotto, M. McLoughlin, R. Gallily, C. K. Edwards III, E. H. Schuchman, Z. Fuks, and R. Kolesnick. 1997. Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J. Exp. Med.* 186:1831.
- Kanto, T., P. Kalinski, O. C. Hunter, M. T. Lotze, and A. A. Amoscatto. 2001. Ceramide mediates tumor-induced dendritic cell apoptosis. *J. Immunol.* 167:3773.
- Sciorati, C., P. Rovere, M. Ferrarini, S. Heltai, A. A. Manfredi, and E. Clementi. 1997. Autocrine nitric oxide modulates CD95-induced apoptosis in  $\gamma\delta$  T lymphocytes. *J. Biol. Chem.* 272:23211.
- Sciorati, C., P. Rovere, M. Ferrarini, C. Paolucci, S. Heltai, R. Vaiani, E. Clementi, and A. A. Manfredi. 1999. Generation of nitric oxide by the inducible nitric oxide synthase protects  $\gamma\delta$  T cells from *Mycobacterium tuberculosis*-induced apoptosis. *J. Immunol.* 163:1570.
- De Nadai, C., P. Sestili, O. Cantoni, J. P. Liévreumont, C. Sciorati, R. Barsacchi, S. Moncada, J. Meldolesi, and E. Clementi. 2000. Nitric oxide inhibits tumor necrosis factor- $\alpha$ -induced apoptosis by reducing the generation of ceramide. *Proc. Natl. Acad. Sci. USA* 97:5480.
- Bogdan, C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* 2:907.
- Barsacchi, R., C. Perrotta, P. Sestili, O. Cantoni, S. Moncada, and E. Clementi. 2002. Cyclic GMP-dependent inhibition of acid sphingomyelinase by nitric oxide: an early step in protection against apoptosis. *Cell Death Differ.* 9:1248.
- Hurwitz, R., K. Ferlinz, and F. Sandhoff. 1994. The antidepressant desipramine causes proteolytic degradation of lysosomal sphingomyelinase in human fibroblasts. *Biol. Chem. Hoppe Seyler* 375:447.
- Grassmé, H., E. Gulbins, B. Brenner, K. Ferlinz, K. Sandhoff, K. Harzer, F. Lang, and T. F. Meyer. 1997. Acid sphingomyelinase mediates entry of *N. gonorrhoeae* into nonphagocytic cells. *Cell* 91:605.
- Paolucci, C., P. Rovere, C. De Nadai, A. A. Manfredi, and E. Clementi. 2000. Nitric oxide inhibits the tumor necrosis factor  $\alpha$ -regulated endocytosis of human dendritic cells in a cyclic GMP-dependent way. *J. Biol. Chem.* 275:19638.
- Paolucci, C., S. E. Burastero, P. Rovere-Querini, C. De Palma, S. Falcone, C. Perrotta, A. Capobianco, A. A. Manfredi, and E. Clementi. 2003. Synergism of nitric oxide and maturation signals on human dendritic cells occurs through a cyclic GMP-dependent pathway. *J. Leukocyte Biol.* 73:253.
- Camporeale, A. A. Boni, G. Iezzi, E. Degl'Innocenti, M. Grioni, A. Mondino, and M. Bellone. 2003. Critical impact of the kinetics of dendritic cells activation on the *in vivo* induction of tumor-specific T lymphocytes. *Cancer Res.* 63:3688.
- Barsacchi, R., C. Perrotta, S. Bulotta, S. Moncada, N. Borgese, and E. Clementi. 2003. Activation of endothelial nitric oxide synthase by tumor necrosis factor- $\alpha$ : a novel pathway involving sequential activation of neutral sphingomyelinase, phosphatidylinositol-3' kinase, and Akt. *Mol. Pharmacol.* 63:886.
- Clementi, E., G. C. Brown, M. Feelisch, S. Moncada. 1998. Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl. Acad. Sci. USA* 95:7631.
- Wiegmann, K., S. Schütze, T. Machleidt, D. Witte, and M. Krönke. 1994. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* 78:1005.
- Karaghiosoff, M., R. Steinborn, P. Kovarik, G. Kriegshäuser, M. Baccarini, B. Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, et al. 2003. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. *Nat. Immunol.* 4:471.
- Schütze, S., T. Machleidt, D. Adam, R. Schwandner, K. Wiegmann, M. L. Kruse, M. Heinrich, M. Wickel, and M. Krönke. 1999. Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signaling. *J. Biol. Chem.* 274:10203.
