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Sepsis-Induced Apoptosis Causes Progressive Profound Depletion of B and CD4$^+$ T Lymphocytes in Humans[1]

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Patients with sepsis have impaired host defenses that contribute to the lethality of the disorder. Recent work implicates lymphocyte apoptosis as a potential factor in the immunosuppression of sepsis. If lymphocyte apoptosis is an important mechanism, specific subsets of lymphocytes may be more vulnerable. A prospective study of lymphocyte cell typing and apoptosis was conducted in spleens from 27 patients with sepsis and 25 patients with trauma. Spleens from 16 critically ill nonseptic (3 prospective and 13 retrospective) patients were also evaluated. Immunohistochemical staining showed a caspase-9-mediated profound progressive loss of B and CD4 T helper cells in sepsis. Interestingly, sepsis did not decrease CD8 T or NK cells. Although there was no overall effect on lymphocytes from critically ill nonseptic patients (considered as a group), certain individual patients did exhibit significant loss of B and CD4 T cells. The loss of B and CD4 T cells in sepsis is especially significant because it occurs during life-threatening infection, a state in which massive lymphocyte clonal expansion should exist. Mitochondria-dependent lymphocyte apoptosis may contribute to the immunosuppression in sepsis by decreasing the number of immune effector cells. Similar loss of lymphocytes may be occurring in critically ill patients with other disorders. *The Journal of Immunology,* **2001, 166: 6952–6963.**

Sepsis and the resultant multiple organ failure that it induces are the most common causes of death in many intensive care units. The CDC currently estimates that >500,000 people develop sepsis and 200,000 die annually in the U.S. alone (1). Sepsis is now the 12th most common cause of death in America (1). A current hypothesis is that sepsis represents a state of uncontrolled activation of the inflammatory cascade resulting in cell and organ injury (2, 3). A recent consensus panel definition of sepsis as "the systemic inflammatory response syndrome due to infection" (4) reflects the concept that sepsis is the result of an uncontrolled inflammatory cascade. Because of this hypothesis, a major focus of sepsis research has been the development of antiinflammatory therapies, e.g., corticosteroids, anti-endotoxin Abs, anti-cytokine Abs, anti-platelet-activating factor, etc. The surprising failure of antiinflammatory strategies (with the possible recent exception of activated protein C (5)) has led to a rethinking of the concept of sepsis as a disorder due to unbridled inflammation (6, 7).

Recent studies in animal models of sepsis as well as in patients who died of sepsis and multiple organ failure have shown that sepsis induces extensive loss of lymphocytes via apoptosis (8–11). Because lymphocytes produce proinflammatory cytokines and activate macrophages, loss of lymphocytes may be beneficial to survival by down-regulating the excessive inflammatory response (10, 12). Alternatively, loss of lymphocytes in sepsis may be detrimental by impairing the ability of the immune system to combat pathogens (10, 12). In support of the concept that lymphocyte apoptosis is detrimental to host survival are a number of studies showing that patients with sepsis are immunologically impaired. Patients with sepsis become anergic, i.e., have no response to skin testing with Ags derived from microbes to which previous exposure would be expected (positive controls) (13, 14). There is also indirect evidence for an immunodeficient state in patients with sepsis. Studies show that trauma patients develop a decrease in circulating lymphocytes that is maximal at day 3. The lowest number of lymphocytes occurred in those trauma patients who developed infection or death (15). Furthermore, intensive care patients who develop a decreased lymphocyte count for >3 days are at a greatly increased risk of nosocomial sepsis (16).

Importantly, animal studies show that prevention of lymphocyte apoptosis either by overexpression of the antiapoptotic protein Bcl-2 or by administration of drugs that prevent activation of caspases (proteases that are activated in response to proapoptotic stimuli) improve survival in sepsis (17). To gain insight into the potential impact of lymphocyte apoptosis in sepsis, it is essential to determine both the extent of loss and type of lymphocytes that are being affected in the disorder. In the present study, the effect of sepsis on the various lymphocyte subsets, i.e., B cells, CD4 T cells, CD8 cytotoxic cells, and NK cells, was investigated. Defects in these lymphocyte subsets impair specific aspects of the host immune response and predispose to various pathogens. In addition to sepsis, other noxious stimuli such as ischemia/reperfusion or hypoxia can induce apoptosis (18). Therefore, nonseptic patients who were critically ill were also examined. Finally, we examined the role of active caspase-9 in lymphocyte apoptosis in sepsis. Apoptosis can proceed by two mechanistically distinct pathways, i.e., a receptor-mediated pathway that proceeds by activation of caspase-8 or a mitochondrial pathway that proceeds by caspase-9 (18, 19). Knowledge of the precise pathway of apoptosis will help identify stimuli that trigger cell death in sepsis and may allow for a more rational therapeutic approach.

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Materials and Methods

Septic patient population

The present work represents in part an extension of a previous study from this laboratory in which we reported the autopsy findings from 15 of the 27 patients with sepsis and 8 of the 25 patients with trauma who are included in the current examination (10). In the previous study, we investigated which of the two mechanisms of cell death (apoptosis or necrosis) occur in a variety of organs (brain, heart, lung, liver, spleen, colon, small intestine, kidney, and muscle) from patients dying of sepsis and multiple organ failure (10). As reported, spleen and colon were the two organs exhibiting the greatest degree of cell death, with apoptosis being the overriding mechanism of death in these organs (10). The aim of the current study was to characterize the immunologic status of septic patients by determining the extent of lymphocyte depletion and by identifying cell phenotypes that might be preferentially affected by apoptosis. In addition to immunohistochemical methods, flow cytometry was used to detect apoptosis and to corroborate immunohistochemical findings.

Spleen sampling in patients with sepsis

The method of obtaining spleen samples was described previously (10) and is discussed briefly. Spleen samples were obtained from 24 septic patients rapidly postmortem, whereas spleen samples were obtained intraoperatively from 3 patients with sepsis during a procedure to remove spleens with abscess formation. In the spleen samples obtained postmortem, a protocol for immediate tissue sampling allowed for tissue harvesting in the intensive care unit as soon as informed consent could be obtained from next of kin. The spleen sample was placed in 10% buffered formalin for 24 h. before paraffin-embedding and sectioning. The length of time between onset of death and tissue sampling ranged from 15 min to 6 h, with the vast majority obtained between 30 and 90 min (10). In the three patients with sepsis whose spleens were removed intraoperatively, the sample was obtained from a grossly normal appearing section of the spleen that was not directly contiguous with the site of infection. Microscopic examination was used to confirm the lack of infectious organisms and abscess formation in the spleen samples obtained intraoperatively. Three other septic patients were excluded from the study because they were taking chronic high doses of immunosuppressive medication, i.e., corticosteroids and/or cyclosporin. Four patients with sepsis who were being treated with corticosteroids were included in the study (see Table I). Two of these four patients had corticosteroids initiated in the 24–36 h preceding their death. Low physiologic doses of corticosteroids were used in the other two (see Table I).

The protocol for tissue sampling was approved by the Human Studies Committee at Washington University School of Medicine.

Criteria of sepsis

Patients were classified as septic based on one of the three following criteria: 1) positive blood, abdominal fluid, or tissue cultures for bacteria or fungi (see Table I); 2) intraoperative evidence of infection, e.g., perforated large bowel with peritoneal contamination, ischemic bowel with purulent peritoneal fluid; 3) a histopathologic diagnosis of infection at postmortem examination (e.g. bronchopneumonia, intraabdominal abscess). All patients also had premortem clinical evidence of sepsis with signs and symptoms of sepsis consisting of hypo- or hyperthermia, altered mental status, and hemodynamic instability usually requiring vasopressors (see Table I).

Critically ill nonseptic patients

Spleens were examined from 16 patients who were critically ill from a variety of nonseptic causes (Table II). Three patients were examined prospectively, and 13 patients were examined retrospectively. The 13 patients who were examined retrospectively were selected from the autopsy files at Barnes Jewish Hospital. Patients were selected on the following criteria: 1) no high clinical suspicion of sepsis and no autopsy evidence of infection; 2) hospitalization in the medical or surgical intensive care units; 3) no evidence of immunosuppression and no immunosuppressive medication; 4) autopsy performed within 24 h after death. We selected a time limit of 24 h for the postmortem examination because previous studies from our laboratory demonstrated adequate preservation of cellular morphology (without autolysis) and no increase in histologic evidence of apoptosis (via light microscopic examination) in spleens that were maintained at room temperature and sampled at consecutive time points for 24 h (10). Additional studies from our laboratory have shown no effect of a 24-h delay in tissue fixation on cell surface marker (CD) staining (our unpublished observations). In the present study, the duration of time between death and autopsy ranged from 0.5 to 21 h with a mean delay of 12.5 h.

