Accelerated Lymphocyte Death in Sepsis Occurs by both the Death Receptor and Mitochondrial Pathways

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Accelerated Lymphocyte Death in Sepsis Occurs by both the Death Receptor and Mitochondrial Pathways

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Patients with sepsis are immune compromised, as evidenced by their frequent inability to eradicate the primary infection, their propensity to acquire new secondary infections, and their failure to respond to skin testing with positive controls (1–4).

The purpose of this study was to quantitate lymphocyte apoptosis in critically ill patients with sepsis, including a shift from a proinflammatory (Th1) to an anti-inflammatory (Th2) cytokine profile, increased production of the anti-inflammatory cytokine IL-10, monocyte deactivation with low HLA-DR expression, and apoptosis of lymphocytes and dendritic cells (4–9). The sepsis-induced loss of lymphocytes may be particularly important not only because of the extensive depletion of critical immune effector cells, but also because of the potential immunosuppressive effect of apoptotic cells on the immune system (10). Animal studies indicate that the immune defects in sepsis may be critical to pathogenesis and mortality and that prevention of lymphocyte apoptosis may improve survival (11–13).

Apoptosis is a relatively recently identified type of cell death in which the cell initiates a highly regulated and evolutionary conserved molecular program, resulting in its own death (14–16). Apoptosis is essential for organ homeostasis and maintains a balance between newly formed cells and dying cells. Apoptosis is activated pathologically in certain diseases, including AIDS, neurodegenerative disorders, and sepsis (16). Although studies in patients with sepsis have demonstrated extensive apoptosis in lymphocytes in spleen and gastrointestinal-associated lymphoid tissue, few studies have examined apoptosis in circulating lymphocytes or investigated the death pathways in apoptotic lymphocytes (17–21). Apoptosis can occur by two divergent pathways, i.e., a death receptor pathway and a mitochondrial-mediated pathway. The death receptor pathway can be mediated by a number of death receptors, including Fas, TNF receptor type 1, etc., that result in activation of caspase 8. An alternative death pathway involves the release of proapoptotic factors from the mitochondria and results from a variety of noxious stimuli, e.g., reactive oxygen species and ischemia/reperfusion. The mitochondrial pathway results in activation of caspase 9 (22, 23). Both active caspases 8 and 9 induce activation of caspase 3, which is in the final common cell death pathway. Clinical trials of drugs that block caspase-mediated apoptosis have been conducted in patients with liver disease with evidence of efficacy (24).

It is important to identify the particular pathway involved in lymphocyte apoptosis in sepsis because it provides insight into potential factors responsible for initiating cell suicide and may allow for the development of a more targeted therapy. The purpose of this study was to quantitate lymphocyte apoptosis in critically ill septic and nonseptic patients and to determine the cell death pathways and potential mechanisms of apoptosis.

Materials and Methods

Patient groups

Patients were classified as having severe sepsis based upon the criteria developed by Bone et al. (25) and as used in the Prowess Study (26).
Prowess criteria include three categories: infection, systemic inflammatory response, and acute organ dysfunction. The patients must have one or more criteria under the infection category plus two or more criteria under the systemic inflammatory response category plus or more criteria under the acute organ dysfunction category to be qualified as having sepsis. Under the infection category, the five possible criteria include 1) documented infection via positive culture results from blood, sputum, etc.; 2) anti-inflammatory therapy consisting of antibiotics; 3) presence of white blood cells in normally sterile fluid; 4) pneumonia, documented by chest radiograph or other findings; and 5) perforated viscus. The systemic inflammatory response includes four possible criteria: 1) body temperature >38 or <36°C; 2) heart rate >90 beats/min; 3) respiratory rate >20 breaths/min; and 4) white blood cell count >12,000/mm³. The seven criteria for diagnosing acute organ dysfunction included the following: 1) respiratory, requiring mechanical ventilation with a partial pressure of oxygen to fraction of inspired oxygen ratio <250 mm Hg and positive end expiratory pressure >7.5 mm Hg; 2) cardiovascular, requiring vasopressor support (systolic blood pressure <90 mm Hg or mean arterial pressure <70 mm Hg for 1 h despite fluid bolus); 3) renal, low urine output (<0.5 ml/kg/h), increased creatinine, or acute dialysis; 4) hematologic, low platelet count (<100,000/mm³) or prothrombin/thromboplastin time greater than upper limit of normal; 5) metabolic, low pH with high lactate; and 6) CNS, altered consciousness. Patients classified as having sepsis fulfilled these Prowess criteria.

