Monocyte Anergy in Septic Shock Is Associated with a Predilection to Apoptosis and Is Reversed by Granulocyte-Macrophage Colony-Stimulating Factor Ex Vivo

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The effects of priming monocytes from septic patients with granulocyte-macrophage colony-stimulating factor (GM-CSF) ex vivo were investigated. Monocytes from septic patients had depressed plasma GM-CSF and dysregulated levels of other cytokines compared with normal subjects. Membrane expression of CD71 and HLA-DR were depressed, and monocytes were anergic to lipopolysaccharide (LPS) stimulation in vitro, which was associated with spontaneous and accelerated activation-induced apoptosis by LPS. Priming monocytes with GM-CSF ex vivo augmented membrane cytokine expression, CD71, and HLA-DR. GM-CSF priming augmented cytokine secretion in response to LPS stimulation, restored cytokine secretion in monocytes from septic patients, and reversed their predilection to undergo apoptosis. Thus, monocyte dysfunction in septic shock is associated with depressed plasma levels of GM-CSF and enhanced apoptosis; however, GM-CSF stimulation ex vivo restored normal monocyte function and cytokine secretion by a mechanism that may depend on abrogating apoptosis.

Circulating monocytes serve an important function in host defense against infection and malignancy [1]. Infection with gram-negative and gram-positive bacteria as well as fungal pathogens may lead to septic (endotoxic) shock and is a major cause of mortality in injured patients who survive their initial trauma [2, 3]. Septic shock is secondary to the primary insult induced by toxic molecules, such as endotoxin, released by gram-negative bacteria. Endotoxin is a lipopolysaccharide (LPS)-containing moiety that is systemically released after phagocytic lysis of the invading gram-negative bacteria. LPS interacts with LPS-binding protein, provoking monocyte activation via occupation of the CD14 membrane receptor [4]. Infusion of LPS replicates septic shock [5] and is associated with the secretion of pro- and anti-inflammatory cytokines, including tumor necrosis factor (TNF) [5, 6], interleukin-1 (IL-1) [7], and interferon-γ (IFN-γ) [8], as well as the systemic release of reactive oxygen and nitrogen free radical species [9, 10].

The anti-inflammatory response is an important component of cellular immunity that modulates the toxic effects of systemic and potentially toxic cytokine secretion. In vitro, IL-10 down-regulates the proinflammatory functions of the monocyte by attenuating TNF and IL-1 secretion, induction of procoagulant activity, and free radical species secretion [11, 12]. Similarly, soluble cytokine antagonists, such as IL-1 receptor antagonist (IL-1Ra) and TNF receptor forms (TNFR-Ip55 and TNFR-Ip75), may reversibly block amplification of proinflammatory cytokine secretion by preventing IL-1 and TNF, respectively, from occupying their cell membrane receptors [13, 14]. Activated monocytes in the septic patient exhibit a hyperinflammatory state that may proceed for hours to days. This is associated with subverted monocyte function and subsequently a hypoinflammatory state that is characterized by anergy to stimulation by endotoxin in vitro [15]. The dysfunctional monocyte exhibits attenuated cytokine secretion in response to LPS and thus may no longer contribute to an effective defense against the disease-causing microorganism [16, 17]. Moreover, activated monocytes are regulated by anti-inflammatory cytokines that may induce senescence or apoptosis [18, 19].

Apoptosis is a process whereby cells undergo programmed cell death during embryonic, hemopoietic, and germinal center growth and differentiation [20]. Apoptosis is a constitutive process, but it can be induced by agents associated with an inflammatory response [21, 22]. Macrophages from septic mice

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failed to release cytokines in response to LPS in vitro, which was associated with a predilection to apoptosis [23]. Immune dysfunction and apoptosis have been observed in murine thymocytes following cecal ligation and puncture [24]. However, IL-10 may be protective in models of sepsis by preventing LPS-induced increases in hyperinflammatory neutrophil survival and by promoting the clearance of apoptotic neutrophils by macrophages in rats challenged intratracheally with LPS [25].

However, the mechanisms that regulate monocyte apoptosis are incompletely understood, although it has been shown that inflammatory mediators and secreted products from microorganisms may modulate the apoptotic process of monocytes [18, 26], and monocyte apoptosis may occur constitutively in cultures supplemented with reduced serum in vitro [18, 26]. The addition of LPS- or monocyte-activating proinflammatory cytokines, such as TNF or IL-1, prolonged monocyte survival and abrogated apoptosis [26]. Under normal conditions, senescent monocytes are phagocyted by resident macrophages [27], preventing the nonspecific release of toxic inflammatory mediators from dying cells that may otherwise be harmful to the host [28]. Monocytes are important in the adaptive immune response to sepsis and can compensate for defects in lymphocyte and neutrophil function in patients with _Candida_ infections [29] or the early removal of phagocytic neutrophils that undergo apoptosis following ingestion of bacterial particles [30].