Trauma patients as a control population

Due to the inability of obtaining normal human spleens, patients with blunt or penetrating abdominal trauma necessitating a splenectomy were used as a control population for comparison with the patients with sepsis. None of the trauma patients had significant comorbidities or was taking immunosuppressive medication; presumably, findings from this group are representative of those of a normal population. Spleens were removed rapidly after injury (usually within 3–8 h); therefore, values for the lymphocyte subsets should reflect those of a normal spleen.

Immunohistochemical staining for cell surface markers

An abbreviated description of the methods is provided. The complete description of immunohistochemical staining protocols is available on the website: *http://elysium.wustl.edu/rhlab/*

Abs against human CD3, CD8, CD20, and NK cell-like were from Dako (Carpinteria, CA). The mouse monoclonal anti-human CD4 Ab was from Vector (Burlingame, CA). Slides were heated and rinsed in Hemo De (Fisher Scientific, St. Louis, MO) and rehydrated. Endogenous peroxidase activity was blocked and Ag retrieval performed with Dako solution. CD4 Ag retrieval required microwave treatment in 1 mM EDTA, pH 8.0.

Anti-human CD3, CD8, and CD20 Abs were prediluted by the manufacturer and applied to sections. Next, HRP polymer (Dako EnVision) was added, and slides were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB).

Incubation with mouse anti-human NK cell-like Ab, diluted 1:75 in PBS, was done for 1 h after blocking with 10% rabbit serum in PBS. Slides were rinsed, incubated with a biotinylated rabbit anti-mouse IgM, rinsed, and incubated with an avidin-biotin peroxidase complex (VectaStain ABC Elite; Vector).

For CD4, primary Ab was diluted 1:75 and incubated for 1 h. After rinsing, secondary Ab (biotinyl-horse anti-mouse IgG; Vector) was added. Slides were rinsed, incubated with ABC, and developed with DAB.

Evaluation and image analysis of immunohistochemical stains

Slides were examined in a blinded fashion at 2× magnification to include as much of the tissue specimen for study as possible. Images were obtained using a Nikon (Melville, NY) COOLPIX 900 digital camera.

B cells and lymphoid follicles. The area of the spleen positively stained for B cells (CD20) was calculated using Image Pro (Media Cybernetics, Silver Spring, MD). Because expansion of B cell zones in splenic white pulp occurs in response to antigenic challenge, the number and area of the lymphoid follicles (which comprise the white pulp) are indications of the immunologic response/competence of the host. The number of lymphoid follicles was calculated and expressed per square centimeter of the spleen sample. The total area of the sample (red and white pulp) occupied by CD20-positive lymphoid tissue was also calculated.

CD4, CD8, CD3, and NK cells. The area of the spleen staining positive for CD4, CD8, CD3 or NK cells was calculated using Metamorph (Universal Imaging, West Chester, PA).

Immunohistochemical staining for active caspase-9

The affinity-purified rabbit anti-active caspase-9 Ab was a gift from Dr. Donald Nicholson (Merck Frosst Labs, Point Claire, Quebec, Canada). Briefly, tissues were deparaffinized, rehydrated, incubated in 3% H_2O_2, and rinsed. Ag retrieval was done in 0.1 M citrate buffer (pH 6.0) which was brought to a boil in a microwave oven. The tissues were blocked with a nonimmune serum (Zymed, San Francisco, CA). Primary Ab was diluted 1:1000 in PBS and incubated for 1 h at 37°C. Secondary Ab was added (Zymed). After incubation and washes, streptavidin complex was added (Zymed). Tissues were developed with metal-enhanced DAB (Pierce, Rockford, IL).

Flow cytometry of splenocytes from patients with sepsis or trauma

In addition to immunohistochemistry, spleens from five septic and six trauma patients were examined by flow cytometry (20, 21). Two of the five spleens included in the septic group were obtained intraoperatively during splenectomy; the three remaining spleens were obtained postmortem. Spleens from the six trauma patients were obtained intraoperatively. At the time of tissue harvesting, a small piece of spleen (~250–500 mg.) was placed in PBS containing glutamine (2 mM), glucose (10 mM), and 1%

[3] Abbreviations used in this paper: DAB, 3,3′-diaminobenzidine tetrahydrochloride; H&E, hematoxylin and eosin.

Table I. *Profiles of patients with sepsis*

Patient	Age (yr)/ Gender	Diagnosis	No. of Days in ICU[a]	No. of Days Septic	Absolute Lymphocyte Count[b]	Comorbidities	Inotropes	Miscellaneous
1	50/M	Necrotic bowel (cecum), peritonitis	6	7	1.0	Chronic renal failure on hemodialysis	Yes	Lactic acidosis
2	28/M	Peritonitis, cardiac arrest with anoxic injury	5	3	1.0	Pancreatitis; chronic renal failure s/p membranous glomerulonephritis	Yes	*P. aeruginosa* in peritoneal fluid
3	50/M	Peritonitis	11	11	5.5	Hepatitis C, cirrhosis	Yes	Hepatorenal syndrome developed terminally
4	49/M	Thigh abscess, aspiration pneumonia	16	16	0.9	Non-insulin-dependent diabetes	Yes	Enterococci and yeast in wound culture
5	53/M	Community pneumonia	1	1	0.4	Alcoholic	Yes	Blood cultures (+) for *Streptococcus pneumoniae*, malnourished
6	80/F	Perforated colon from malignancy, peritonitis	27	27	1.4	None	Yes	Blood cultures (+) for *Candida torulopsis*; nocosomial bacterial pneumonia
7	52/F	Intraabdominal abscess secondary to diverticulitis	Multiple admissions	77	1.0	Sepsis-induced diabetes	Yes	*Citrobacter freundii* and *Enterococcus faecium* in peritoneal fluid
8	63/M	Ischemic bowel, septic shock	4	4	0.8	Valvular heart disease	Yes	Replacement aortic and mitral valve 1 mo prior
9	92/M	Nosocomial pneumonia s/p repair aortic aneurysm	12	6	0.5	Emphysema	No	Developed renal failure requiring dialysis
10	77/F	Pseudomembranous colitis, nosocomial pneumonia	15	4	0.7	Previous inferior MI; old stroke	No	s/p resection of gastric carcinoma; malnourished
11	80/M	Peritonitis, gastrointestinal bleeding	4	4	0.6	Pancreatic cancer, COPD	Yes	Blood cultures (+) for *Enterococcus faecalis*; malnourished
12	60/M	Cellulitis	5	5	0.2	Crohn's disease, insulin-dependent diabetes, chronic steroids	Yes	Blood cultures (+) for β-hemolytic streptococci; hydrocortisone 50 mg q8h
13	45/M	Community pneumonia, aspiration	19	19	1.9	Alcoholic liver disease	Yes	*Haemophilus influenzae* pneumonia
14	49/F	Acute bilateral bronchopneumonia	3	3	N.A.	Transferred from outside hospital for worsening respiratory failure	Yes	Methylprednisolone 60 mg tid for cerebrovascular accident started 24 h before death; acute MI
15	82/M	Nosocomial pneumonia s/p repair leaking aortic aneurysm	23	10	0.8	None	Yes	*Serratia marcescens*; pneumonia; developed renal failure requiring dialysis
16	38/F	Intraabdominal abscesses secondary to perforated colon cancer	9	9	0.3	None	Yes	Splenic abscess, peritoneal fluid (+) for *P. aeruginosa*
17	18/M	Peritonitis/pneumonia after blunt abdominal trauma with right colon injury	34	32	0.3	None	Yes	Splenic abscess, *P. aeruginosa* and *Aspergillus fumigatus* in wound
18	60/M	Intraabdominal abscess, bile leak from hepatic resection for colon metastasis	31	31	1.9	None	Yes	Intraabdominal abscess, *Candida albicans* and *Acinetobacter baumannii* in blood
19	81/M	Nosocomial pneumonia after MVA with pulmonary contusion and femur fracture	7	3	0.6	None	Yes	Jehovah's Witness who refused blood, hematocrit of 15%
20	75/M	Endocarditis and sepsis	Multiple admissions	20	0.5	Valvular heart disease	Yes	*Candida parapsilosis* splenic abscess, sepsis after cardiac surgery for aortic valve repair
21	51/M	Necrotizing fasciitis after abdominal surgery for adhesiolysis	3	4	0.5	None	Yes	Gas gangrene
22	83/F	Ischemic bowel with peritonitis, secondary anastomosis leak	3	3	0.4	None	No	Peritoneal fluid (+) for Gram-positive cocci and *Bacteroides fragilis*
23	60/F	Bilateral bronchopneumonia	5	5	0.3	SLE, recurrent pneumonias	Yes	Hydrocortisone 25 mg tid, on chronic steroids, *Staphylococcus aureus*; malnourished
24	85/F	MVA with perforated intestine, long bone fractures, cardiac contusion	8	8	0.9	None	Yes	*Escherichia coli* and mixed organisms in peritoneal fluid
25	71/F	Nosocomial pneumonia	Multiple admissions	24	1.0	COPD	Yes	Blood cultures (+) for pneumococci and *Acinetobacter*; malnourished
26	74/F	Ischemic bowel	3	3	0.2	None	No	History of mesenteric ischemia
27	73/F	Urosepsis	2	2	0.3	None	Yes	Found at home; likely sick for additional days, hydrocortisone 20 mg tid started 24 h before demise

[a] ICU, intensive care unit; MVA, motor vehicle accident; COPD, chronic obstruction lung disease; SLE, systemic lupus erythematosus; MI, myocardial infarct; NA, not available; (+), positive; q8h, every 8 h; tid, three times a day; s/p, surgical procedure.
[b] Lower limit of absolute lymphocyte count at Barnes Jewish Hospital is 1.2 k/mm^3.