All patients were hospitalized in the surgical or medical intensive care units of Barnes Jewish Hospital, a teaching hospital for Washington University School of Medicine (see Table I and supplemental data online) for description of individual patient population: age, sex, primary diagnosis, comorbidities, days in intensive care unit, presence of shock vs nonshock, absolute lymphocyte count, bacteriologic cultures, and outcome. Critically ill patients without sepsis who were hospitalized in the intensive care units were a second group of study individuals. The majority of the critically ill, nonseptic patients were hospitalized in the intensive care unit for recovery after major surgery or traumatic injury (motor vehicle accident, blunt or penetrating trauma, etc.); supplemental data available online. Seven of the critically ill patients who did not have sepsis on initial presentation to the intensive care unit subsequently developed sepsis. When these patients developed sepsis, they were included in the septic group. Consequently, data from these seven patients were included in both the critically ill, nonseptic group and the septic group, appropriate for their conditions (i.e., critically ill, nonseptic, or septic) at a particular time. Exclusion criteria for the study included immunocompromised patients, patients taking immunosuppressive medication, and patients with cancer who had received chemotherapy agents or irradiation within the last year. The sources of infection for the patients were respiratory tract (n = 16), intra-abdominal (n = 29), wound (n = 5), intravascular catheter (n = 1), and unknown (n = 6). Seven patients had both abdominal and respiratory sources of sepsis (see Table II). Based upon a recent study purportedly showing improved survival in patients with sepsis who have an inadequate response to the corticosteroid stimulation test (27), 19 patients with sepsis did receive corticosteroids according to this study recommendation. These patients were not excluded from entry into the study. Evaluation of the percentage of lymphocyte apoptosis in septic patients receiving corticosteroid replacement therapy vs septic patients not receiving corticosteroid therapy showed no difference (see Results). Six healthy adult volunteers who had no existing comorbidities were included as a third group.

Blood sampling

After obtaining consent from the patient or the patient’s nearest related kin, whole blood was obtained from an indwelling arterial or central venous catheter into heparinized Vacutainer tubes (BD Vacutainer Systems). In 56 patients (predominantly patients with sepsis), depending upon the length of stay in the intensive care unit, time of discharge or death, availability of study nurse, change in clinical status, etc., an additional two or three blood samples were obtained serially. In patients with sepsis who had two or three blood samples obtained during their septic conditions, the septic values presented are the average of all samples during the sepsis. Note that not every patient had all tests performed. In some instances, particular tests, i.e., Western blotting, almost the entire blood sample was used for the assay, and therefore, few cells were available for other assays. The human studies committee at Washington University School of Medicine approved the protocol.

Cytokine assays

The proinflammatory cytokines, IFN-γ and TNF-α, and the anti-inflammatory cytokine, IL-10, were measured in a subset of septic and critically ill, nonseptic patients. DuoSet kits from R&D Systems were used for all ELISAs of cytokines, and the manufacturer’s protocol was followed, as described previously (10).

Detection of apoptosis

**Western blotting.** Heparinized whole blood was treated with RosetteSep Ab Cocktail (StemCell Technologies), and the manufacturer’s protocol for isolation of lymphocytes was followed. The purity of the specimens was verified by flow cytometry, which demonstrated that the separated samples were ~85–90% lymphocytes (data not shown). Equal amounts of protein (50 µg) were loaded on 12% SDS-PAGE. Next, protein was transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was placed in blocking buffer containing the respective primary Ab (1/1000). The anti-active caspase 8 and anti-active caspase 9 polyclonal Abs were obtained from Merck Frosst Laboratories. Both Abs are directed against the cleaved caspase caspase, are specific for the active caspases, and do not recognize the procaspase forms. Positive controls for active caspase 8 and active caspase 9 were lyzed apoptotic Jurkat cells (BD Pharmingen; catalog no. 51-16606N), which were included in each gel. In addition, molecular markers of characteristic molecular weights were included to verify that the active caspases were at appropriate positions on the gel.

**Flow cytometry.** Apoptosis was quantified using a commercially available fluorescein-labeled annexin V product (Apoptosis Detection kit; R&D Systems) as previously described (8, 10). Human lymphocyte subsets (CD3, CD4, CD8) were analyzed and plotted using FlowJo software. Abs were obtained from BD Pharmingen. The number of deaths caused by the sepsis was determined by a blinded reviewer of the data.

