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Lipopolysaccharide and Ceramide Use Divergent Signaling Pathways to Induce Cell Death in Murine Macrophages

Viktor Lakics and Stefanie N. Vogel

Ceramide is a well-known apoptotic agent that has been implicated in LPS signaling. Therefore, we examined whether LPS-induced macrophage cytotoxicity is mediated by mimicking ceramide. Both LPS and the cell-permeable ceramide analogue, C2 ceramide, induced significant cell death in IFN-γ-activated, thioglycollate-elicited peritoneal macrophages after 48 and 24 h, respectively. Ceramide-induced cell death was neither accompanied by DNA fragmentation nor phosphatidylserine externalization, characteristics of apoptosis. In contrast, LPS induced a significant fraction of cells to undergo apoptosis, as demonstrated by DNA fragmentation and quantified by DNA analysis on FACS, yet the majority of the cells died in a necrotic fashion. C3H/HeJ Lps<sup>d</sup> macrophages were resistant to LPS-induced cell death and less sensitive to C2 ceramide-evoked cytotoxicity, when compared with Lps<sup>d</sup> macrophages. C2 ceramide plus IFN-γ failed to activate release of nitric oxide (NO), whereas LPS-induced cell death, but not C2-induced cytotoxicity, was blocked by an inhibitor of inducible NO synthase (iNOS), N<sup>5</sup>-monomethyl-l-arginine. Macrophages from IFN regulatory factor-1 (−/−) mice shown previously to respond marginally to LPS plus IFN-γ to express iNOS mRNA and NO, were refractory to LPS plus IFN-γ-induced cytotoxicity and apoptosis. These data suggest that although LPS may mimic certain ceramide effects, signal transduction events that lead to cytotoxicity, as well as the downstream mediators, diverge. The Journal of Immunology, 1998, 161: 2490–2500.

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3 Abbreviations used in this paper: PKC, protein kinase C; NF-κB, nuclear factor-κB; SPPK/JNK, stress-activated protein kinases; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; SMase, sphingomyelinase; TNF-α, tumor necrosis factor-α; CPP32, 32-kDa cysteine protease; ANOVA, analysis of variance.
critical element in the activation of iNOS, and therefore LPS-induced apoptosis. However, controversy exists in the literature as to the ability of ceramide analogues or SMase to trigger the nuclear translocation of NF-κB (33–37).

Although LPS-induced apoptosis has been associated with iNOS activation, little is known about upstream events. In this study, the question of whether LPS induces cell death in peritoneal macrophages by mimicking ceramide was examined. The cytotoxicity of ceramide analogues in a variety of cell types is well established, but their possible toxic effects on peritoneal exudate macrophages has not yet been described. Here we characterize cell death induced by N-acetylsphingosine (C2 ceramide) in peritoneal macrophages, showing differences compared with LPS-induced toxicity. In IFN-γ-treated mouse peritoneal macrophage cultures, LPS was found to induce both necrosis and apoptosis, whereas C2 ceramide-induced cell death was predominantly necrotic. We also show that C3H/HeJ macrophages are resistant to both LPS plus IFN-γ-induced apoptosis and necrosis, and less sensitive to C2 ceramide plus IFN-γ-induced cell death, compared with macrophages from fully LPS-responsive C3H/OuJ mice. IFN regulatory factor-1 (IRF-1) knockout mice, whose macrophages have been shown previously to be refractory to LPS plus IFN-γ to release NO (38) and whose T cells are refractory to certain inducers of apoptosis (39), failed to exhibit cytotoxicity or apoptosis in response to LPS plus IFN-γ. Finally, we compared the involvement of iNOS and the role of soluble TNF-α in C2 ceramide- and LPS-induced macrophage cytotoxicity. Taken collectively, the data demonstrate that LPS-induced cytotoxicity is, in part, apoptotic and NO-mediated and distinct from the pathway evoked by ceramide in murine macrophages.

Materials and Methods

Reagents

Escherichia coli

K235 LPS was prepared using the method of McIntyre et al. (40). Murine rIFN-γ is kindly provided by Genentech (South San Francisco, CA). Recombinant soluble TNF receptor Fc conjugate (TNFR:Fc) was provided by Immunix (Seattle, WA). Annexin-V-propidium iodide apoptosis detection kit was purchased from Boehringer Mannheim (Indianapolis, IN). N-acetyl sphingosine (C2 ceramide), dihydro-C2 ceramide, and N-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanamide (PPMP) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). N3-monomethyl-L-arginine (L-NMMA) was purchased from Sigma (St. Louis, MO). All reagents were solubilized according to the manufacturers’ instructions DMSO for ceramides and PPMP; cell culture medium for L-NMMA. The endothelial content of 25 μM C2 ceramide and PPMP was measured by Limulus amebocyte lysate assay and found to be less than 0.05 ng/ml in both cases. All other chemicals were obtained from Sigma and were the highest analytical grade possible.

Animals

C3H/OuJ (LPS-responsive) and C3H/HeJ (LPS-hyporesponsive mouse (female, 5 to 6 wk old) were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice with a targeted mutation in the IRF-1 gene (IRF-1 (−/−) gene homozygous “knockout” mice and their heterozygotic (+/−) littermates) were originally obtained from Dr. Tak Mak (Amgen Institute, Toronto, Canada) (41). Mice were housed in a virus Ab-free facility. The IRF-1 (−/−) colony was produced by mating IRF-1 (−/−) mice to either wild type (+/+) or IRF-1 (−/−) mice. The IRF-1 wild-type (+/+) colony was maintained by mating IRF-1 (+/+ ) mice that were derived from heterozygous matings to either IRF-1 (−/−) or IRF-1 (+/+ ) mice. To prevent the background of the IRF-1 (−/−) and (+/+ ) colonies from straying, all new breeding pairs were the progeny of IRF-1 (−/−) × IRF-1 (−/−) matings. The genotype of all IRF-1 mice was determined by PCR using the primers and methods described previously (42). The IRF-1 primers amplify a 300-bp sequence from genomic DNA.