FCS. The tissue section was promptly prepared for flow cytometry as previously described (20, 21).

Generally, flow cytometry was performed within 4–8 h after obtaining tissue, and in no instances did the delay in examination exceed 24 h. Previous studies showed no differences in the degree of apoptosis in splenocytes examined immediately vs 24 h later providing that the splenocytes had been dissociated and stored at 4–8°C in appropriate enriched buffer as described above (our unpublished observations). Apoptosis was quantified using a commercially available fluorescein-labeled annexin V/propidium iodide kit (Apoptosis Detection Kit; R&D Systems, Minneapolis, MN) as described previously (20, 21). The various lymphocyte phenotypes were identified using fluorescently labeled mAbs directed against lymphocyte surface markers (PharMingen, San Diego, CA): B cells, CD20; CD4 T cells; CD8 T cells; CD3 T cells; NK cells.

Statistical analysis

Data are means ± SEM. Data were analyzed with a statistical software program, Prism (GraphPad Software, San Diego, CA). Data involving two groups only 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

Patient profiles and intensive care unit course

Pertinent clinical and laboratory findings for the septic and critically ill nonseptic patients are presented in Tables I and II, respectively. With two exceptions (septic patient 16 and critically ill nonseptic patient 10), the patients died. Twenty-three of the 27 septic patients and 11 of 16 critically ill nonseptic patients had a documented period of shock in which mean arterial pressure was <60 mm Hg and/or vasopressor therapy (dopamine, phenylephrine, and/or norepinephrine) was needed to maintain mean arterial pressure of >60 mm Hg. (The time period immediately before the patient's demise (generally ~30–90 min) was not included in the assessment of mean arterial blood pressure.)

Patient circulating lymphocyte counts

Most of the septic patients had a persistently low absolute lymphocyte count during their illness (data not shown). Values for the lowest absolute lymphocyte count of the patients during the 48 h preceding spleen harvesting are presented in Tables I and II. Twenty-two of the 26 septic patients and 11 of 16 critically ill nonseptic patients had an absolute lymphocyte count of <1200/mm^3 (the lower limit of normal at Barnes Jewish Hospital) (Tables I and II).

Immunohistochemical staining

B cells (CD20). Several parameters were used to evaluate the effect of the disorders on B cells: 1) the total area of spleen (red and white pulp) occupied by B cells was determined; 2) the area of the spleen occupied by lymphoid follicles and the number of lymphoid follicles per cm^2 of spleen were calculated. These two determinations provide complementary data on the effect of sepsis or critical illness on B cells.

Evaluation of spleens from septic patients showed a marked loss of B cells compared with spleens from both trauma patients and critically ill nonseptic patients (Figs. 1–3; Table III). In many cases, spleens from septic patients could be distinguished from spleens from trauma patients by gross visual examination, i.e., with the naked eye, of the microscopic slide (Fig. 1). There was a marked loss in the number and size of dark stained foci representing the lymphoid follicles (Fig. 1). Microscopic examination confirmed the gross visual examination and demonstrated a 43% decrease in area of lymphoid follicles (white pulp) and a 38% decrease in total B cell area (red and white pulp) in septic vs trauma patients (Figs. 2 and 3 and Table III).

Although there was no statistical difference in either the area of lymphoid follicles or the total B cell area in critically ill nonseptic vs trauma patients (Fig. 3 and Table III), there were individual critically ill nonseptic patients who did demonstrate obvious decreases in B cells (Fig. 4*B*).

In reviewing results of B cell staining, it was apparent that patients who had been septic for prolonged periods had a greater loss of B cells. Furthermore, it seemed logical that the severity of lymphocyte loss might be related to duration of sepsis. To assess this possibility, patients were divided into two groups, i.e., those who

Table II. *Profiles of critically ill nonseptic patients*

Patient	Age (yr)/ Gender	Diagnosis	No. of Days in ICU[a]	Absolute Lymphocyte Count[b]	Comorbidities	Inotropes	Miscellaneous
1	80/M	Cardiac arrest and shock after pneumonectomy	3	1.0	COPD and lung cancer	Yes	Developed shock; liver and renal failure
2	83/F	Cardiac arrest with hypoxic encephalopathy after repair of humeral fracture	2	0.2	None	Yes	Developed renal failure
3	74/M	Heart failure after MI and cardiac surgery	2	1.4	CHF and diabetes	No	
4	64/M	Heart failure after MI; arrhythmia	8	0.5	CASHD, COPD, diabetes	Yes	Intraaortic balloon pump, respiratory failure, ileus
5	46/F	Cardiac arrest with hypoxic encephalopathy	4	0.5	CRF on dialysis, diabetes, CASHD	No	
6	37/M	Massive pulmonary embolism, DKA	1	1.1	Diabetes	Yes	Severe acidosis
7	32/F	Cardiac arrest with hypoxic encephalopathy	2	1.3	Diabetes, depression on lithium	Yes	Acute renal failure
8	77/F	Massive pulmonary emboli	2	1.5	CHF, Parkinson's	Yes	Cardiogenic shock, renal failure
9	67/M	MI	3	1.4	CASHD	Yes	Intraaortic balloon pump
10	60/M	Pancreatitis, portal hypertension	1	0.5	Alcohol abuse, MI	Yes	Gastrointestinal bleeding
11	42/M	Massive gastrointestinal bleeding	1	0.2	Glomerular nephritis, alcohol abuse	No	
12	80/F	Cardiac arrest with anoxic injury after orthopedic surgery	3	0.7	None known	No	Pedestrian injury with ankle and humerus fracture
13	69/F	Cardiac arrest after aspiration	2	1.3	MI	Yes	Developed renal failure
14	52/M	MI and shock	7	0.7	None	Yes	Developed renal failure requiring dialysis
15	61/F	Pulmonary emboli and hemorrhage s/p hysterectomy	3	0.8	Previous pulmonary emboli	Yes	Renal failure
16	83/M	Massive gastrointestinal bleeding	2	0.6	Cerebrovascular disease	Yes	Liver and kidney failure

[a] ICU, Intensive care unit; COPD, chronic obstruction lung disease; CHF, congestive heart failure; CASHD, coronary atherosclerotic heart disease; CRF, chronic renal failure; DKA, diabetic ketoacidosis; MI, myocardial infarct; s/p, surgical procedure.

[b] Lower limit of absolute lymphocyte count at Barnes Jewish Hospital is 1.2 k/mm^3.

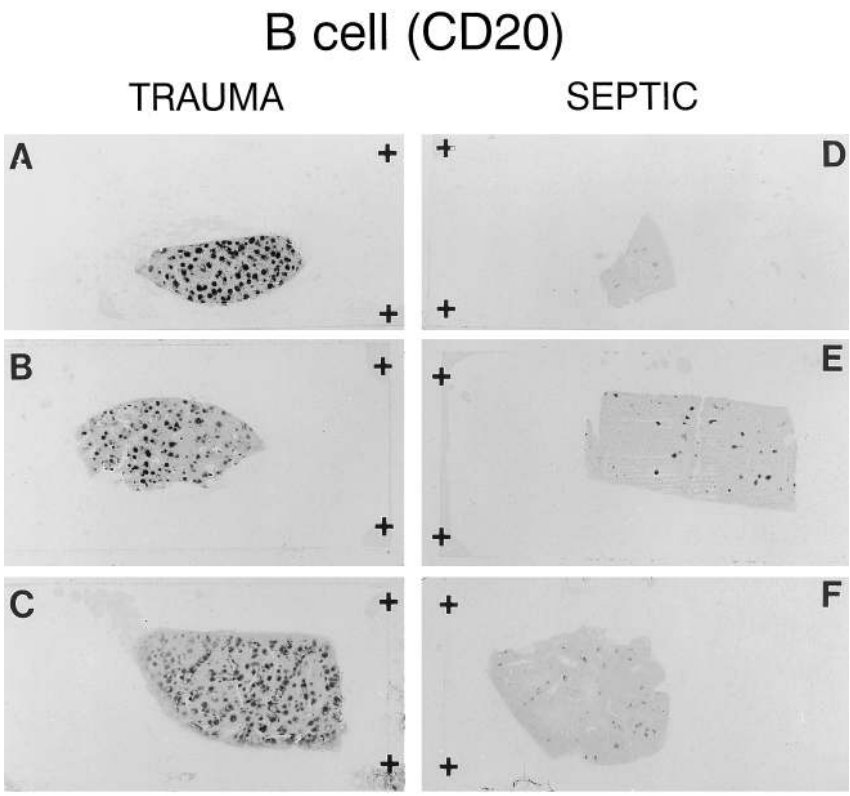

FIGURE 1. Unmagnified view of six microscope slides stained for B cells (CD20) from trauma patients (*A–C*) and septic patients (*D–F*). The dark staining regions are concentrations of B cells in lymphoid follicles visible to the naked eye. Note the dramatic decrease in the size and number of lymphoid follicles in the septic patients vs the trauma patients. *D, E,* and *F* are septic patients 7, 9, and 17, respectively, in Table I. The spleen from patient 17 was removed intraoperatively.