The protooncogene Bcl-2 encodes a 26-kDa protein that promotes cell survival by blocking apoptosis and is localized to the inner and outer mitochondrial membranes, nuclear envelope, and endoplasmic reticulum [31]. By contrast, the Bax oncoprotein is a homologue of Bcl-2 that heterodimerizes and acts in opposition to it [32]; that is, Bax accelerates apoptosis, and the cellular ratio of the Bcl-2/Bax rheostat could be important in determining the survival of the cell under conditions that promote apoptosis.

Few studies have investigated monocyte dysfunction and apoptotic dysregulation in human disease. However, the regulatory role of LPS and cytokines on human alveolar macrophage apoptosis has been studied [33]. LPS induced apoptosis, whereas cytokines such as IL-1β, IL-4, IL-6, IL-10, TNF, transforming growth factor-β (TGF-β), IFN-γ, or myeloid colony-stimulating factors did not promote apoptosis of these cells. Interestingly, IFN-γ enhanced, while IL-4, IL-10, and TGF-β attenuated, LPS-induced apoptosis in alveolar macrophages [33].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) primes the antimicrobial activation of monocytes in vitro [34] and restores monocyte function when administered to patients after chemotherapy [35, 36]. When administered to animals, GM-CSF was protective against supralethal doses of bacteria or _Candida_ species [37], although when given after the onset of abdominal sepsis, GM-CSF did not confer benefit [38]. In addition, conditioned culture medium containing GM-CSF secreted by human alveolar macrophages delayed neutrophil apoptosis from the same subjects [39]. By contrast, both GM-CSF and IFN-γ maintained the viability of human blood monocytes by a mechanism that was dependent on the diminution in IL-10 secretion [39]. Moreover, GM-CSF contributes to monocyte survival in chronic atopic dermatitis, and these monocytes may contribute to chronic inflammatory diseases [40].

In this study, we investigated aspects of monocyte function in patients with septic shock.

### Materials and Methods

**Patients and control subjects.** Twelve patients selected for the study presented with a focus of infection or were considered to be at high risk of exposure to subsequent hospital-acquired infection. Clinical details have previously been reported [41]. All of the patients selected for the study fulfilled the defined clinical criteria for septic shock as previously described [41]. In total, 64 sex-matched healthy control subjects were studied: 28 men (median age, 34.6 years; range, 21–60) and 36 women (median age, 33.8 years; range, 22–60). Where possible, age-matching was also done. At least 1 control subject was studied simultaneously for each assay. All blood samples were taken from patients within 12–18 h of the initial diagnosis of septic shock by the referring clinician according to the criteria as previously described [41].

**Preparation of human peripheral blood mononuclear cells.** Whole venous blood in 10 IU/mL preservative-free heparin was used to enrich for plastic-adherent monocytes as previously reported [35, 36]. Physically harvested monocytes were >90% pure (range, 86.5%–97.0%) by morphologic and immunophenotypic criteria (<5% CD3, <5% CD56, 90.5% ± 4.5% CD14, and 92.8% ± 3.7% CD64 cells); they were >95% viable for normal monocytes and >90% viable for septic patient monocytes by 0.2% (vol/vol) trypan blue exclusion in Hanks’ balanced saline solution (HBSS). The mean yield of monocytes obtained from normal subjects was 9.8 ± 10⁶ (range, 7.6–11.7 ± 10⁶) and from septic patients was 9.3 ± 10⁶ (range, 7.9–14.6 ± 10⁶). Since we wished to study only the function of monocytes, enriched monocyte cultures were used in assays of cytokine secretion and bioactivity so as to exclude the contaminating element of other cell types.

**Quantitation of inflammatory cytokines by ELISA.** Whole blood anticoagulated in K, EDTA was separated at 2000 g for 15 min at 4°C, and the plasma was heat-inactivated at 56°C for 30 min. Monocytes enriched as described above were seeded into 24-well culture plates at 5 × 10⁵ cells/well. Cytokine secretion was assessed before and after stimulation with LPS ( _Escherichia coli_ O55:B5; Sigma, Dorset, UK) at 1.0 μg/mL with or without monocyte priming with GM-CSF (Schering-Plough Pharmaceuticals, Perterborough, UK) at 5 ng/mL for 2 h (a time that gave optimal pro- and anti-inflammatory cytokine secretion in preliminary dose- and time-dependent studies in normal subjects) at 37°C. The cytokines were quantified in plasma and monocyte supernatants by commercially obtained ELISA kits. TNF-α, IL-1β (low- and high-sensitivity formats), and IL-6 ELISA kits were obtained from R&D Systems (Ox-
ford, UK); and IL-10 ELISA kits were obtained from Genzyme Diagnostics (Cambridge, MA). Plasma cytokines were quantified as picograms per milliliter and monocyte-secreted cytokines as picograms per million cells.