Culture of peritoneal macrophages

Mice were injected i.p. with 3 ml of 3% thioglycollate medium (Difco, Detroit, MI). Peritoneal exudate cells were collected by lavage 4 days after injection. Thioglycollate-elicited macrophages were plated in RPMI 1640 medium supplemented with 2% FCS (HyClone, Logan, UT), 2 mM glutamine, 30 mM HEPES, 0.4% sodium bicarbonate, and penicillin-streptomycin (100 IU/ml and 100 μg/ml). After 3 h of adherence, cells were washed two times and then treated with various agents for the indicated time periods, using the serum-free version of the above-mentioned medium at 37°C in 5% CO2 atmosphere in a humidified incubator. LDH was used for different measurements. For cytotoxicity studies (lactate dehydrogenase (LDH) assay and trypan blue exclusion), 24-well plates (5.4 × 105 cells/well, in a volume of 1 ml) (Corning, Corning, NY) and six-well plates (3 × 106 cells/well, in a volume of 1.5 ml) (Corning) were used. For DNA analysis by FACS and DNA fragmentation experiments, cells were cultured in 6-well plates as described above. To study phosphatidylserine externalization, cells were plated in Teflon beakers (Pierce, Rockford, IL; 3 × 106 cells/beaker in a volume of 1.5 ml) and because of the weak adherence of macrophages to Teflon beakers, the washing steps were omitted. Although throughout this study we used serum-free medium for treatments, addition of 2% FCS did not significantly change the kinetics, the type (i.e., apoptotic vs necrotic), or the extent of cell death caused by ceramide or LPS (data not shown).

Evaluation of cell death

Cell death was evaluated qualitatively and quantitatively by phase contrast microscopy of trypan blue-stained cells, and quantified by the measurement of LDH leakage from damaged cells, according to the method of Wrobleski and La Due (43). Cell injury was expressed as a ratio of LDH activity released into the media and the total LDH activity after detergent treatment of cells in the very same wells. For comparison, in some cases, the percent cell death was also assessed by trypan blue exclusion. For this method, at least 200 cells were counted in triplicate wells, and the percent of trypan blue-positive cells was calculated. Unless otherwise stated, results were expressed as arithmetic means of triplicate samples ± SEM. Wherever statistically significant differences are mentioned in the text, one-way analysis of variance (ANOVA), combined with Tukey’s test was used at the significance level of p < 0.05.

DNA analysis by flow cytometry

For selective and quantitative determination of apoptosis, flow cytometric DNA analysis described by Nicoletti et al. (44) was utilized. Accordingly, the percentage of apoptotic cells whose DNA content is lower than that of diploid cells is calculated. Briefly, cells were harvested by using a rubber policeman, then centrifuged at 400 × g for 10 min. The pellet was gently resuspended in 1 ml hypotonic fluorochrome solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton-X-100) in polypropylene tubes. The sample was kept at 4°C in the dark, at least overnight, and then red fluorescent (620 nm) of individual nuclei was measured by using a Coulter Epics XL-MCL flow cytometer equipped with System II acquisition software (Coulter, Hialeah, FL). The forward scatter and side scatter of particles were simultaneously measured. Cell debris were excluded from analysis by appropriately raising the forward scatter threshold. All measurements were done under the same instrument conditions. For this, 10,000 cells were measured in every sample. Because of DNA loss, apoptotic cells are represented by a distinct and quantifiable subdiploid peak in the fluorescence histogram.

Evaluation of apoptosis by measurement of phosphatidyl serine externalization

The externalization of phosphatidyl serine in the cell membrane is one of the earliest events in apoptosis and seems to be a cell type- and inducer-independent process. Using simultaneous annexin-V-FITC and propidium iodide staining, the percentage of cells that underwent apoptosis and necrosis was quantitatively determined. Fluorescin-labeled annexin-V and propidium iodide from Boehringer Mannheim were used for detection of apoptosis according to the manufacturer’s instructions. Because the macrophages adhere so strongly to tissue culture plates, Teflon beakers were used for all of these experiments to minimize induction of cell death by mechanical shearing. In brief, after incubation with various cell death-inducing agents, macrophages were detached by gentle aspiration from the Teflon beaker surface and centrifuged at 400 × g for 10 min. The pellet was then resuspended in 100 μl labeling solution (140 mM NaCl, 10 mM HEPES, 5 mM CaCl2, 1 μg/ml propidium iodide, 1/50 volume of annexin-V-FITC), and then incubated for 10 min in the dark at 4°C. Samples were analyzed on a Coulter Epics XL-MCL flow cytometer using 488-nm excitation and 525-nm bandpass filters for fluorescein detection and a filter of 620 nm for PI detection, after electronic compensation of the instrument to exclude overlap of the two emission spectra. Propidium iodide positive
(necrotic) cells were gated out, and results are shown as annexin-V binding histograms, where at least 10,000 cells were measured in each sample.

**Measurement of NO and TNF Bioassay**

Secretion of NO by macrophages was measured by spectrophotometric determination of nitrite, the stable end product of NO oxidation, as described previously (45). Supernatants (or their appropriate dilutions) from macrophage cultures were mixed with an equal volume of Griess reagent (one part 0.1% N-(1-naphthyl)-ethylenediamine in water and one part 1% sulfanilamide in 5% phosphoric acid), and the absorbance at 570 nm was measured and compared with a NaNO~2~ (1–100 μM) standard curve.

To measure bioactive TNF-α/β released into the cell culture medium, a previously described, standard TNF cytotoxicity assay was used (46).