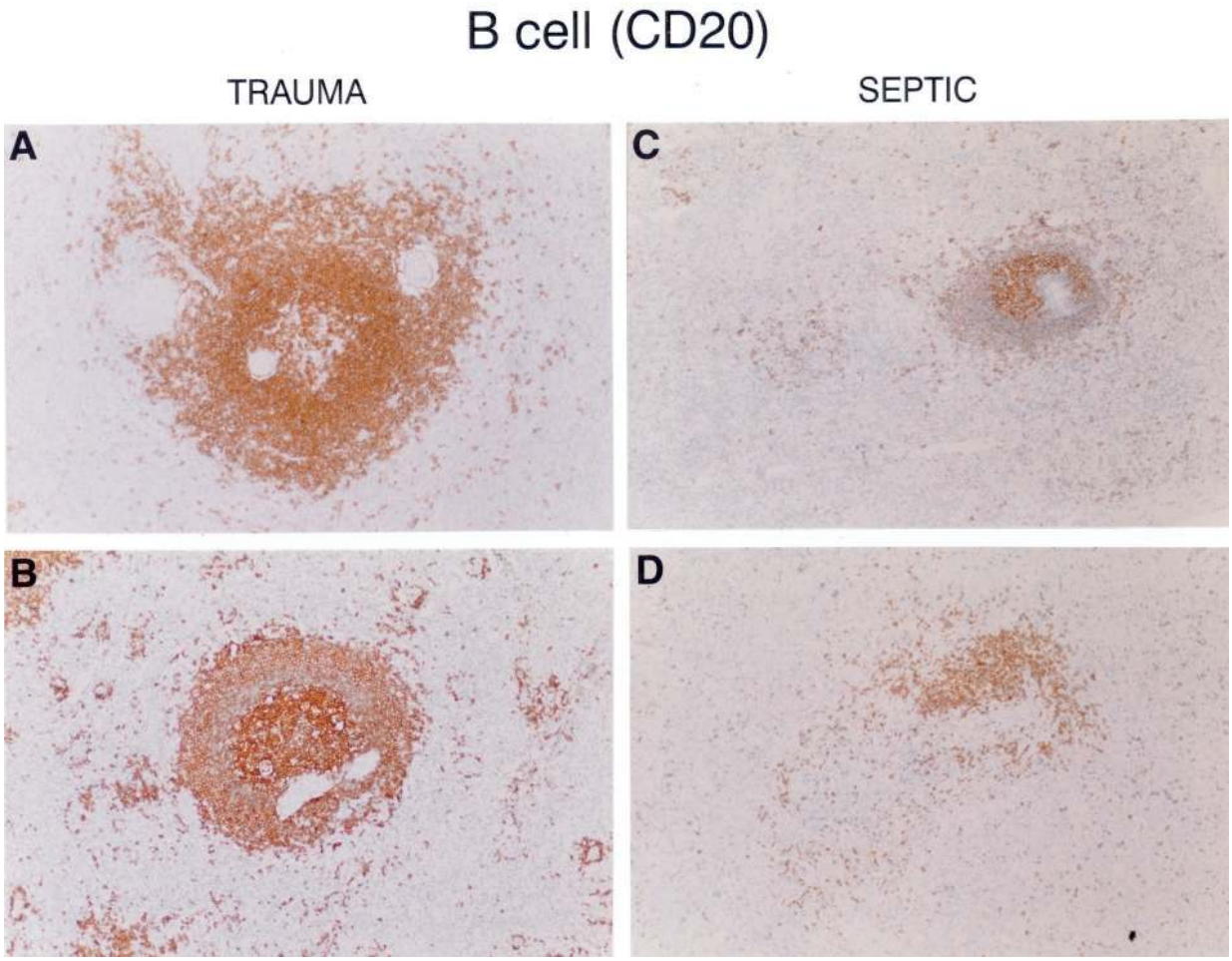

FIGURE 2. Immunohistochemical staining for B cells (CD20) in a lymphoid follicle. ×200. Trauma patients (*A* and *B*) have considerably larger follicles and many more B cells than the septic patients (*C* and *D*). *C* and *D* are septic patients 17 and 20, respectively, in Table I.

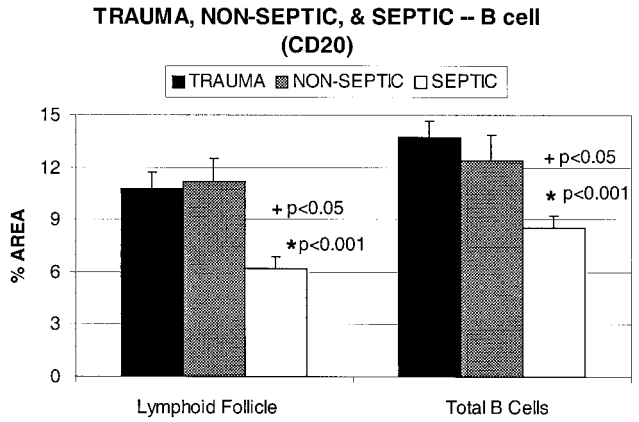

FIGURE 3. Comparison of immunohistochemical staining for B cells (CD20). The B cell-rich lymphoid follicles (white pulp) and total B cell area (red and white pulp) as a percentage of the total spleen area were determined for septic, critically ill nonseptic (nonseptic), and trauma specimens using an image analysis program as described in *Materials and Methods*. Note the marked decrease in the percentage area of lymphoid follicles and total area for B cells in septic vs trauma patients ($p < 0.001$). *, p compares septic to trauma; +, p compares septic to nonseptic.

were septic for <7 days and those who were septic for 7 or more days. All 4 patients on corticosteroids were in the group of patients who were septic for <7 days (see Table I). As shown in Fig. 5*A*, there was a more profound loss in B cells with increased duration of sepsis; compared with trauma patients, there was a 25 and a 52% decrease in B cell area in patients with sepsis for <7 and ≥7 days, respectively ($p < 0.05$ and $p < 0.001$, respectively). The decrease in B cell area at ≥7 days was greater than the decrease in B cell area at <7 days, $p < 0.05$ (Fig. 5*A*). In addition to the decrease in total B cell area, sepsis caused a quantitatively similar progressive decrease in the area of lymphoid follicles (Fig. 5*A*). Finally, the number of lymphoid follicles was decreased in patients with sepsis by ~40% compared with trauma patients, and this difference did not change with duration of sepsis, i.e., 101.6 ± 9.2, 58.7 ± 6.9, and 61.6 ± 11.6 lymphoid follicles/cm^2 for trauma patients, patients with sepsis for <7 days, and patients with sepsis for ≥7 days, respectively ($p < 0.01$ and $p < 0.05$, respectively).

CD4 T cells. Similar to the findings with B cells, sepsis caused a significant loss in CD4 T cells which were decreased by 40% compared with trauma patients (Figs. 6, *C* and *D*, and 7 and Table III). The loss in CD4 T cells also was examined for the effect of duration of sepsis. Compared with trauma patients, there was a 34% and a 46% decrease in CD4 T cells in patients with sepsis for <7 days and ≥7 days, respectively (Fig. 5*B*; $p < 0.001$ for both). However, there was no difference in percentage area of CD4 T cells in patients in sepsis for <7 vs ≥7 days (Fig. 5*B*).

There was no difference in CD4 T cells in critically ill nonseptic patients vs trauma patients (Table III). Similar to the B cell data, individual critically ill nonseptic patients exhibited markedly decreased CD4 T cell staining (Fig. 4, *C* and *D*).

CD8 T cells and NK cells. In contrast to the decrease in B cells and CD4 T cells, septic patients had a 60% increase in CD8 T cells compared with trauma patients ($p < 0.05$) (Table III). Similarly, critically ill nonseptic patients had a 130% increase in CD8 T cells vs trauma, ($p < 0.05$). There was a 44% increase in NK cells in septic vs trauma patients, but this difference did not achieve significance ($p > 0.05$) (Table III). Interestingly, the CD4:CD8 ratio, a measure of immune status, was decreased from 3:1 in trauma patients to 1:1 in septic patients ($p < 0.01$ (Table III)).