**Assay of bioactive TNF, IL-1β, and IL-6 by secretory monocytes.** The ELISA described above quantitated net immunoreactive cytokine only. However, we also wished to quantify the bioactive fraction of proinflammatory cytokines that was quantitated by immunoreactivity. TNF bioactivity was quantitated by the well-established TNF-mediated cytolyis of TNF-sensitive murine L929 fibroblasts. Logarithmic growth-phase L929 cells were seeded at 5 x 10^5 cells/mL, as 100-μL aliquots in complete RPMI 1640 containing 2 μg/mL actinomycin D (to sensitize target cells to the effects of TNF), to each well of a 96-well flat-based microtiter plate before the addition of 100 μL of diluent (complete RPMI 1640), standard recombinant human TNF (10 pg to 1 μg/mL), or test supernatant. After 24 h, the cells that survived the interaction with TNF were estimated by staining with 1% (vol/vol) crystal violet vital stain for 20 min. After solubilization of the stained product in 10% (vol/vol) aqueous acetic acid, the stained viable cells were optically measured at a wavelength of 620 nm. Data were recorded as percentage of specific lysis with respect to the negative (diluent alone) and positive (1 μg/mL recombinant human TNF) controls and the TNF standard.

IL-1 bioactivity was quantified by an indirect bioassay of IL-2 secretion by an IL-1-responsive cell line [42]. The NOB-1 subclone of the murine thymoma cell line EL4.6.1 was used as a sensitive responder to IL-1 in the culture supernatants. This cell line produces IL-2 in response to IL-1, which is measured using the CTRLL-2 murine T cell line [43]. EL4/NOB-1 cells were seeded into 96-well microtiter plates at 10^5 cells/mL as 100-μL aliquots in complete RPMI 1640, standard recombinant human IL-1β (10 pg to 1 μg/mL), or test supernatant. After 24 h at 37°C in 5% CO2/95% air, the resultant blue-purple formazan product was solubilized as before. Plates were read at a test wavelength of 570 nm and a reference wavelength of 690 nm. A standard curve of OD at 540 nm minus that at 690 nm versus concentration of standard IL-6 was plotted, and the concentration of IL-6 in the test supernatants was quantitated by comparison to the standard curve of known IL-6 concentration.

**Flow cytometric assessment of cell membrane antigens, cytokines, and cytokine receptors.** To study the expression of activation antigens on the surface of monocytes and cytokines or cytokine receptors, whole blood samples anticoagulated in preservative-free heparin (10 IU/mL) were used, since isolation techniques may alter the expression of membrane antigens. Samples were incubated with diluent (HBSS supplemented with 10 mM HEPES buffer, pH 7.4, 2 mM L-glutamine, and 0.5% bovine serum albumin) and/or LPS (1 μg/mL) at 37°C in a shaking water bath for 150 min. This procedure was repeated in samples that were primed with GM-CSF (5.0 ng/mL) at 37°C for 120 min, and then stimulated with LPS (1.0 μg/mL) at 37°C in a shaking water bath for 150 min. Blood (50 μL) was removed and incubated with 50 μg of the following monoclonal antibodies/mL by direct staining: phycoerythrin (PE)-conjugated anti-IL-1α, FITC-conjugated anti-IL-1β, and FITC-conjugated anti-TNF (R&D Systems). Indirect staining of membrane TNF receptors was carried out with unconjugated monoclonal anti-human TNFR-Ip55 or unconjugated monoclonal anti-human TNFR-IIp75 (Genzyme) essentially as described before [35]. Samples were washed twice and stained with a 1:10 dilution of PE-conjugated goat anti-mouse F(ab')2 immunoglobulins at 4°C. Samples were lysed with 10% aqueous FACslyse solution (Becton Dickinson) and washed in HBSS supplemented with 10 mM HEPES buffer, pH 7.4, 0.5% bovine serum albumin, and 0.01% sodium azide solution at 400 g and 4°C and suspended in HBSS containing 1% (vol/vol) formaldehyde solution. A Becton-Dickinson FACScan flow cytometer and Consort 32 LYSYS II version 1.02 software were used to assess the percentage positive and mean fluorescence intensity of membrane antigens. Monocyte gates were confirmed by anti-CD14-PE staining and light scatter characteristics.