**DNA Isolation and Electrophoresis**

DNA isolation was performed by the method of Miller et al. (47), with minor modifications. Briefly, macrophages were cultured in 6-well plates and treated for 8 to 48 h, depending on the cell death-inducing agent, then cells were harvested and centrifuged, and pellets were rapidly frozen in liquid nitrogen. After thawing the pellets on ice, cells were resuspended in a buffer containing 200 μg/ml proteinase-K (Boehringer Mannheim), 10 mM Tris-HCl (pH 8), 10 mM EDTA, and 0.5% SDS and incubated for 3 h at 55°C. Chromosomal DNA and proteins were precipitated overnight at 4°C in the presence of 1 M NaCl. After centrifugation (30 min at 5000 × g), supernatants were treated with 25 μg/ml RNase (Sigma) for 1 h, then DNA fragments were precipitated overnight by two volumes of absolute ethanol at −20°C. Samples were centrifuged for 30 min at 10,000 × g. DNA pellets were air-dried and then resuspended in 10 mM Tris-HCl (pH 8), and 1 mM EDTA, and were subjected to horizontal gel electrophoresis (agarose, 2%). Images of ethidium bromide-stained gels were captured by the Eagle Eye gel documentation system (Stratagene, La Jolla, CA) and were processed by subtracting the background, defined by medium-treated samples.

**Results**

**LPS and Ceramide Induce Cell Death in Peritoneal Macrophages**

It is well documented that LPS can exert direct toxic effects on peritoneal macrophages, macrophage cell lines, endothelial cells, and many other cell types. Although early studies utilized very high LPS concentrations (20–50 μg/ml) to demonstrate direct macrophage cytotoxicity (10, 48, 49), simultaneous stimulation with IFN-γ or cycloheximide has been shown to potentiate the toxicity of much lower LPS concentrations (<1 μg/ml) (12, 13). In an initial series of studies (Fig. 1) in which cell death was assessed by release of LDH into the culture supernatants in 24-well plates, we found that 1 μg/ml LPS alone was not significantly cytotoxic for LPS-responsive, C3H/OuJ macrophages, even after 48 h of incubation. The presence of IFN-γ potentiated the cell death, as previously reported (12, 13). No measurable cell death was observed with LPS alone or LPS plus IFN-γ at 24 h (data not shown).

The cell-permeable ceramide analogue N-acetylphosphoginsine (C2-ceramide) at a dose of 25 μM, a dose shown in previous studies to induce gene expression in these macrophages (19), was found to be directly cytotoxic by 24 h of exposure, and like LPS, ceramide-induced cell death was potentiated by the presence of IFN-γ.

These findings were extended by experiments shown in Figures 2–4. Using both trypan blue exclusion and LDH as measurements of macrophage cell death, Figure 2 illustrates that both LPS- (Fig. 2A) and C2 ceramide- (Fig. 2B) induced cytotoxicity are dose-dependent in the presence of IFN-γ. Even when very high concentrations of LPS (25–50 μg/ml) plus IFN-γ were used, cell death rarely exceeded 50% (as measured in the LDH assay), whereas 1 μg/ml LPS plus IFN-γ evoked suboptimal, but statistically significant, cell death. IFN-γ alone failed to induce measurable cytotoxicity, even after 72 h of incubation (data not shown). LPS-hyporesponsive C3H/HeJ macrophages were highly refractory to LPS plus IFN-γ-induced cytotoxicity (Fig. 2A). Under identical conditions, 25 and 50 μM C2 ceramide were also toxic, causing ~40% and ~60% cell death, respectively, after only 24 h of incubation in the presence of IFN-γ (Fig. 2B). The inactive ceramide analogue, dihydro-C2 ceramide, plus IFN-γ did not affect the viability of macrophages, even after 48 h (data not shown). Elevation of intracellular ceramide levels by inhibition of glucosylceramide synthase with 25 to 50 μM PPMP (50), which inhibits the further incorporation of ceramide into glycolipids, also induced cell death in macrophages after 24 h of treatment, and similarly to C2 ceramide-induced cell death, this was potentiated by the presence of IFN-γ (Table I). LPS-hyporesponsive C3H/HeJ macrophages were highly refractory to very high concentrations of LPS (i.e., 50 μg/ml) plus IFN-γ-induced cell death (Fig. 2A), and they were also less sensitive to C2 ceramide (25 μM) plus IFN-γ-induced cell death (Fig. 2B), although at 50 μM, they were equivalently sensitive to C3H/OuJ macrophages.

LPS plus IFN-γ-induced cell death was highly dependent on the volume of medium used in cell culture wells. The data presented in Figures 1 and 2 were measured in 24-well plates, with 5.4 × 10⁵ cells in 1 ml of medium per well. Under conditions where the cells were cultured in 6-well plates in a final volume of 1.5 ml medium (i.e., 3 × 10⁶ cells/well; the same cell density as in the 24-well plates, but in a relatively smaller volume of medium), the sensitivity of the macrophages to LPS plus IFN-γ was greatly increased. Figure 3A illustrates that under these culture conditions, as little as...
0.1 ng/ml LPS induced macrophage cytotoxicity in the presence of IFN-\(\gamma\). Again, LPS-induced cytotoxicity was not evident at 24 h of incubation, whether or not IFN-\(\gamma\) was present. Under these conditions of increased LPS sensitivity, the macrophages remained comparably sensitive to C2 ceramide (data not shown). Figure 3B demonstrates that the pattern of cytotoxicity induced by LPS plus IFN-\(\gamma\) was paralleled by the release of NO\(_2\) into culture supernatants.