CD3 T cells. The CD3 surface marker is associated with the TCR in both CD4 and CD8 T cells. In spleen, therefore, CD3 staining can be used to corroborate CD4 and CD8 immunohistochemical findings, because the number of CD3-positive T cells is an approximation of the sum of CD4 and CD8 T cells. In the present study, the sum of the areas of CD4 and CD8 approximately equaled the area of CD3 in all three groups of patients (Fig. 7 and Table III). Patients with sepsis had a 35% decrease in the CD3 area compared with the critically ill nonseptic patients (Fig. 7; $p < 0.05$).

Caspase-9-positive apoptosis in spleens from septic patients

Previously, our laboratory has shown that patients with sepsis (including some of the patients in the present study) have a significant increase in caspase-3-mediated lymphocyte apoptosis in spleen and other organs (10). In the former and current studies, the characteristic morphologic changes of apoptosis, i.e., condensed and compacted nuclei with apoptotic bodies, were seen in routine hematoxylin and eosin (H&E) stains of spleens from septic patients (Fig. 8). In the present study, slides were evaluated in a blinded manner at ×400. With a photomask eyepiece to overlay the area of interest, the number of mantle and marginal cells in splenic white pulp that were positive for active caspase-9 within the specified area (0.0072 mm^2) were counted.

Many of the cells that were positive for active caspase-9 had typical apoptotic features of nuclear compaction and fragmentation (Fig. 9*D*). The number of cells that were positive for active caspase-9 were 41.6 ± 15.4, 32.9 ± 15.4, and 5.8 ± 3.3 for critically ill nonseptic, septic, and trauma patients, respectively. Both critically ill nonseptic and septic patients were statistically different from trauma patients ($p < 0.05$) but not from each other.

To determine whether delay in tissue fixation could impact active caspase-9 positivity, spleen specimens were left at room temperature and sampled at multiple time points during the next 6 h. There was no change in the number of cells positive by caspase-9 at the initial vs final time point (data not presented).

Table III. *Percent area of splenic lymphocyte subtypes determined via immunohistochemistry*

	CD20 (B cells)	Lymphoid Follicle	CD4 (T cell subset)	CD8 (T cell subset)	CD3 (T cells)	NK
Trauma (n = 25–26)	13.8 ± 0.9	10.9 ± 1.0	3.1 ± 0.1	1.0 ± 0.2	4.4 ± 0.4	1.1 ± 0.2
Critically ill nonseptic (n = 15–16)	12.4 ± 1.5	11.2 ± 1.4	3.0 ± 0.4	2.3 ± 0.3*[a]	5.3 ± 0.6	1.2 ± 0.2
Septic (n = 25–27)	8.5 ± 0.7*†	6.2 ± 0.7*†	1.8 ± 0.2*†	1.6 ± 0.1*	3.4 ± 0.2†	1.7 ± 0.2
% Change in trauma vs critically ill nonseptic	10 ↓	4 ↑	3 ↓	130 ↑	20 ↑	6 ↑
% Change in septic vs trauma	38 ↓	43 ↓	40 ↓	60 ↑	23 ↓	44 ↑

[a] *, $p < 0.05$ vs trauma; †, $p < 0.05$ vs critically ill nonseptic.

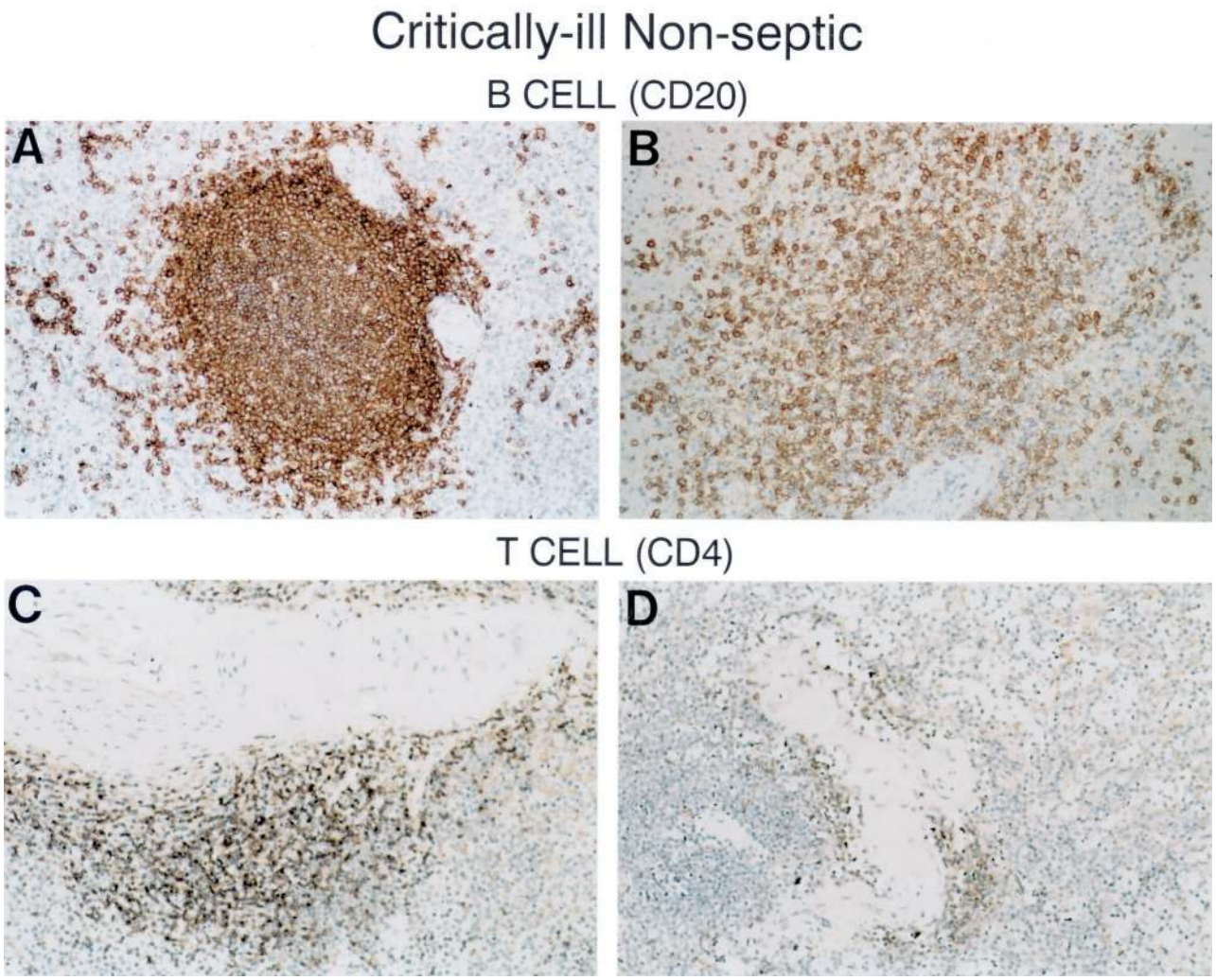

FIGURE 4. Immunohistochemical stain for splenic B and T cells in critically ill nonseptic patients, showing that some critically ill nonseptic patients had normal lymphocytes whereas other critically ill nonseptic patients had lymphocyte depletion. *A,* Robust follicle stained for B cells (CD20); *B,* follicle that is relatively depleted of B cells. ×200. *C,* Normal CD4 T cells; *D,* decreased density of CD4 T cells. ×400. *A, B, C,* and *D* represent patients 12, 4, 14, and 15, respectively, in Table II.

Lymphocyte cell typing and quantitation of apoptosis by flow cytometry

No spleens were available from critically ill nonseptic patients; therefore, only septic and trauma patients were compared. The findings from flow cytometry closely paralleled the results from the immunohistochemical staining studies. There was a 42% loss in B cells and a 45% loss in CD4 T cells in septic patients compared with trauma patients, $p < 0.001$ and $p < 0.01$, respectively (Fig. 10*A*). In contrast to the decrease in CD4 T cells and B cells, there was a 57% increase in NK cells in patients with sepsis vs those with trauma, $p < 0.02$. There was no difference in the percentage of CD8 T cells in septic vs trauma patients (Fig. 10A). The percentage of CD3 T cells was decreased by 38% in septic patients compared with trauma patients, $p < 0.003$.

Annexin V and propidium iodide staining revealed apoptosis in 4% of lymphocytes in trauma patients compared with 12% in septic patients, $p < 0.002$ (Figs. 10*B* and 11).