**Quantitation of monocyte viability by lactate dehydrogenase (LDH) release assay.** Spontaneous and activation-induced cytolysis of monocytes from septic and normal subjects was determined by the LDH release assay (Cytox-tox-96 assay; Promega, Southampton, UK) essentially as previously reported [36]. Monocytes were seeded in duplicate at 8 x 10^4 cells/mL in a total volume of 200 μL in 96-well tissue culture microtiter plates in RPMI 1640 that contained reduced fetal calf serum at 2.0% (vol/vol), 2 mM L-glutamine, 2.0% (vol/vol) bovine serum albumin, 20 mM HEPES buffer, 0.05 mM 2-mercaptoethanol, and no antibiotics. Monocytes were stimulated in situ with control diluent (HBSS supplemented with 10 mM HEPES buffer, pH 7.4) or LPS at 1 μg/mL. This assay was repeated in monocytes that were primed with GM-CSF (5 ng/mL, 2 h) and stimulated with control diluent or LPS. Monocyte supernatants as 100-μL aliquots were centrifuged at 2000 g
for 5 min at 4°C at 0, 2, 6, 24, and 48 h after stimulation and assayed for viability with respect to the maximal release of LDH in the presence of 0.50% Triton X-100 solution. The 0 h time point was normalized with respect to the GM-CSF–primed samples so that all monocyte cultures had been incubated for exactly 2 h in the presence of diluent or GM-CSF before initiation of the assay. Monocyte cytolysis was calculated as % monocyte cytolysis = \((O - B)/(M - (B + V))\) \times 100\%, where \(O\) represents the OD of the test monocyte cultures, \(M\) represents the OD of the maximal released LDH in the presence of 0.50% Triton X-100, \(B\) represents the OD of the background levels of LDH present in culture medium alone, and \(V\) represents the volume correction control OD of culture medium plus 0.50% Triton X-100 solution.

Monocyte apoptosis by assessment of intranucleosomal DNA fragmentation. Monocyte apoptosis was assessed after a 48-h incubation period in serum-reduced RPMI 1640 containing 2.0% (vol/vol) fetal calf serum (which is normally at 10% [vol/vol] for cultures of mononuclear cells). Monocytes were seeded at 1.5 \times 10^6 cells/mL in 24-well tissue culture receptacles in a total volume of 1.0 mL, and DNA was extracted by a commercial DNA extraction kit (Genosys Biotechnologies, Cambridge, UK). Monocytes were homogenized in DNA isolation agent for 5 min at room temperature, and DNA was extracted with chloroform for 5 min at 4°C. After centrifugation at 12,000 g for 5 min, the aqueous phase was collected, and 2 vol of ice-cold isopropanol was added to precipitate the DNA on ice, which was washed in 70% ice-cold ethanol, air-dried, and solubilized in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Samples were treated with 10 IU/mL DNase-free RNase (Sigma) for 1 h at 37°C, and the reaction was quenched with 250 mM EDTA. DNA was electrophoresed on a 1.5% agarose gel at 50 V for 3 h in the presence of 0.1 μg of ethidium bromide. Intranucleosomal DNA fragments of 180 bp were visualized by UV transillumination and photographed using a Polaroid DS 34 direct screen instant camera mounted on a DS H-8/0.8X hood. In 4 patients, the effect of priming monocytes with or without GM-CSF at 5 ng/mL over the 48-h period was assessed, and DNA was subjected to agarose gel electrophoresis as described above.

Semiquantification of Bcl-2 and Bax expression by Western immunoblotting. In 6 of the 12 patients and in 6 normal subjects, Bcl-2 and Bax expression was assessed. Briefly, monocytes were homogenized in buffer consisting of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1 mM sodium orthovanadate, 0.01 mg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 4 mM sodium azide for 30 min on ice with occasional agitation. Cell lysates were cleared of cellular and nuclear debris by centrifugation at 15,000 g for 10 min at 4°C. Next, 250 μg of protein was loaded per well per sample and resolved in a 12% (vol/vol) acrylamide-bisacrylamide SDS-PAGE system at a constant current of 20 mA. Proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham-Pharmacia Biotech, Amersham, UK) using a semidry transfer cell transfer cell (Bio-Rad Laboratories, Hemel Hempstead, UK). Equivalent loading and transfer was confirmed by staining SDS-polyacrylamide gels in 0.06% Coomassie blue G-250 in 10% (vol/vol) acetic acid and reversible Ponceau-S reagent staining (Bio-Rad) of nitrocellulose membranes. Nonspecific binding sites were blocked in 5% nonfat milk proteins in PBS with 0.02% (vol/vol) Tween 20. Specific protein bands were detected with monoclonal anti-human Bcl-2 (clone 100) or polyclonal anti-human Bax (clone N-20) from Santa Cruz Biotechnology (Santa Cruz, CA) at 1 μg/mL in 1% (vol/vol) nonfat milk proteins in PBS with 0.02% (vol/vol) Tween 20. Bcl-2 and Bax protein bands were visualized with a 1/250 dilution of goat anti-mouse or goat anti-rabbit horseradish peroxidase–IgG conjugate, respectively, followed by ECL detection reagent, and permanent records were obtained by exposing the ECL-nitrocellulose blots to Hybond Hyperfilm-ECL (Amersham-Pharmacia). The Hyperfilm-ECL autoradiograms were then subjected to densitometric spectrophotometry using the GS-670 imaging densitometer and molecular analyst software 1.2 (Bio-Rad).