Involvement of iNOS and autocrine TNF production in LPS- and ceramide-induced cell death

A comparison of LPS- and C2 ceramide-induced cytotoxicity and NO\(_2\) production was next studied. As shown in Figure 3, Figure 4A again illustrates that LPS plus IFN-\(\gamma\) induced both cell death and NO\(_2\) release in parallel in C3H/OuJ macrophages, whereas C3H/HeJ macrophages were highly refractory to LPS-induced cell death and produced minimal levels of NO\(_2\). In C3H/OuJ macrophages, L-NMMA, an inhibitor of iNOS, blocked both cell death and NO\(_2\) release in response to LPS and IFN-\(\gamma\). In contrast, 25 \(\mu\)M C2 ceramide plus IFN-\(\gamma\) did not induce NO\(_2\) release into the culture media (Fig. 4B), nor did L-NMMA protect C3H/OuJ macrophages from C2 ceramide plus IFN-\(\gamma\)-induced cell death under conditions where it protected against LPS plus IFN-\(\gamma\)-induced cytotoxicity. Moreover, PPMP, an agent that

Table I. PPMP induces cell death without concurrent nitrite release in peritoneal macrophage cultures

<table>
<thead>
<tr>
<th>Treatment(^a)</th>
<th>% Cell Death (LDH release)</th>
<th>Nitrite Release ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without IFN-(\gamma)</td>
<td>Without IFN-(\gamma)</td>
</tr>
<tr>
<td>Medium</td>
<td>7.3 ± 1.9</td>
<td>7.4 ± 1.2</td>
</tr>
<tr>
<td>PPMP (12.5 (\mu)M)</td>
<td>11.0 ± 1.5</td>
<td>16.0 ± 1.5*</td>
</tr>
<tr>
<td>PPMP (25 (\mu)M)</td>
<td>19.6 ± 1.6*</td>
<td>27.8 ± 1.9*</td>
</tr>
<tr>
<td>PPMP (50 (\mu)M)</td>
<td>35.1 ± 1.0*</td>
<td>47.6 ± 0.1*</td>
</tr>
</tbody>
</table>

\(^a\)Cells were cultured and treated with PPMP for 24 h on 24-well plates in the presence or absence of IFN-\(\gamma\). Cell death and nitrite release into culture supernatants were measured as described in Materials and Methods. Data represent arithmetic means ± SEM of triplicate samples from a representative experiment (\(n = 3\)).

\*Statistically significant difference, compared to the medium or IFN-\(\gamma\)-treated control, respectively (\(n = 3\), \(p < 0.05\), one-way ANOVA, combined with Tukey’s test).
the bioactive TNF-
LPS plus IFN-
g induced cell death (Table II).

To study whether the C2 ceramide plus IFN-
g-induced cell death were the result of apoptosis or necrosis, DNA fragmentation studies, as well as quantitative flow cytometric approaches, were employed. After 48 h of treatment, 1 μg/ml LPS plus IFN-γ caused detectable apoptotic DNA fragmentation in peritoneal macrophage cultures (Fig. 5). No DNA laddering was observed after 24 h of treatment. Blocking iNOS activity with l-NNMA eliminated the apoptotic DNA laddering caused by LPS plus IFN-γ (Fig. 5). The positive control, 3 μM gliotoxin (13, 51), resulted in much stronger DNA fragmentation, even after 8 h, compared with that induced by LPS plus IFN-γ. In contrast, 25 μM C2 ceramide plus IFN-γ did not induce any measurable DNA fragmentation even after 24 h (Fig. 5), the time point when ~40 to 60% of the macrophages were dead (Figs. 1 and 2).

To quantify macrophage apoptosis caused by LPS plus IFN-γ, the percentage of subdiploid nuclei, characteristic for apoptotic cell populations, was measured by flow cytometry. After 48 h of incubation with 1 μg/ml LPS plus IFN-γ, which resulted ~70% macrophage death in 6-well plates (Fig. 4A), ~20% of the cells exhibited subdiploid nuclei, in contrast to the positive control, 3 μM gliotoxin, which induced >90% apoptosis within 24 h (see Fig. 6A for a typical experiment, and Table III for statistical analysis). Without IFN-γ, LPS did not increase the percentage of apoptotic cells, compared with medium-treated controls (Fig. 6A). Again, consistent with the DNA fragmentation studies (Fig. 5), l-NNMA blocked the appearance of cells with subdiploid nuclei in LPS plus IFN-γ-treated samples. Macrophages from Lpsd mice proved to be resistant to apoptotic insult by LPS plus IFN-γ (Fig. 6A).

In contrast to gliotoxin, freeze-thawing of the cultures (Fig. 6B) or killing the macrophages by hypotonic lysis with distilled water (data not shown) did not produce a hypodiploid peak in the histogram, showing that this method distinguishes clearly between apoptosis and necrosis. In agreement with the DNA fragmentation studies (Fig. 5), 25 μM C2 ceramide plus IFN-γ failed to elicit any apoptosis after 24 h of treatment, as measured by flow cytometric DNA analysis (Fig. 6B, Table III). No increase in the percentage of subdiploid nuclei was measurable even at a C2 ceramide concentration of 50 μM, in the presence of IFN-γ (Fig. 6B).

One of the earliest events in apoptosis is the externalization of phosphatidyl serine in the plasma membrane as a consequence of apoptotic injury. Besides DNA fragmentation, this process is thought to be one of the hallmarks of apoptosis in a wide variety of experimental systems and, in fact, represents an independent event from nuclear changes. Because DNA fragmentation and analysis of subdiploid nuclei failed to suggest evidence for C2 ceramide plus IFN-γ-induced apoptosis, we also measured the binding of annexin-V to macrophages, concurrent with propidium iodide staining, in an attempt to detect possible phosphatidyl serine externalization. A relatively low dose of gliotoxin (0.5 μM) elicited apoptosis by 24 h, as measured by an increase in annexin-V binding to macrophages from 11% to 75%. After 4 h of treatment with 25 μM C2 ceramide plus IFN-γ, no increase was observed in the percentage of annexin-V binding cells (12% of C2 ceramide plus IFN-γ vs 11% of medium-treated control). Even after 24 h of incubation with C2 ceramide plus IFN-γ, the proportion of annexin-V positive cells was only 13% vs 11% of medium-treated controls, indicating that the cell death caused by C2 ceramide plus IFN-γ is not apoptotic. This is illustrated in Figure 7, A (medium- and gliotoxin-treated cells) and B (medium and C2 ceramide plus IFN-γ-treated cells), where the respective annexin-V-binding histograms are shown in an overlayed fashion.