Discussion

Perhaps the most significant finding in the present study is the remarkable degree of loss of B cells and CD4 T cells in spleens of septic patients. The loss in splenic lymphocytes was associated with a significant and often profound decrease in circulating lymphocytes in 22 of the 26 septic patients. In spleens, the loss of B cells (which are concentrated in large lymphoid follicles) was so prominent that, in some cases, it was readily detected by gross visual examination of the microscopic slides by the naked eye (Fig. 1). During infection, B cells do not migrate to the site of pathogens but rather proliferate in spleen and other organized lymphoid tissues and release Abs that are transported to the focus of disease (22, 23). Therefore, the decrease in B cells observed in the spleens of septic patients has particular importance. The loss in CD4 T cells in septic patients was also striking (Fig. 6). The loss in lymphocytes (both B cells and CD4 T cells) is even more significant considering that it occurs in the context of overwhelming infection, a condition in which massive clonal expansion of B and T lymphocytes should be occurring.

A hallmark of sepsis is immunosuppression that is characterized by loss of delayed-type hypersensitivity, failure to eradicate a primary infection, and propensity to acquire new secondary infections (2, 3). The documented loss in CD4 T and B cells (rather than the anticipated increase in lymphocytes) may be contributing to the immunosuppression by decreasing the number of immune cells available to combat infection. In addition to causing a decrease in the

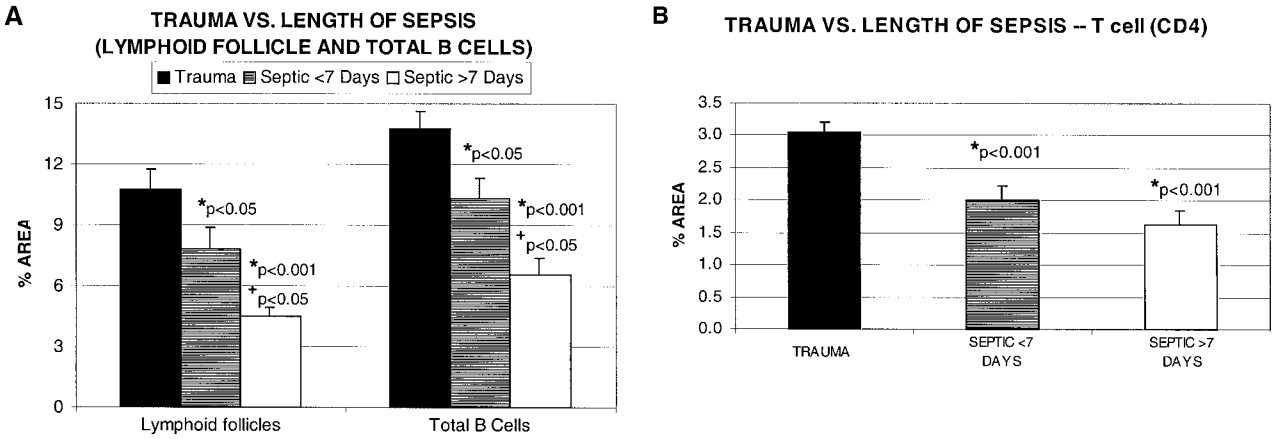

FIGURE 5. *A,* Comparison between septic and trauma for lymphoid follicles and total B cell area based on the duration of sepsis. Patients who were septic for <7 days had smaller lymphoid follicle area and smaller B cell area; *, $p < 0.05$. Patients who were septic for >7 days had an even smaller lymphoid follicle area and a B cell area that was less than the areas for trauma patient (*, $p < 0.001$) and also less than the areas for the patients septic for <7 days (+, $p < 0.05$). *B,* Effect of the duration of sepsis on CD4 T cell area. Both groups of septic patients, i.e., patients who were septic for <7 days and patients who were septic for >7 days had less area of spleen occupied by CD4 T cells (*, $p < 0.001$), but they were not different from each other.

number of immune effector cells, lymphocyte apoptosis may contribute to immunosuppression by other mechanisms as well. Recent research has shown that apoptotic cells actively suppress the inflammatory response (24–26). Voll et al. (24) demonstrated that addition of apoptotic lymphocytes to endotoxin-stimulated PBMC caused a shift from secretion of proinflammatory cytokines (TNF-α, IL-1β, and IL-12) to antiinflammatory cytokines (IL-10). Barker et al. (25) showed that macrophages that ingested apoptotic cells had increased

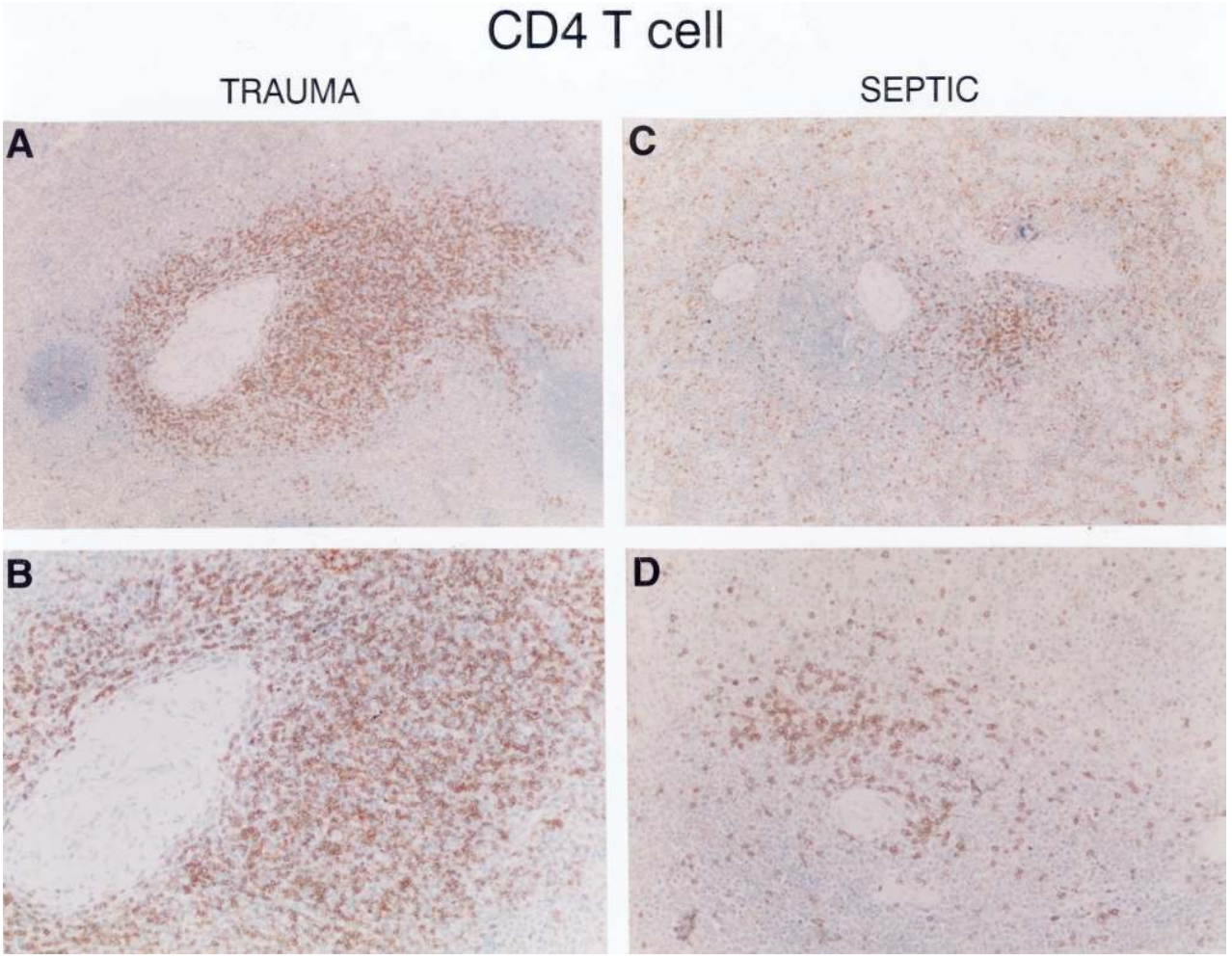

FIGURE 6. Immunohistochemical staining for splenic CD4 T cells. The T cell-rich periarteriolar zone is demonstrated in the trauma patient (*A,* ×200; *B,* ×400 magnification of the same patient) and the septic patients (*C,* ×200; *D,* ×400). *C* and *D,* Septic patients 6 and 25, respectively, in Table I. Note the decreased number of CD4 T cells in septic vs trauma patients.