Table I. Patient profiles

<table>
<thead>
<tr>
<th>Number of Patients</th>
<th>Septic</th>
<th>Critically Ill Nonseptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>71</td>
<td>55</td>
</tr>
<tr>
<td>Male, female</td>
<td>35 male, 36 female</td>
<td>34 male, 21 female</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>57 male, 59 female</td>
<td>60 male, 62 female</td>
</tr>
<tr>
<td>Age (median)</td>
<td>62 male, 60 female</td>
<td>61 male, 59 female</td>
</tr>
<tr>
<td>Age (range)</td>
<td>19–83 male, 22–86 female</td>
<td>21–90 male, 36–83 female</td>
</tr>
<tr>
<td>Percent of patients in shock (%)</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>Percent of patients who survived and were discharged from ICU (%)</td>
<td>69</td>
<td>98</td>
</tr>
</tbody>
</table>

Note: Percentages are rounded to the nearest whole number.

Table II. Sources of infection in septic patients

<table>
<thead>
<tr>
<th>Infection</th>
<th>Septic</th>
<th>Critically Ill Nonseptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sources of infection</td>
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<td>29</td>
</tr>
<tr>
<td>Respiratory tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-abdominal</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Wound (other than abdominal)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Intravascular catheters</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Multiple sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Septic</th>
<th>Critically Ill Nonseptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal/respiratory</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Abdominal/urinary</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Wound/respiratory</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Note: Percentages are rounded to the nearest whole number.

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3 The online version of this article contains supplemental material.

4 Wounds often extensive, including necrotizing fasciitis, gangrene, and second and third degree infected burns.

5 No clearly identified source of infection despite the patient having hallmarks of sepsis.
CD4, CD8, B, and NK cells) were identified using characteristic phenotypic markers (BD Pharmingen). Identifications of apoptotic cells by annexin V and cell phenotyping were performed simultaneously using three-color analysis. Flow cytometric analysis (50,000 events/sample) was performed on FACSscan (BD Biosciences).

For staining of active caspases 3, 8, and 9, cells that had been fixed with FACS lysing solution (BD Biosciences) were incubated with their respective primary Abs and stained with propidium iodide-conjugated donkey anti-rabbit Ab (Jackson ImmunoResearch Laboratories) at a 1/500 concentration. The primary Ab (1/100 concentration) for active caspase 3 was purchased from Cell Signaling Technology (catalog no. 9661); this Ab is specific for the cleaved fragment of caspase 3 and does not recognize the procaspase 3 form. The primary Abs for active caspases 8 and 9 were generously provided by Merck Frosst (1/10,000 concentration) and, as described previously, were specific for the active caspase. For Bcl-2 quantitation, a fluorescein-conjugated anti-human Bcl-2 mAb (DakoCytomation) was used to label fixed cells according to the manufacturer’s instructions.

Statistical analysis

Data reported are the mean ± SEM. Data were analyzed with the statistical software program PRISM (GraphPad). Data involving only two groups were analyzed by Student’s t test, whereas data involving more than two groups were analyzed using one-way ANOVA with Tukey’s multiple comparison test. Significance was accepted at p < 0.05.

Results

Pro- and anti-inflammatory cytokines in critically ill, nonseptic, and septic patients

Circulating plasma cytokines were determined in 37 critically ill, nonseptic patients and 35 patients with sepsis. Levels of the anti-inflammatory Th2 cytokine, IL-10, were 7.9 and 145.9 pg/ml in critically ill, nonseptic and septic patients, respectively; this 54% increase in sepsis was statistically significant (p < 0.05). The proinflammatory Th1 cytokine, IFN-γ, was increased ~2-fold in septic vs critically ill nonseptic patients, i.e., 92.6 ± 24.0 vs 46.2 ± 9.9 pg/ml, respectively (p < 0.05). Although TNF-α was also increased ~2-fold in septic vs critically ill, nonseptic patients, i.e., 128.3 ± 38.2 vs 63.4 ± 14.1 pg/ml, respectively, this difference was not statistically significant (p = 0.11).