Biometry and data analysis. Data are expressed as mean ± SD. Independent analysis between the different treatments was done by multi-parameter analysis of variance with post hoc analysis (Scheffe test) and Kruskal-Wallis two-way and three-way analysis of variance (fixed effects and repeated measures) for multiple comparisons between and within groups. For comparisons between paired samples, the Student’s t test and the analysis of variance F test were used. Statistical significance was set at an α value of P < .05.

Results

Plasmaborne inflammatory cytokines are abnormal in septic shock patients. Plasma cytokine levels from normal and septic subjects are shown in table 1. Both TNF and GM-CSF levels were much lower in septic than in normal subjects, whereas IL-1β and IL-6 levels were markedly increased. In addition, IL-10, IL-1Ra, and soluble TNF receptors were also significantly increased in the plasma of septic subjects compared with levels in normal subjects. IL-1α was not detected.

Monocytes exhibit anergy and dysfunctional cytokine secretion in septic shock. We investigated time-dependent secretion of proinflammatory cytokines (figure 1) and anti-inflammatory cytokines and antagonists (figure 2) by ex vivo–stimulated monocytes from both normal and septic subjects. Spontaneous secretion of all cytokines was low and did not differ between

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normal subjects</th>
<th>Septic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Median 59.0</td>
<td>Median 2.50</td>
</tr>
<tr>
<td></td>
<td>Range 15.0–140.0</td>
<td>Range 0–12.50</td>
</tr>
<tr>
<td>IL-13</td>
<td>Median 9.25</td>
<td>Median 120.2</td>
</tr>
<tr>
<td></td>
<td>Range 0–45.0</td>
<td>Range 45.2–285.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>Median 2.35</td>
<td>Median 1241</td>
</tr>
<tr>
<td></td>
<td>Range 0–12.5</td>
<td>Range 656–2568</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Median 208.5</td>
<td>Median 13.4</td>
</tr>
<tr>
<td></td>
<td>Range 82.2–356.6</td>
<td>Range 4.6–61.3</td>
</tr>
<tr>
<td>IL-10</td>
<td>Median 1.03</td>
<td>Median 56.6</td>
</tr>
<tr>
<td></td>
<td>Range 0–10.3</td>
<td>Range 12.3–122.3</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Median 160</td>
<td>Median 422.5</td>
</tr>
<tr>
<td></td>
<td>Range 10.2–242</td>
<td>Range 98.6–806.5</td>
</tr>
<tr>
<td>TNFR-Ig5</td>
<td>Median 764</td>
<td>Median 1222</td>
</tr>
<tr>
<td></td>
<td>Range 388.5–1645</td>
<td>Range 256.5–512</td>
</tr>
<tr>
<td>TNFR-Ig5</td>
<td>Median 1622</td>
<td>Median 3655</td>
</tr>
<tr>
<td></td>
<td>Range 864–2688</td>
<td>Range 1014–7148</td>
</tr>
</tbody>
</table>

NOTE. Supernatants were assessed for levels of soluble cytokines and cytokine antagonists (TNF, tumor necrosis factor; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; TNFR, TNF receptor) by antibody-capture ELISA and quantified by extrapolation against standard curve of respective purified cytokine or cytokine antagonist. Plasma samples were enumerated in duplicate and are given as pg/mL.

a P < .001 vs. normal subjects.
b P < .01 vs. normal subjects.
normal and septic subjects. Ex vivo stimulation of monocytes with LPS induced a time-dependent increase in the secretion of all cytokines measured (figures 1, 2). However, cytokine secretion was reduced in septic subjects compared with that in normal subjects. In addition, secretion of IL-1α, IL-1β, and TNF was delayed in septic subjects.

GM-CSF alone induced minimal secretion of all cytokines except IL-1β. Secretion of IL-1α, IL-10, and TNFR-Ip55 in response to GM-CSF was reduced in septic subjects compared with normal subjects, whereas no difference was detected in the levels of other cytokines. Priming of monocytes with GM-CSF and secondary stimulation with LPS enhanced cytokine secretion. In all cases, GM-CSF priming partially restored the ability of monocytes from septic patients to respond to LPS, although secretion was lower than observed in normal subjects. The secretion of immunoreactive mediators as measured by ELISA was confirmed by use of a bioassay for TNF, IL-6, and IL-1 (figure 3). This was important information, as it confirmed that proinflammatory cytokines secreted by activated monocytes exhibited bioactivity in addition to their immunoreactivity by ELISA.