FIGURE 3. LPS-induced cell death and NO release in the presence or absence of IFN-γ. LPS-induced cell death and NO release were measured in the absence or presence of 50 IU/ml IFN-γ at various LPS concentrations after 24 and 48 h of incubation. C3H/OUJ macrophage cultures were prepared as described in Materials and Methods in 6-well culture plates, in a final volume of 1.5 ml. Cell death was measured by LDH activity released to the media by the injured cells (A), and nitrite concentration was assessed by Griess reaction (B). Data are from duplicate samples from a representative experiment (n = 3).

LPS plus IFN-γ causes necrosis and apoptosis, whereas C2 ceramide plus IFN-γ induces necrosis only

Because TNF-α, induced by LPS treatment, plays a key role in development of sepsis syndrome in vivo, and because TNF-α is a well-known inducer of apoptosis, we investigated a potential role for TNF-α in LPS plus IFN-γ- and C2 ceramide plus IFN-γ-induced cell death in vitro. Table II illustrates that LPS plus IFN-γ induced a large accumulation of bioactive TNF. Although, inclusion of 1 x 10^-5 M soluble TNF-receptor (TNFR:Fc) eliminated the bioactive TNF-α induced by LPS plus IFN-γ, it was not protective against the cell death induced under the same conditions. A total of 25 μM C2 ceramide plus IFN-γ failed to induce the release of bioactive TNF-α, as measured by TNF bioassay. TNFR:Fc conjugate did not protect the cells against C2 ceramide plus IFN-γ-induced cell death (Table II).

LPS plus IFN-γ causes necrosis and apoptosis, whereas C2 ceramide plus IFN-γ induces necrosis only
LPS plus IFN-γ-induced cytotoxicity and apoptosis are IRF-1-dependent

Previous studies have indicated that mice with a targeted mutation in the gene that encodes the IRF-1 possess macrophages that respond poorly to LPS plus IFN-γ to induce iNOS gene expression and NO release (38). Moreover, the T cells of these mice have also been reported to be refractory to apoptosis induced by DNA damage (e.g., γ-irradiation, etoposide, adriamycin) (39). Therefore, we sought to evaluate the responsiveness of macrophages derived from IRF-1 (−/−) mice and IRF-1 (+/+) controls to LPS plus IFN-γ-induced cell death and NO release. Figure 8, A and B illustrate that LPS plus IFN-γ induces both cell death and NO release in parallel in IRF-1 (+/+ ) macrophages within 48 h, whereas IRF-1 (−/−) macrophages were highly resistant to LPS-induced cell death and produced low levels of NO. In IRF-1 (+/+ ) macrophages, L-NNMA blocked both cell death and NO release in response to LPS and IFN-γ. Also, as was observed in C3H/OuJ macrophage cultures (Fig. 6A), a significant portion of IRF-1 (+/+) macrophages underwent apoptosis (∼30%, Fig. 8C) in response to LPS plus IFN-γ, although necrotic cell death remained dominant. In contrast, IRF-1 (−/−) peritoneal macrophages did not show nuclear DNA loss as measured by the appearance of subdiploid nuclei by FACS (Fig. 8C). These data demonstrate that both the LPS plus IFN-γ-induced cell death and apoptosis are IRF-1-dependent in peritoneal macrophages.

Discussion

In addition to its systemic effects, LPS also exerts cellular toxicity that may be particularly important as one of the causes of multiple organ failure in sepsis. Indeed, LPS-induced endothelial cell death has been proposed as a major cause of mortality in a murine model of endotoxic shock (52). Although a variety of cell types such as T cells, B cells, and neutrophils are reported to be sensitive to LPS toxicity in vivo, little is known about whether macrophages undergo apoptosis during the process of endotoxic shock. Recently, Ayala et al. (53) showed increased peritoneal macrophage apoptosis in vivo in a cecal...
ligation and puncture sepsis model (CLP), compared with sham-CLP animals. This increased apoptosis rate during sepsis appears to be correlated with an “acquired immunosuppression” (dysfunction of macrophages) in the late phase of sepsis. Moreover, it has also been reported that treatment of macrophages from CLP mice in vitro with LPS can further increase apoptosis, and the process is NO-dependent (54). In spite of these in vivo and the aforementioned in vitro studies, the exact contribution of the macrophage cell death to endotoxicity is still not known.

Involvement of ceramide pathway in signal transduction of certain LPS effects has recently been suggested (21). It has also been reported that in vivo, LPS-induced toxicity is mediated by TNF release and subsequent ceramide generation in endothelial cells (52). However, the possible role of the ceramide pathway in LPS-induced cell death in macrophages remained to be elucidated. To address this question, the in vitro toxicity of LPS and C2 ceramide in peritoneal exudate macrophage cultures was compared in the presence of IFN-γ. In vivo, IFN-γ has a very important role in LPS-induced toxicity. In fact, injection of anti-IFN-γ Abs has protective effect of LPS-induced thymocyte cell death in vivo, showing that LPS and IFN-γ may act in concert to induce toxicity (16).