Absolute lymphocyte counts and percentage of lymphocyte subsets

The lower limit of normal for the blood absolute lymphocyte count at Barnes Jewish Hospital is 1.2 K/mm³. The mean value for the absolute lymphocyte count was comparably decreased in both patients with sepsis as well as critically ill, nonseptic patients, i.e., 1.1 ± 0.1 (n = 71) and 0.9 ± 0.1 (n = 55), respectively. The percentage of CD3 T, CD4 T, CD8 T, B (CD20), and NK cells (CD56) in healthy controls was comparable to values reported for other normal individuals (Fig. 1) (28). There was a statistically significant decrease in the percentage of CD3 T cells in both critically ill, nonseptic patients and septic patients compared with healthy volunteers (p < 0.05). In addition, the percentage of CD4 T cells (a subtype of CD3 T cells) in septic patients, but not in critically ill, nonseptic patients, was decreased compared with that in healthy controls (p < 0.05; Fig. 1).

Flow cytometry and apoptosis

The characteristic forward and side scatter properties of lymphocytes were used to identify the lymphocyte gate. Back-gating of labeled lymphocytes (CD3 and CD20) was used to confirm the lymphocyte gate. The percentage of apoptotic cells within the lymphocyte gate was quantitated via annexin V and 7-aminoactinomycin (7-AAD) labeling. Annexin V-/7-AAD staining for apoptosis showed that the percentages of apoptotic lymphocytes in

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* Abbreviation used in this paper: 7-AAD, 7-aminoactinomycin.

FIGURE 1. Percentage of peripheral mononuclear white blood cells. The percentage of the various lymphocyte subsets and monocytes in blood was determined via flow cytometry and CD markers. Healthy controls, n = 6; critically ill, nonseptic, n = 25; septic patients, n = 44. *, p < 0.05 for comparison of septic patients vs healthy controls.

blood were 4.8 ± 0.8 (n = 6) and 5.1 ± 0.2 (n = 48) for healthy volunteers and critically ill, nonseptic patients, respectively; these values were not statistically different (Fig. 2A). In contrast, lymphocyte apoptosis was increased in patients with sepsis to 10.0 ± 0.4 (n = 64); this was statistically different compared with both other groups (p < 0.01; Fig. 2). (Note that there was no statistical difference in the percentage of lymphocyte apoptosis in 19 septic patients receiving corticosteroids for adrenal insufficiency (10.5 ± 0.7%) vs septic patients who were not being treated for adrenal insufficiency). The degree of lymphocyte apoptosis in patients with sepsis vs patients with septic shock showed no difference, i.e., 9.4 ± 0.1 (n = 35) and 10.6 ± 0.1 (n = 29), respectively (Fig. 2B).

Analysis of apoptosis in specific lymphocyte subsets revealed that sepsis increased the percentage of lymphocyte apoptosis in CD3 T cells, B cells (CD20), and CD56 (NK) cells compared with both

FIGURE 2. Lymphocyte apoptosis determined by annexin V and 7-AAD. A. Lymphocytes were identified based upon characteristic forward and side scatter properties. The percentage of lymphocyte apoptosis (based upon the lymphocyte gate) was determined by annexin V and 7-AAD labeling in healthy controls (n = 6); critically ill, nonseptic patients (n = 49); and septic patients (n = 64). *, p < 0.01, septic patients vs both other groups. B. Percentage of apoptotic lymphocytes in critically ill, nonseptic patients (n = 49); patients with sepsis and without shock (n = 35); and patients with sepsis and shock (n = 29). *, and †, p < 0.01 for both septic groups vs critically ill, nonseptic.
healthy volunteers and critically ill, nonseptic patients (Figs. 3 and 4). Apoptosis was increased in both subtypes of CD3 T cells, i.e., CD4 and CD8 T cells (Fig. 3).

In 10 patients, three blood samples were obtained during each patient’s hospital course, and the degree of CD3 T cell apoptosis was compared during the time that he/she was septic or had resolved his/her septic condition. In nine of the 10 patients, the degree of CD3 T cell apoptosis correlated with the activity of his/her sepsis, i.e., the degree of CD3 T cell apoptosis was increased when the patient became septic and/or was decreased when the patient resolved his/her sepsis (Fig. 5).