Monocytes exhibit dysregulated membrane-bound antigen expression in septic shock. The expression of HLA-DR (major histocompatibility complex class II), CD71 (the transferrin receptor), transmembrane TNF, and membrane-associated IL-1α on monocytes from normal and septic subjects were quantitated by flow cytometry (table 2). Monocytes from normal subjects exhibited higher levels of HLA-DR and CD71 than did monocytes from septic patients ($P < .001$). In addition, whereas LPS stimulation up-regulated the expression of both HLA-DR and CD71, LPS was without effect on monocytes from septic patients. GM-CSF increased the expression of HLA-DR and CD71 on normal subjects and primed them for further regulation on secondary stimulation with LPS. GM-CSF also re-
stored the response of monocytes from septic subjects to secondary stimulation with LPS.

The expression of transmembrane TNF on monocytes from septic subjects was also higher than was observed for monocytes from normal subjects ($P<.001$; table 2). This was in contrast to the higher levels of membrane-associated IL-1α present on monocytes from normal subjects compared with septic patients. Monocytes from normal subjects exhibited an increased expression of both TNF and IL-1α following stimulation with LPS, whereas monocytes from septic patients were unresponsive. Again, GM-CSF increased the expression of both of these membrane-associated cytokines on monocytes from normal subjects and septic patients. In addition, GM-CSF primed both sets of monocytes for further up-regulation of cytokine expression in response to LPS, thus restoring the response of monocytes from patients with septic shock to LPS.

### Monocyte anergy is associated with spontaneous or activation-induced apoptosis

Monocytes from septic patients responded abnormally to LPS stimulation, which could be reversed by GM-CSF. Thus, we investigated whether this refractoriness was associated with defective viability of monocytes from septic subjects. Spontaneous release of LDH from normal monocytes was low (median, 2.5%; range, 0–4.2%; figure 4). LPS-stimulated monocytes released higher levels of LDH, which was detectable as early as 6 h after stimulation and peaked at 48 h, although LDH release was always ≤8.0% (median, 5.5%; range, 2.5%–7.6%). GM-CSF alone exhibited no effect and delayed monocyte cytolysis in response to LPS (figure 4A).

Interestingly, monocytes from septic shock patients exhibited a greater predilection toward cell death (figure 4B). Spontaneous release of LDH by monocytes from septic patients was detectable as early as 2 h following culture and peaked at 18.5%.

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**Figure 2.** Time-dependent secretion of immunoreactive anti-inflammatory mediators interleukin-10 (IL-10) (A), IL-1 receptor antagonist (IL-1Ra) (B), and soluble tumor necrosis factor (TNF) receptor forms TNFR-Ip55 (C) and TNFR-IIp75 (D) by enriched monocyte cultures of normal control subjects and septic shock patients. Data are expressed as pg/10⁶ monocytes (SD). Monocytes were stimulated with control diluent (■), GM-CSF at 5 ng/mL (▲), lipopolysaccharide (LPS) at 1.0 μg/mL (●), or GM-CSF and LPS (◇), and supernatants were assayed for cytokine content and soluble TNF receptors by ELISA.
representing a 700% increase in cytolysis compared with normal monocytes. Stimulating monocytes from septic patients with LPS accelerated and enhanced the LDH release, which peaked at 33%, 6-fold greater than that observed in normal subjects (figure 4). Priming monocytes from septic patients with GM-CSF abrogated both spontaneous and LPS-induced monocyte death. Apoptosis in monocytes undergoing LDH release was confirmed by agarose gel electrophoresis of genomic DNA taken from monocytes of 3 normal subjects and comparing results with those from 12 septic patients (figure 5A). Characteristic intranucleosomal genomic DNA fragments (or apoptotic DNA ladders) were not observed in normal subjects. However, intranucleosomal DNA fragmentation was observed in monocytes of all 12 septic patients after culture for 48 h in the absence of in vitro stimulation. Thus, monocytes from septic patients, in contrast to monocytes from normal subjects, exhibited a predilection towards apoptosis. This finding was concordant with the observed spontaneous release of LDH by monocytes from septic patients and suggests that monocyte dysfunction in septic shock may be associated with apoptosis.

Moreover, monocytes from 4 patients with septic shock were stimulated with GM-CSF, which was shown to abrogate the onset of apoptosis after 48 h of culture compared with untreated monocytes (figure 5B). This absence of intranucleosomal DNA fragmentation in the presence of GM-CSF was concordant with the absence of DNA fragmentation in monocytes from normal control subjects. Importantly, this was associated with a restoration of monocyte responsiveness to in vitro stimulation with LPS.