In our studies, LPS- and ceramide-induced cytotoxicity was quantified by two different methods (trypan blue exclusion and LDH release), and these assays gave comparable results (Fig. 2). High concentrations of both LPS (25–50 μg/ml) and C2 (25–50 μM) ceramide were directly toxic for peritoneal exudate macrophages derived from C3H/OuJ mice. The presence of IFN-γ potentiated this effect, but more strikingly for LPS than for C2 ceramide. Under conditions of high cell number and low supernatant volume (e.g., 6-well plates), concentrations of LPS as low as 1 ng/ml could be demonstrated to be highly toxic in the presence of IFN-γ (Fig. 3A). This suggests that a factor(s) that accumulate(s) within the supernatant contribute(s) to LPS-induced cell death. Previous studies have demonstrated that LPS plus IFN-γ-induced cell death is NO-mediated (12, 13, 15, 17). Indeed, this was confirmed in these studies by the demonstration that inactive arginine analogue, L-NMMA, blocked LPS plus IFN-γ-induced cytotoxicity with a concurrent inhibition of NO release into the culture supernatant. Thus, the enhancement of LPS-induced cell death at low LPS concentrations by the presence of IFN-γ is likely to be related to the well-characterized synergy between these two compounds for the induction of iNOS gene expression (45). In contrast, the cytotoxic effects of C2 ceramide or elevation of endogenous ceramide levels by PPMP (in the absence or presence of IFN-γ) appear to be independent of NO release: C2 ceramide and PPMP (±IFN-γ) failed to stimulate release of NO in C3H/OuJ peritoneal exudate macrophages, and L-NMMA failed to protect the cells from C2 ceramide-induced cytotoxicity. These observations, coupled with the failure of the inactive dihydro-ceramide analogue to trigger cytotoxicity, further support the notion that macrophage cytotoxicity induced through the activation of the ceramide pathway is mechanistically distinct from that induced by LPS.

In addition to the lack of evidence for participation of NO in ceramide-induced cytotoxicity, other important differences in kinetics, the type of cell death, and the relative sensitivity of Lps- macrophages to LPS- vs C2 ceramide-induced cytotoxicity were observed. Although C2 ceramide plus IFN-γ induced massive cell death after 24 h of incubation, even high concentrations of LPS (>10 μg/ml) plus IFN-γ did not induce cell death at the same time point. For LPS plus IFN-γ-induced toxicity, at least 48 h of incubation was necessary, even with increased cell number/media volume ratio, which remarkably increased the extent of cell death (~70% compared with ~30%), but without an alteration in kinetics. This finding suggests important differences in the signal transduction and/or the effector mechanisms of cell death for these two compounds.

**Table II.** LPS- and C2 ceramide-induced TNF release and cell death in peritoneal macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Cell Death (LDH release)</th>
<th>TNF-α (IU/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>LPS-induced releasea</td>
<td>Medium</td>
<td>13.1</td>
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<tr>
<td></td>
<td>IFN-γ (50 IU/ml)</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>LPS (1 μg/ml) + IFN-γ</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁻³ M TNFR-Fc + LPS + IFN-γ</td>
<td>87.2</td>
</tr>
<tr>
<td>C2 ceramide-induced releaseb</td>
<td>Medium</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>IFN-γ (50 IU/ml)</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>C2 (25 μM) + IFN-γ</td>
<td>42.0</td>
</tr>
<tr>
<td></td>
<td>TNFR-Fc (1 × 10⁻³ M) + LPS + IFN-γ</td>
<td>42.7</td>
</tr>
</tbody>
</table>

a Cells were cultured and treated in 6-well plates (1 μg/ml LPS + IFN, 1 × 10⁻³ M TNFR-Fc) for 48 h, and cell death was measured by LDH assay in parallel with measurement of TNF released to the culture medium.

b Macrophages were cultured in 24-well plates for 24 h. Cell death was evaluated by LDH assay, as described in Materials and Methods, in parallel with measurement of TNF released to the culture medium. Data shown are means of triplicate samples from two independent experiments.

**FIGURE 5.** Comparison of LPS plus IFN-γ and C2 ceramide plus IFN-γ-induced apoptotic DNA laddering. Cells were plated in 6-well plates and treated with medium (lane D, for 48 h), 1 μg/ml LPS plus IFN-γ (lane E, for 48 h), 1 μg/ml LPS plus IFN-γ in the presence of 0.5 mM L-NMMA (lane F, for 48 h), 25 μM C2 ceramide plus IFN-γ (lane B, for 24 h), or 3 μM gliotoxin (lane C, for 8 h). DNA was extracted from macrophages and subjected to electrophoresis (see Materials and Methods). As a m.w. marker, HindIII digested A-phage DNA, was used (lane A). Similar data were obtained in another separate experiment.
It is well known that macrophages from Lps<sup>d</sup> C3H/HeJ mice are hyporesponsive to LPS effects in vivo and in vitro (2). In the interim, it has been demonstrated that not only are macrophages derived from C3H/HeJ mice hyporesponsive to LPS, but also, they fail to respond to soluble ceramide analogues and exogenously added SMase to induce expression of a subset of LPS-inducible genes or to secrete cytokines in response to SMase stimulation. Interestingly, C3H/HeJ macrophages were profoundly refractory to LPS-induced cytotoxicity, even in the presence of IFN-γ, whereas longer (>24 h) incubation or high concentrations of C2 ceramide (50 μM) plus IFN-γ induced cytotoxicity comparable to that observed in the normoresponsive macrophages. Thus, in C3H/HeJ macrophages, the longer incubation time or high concentrations of C2 ceramide can apparently circumvent the mutation that protects these cells from cytotoxicity induced by high concentrations of LPS, in the absence or presence of IFN-γ. It is interesting to note that similar observation was made by Thieblemont and Wright (20) with respect to defective ceramide uptake in C3H/HeJ macrophages; i.e., at longer incubation times, the initial defect in ceramide uptake became normalized to that observed in C3H/OuJ macrophages.