**FIGURE 3.** A, Percentage of apoptosis in lymphocyte subsets determined by flow cytometry with annexin V and 7-AAD labeling. Healthy controls, \( n = 6 \); critically ill, nonseptic, \( n = 25 \); septic patients, \( n = 44 \). *p < 0.05, septic patients vs both other groups; +, p < 0.05, septic patients vs critically ill, nonseptic patients; \( \triangledown \), p < 0.05 septic vs healthy controls. Note the increase in apoptosis in the patients with sepsis compared with the other two patient groups. B, Apoptosis in CD3 T cells detected by active caspase staining. Apoptosis was determined by flow cytometry and staining for active caspase 3, active caspase 8, and active caspase 9 in healthy controls (\( n = 6 \)); critically ill, nonseptic (\( n = 9 \)); and septic patients (\( n = 34 \)). *p < 0.05, values in septic patients greater than those in healthy controls and critically ill, nonseptic patients. C, Sepsis increases apoptosis in cells with normal and decreased Bcl-2 levels. Lymphocytes from healthy controls (\( n = 6 \)); critically ill, nonseptic patients (\( n = 6 \)); patients with sepsis (\( n = 12 \)); and patients with septic shock (\( n = 8 \)) were determined by flow cytometry and staining for active caspase 3 and Bcl-2. Note that in patients with sepsis with and without shock, there was an increase in apoptosis in lymphocytes with decreased Bcl-2 and normal amounts of Bcl-2.

**FIGURE 4.** Apoptosis in CD3 T cells. Apoptosis was determined in CD3 T cells (identified by an anti-CD3 surface marker) using annexin V labeling in four patients. Apoptotic annexin V-positive CD3 T cells are located in upper right quadrant of each scattergram. The numbers in parentheses represent the percentage of cells in the individual quadrants. Note the increase in apoptosis in CD3 T cells in patients with sepsis compared with the other two patients.

**Determination of apoptotic death pathway via flow cytometry**

To determine whether lymphocyte apoptosis was preceding via a mitochondrial or death receptor-induced pathway, CD3 T cells were stained for active caspases 3, 8, and 9 (Figs. 3B and 6). (CD3 T cells were chosen because there were sufficient numbers of circulating CD3 T cells for quantitation.) Patients with sepsis had a marked increase in active caspases 3, 8, and 9 compared with both healthy volunteers and critically ill, nonseptic patients (Figs. 3B and 6). There was no difference in active caspases in healthy volunteers vs critically ill, nonseptic patients.

**Determination of apoptotic death pathway via Western blotting**

The protein isolate prepared from the whole blood lymphocyte fraction (obtained by RosetteSep Ab Cocktail; StemCell Technologies; see Materials and Methods) underwent Western blotting. Samples from 13 patients with sepsis and seven critically ill, nonseptic patients were analyzed by Western blotting (Fig. 7). Equal amounts of protein (50 \( \mu \)g) were loaded in each lane, and a positive control (apoptotic Jurkat cells) was included in each gel. In addition, the identities of the bands for active caspase 8 and active caspase 9 were confirmed based upon the characteristic molecular weights of the activated, i.e., cleaved, caspases using molecular markers (Fig. 7). Only a limited number of specimens were examined because of the large amount of protein (requiring large numbers of lymphocytes and use of almost the entire blood specimen) necessary to achieve adequate sensitivity for detection via Western blotting. The bands identified as corresponding to active caspases 8 and 9 were at appropriate positions for their molecular weights based upon the location of reference molecular markers (Fig. 7). Active caspase 8 was positive in six of 13 patients with sepsis and in only one of seven critically ill, nonseptic patients. Active caspase 9 was positive in 10 of 13 patients with sepsis and five of seven critically ill, nonseptic patients. These values were not significantly different, although trends are apparent.
**Loss of Bcl-2 in a subset of apoptotic lymphocytes**

Flow cytometric evaluation of CD3 T cells that had dual staining for active caspase 3 and Bcl-2 demonstrated an increase in active caspase 3 and a decrease in Bcl-2 (Figs. 3C and 8). Interestingly, the increase in caspase 3-mediated apoptosis occurred in lymphocytes that had normal concentrations of Bcl-2 (Fig. 3C and upper right quadrant of Fig. 8) as well as in lymphocytes that had decreased concentrations of Bcl-2 (Fig. 3C and upper left quadrant of Fig. 8). This finding suggests that both receptor and mitochondrial-mediated death pathways occur in different CD3 T cell populations, as indicated by the caspase 3-positive/Bcl-2-positive apoptotic cells and the caspase 3-positive/Bcl-2-negative apoptotic cells, respectively. As apparent in Fig. 8, however, there was a generalized decrease in Bcl-2 even in cells considered to be Bcl-2 positive (located in lower right quadrant).