This finding was explored further by investigating the ex-
Table 2. Phenotypic expression of monocyte function-associated molecules.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HLA-DR</th>
<th>CD71</th>
<th>TNF-α</th>
<th>IL-1α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Septic</td>
<td>Normal</td>
<td>Septic</td>
</tr>
<tr>
<td>Resting monocytes</td>
<td>247.8 ± 26.3</td>
<td>47.6 ± 11.3</td>
<td>86.8 ± 9.1</td>
<td>21.3 ± 5.4a</td>
</tr>
<tr>
<td>LPS stimulation</td>
<td>415 ± 33.2</td>
<td>45.3 ± 10.6</td>
<td>143.3 ± 10.3</td>
<td>19.8 ± 4.9a</td>
</tr>
<tr>
<td>GM-CSF stimulation</td>
<td>331.2 ± 13.6</td>
<td>149.4 ± 56.9</td>
<td>133.4 ± 24.4</td>
<td>81.4 ± 10.6</td>
</tr>
<tr>
<td>GM-CSF plus LPS</td>
<td>746.3 ± 22.8</td>
<td>399.6 ± 42.3</td>
<td>299.6 ± 36.5</td>
<td>172.3 ± 14.6</td>
</tr>
</tbody>
</table>

NOTE. Data are mean fluorescence index ± 1 SD in unstimulated (resting) monocytes or after stimulation with lipopolysaccharide (LPS) at 1 μg/mL for 150 min. Analyses were repeated in samples primed with GM-CSF at 5 ng/mL for 120 min followed by stimulation with LPS for 150 min. GM-CSF priming augmented expression of functional molecules above levels observed for non-GM-CSF-treated monocytes (P < .01).

Expression of functional molecules was lower than in normal subjects (P < .001).

Expression of functional molecules was greater than in normal subjects (P < .01).

Discussion

We have clearly demonstrated that monocytes are dysfunctional in septic shock, and we hypothesize that this is associated with dysregulated apoptosis. Monocytes from septic patients exhibited dysregulated surface expression of certain antigens that are important for host defense. In addition, secretion of inflammatory cytokines in response to physiologically relevant stimuli was attenuated. This observation was in agreement with the low levels of plasmaborne TNF and GM-CSF that were present in septic patients. Other cytokines were either undetectable or elevated compared with those in normal subjects. Thus, monocytes from septic patients exhibited tolerance to LPS stimulation.

Induction of tolerance appears to require a degree of activation of the monocyte on initial contact with endotoxin [15]. The phenomenon of LPS tolerance appears to be switched on to prevent excessive inflammation. On secondary contact with LPS, the expression of a number of genes for cytokines and other inflammatory mediators is attenuated. By contrast, LPS tolerance is associated with an up-regulation in the synthesis of IL-10, soluble TNFR-IIp75, and the p50 component of the

Figure 4. Spontaneous and lipopolysaccharide (LPS)-induced monocyte lactate dehydrogenase (LDH) release in normal subjects (A) and septic patients (B). Time-dependent LDH release was measured by specific colorimetric assay in monocytes cultured with control diluent alone (spontaneous release) or in presence of GM-CSF at 5 ng/mL, LPS at 1.0 μg/mL, or GM-CSF and LPS. Data are % monocyte cytolyis (mean ± 1 SD) with respect to maximal LDH release by monocytes cultured for 48 h followed by lysis with 0.5% (vol/vol) Triton X-100 solution.
NF-κB homodimer [16]. IL-10 and TNFR-IIp75 exhibit a negative feedback to exert anti-inflammatory effects on the monocyte [44, 45].

Activation-induced apoptosis could be a major mechanism for down-regulating an active immune response in the interest of maintaining immunologic homeostasis and deleting T cells with functional specificities that may be harmful [46]. Moreover, apoptosis of peripheral blood leukocytes is able not only to regulate cell number at the inflammatory lesion but also to regulate the systemic secretion of potentially toxic mediators [28]. The present study suggests that monocyte dysfunction may be associated with enhanced apoptosis, which may represent a normal physiologic response. This observation is in agreement with the work of others [23–25]. We also observed a coordinate down-regulation in the expression of Bcl-2 protein with no obvious effect on the expression of Bax. This observation was also associated with a diminution in the Bcl-2/Bax rheostat.

A number of soluble cytokines and oncoproteins regulate the...
expression of Bcl-2 and Bax in primitive and mature hematopoietic cells [47–49]. GM-CSF has been shown to induce DNA synthesis and suppress apoptosis of hematopoietic cells [50, 51]. This action of GM-CSF is mediated via the activation of multiple signaling pathways. Janus protein kinase 2 (Jak2), which binds to the membrane proximal domain of the GM-CSF receptor β chain, induces Bcl-2 expression and prevents cell death [50]. This function of Jak2 is dependent on tyrosine phosphorylation. Moreover, the Ras signaling pathway may also play a role in regulating Bcl-2 expression [51]. Activation of Ras resulted in a rapid up-regulation of Bcl-2 with no effect on Bax [51]. One may thus infer that in monocytes from septic patients, in whom the plasma levels of GM-CSF were markedly lower than those levels observed in normal subjects, the Ras pathway and Jak2 activation may be less active than in normal monocytes. Thus, cytokines such as GM-CSF secreted by leu-