In sepsis, or in vivo models of endotoxemia, varieties of cell types such as thymocytes, B cells, and endothelial cells have been reported to undergo apoptosis in response to bacterial LPS. Whether a cell death process is apoptotic or necrotic has particular importance in vivo, because in apoptosis, the cytoplasm of dying cells is preserved in “membrane-packed” apoptotic bodies; therefore, the content of the cell is not released into the extracellular

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Subdiploid Nuclei</th>
<th>Number of Independent Experiments</th>
</tr>
</thead>
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<tr>
<td>Medium</td>
<td>6.8 ± 0.7</td>
<td>9</td>
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<tr>
<td>LPS (1 μg/ml) + IFN-γ (50 U/ml)</td>
<td>18.5 ± 1.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>9</td>
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<tr>
<td>NMMA + LPS + IFN-γ</td>
<td>10.4 ± 2.5&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>C2 (25 μM) + IFN-γ</td>
<td>9.8 ± 1.9&lt;sup&gt;†&lt;/sup&gt;</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> DNA analysis experiments were done as described in Materials and Methods. Representative experiments were shown in Figure 6A and B. Data are arithmetic means ± SEM from independent experiments.

<sup>*</sup> Statistically significant difference, (p < 0.05) compared to medium-treated control.

<sup>†</sup> No statistically significant difference compared to medium treated control (one-way ANOVA, combined with Tukey’s test).

FIGURE 6. Quantitative determination of apoptosis by flow cytometric DNA analysis following propidium iodide staining. A, DNA analysis of LPS plus IFN-γ-induced cell injury. Macrophages from Lps<sup>d</sup> C3H/HeJ and Lps<sup>n</sup> (C3H/OuJ) mice were cultured in 6-well plates, treated with 1 μg/ml LPS alone, LPS plus IFN-γ, or 0.5 mM NMMA plus LPS plus IFN-γ for 48 h and 3 μM gliotoxin for 24 h. Bars represent percentage values of apoptotic cells with subdiploid nuclei that correspond to the “M2” marker in the histograms presented in B. At least 10,000 cells were analyzed at each point. Repeated experiments (at least n = 3) gave similar results. B, DNA analysis of C2 ceramide plus IFN-γ-induced cell death. Cells were treated with medium, 50 U/ml IFN-γ, 25 or 50 μM C2 ceramide plus IFN-γ for 24 h, and 3 μM gliotoxin for 16 h. Freeze-thawing of macrophage cultures served as a positive control for necrosis. In the latter, macrophages were frozen at −20°C for 24 h, then were thawed and used for measurements. “M1” and “M2” markers represent the nonapoptotic diploid (viable or necrotic) and hypodiploid (apoptotic) nuclei, respectively. The numbers next to the M1 and M2 markers represent the percentage values of apoptotic and nonapoptotic cells in the graph.
space. In contrast, cytoplasmic content of necrotic cells can act as an additional inflammatory signal to enhance the uncontrolled inflammation that is characteristic of septic shock. To study further the cell death induced by LPS and C2 ceramide, we examined whether these compounds induced apoptosis or necrosis in peritoneal macrophages in vitro. It has been reported that LPS plus IFN-γ triggers apoptosis in peritoneal macrophages (12, 13) and macrophage-like cells (15) through an iNOS-dependent pathway. In agreement with these publications, LPS plus IFN-γ induced detectable DNA fragmentation in our macrophage cultures (Fig. 5). To quantify the extent of apoptosis, we used DNA analysis by FACS. In contrast to previous reports (12, 13, 17), our data show that LPS plus IFN-γ-induced cell death is predominantly necrotic, although a significant fraction of cells underwent apoptosis. A possible explanation for this contradiction is that in previous reports mentioned above, apoptotic cell death was demonstrated by morphologic evidence and DNA fragmentation with no precise quantification of apoptosis. Supportive of our findings, Albina et al. (13) noted that only 10 to 15% of the cells showed apoptotic morphology at 48 h of incubation in their macrophage cultures treated with LPS plus IFN-γ. As an explanation, they hypothesized that although the process as a whole is apoptotic, this may be a result of efficient removal of apoptotic cells by neighboring macrophages or progression of the apoptosis into a phase of secondary necrosis. Our results demonstrated that only 20 to 30% of cells were apoptotic, even when ~70 to 80% cell death was induced by LPS plus IFN-γ at 48 h. The necrotic cells still had intact nuclei as measured by DNA analysis using FACS, so the cell death described here cannot be attributed to secondary necrosis. Moreover, in the case of gliotoxin-treated macrophages, which are clearly apoptotic (according to DNA fragmentation, appearance of subdiploid nuclei, and increased annexin-V binding), the phagocytosis of apoptotic macrophages by neighboring cells did not interfere with the detection of apoptosis. The appearance of necrosis and apoptosis at the same time in response to cell death inducers has been reported in other cell types in vitro and in vivo (55–57). Similarly, in peritoneal macrophage cultures, different subpopulations may exist that respond differently to cell death inducers. Interestingly, both the apoptotic and necrotic cell death of macrophages were antagonized by the iNOS inhibitor, L-NMMA. Although NO-mediated cell death is generally considered as to be apoptotic (13, 15, 58),

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** C2 ceramide plus IFN-γ does not induce apoptosis as assessed by annexin-V staining in peritoneal macrophage cultures. C3H/OuJ macrophages were treated with medium, C2 ceramide plus IFN-γ (4 and 24 h), or 0.5 μM gliotoxin (24 h). Phosphatidyl serine externalization was measured by annexin-V-FITC binding. Necrotic cells were gated out on the basis of their ability to uptake propidium iodide. A and B, Respective annexin-V binding histograms that are overlayed with medium-treated control. Note the increased annexin-V binding induced by gliotoxin (A, histogram is shifted, compared with medium treated control) and the lack of phosphatidyl serine externalization by 4 and 24 h of treatment with C2 ceramide plus IFN-γ (B, completely overlapping histograms). M1 and M2 markers were used to calculate the percentages (see Results) for viable and apoptotic populations, respectively. Data are derived from a representative experiment (n = 3).