- Tanaka, M., F. Nara, K. Suzuki-Konagai, T. Hosoya, and T. Ogita. 1997. Structural elucidation of scyphostatin, an inhibitor of membrane-bound neutral sphingomyelinase. *J. Am. Chem. Soc.* 116:7871.
- Arenz, C., M. Thutewohl, O. Block, H. Waldmann, H. J. Altenbach, and A. Gianni. 2001. Manumycin A and its analogues are irreversible inhibitors of neutral sphingomyelinase. *Chembiochemistry* 2:141.
- Garthwaite, J., E. Southam, C. L. Boulton, E. B. Nielsen, K. Schmidt, and B. Mayer. 1995. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.* 48:184.
- Gadbois, D. M., H. A. Crissman, R. A. Tobey, and E. M. Bradbury. 1992. Multiple kinase arrest points in the G<sub>1</sub> phase of nontransformed mammalian cells are absent in transformed cells. *Proc. Natl. Acad. Sci. USA* 89:8626.
- Banchereau, J., B. Schuler-Thurner, A. K. Palucka, and G. Schuler. 2001. Dendritic cells as vectors for therapy. *Cell* 106:271.
- Underhill, D. M. 2003. Toll-like receptors: networking for success. *Eur. J. Immunol.* 33:1767.
- Muzio, M., D. Bosisio, N. Polentarutti, G. D'Amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. P. Ruco, P. Allavena, et al. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.* 164:5998.
- Horinouchi, K., S. Erlich, D. P. Perl, K. Ferlinz, C. L. Bisgaier, K. Sandhoff, R. J. Desnick, C. L. Stewart, and E. H. Schuchman. 1995. Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease. *Nat. Genet.* 10:288.
- Santana, P., L. A. Pena, A. Haimovitz-Friedman, S. Martin, D. Green, M. McLoughlin, C. Cordon-Cardo, E. H. Schuchman, Z. Fuks, and R. Kolesnick. 1996. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell* 86:189.
- Kirschnek, S., F. Paris, M. Weller, H. Grassmé, K. Ferlinz, A. Riehle, Z. Fuks, R. Kolesnick, and E. Gulbins. 2000. CD95-mediated apoptosis *in vivo* involves acid sphingomyelinase. *J. Biol. Chem.* 275:27316.

54. Dhami, R., X. He, R. E. Gordon, and E. H. Schuchman. 2001. Analysis of the lung pathology and alveolar macrophage function in the acid sphingomyelinase-deficient mouse model of Niemann-Pick disease. *Lab Invest.* 81:987.
55. Paris, F., Z. Fuks, A. Kang, P. Capodiceci, G. Juan, D. Ehleiter, A. Haimovitz-Friedman, C. Cordon-Cardo, and R. Kolesnick. 2001. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* 293:293.
56. Paris, F., H. Grassmé, A. Cremesti, J. Zager, Y. Fong, A. Haimovitz-Friedman, Z. Fuks, E. Gulbins, and R. Kolesnick. 2001. Natural ceramide reverses Fas resistance of acid sphingomyelinase<sup>-/-</sup> hepatocytes. *J. Biol. Chem.* 276:8297.
57. Furuke, K., and E. T. Bloom. 1998. Redox-sensitive events in Fas-induced apoptosis in human NK cells include ceramide generation and protein tyrosine dephosphorylation. *Int. Immunol.* 10:1261.
58. Cifone, M. G., G. Migliorati, R. Parroni, C. Marchetti, D. Millimaggi, A. Santoni, and C. Riccardi. 1999. Dexamethasone-induced thymocyte apoptosis: apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. *Blood* 93:2282.
59. Deigner, H. P., R. Claus, G. A. Bonaterra, C. Gehrke, N. Bibak, M. Blaess, M. Cantz, J. Metz, and R. Kinscherf. 2001. Ceramide induces aSMase expression: implications for oxLDL-induced apoptosis. *FASEB J.* 15:807.
60. Hotchkiss, R. S., and I. E. Karl. 2003. The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* 348:138.
61. Krabbe, K. S., H. Bruunsgaard, J. Qvist, L. Fonsmark, K. Møller, C. M. Hansen, P. Skinhøj, and B. K. Pedersen. 2002. Activated T lymphocytes disappear from circulation during endotoxemia in humans. *Clin. Diagn. Lab. Immunol.* 9:731.