**Discussion**

Previous studies have shown that sepsis induces extensive lymphocyte apoptosis in diverse organs, including spleen and intestine (8, 17, 20, 29). Although most studies focused on lymphocyte apoptosis in tissues, few studies have examined the effects of sepsis on circulating lymphocytes (18). Apoptotic cells are rapidly cleared within a few hours by phagocytic cells (30). Consequently, the presence of a large percentage of apoptotic cells (between 10 and 25% depending upon the assay) in the blood of septic patients documented in the present study probably represents an extremely accelerated degree of cell death of the patients’ immune cells. The profound lymphocyte depletion reported in tissues from patients with sepsis provides confirmatory evidence for the impact of the high degree of lymphocyte apoptosis measured in blood (8). Increased lymphocyte apoptosis in circulating blood also has been reported not only for sepsis, but for other infectious diseases as well (18–21). Baize et al. (21) described massive intravascular apoptosis in lymphocytes of patients dying from ebola virus and argued that the loss of lymphocytes resulted in impaired humoral and cellular immune responses, thus contributing to the fatal outcome.

Interestingly, the absolute lymphocyte count was depressed equally in critically ill, nonseptic patients and patients with sepsis despite the fact that the former did not have accelerated lymphocyte apoptosis. The decrease in absolute lymphocyte count observed in both septic and critically ill, nonseptic patients is due to two factors: 1) recruitment of lymphocytes from the bloodstream to peripheral tissues and 2) apoptosis. Critically ill, nonseptic patients tend to have a more rapid return of their absolute lymphocyte counts toward normal values, whereas patients with sepsis tend to
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have a persistently decreased absolute lymphocyte count throughout the course of their sepsis (17, 18). Furthermore, in the individual patient with sepsis, the degree of lymphocyte apoptosis is a marker for the activity of the disease (18). As demonstrated in Fig. 6, the degree of lymphocyte apoptosis tracked the patient’s recovery from or development of sepsis. This correlation was a consistent finding in the patient subgroup. Although there was no difference in the circulating lymphocyte count of septic vs critically ill, nonseptic patients, it is important to note that at the tissue level there is marked distinction. Patients with sepsis have a severe depletion of lymphocytes in spleen and lymph nodes, and this abnormality does not occur in critically ill patients who are not septic (8, 17).

The concentrations of IFN-γ, TNF-α, and IL-10 were elevated in both septic and critically ill, nonseptic patients compared with healthy volunteers in whom the cytokine concentrations are usually undetectable. The results for TNF-α in septic patients in the present study are consistent with numerous other studies of patients with sepsis that report serum TNF-α concentrations ranging from 40 to 200 pg/ml (31). The elevated TNF-α levels in the critically ill, nonseptic patients, which were not statistically different from those in septic patients, are indicative of the high severity of illness in the critically ill patients who were not septic. TNF-α can induce death receptor-mediated apoptosis in particular types of cells under specific situations. However, a previous study from our laboratory using anti-TNF-α Abs failed to block lymphocyte apoptosis in a murine model of sepsis (32). Therefore, we believe that TNF-α is unlikely to be driving apoptotic cell death in patients with sepsis. Both the Th1 cytokine, IFN-γ, and the Th2 cytokine, IL-10, were elevated in plasma from patients with sepsis. IFN-γ may also be secreted by NK cells, and therefore, circulating concentrations are not specific for the Th1 response.

Although, as a group, annexin V labeling showed that there was no increase in lymphocyte apoptosis in critically ill, nonseptic patients compared with healthy control patients, individual critically ill, nonseptic patients did have increased lymphocyte apoptosis that was elevated several-fold over the mean value for healthy control patients and was comparable to values in septic patients (Fig. 2A). Flow cytometric evaluation of active caspases 8 and 9 demonstrated a similar trend (although not statistically different) for increased apoptosis in critically ill, nonseptic patients compared with normal healthy volunteers (Fig. 3B). Also, previous studies from our laboratory showed that an occasional critically ill,
nonseptic patient had significant loss of splenic lymphocytes comparable to the loss occurring in patients with sepsis (8). Furthermore, patients with trauma and ischemia reperfusion injury have extensive focal apoptosis of gastrointestinal-associated lymphocytes (30, 33). In summary, although increased circulating lymphocyte apoptosis does occur in the occasional critically ill, nonseptic patient, it is not typical of critically ill, nonseptic patients when they are considered as a group.

The present results showing that sepsis induces an increase in apoptosis in various human lymphocyte subsets are consistent with published findings in animals, including studies from our own laboratory showing that sepsis causes apoptosis in both subsets of CD3 T cells, i.e., CD4 and CD8 T cells, as well as B cells (10, 11, 13, 34). Furthermore, NK cells are known to undergo apoptosis in response to a variety of stress conditions, including infection. The loss of these lymphocytes as well as the anti-inflammatory impact of the uptake of apoptotic cells on phagocytic cells may be a significant cause of immune suppression and failure to clear the infectious process.