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** IRF-1 (−/−) macrophages are refractory to LPS plus IFN-γ-induced cell death and apoptosis. Cells were treated with 50 IU/ml IFN-γ alone and 1 μg/ml LPS plus IFN-γ in the presence or absence of 0.5 mM L-NMMA for 48 h. IRF-1 (−/−) and IRF-1 (+/+) macrophages were cultured in 6-well plates in a volume of 1.5 ml, and cell death was measured by LDH release (A). From the very same wells, apoptosis was assessed by DNA-analysis (C) and NO release was also measured in parallel (B), as described in Materials and Methods. *Statistically significant difference compared with LPS plus IFN-γ-treated IRF-1 (+/+) samples (n = 3, p < 0.05, one-way ANOVA, combined with Tukey’s test). Data are from a representative experiment, repeated twice.
NO-induced necrosis has also been reported in oligodendrocytes (59) and in an epithelial cell line (60). As a possible mechanism for NO-induced apoptosis, direct DNA damage (61) and as a consequence, induction of the tumor suppressor gene, p53, were suggested (15). Although p53 activation has been demonstrated in response to LPS plus IFN-γ treatment in RAW 264.7 cells, a yet unidentified p53-independent pathway is also likely to play a role in this process (15). It has also been reported that p53 activates IL-1β-converting enzyme-like proteases, specifically Caspase-3/CPP32 (32-kDa cysteine protease), which is an important mediator of apoptosis (61). As a mechanism for NO-mediated necrosis, damage caused by free radicals, ATP depletion, and mitochondrial membrane injury have been proposed (59).

In contrast to LPS plus IFN-γ-induced cell death, C2 ceramide plus IFN-γ caused purely necrotic cell death in peritoneal macrophages as measured by DNA fragmentation, DNA analysis by FACs, and annexin-V binding. This was very surprising, given the number of reports that demonstrate that ceramide is a potent inducer of apoptosis (22, 25). A growing body of evidence has implicated possible mediators and regulatory molecules that contribute to ceramide-induced apoptosis. Among others, ceramide-activated proteases (e.g., IL-1β-converting enzyme-like family proteases, especially Caspase-3/CPP32) (62), SAPK/JNK (63), and cytosolic translocation of certain PKC isoforms (64) appear to be important in mediating ceramide-induced apoptosis. Moreover, Bel-2 has regulatory role in this process (32). Much less is known about the mechanisms of ceramide-induced necrosis. Recently, Arora et al (26) showed that ceramide analogues cause mitochondrial membrane permeability transition (MOMP), ATP depletion, and subsequent necrotic cell death. MOMP has been proposed as a final common pathway of cell death.

Among other factors, TNF-α is believed to play fundamental role in septic shock induced by LPS (2). Under in vivo conditions, LPS-induced TNF-α secretion and subsequent TNF-α-induced apoptosis is one of the most important factors in LPS toxicity (52, 65, 66). To examine the role of TNF-α in LPS- and C2 ceramide-induced cell death in macrophages in vitro, we used a soluble TNF receptor (TNFR:Fc). A total of $1 \times 10^{-5}$ M TNFR:Fc neutralized most of the bioactive TNF-α induced by LPS plus IFN-γ (Table II). However, TNFR:Fc did not protect peritoneal macrophages against LPS plus IFN-γ-induced cell death, suggesting that TNF-α is not involved in LPS cytotoxicity in vitro. Similar results were published by Yamamoto et al. (17) and Amano et al. (14). In contrast to Smase stimulation of macrophages (19), C2 ceramide failed to induce bioactive TNF-α in peritoneal macrophage cultures, and TNFR:Fc was not protective against C2 ceramide plus IFN-γ-induced cell death (Table II), suggesting that TNF-α does not mediate this toxicity.

Since IRF-1 was shown previously to be important in certain types of apoptosis (39) and its role in induction of iNOS is well documented (38), we next investigated the participation of IRF-1 in LPS plus IFN-γ-induced cell death and apoptosis. Our experiments with IRF-1 (−/−) mice show that not only the LPS plus IFN-γ-induced apoptosis (~30% of the cells), but cell death in general, are IRF-1-dependent (Fig. 8, A and C). Because a functional IRF-1 gene is essential for activation of iNOS (Fig. 8B), these data further support the hypothesis that LPS plus IFN-γ-induced cell death is NO-mediated. It is also interesting to note that the IRF-1- and p53-dependent apoptotic pathways are considered to be distinct in T lymphocytes (39), p53-dependent pathways in LPS plus IFN-γ-induced apoptosis were described in RAW 264.7 macrophages (58), and here we show that this process in peritoneal macrophages is IRF-1-dependent. This suggests that distinct pathways can act simultaneously to induce apoptosis, depending on the particular cell type and apoptosis inducer.

Taken collectively, in this study we characterized cell death induced by C2 ceramide in peritoneal macrophages, showing the commonalities, as well as differences, with LPS-induced macrophage cytotoxicity. In the presence of IFN-γ, LPS-induced cell death in peritoneal macrophages is mostly due to necrosis, with a significant fraction of cells undergoing apoptosis. Although differing in extent, both C2 ceramide- and LPS-mediated cell death depend on the presence of a normal Lps gene, suggesting a possible common early mediator in the signaling of cell death. In contrast, we demonstrated that LPS-induced, not C2 ceramide-triggered cell death, is mediated by NO in peritoneal macrophages in vitro. Soluble TNF-α was also ruled out as a possible mediator of ceramide- or LPS-induced toxicity. In summary, our study suggests that although LPS may mimic certain ceramide effects, signal transduction events that lead to cytotoxicity, as well as the downstream mediators, diverge.

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References


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