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**Figure 6.** A. Differential expression and densitometric scans of Bcl-2 and Bax expression in septic shock patients and normal control subjects analyzed immediately after monocyte enrichment and quantitated by SDS-PAGE and enhanced chemiluminescence of whole cell lysates. B. Mean difference in relative Bcl-2 and Bax levels in monocytes from septic patients and normal control subjects. Statistical significance in expression levels between septic patients and normal control subjects was measured by nonparametric Mann-Whitney U test.
kocytes in response to infection may play a role in regulating inflammation by the inhibition of apoptosis. Normal neutrophils rapidly undergo apoptosis in vitro; by contrast, apoptosis is inhibited in neutrophils from patients with severe burns [52]. Moreover, induction of GM-CSF secretion by as-yet-unidentified factors in the plasma of burn patients may inhibit neutrophil apoptosis [52].

In addition to a predisposition to apoptosis in septic patients, the persistent exposure of monocytes to LPS (endotoxin) may have induced apoptosis. It has been shown that alveolar macrophages exhibited a marked increase in their death rate on exposure to LPS [33]. The rate of spontaneous apoptosis was not enhanced by IL-1, IL-4, IL-6, IL-10, TNF, TGF-β, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, or GM-CSF. However, LPS-induced apoptosis was enhanced by IFN-γ and decreased by macrophage-inhibitory cytokines such as IL-4, IL-10, and TGF-β [33]. Although we did not assay for TGF-β in the plasma of septic or normal subjects, septic patients exhibited enhanced levels of IL-10 compared with normal subjects, whereas IL-4 was undetectable (data not shown). Despite the presence of IL-10 in the plasma of septic shock patients, spontaneous monocyte apoptosis was increased compared with that in normal subjects. It has been shown that IL-10 increases Bcl-2 expression in primary human hematopoietic progenitor cells, and moreover, IL-10 promoted their survival [49]. However, in cultures of peripheral blood monocytes, the addition of exogenous IL-10 accelerated cell death, which could be abrogated by coculturing monocytes in the presence of GM-CSF or IFN-γ [39]. This observation is concordant with our findings of enhanced apoptosis of monocytes from septic patients ex vivo that may be explained by their prior exposure to IL-10 in vivo.

It has previously been shown that IL-4 (and possibly IL-10) are key regulators of monocyte-mediated immune functions [53]. The proposed mechanism is one of deactivating the hyperinflammatory monocyte while simultaneously inducing apoptosis. It has been proposed that apoptosis may be switched on by down-regulating the expression of the LPS receptor or CD14 and therefore blocking signal-transducing events responsible for rescuing cells from apoptosis [54]. This is an intriguing observation, since we have previously shown that monocytes from septic patients exhibit a depressed membrane expression of CD14 that can be restored to near normal levels after priming with GM-CSF ex vivo [41]. The depressed levels of CD14 that we have previously reported may be associated with the enhanced predilection for apoptosis as detailed in the present study, in which the same group of septic patients was investigated. Indeed, it has been postulated that occupation of the CD14 receptor by its ligand may represent a previously unreported survival function for this receptor [55], and our work supports this hypothesis. Moreover, occupation of the CD14 receptor induces calcium mobilization, which may in turn be associated with subsequent activation of apoptosis-inhibitory enzymes [54]. Thus, the restoration of CD14 expression by GM-CSF priming that we have previously observed in septic patients appears to be associated with an abrogation of programmed cell death and a restoration of monocyte effector functions that are important in host cell-mediated immunity.

In the present study, GM-CSF priming of monocytes from septic patients restored the secretion of TNF and IL-1 after secondary stimulation with LPS ex vivo. Both TNF and IL-1 are known to rescue monocytes from apoptosis in a paracrine or an autocrine manner. Thus, the apoptosis-abrogating effects of GM-CSF may have acted at least in part through the restoration of cytokine secretion and through the restoration of function-associated molecules such as CD14, as we have previously shown [41].

In conclusion, we have clearly shown that GM-CSF restored the response of monocytes to secondary stimulation with physiologically relevant mediators such as LPS. The key finding of this study was our observation that GM-CSF abrogated the spontaneous and activation-induced (LPS) apoptosis of monocytes from septic patients. This was evidenced by an attenuation in the release of stable cytosolic enzyme LDH from monocytes and a cessation of the intranucleosomal DNA fragmentation that was otherwise observed in the absence of GM-CSF priming. We hypothesize that the dysregulated function of the monocyte system in septic patients may be associated with a depressed physiologic level of GM-CSF, depressed expression of function-associated markers such as CD14 (as we have previously shown), and a predilection toward apoptosis. Moreover, monocyte dysfunction and the enhanced rate of spontaneous monocyte apoptosis in septic shock may be corrected directly, at least in part, by exogenous priming with GM-CSF.

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