Le Tulzo et al. (18) examined circulating lymphocyte apoptosis in human sepsis with the annexin V method used in the present study. They reported that patients with septic shock had an increase in lymphocyte apoptosis to 16.5 ± 3.5%, and this was increased compared with the 7.5 ± 1% lymphocyte apoptosis in patients with sepsis but without shock and the 7.5 ± 1.5% lymphocyte apoptosis in critically ill, nonseptic patients. The values for lymphocyte apoptosis reported by Le Tulzo et al. (18) are different from the values of 5.1 ± 0.2 for critically ill, nonseptic patients, 9.4 ± 0.1 for septic patients, and 10.6 ± 0.1 for patients with septic shock reported in the present study. There are several possible explanations for the discrepancy between the two studies. First, the critically ill, nonseptic population in the study by Le Tulzo et al. consisted of only seven patients compared with the 48 patients in the present study. As noted previously, there is some variability in the values for critically ill, nonseptic patients, and the seven patients in the earlier study may have been more seriously ill, a factor that we postulate has a major impact on the degree of lymphocyte apoptosis. The percent lymphocyte apoptosis in patients with sepsis in Le Tulzo’s study, i.e., 16.5%, was greater than the 10.5% in the present study. A possible explanation for this difference may relate to the timing of blood sampling. In the study by Le Tulzo et al. (18), the first blood sample was obtained on the initial day that the patient developed septic shock. In the present study, the initial sample was not automatically obtained on the first day of septic shock (depending upon the availability of the study nurse and investigators to perform flow cytometry; see Materials and Methods). In other words, in the present study many septic patients had septic shock for several days before the first blood sample was obtained. Work from our laboratory agrees with the findings of Le Tulzo et al. (18), who showed that the initial onset of sepsis was associated with the largest degree of lymphocyte apoptosis. It also should be noted that although the mean value for lymphocyte apoptosis determined by annexin V was only ~10% in patients with septic shock, occasional patients had much higher values (Fig. 2). Finally, staining for active caspase 3 and active caspase 9 and evaluation by flow cytometry, which may be a more sensitive method to detect apoptosis, showed that ~16.8 ± 1.1 and 27.0 ± 2.6% of lymphocytes were apoptotic in patients with sepsis (Fig. 3B).

A major objective of the study was to determine which pathways of cell death were operative in sepsis-induced lymphocyte apoptosis. Defining death pathways is important because of insight provided into the mechanisms of cell death and potential therapy. For example, involvement of the death receptor pathway implicates members of the TNF receptor superfamily, and a number of specific receptor antagonists are capable of blocking these sites (22, 23, 35–37). Involvement of the mitochondrial-mediated pathway suggests stress-induced mitochondrial injury, as occurs during oxidative stress. Antioxidant therapy has shown promise in some models of apoptosis. In the present study both methods used to evaluate apoptosis (flow cytometry and Western blotting) documented that both active caspases 8 and 9 were involved in apoptotic cell death (Figs. 3B, 4, and 6–8). These results indicate that lymphocyte apoptosis may be occurring by both death receptor-mediated injury (the extrinsic pathway) and mitochondrial stress-mediated injury (intrinsic pathway). Other experimental findings are consistent with activation of both pathways in sepsis. As demonstrated in Figs. 3C and 8, sepsis induces an increase in active caspase 3-positive, Bcl-2-negative lymphocytes, suggestive of apoptosis resulting from a mitochondrial-mediated pathway. In addition, Figs. 3C and 8 show an increase in active caspase 3-positive, Bcl-2-positive lymphocytes, suggestive of a death receptor-mediated pathway. Close inspection of Fig. 8 does show a generalized decrease in the quantity of Bcl-2 even in those lymphocytes considered positive for Bcl-2. Other investigators have reported that endotoxin shock leads to decreased Bcl-2 (35, 38).