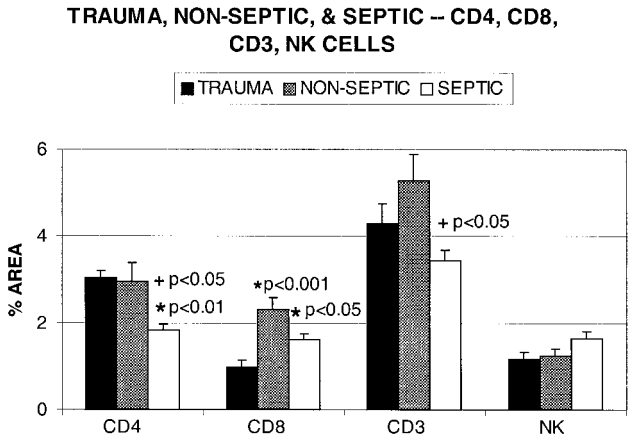

FIGURE 7. Comparison of CD4 T cell subset, CD8 T cell subset, CD3 T cells, and NK. Immunohistochemical staining was performed, and the percentage area of the cell subsets was determined (see *Materials and Methods*). Compared with trauma patients, septic patients had a decrease in CD4 and CD3 and an increase CD8 area of the spleen. ∗, Critically ill nonseptic (nonseptic) had increased CD8 compared with trauma and increased CD3 compared with septic. ∗, p compares septic or nonseptic critically ill to trauma; +, p compares septic to critically ill nonseptic.

production of TGF-β_1, a potent immunosuppressant and antiinflammatory cytokine. Fadok et al. (26) reported that uptake of apoptotic vs necrotic cells caused the macrophage to respond in an antiinflammatory or proinflammatory manner, respectively.

Critically ill nonseptic patients

Although the critically ill nonseptic patients considered as a group did not demonstrate loss of lymphocytes compared with the control population (trauma patients), it was clear that certain individual critically ill nonseptic patients did have significant loss of lymphocytes (Fig. 4, *A* and *B*). The increase in active caspase-9 in critically ill nonseptic patients relative to trauma patients demonstrates that apoptosis of lymphocytes is occurring and will result in cell loss. It is likely that studies examining larger numbers of these patients would disclose subsets of critically ill nonseptic patients, e.g., liver failure, renal failure, congestive heart failure, who did have loss of lymphocytes that are comparable to that in the septic patients. As indicated in septic patients (Fig. 5), it is likely that the longer the duration of illness in the critically ill nonseptic patient, the more likely there is to be loss of lymphocytes. In this regard, the critically ill nonseptic patient who had the most extensive loss of B cells (Fig. 4) was also the patient who had the longest stay in the intensive care unit before death (8 days).

Although both critically ill nonseptic and septic patients had comparable increases in the number of cells that were positive for active caspase-9, many spleens of septic patients had a decreased number of lymphocytes in the lymphoid follicle; i.e., there was a decreased density of cells per unit area (see Fig. 9*B*). This finding of depletion of splenic lymphocytes per unit area was reported previously (10). It is likely that many of the lymphocytes of septic patients had already undergone caspase-9-mediated apoptosis, and this may have been a factor in the lack of difference in septic vs critically ill nonseptic patients. Studies from our laboratory show that there is a marked decrease in active caspase-9-positive cells in patients who were septic for prolonged periods of time vs patients who were septic for brief periods (data not shown).

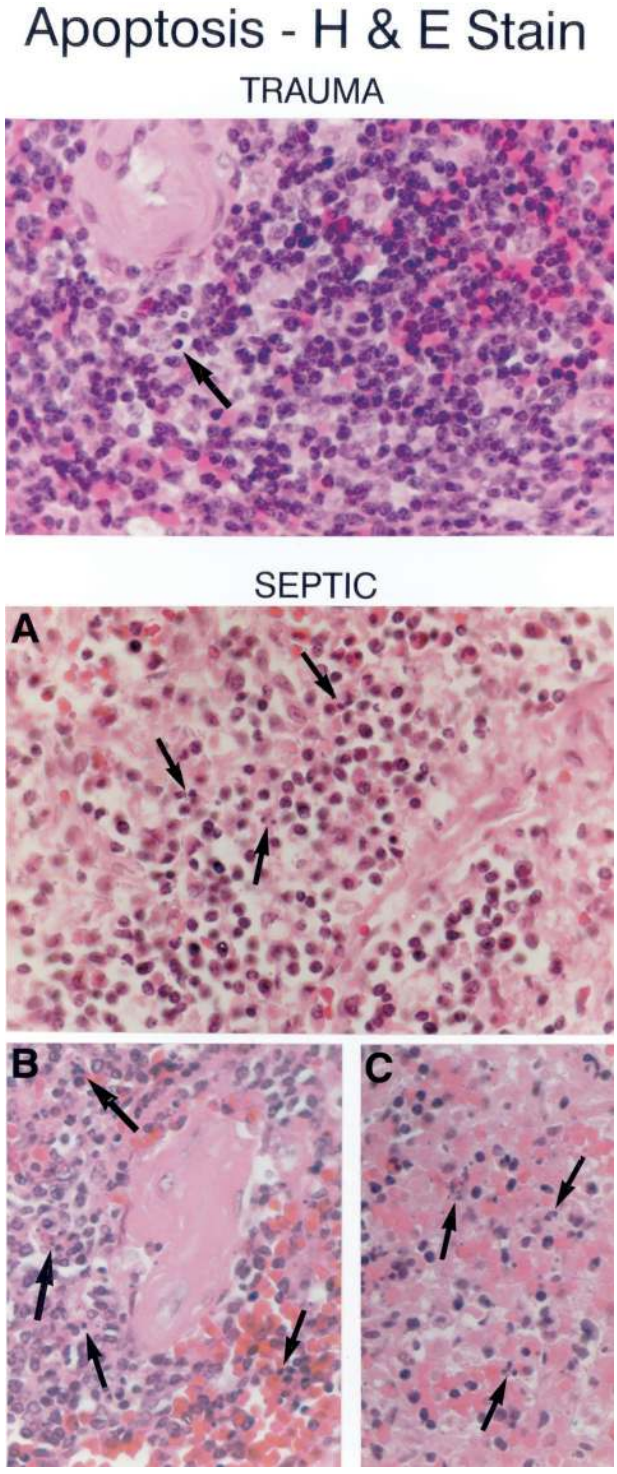

FIGURE 8. H&E stain (×600) of spleens from trauma and septic patients (*A–C*). The trauma patient has a single apoptotic cell (arrow). Septic patients have multiple apoptotic lymphocytes only some of which are indicated by arrows. *A,* Septic patient 21; *B* and *C,* septic patient 26, showing apoptotic cells in white and red pulp of spleen, respectively.

Lymphocyte apoptosis: a key pathophysiologic mechanism in sepsis?

The present results establish an association between decreased lymphocytes and sepsis but do not establish causality between lymphocyte apoptosis and outcome in patients with sepsis. However, a growing body of animal studies supports the hypothesis that

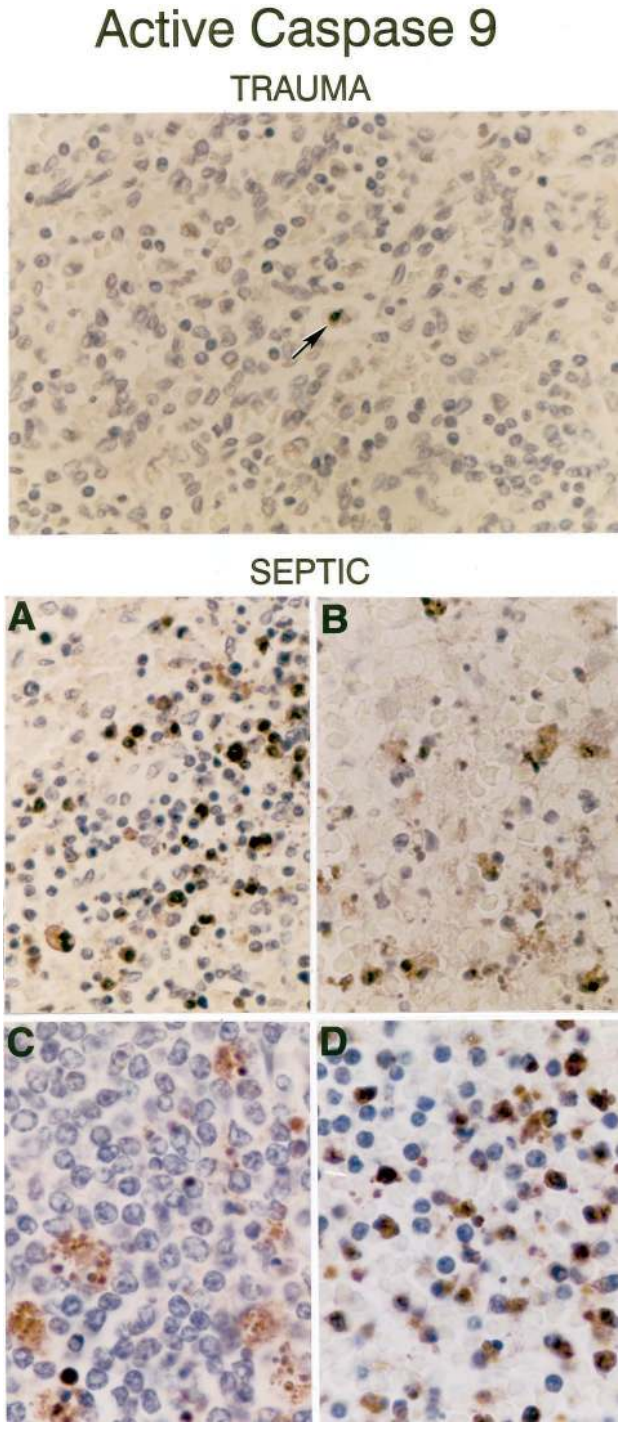

FIGURE 9. Immunohistochemical stain for active caspase-9 in spleen. The trauma patient had a single cell positive for active caspase-9 (identified by arrow). ×400. The septic patients had numerous cells positive for active caspase-9 cells (brown staining); *A* and *B,* ×400; *C* and *D,* ×600. Many of the cells that are positive for active caspase-9 also exhibit the characteristic features of apoptosis, i.e., condensed and or fragmented nuclei. In *C,* macrophages have ingested apoptotic lymphocyte fragments that are positive for active caspase-9. *A,* Septic patient 19; *B,* septic patient 13; *C* and *D,* septic patient 26 (Table I).