62. Ditschkowski, M., E. Kreuzfelder, M. Majetschak, U. Obertacke, U. F. Schade, and H. Grosse-Wilde. 1999. Reduced B cell HLA-DR expression and natural killer cell counts in patients prone to sepsis after injury. *Eur. J. Surg.* 165:1129.
63. Oberholzer, C., A. Oberholzer, M. Clare-Salzler, and L. L. Moldawer. 2001. Apoptosis in sepsis: a new target for therapeutic exploration. *FASEB J.* 15:879.
64. Lin, T., L. Genestier, M. J. Pinkoski, A. Castro, S. Nicholas, R. Mogil, F. Paris, Z. Fuks, E. H. Schuchman, R. N. Kolesnick, et al. 2000. Role of acidic sphingomyelinase in Fas/CD95-mediated cell death. *J. Biol. Chem.* 275:8657.
65. Monick, M. M., R. K. Mallampalli, A. B. Carter, D. M. Flaherty, D. McCoy, P. K. Robeff, M. W. Peterson, and G. W. Hunninghake. 2001. Ceramide regulates lipopolysaccharide-induced phosphatidylinositol 3-kinase and Akt activity in human alveolar macrophages. *J. Immunol.* 167:5977.
66. Han, J., J. D. Lee, L. Bibbs, and R. J. Ulevitch. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265:808.
67. Moncada, S., and E. A. Higgs. 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* 9:1319.
68. Reade, M. C., and J. D. Young. 2003. Of mice and men (and rats): implications of species and stimulus differences for the interpretation of studies of nitric oxide in sepsis. *Br. J. Anaesth.* 90:115.
69. Parratt, J. R. 1998. Nitric oxide in sepsis and endotoxaemia. *J. Antimicrob. Chemother.* 41SA:31.
70. Vincent, J. L., H. Zhang, C. Szabo, and J. C. Preiser. 2000. Effects of nitric oxide in septic shock. *Am. J. Respir. Crit. Care Med.* 161:1781.
71. Wei, X. Q., I. G. Charles, A. Smith, J. Ure, G. J. Feng, F. P. Huang, D. Xu, W. Muller, S. Moncada, and F. Y. Liew. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375:408.
72. MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. W. Xie, K. Sokol, N. Hutchinson, et al. 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81:641.
73. Laubach, V. E., E. G. Shesely, O. Smithies, and P. A. Sherman. 1995. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc. Natl. Acad. Sci. USA* 92:10688.
74. Cobb, J. P., R. S. Hotchkiss, P. E. Swanson, K. Chang, Y. Qiu, V. E. Laubach, I. E. Karl, and T. G. Buchman. 1999. Inducible nitric oxide synthase (iNOS) gene deficiency increases the mortality of sepsis in mice. *Surgery* 126:438.
75. Hollenberg, S. M., M. Broussard, J. Osman, and J. E. Parrillo. 2000. Increased microvascular reactivity and improved mortality in septic mice lacking inducible nitric oxide synthase. *Circ. Res.* 86:774.
76. Taylor, B. S., L. H. Alarcon, and T. R. Billiar. 1998. Inducible nitric oxide synthase in the liver: regulation and function. *Biochemistry* 63:766.
77. Wang, W., S. Jittikanont, S. A. Falk, P. Li, L. Feng, P. E. Gengar, B. D. Poole, R. P. Bowler, B. J. Day, J. D. Crapo, et al. 2003. Interaction among nitric oxide, reactive oxygen species, and antioxidants during endotoxemia-related acute renal failure. *Am. J. Physiol.* 284:F532.
78. Price, S., J. A. Mitchell, P. B. Anning, and T. W. Evans. 2003. Type II nitric oxide synthase activity is cardio-protective in experimental sepsis. *Eur. J. Pharmacol.* 472:111.
79. Rudkowski, J. C., E. Barreiro, R. Harfouche, P. Goldberg, O. Kishta, P. D'Orleans-Juste, J. Labonte, O. Lesur, and S. N. Hussain. 2004. Roles of iNOS and nNOS in sepsis-induced pulmonary apoptosis. *Am. J. Physiol.* 286:L793.
80. Liu, L., and J. S. Stamler. 1999. NO: an inhibitor of cell death. *Cell Death Differ.* 6:937.