Studies in animal models of sepsis support the concept that lymphocyte apoptosis in sepsis results from both death receptor-mediated and mitochondrial-mediated pathways. Work from the laboratory of Ayala et al. (36, 37) has shown that sepsis-induced lymphocyte apoptosis can be decreased and survival improved by administration of compounds that block the Fas death receptor-mediated pathway. Studies from three independent laboratories have shown that lymphocytes from mice that overexpress Bcl-2 are resistant to sepsis-induced apoptosis and have improved survival (12, 39, 40). Most recently, a live Escherichia coli baboon model of sepsis showed that both receptor- and mitochondrial-mediated apoptotic pathways are responsible for cell death in splenocytes (41). Serial sections of the septic baboon spleen showed specific regions containing apoptotic splenocytes positive for Fas ligand (which activates the death receptor pathway), whereas other apoptotic cells in different locations were positive for active caspase 9 and apoptosis-inducing factor (evidence of a mitochondrial-mediated pathway).

One possible complicating factor in trying to decipher specific death pathways involves cross-talk between the death receptor pathway and the mitochondrial-mediated pathway. In certain types of cells, activation of the death receptor pathway results in cleavage of the proapoptotic Bcl-2 family member Bid, which subsequently activates the mitochondrial-mediated death pathway. However, this cross-talk between the two pathways appears to be cell type specific, and although it has been described for hepatocytes, it is currently not believed to occur in lymphocytes (42, 43).

Sepsis is an extremely complex disorder involving activation of numerous intersecting cascades, including proinflammatory, anti-inflammatory, coagulation, and complement systems (44). Thus, it is not surprising that multiple pathways of cell death may be involved in sepsis. Furthermore, different bacteria possess different toxins that may activate unique cell death programs. (The patients in the present study had sepsis due to a variety of Gram-positive and -negative bacteria as well as fungi.) In this regard, however, in the baboon model, a single bacteria, i.e., E. coli, was able to induce both receptor-mediated and mitochondrial-mediated lymphocyte death (41).

A potentially important finding in the present study was the fact that the degree of lymphocyte apoptosis paralleled the presence or the absence of sepsis in the individual patient when apoptosis was
quantitated serially during the patient’s intensive care unit stay (Fig. 5). Although the degree of lymphocyte apoptosis was not useful in discriminating the severity of sepsis between groups of patients (there was no difference in lymphocyte apoptosis in patients with sepsis vs patients with septic shock; Fig. 2B), it was helpful in following the activity of sepsis in the individual patient (Fig. 5). In other words, as patients became septic or recovered from sepsis, there was a corresponding increase or decrease in the degree of lymphocyte apoptosis, respectively (Fig. 5). If this relationship between the presence of sepsis and lymphocyte apoptosis is confirmed in larger studies, it may be that quantitation of lymphocyte apoptosis in the individual patient during his/her hospital course could be used to predict early onset of sepsis or response to therapy. These studies are currently underway in our laboratory. However, there are limitations. Although caspase activation is often assumed to be synonymous with cell death, it is now clear that caspases are multifunctional enzymes that have a number of actions, including regulation of lymphocyte proliferation and differentiation and cell cycle regulation (44–46). Thus, the fact that there is a documented increase in caspase activation does not, a priori, signify that lymphocytes are undergoing apoptosis. In the present case, however, there is additional evidence that strongly suggests that the increased caspase activation results in cell death. First, the marked increase in annexin V-positive staining of lymphocytes from septic vs critically ill, nonseptic patients is highly indicative of apoptosis in the former group compared with the latter group. Secondly, flow cytometry consistently showed a decrease in forward and side scatter in lymphocytes from septic patients vs critically ill, nonseptic patients (data not presented). This feature is typical of cells that are becoming compacted and shrunken, a hallmark of apoptosis. Finally, histologic evaluation of spleens and lymph nodes from patients who died of sepsis shows marked depletion of B and T cells (8), a finding highly consistent with extensive apoptosis. Thus, we are confident that the increase in active caspases 3, 8, and 9 results in cell apoptosis.

In conclusion, sepsis induces extensive apoptosis in circulating lymphocytes that occurs by both death receptor and mitochondrial-mediated pathways. Although apoptosis was not increased in the critically ill, nonseptic patients considered as a group, individual critically ill, nonseptic patients did have markedly increased apoptosis, which was equivalent to that occurring in some septic patients. There is a significant decrease in Bcl-2 in a subpopulation of lymphocytes in sepsis, and this decrease in Bcl-2 may be contributing to the mitochondrial-mediated death. In the individual patient with sepsis, the degree of lymphocyte apoptosis tends to track the activity of his/her sepsis, although additional studies are necessary to confirm this finding. The extensive lymphocyte apoptosis in sepsis may have important adverse effects on immune function and may be a major factor in the immune suppression and accompanying lethality that characterize the disorder.

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Disclosures
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