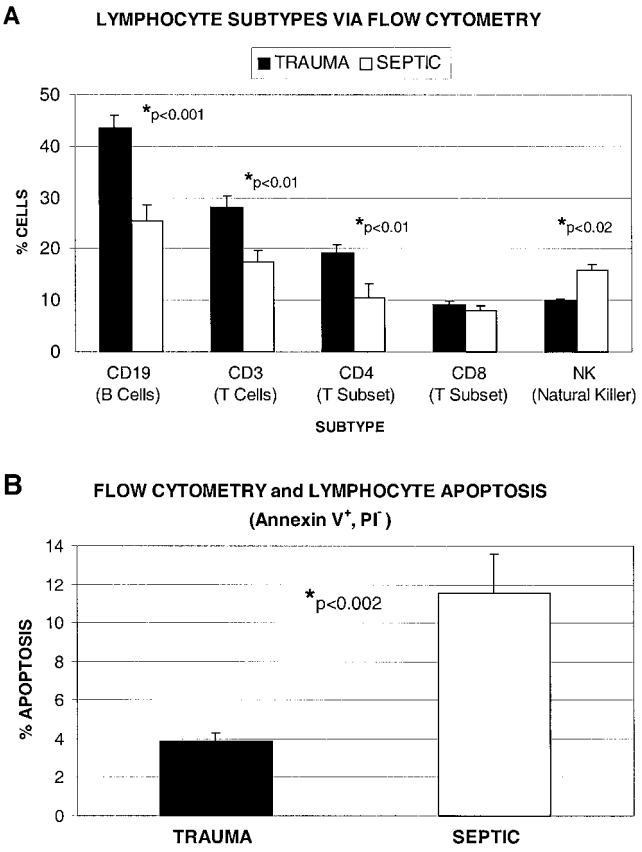

FIGURE 10. Lymphocyte subtyping and quantitation of apoptosis via flow cytometry. Spleens from six septic patients and five trauma patients were dissociated and prepared as described in *Materials and Methods*. Lymphocyte markers specific for the various lymphocyte subsets were added, and flow cytometry was performed. *A* shows the dramatic decrease in B cells (CD19), in CD4 T cells, and in total T cells (CD3) in septic vs trauma patients. In contrast to the decrease in the other lymphocyte subsets, NK cells were increased in sepsis vs trauma (∗, $p < 0.02$). There was no effect of sepsis on CD8 T cells. *B,* Lymphocyte apoptosis was determined using annexin V and propidium iodide (PI) as described in *Materials and Methods*. There was a marked increase in lymphocyte apoptosis (annexin V-positive and propidium iodide-negative staining) in septic patients (∗, $p < 0.002$).

a major pathophysiologic mechanism in sepsis is lymphocyte apoptosis (8–11, 27). Recently, Braun et al. (28) showed that administration of caspase inhibitors, drugs that block apoptosis, provided excellent neuroprotection in a rabbit model of pneumococcal meningitis. Ayala et al. (29) reported that septic mice deficient in FasL^{gld} had decreased mucosal B lymphocyte apoptosis and improved survival compared with controls. Our laboratory has reported that prevention of lymphocyte apoptosis by a variety of compounds that inhibit caspases improves survival in a peritonitis animal model of sepsis (17, 20, 30). In addition to the efficacy of caspase inhibitors, mice in which the antiapoptotic protein Bcl-2 is overexpressed in T or B lymphocytes have decreased blood bacterial counts and improved survival in sepsis (17, 20, 30). Furthermore, adoptive transfer of T cells that overexpress Bcl-2 into nontransgenic mice improves survival in sepsis (30). Finally, caspase inhibition has also been reported to improve survival in an endotoxin model as well (31). Considered together, these studies provide strong supporting evidence in animal models that loss of lymphocytes in sepsis is a central pathogenic event.

Ischemia/reperfusion injury and other potential variables in sepsis-induced apoptosis

There are numerous metabolic and physiologic changes in critically ill patients that may be confounding factors in the sepsis-induced lymphocyte apoptosis. For example, the majority of patients with severe sepsis have episodes of ischemia/reperfusion injury and, in the

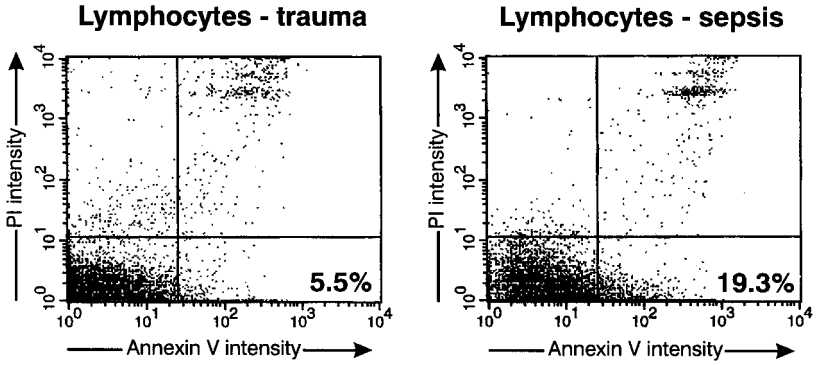

FIGURE 11. Detection of apoptosis via flow cytometry. Cells were isolated from a spleen from a trauma patient and a septic patient and labeled with annexin V and propidium iodide (PI). Flow cytometry was performed with gating on lymphocytes using characteristic forward and side scatter. Lymphocytes were judged to be apoptotic if they were annexin V-positive and PI-negative stained, indicated for cells in the right lower quadrant. In the spleen from the septic patient vs 5.5% of lymphocytes apoptotic in the trauma patient, 19.3% of lymphocytes were apoptotic.

present study, 23 of the 27 septic patients required vasopressor therapy to maintain an adequate mean arterial blood pressure (Table I). Animal studies and some human data indicate that ischemia/reperfusion injury can induce apoptosis in the gastrointestinal tract (32), heart (33), kidney (34), and brain (35).

Four of the patients with sepsis were receiving corticosteroids (see Table I). It is possible that the corticosteroids contributed to lymphocyte apoptosis. However, the dose of corticosteroids in two of four septic patients (patients 23 and 27) was low (20–25 mg hydrocortisone every 8 h) and much less than can be produced by the body under severe stress. Furthermore, it is significant that all four of the septic patients receiving corticosteroids were in the group of patients who had sepsis for <7days. These patients had less loss of B cells and $CD4^+$ T cells than patients who were septic for > 7 days.

Pathways of lymphocyte apoptosis in sepsis

There are two major pathways involved in initiation of apoptosis, i.e., a receptor initiated caspase-8-mediated pathway and a mitochondrial-initiated caspase-9-mediated pathway (18, 19). Activation of caspase-8 or caspase-9 subsequently leads to activation of caspase-3, an effector caspase that is in the final common pathway of the cell death program. Caspase-8 can be activated by a number of ligands including TNF and Fas (18, 36). The mitochondrial-mediated pathway can be activated by a diverse number of stimuli including, e.g., reactive oxygen species (36). Understanding the particular pathway of sepsis-induced apoptosis is important because it provides insight into potential factors responsible for initiating cell suicide and may allow for a more targeted therapy. The present studies showing activated caspase-9 in apoptotic lymphocytes of septic patients supports a mitochondrial-mediated pathway. Although demonstrating that caspase-9 is activated provides supporting evidence for a mitochondrial-initiated pathway, it is not conclusive proof because of recent studies which show that, in some instances, there is "cross-talk" between the various caspases (19, 36). However, a great deal of data in animal models of sepsis are consistent with the concept that sepsis-induced lymphocyte apoptosis proceeds by the mitochondria-initiated pathway. Sepsis-induced thymocyte and splenocyte apoptosis is not blocked in FasR-deficient mice (21) or in TNF p55 or TNF p75 receptor-deficient mice (our unpublished observations). Although controversial, most recent studies show that Bcl-2 inhibits the mitochondrial but not the receptor-mediated apoptosis pathway (19). Therefore, the published studies noting that overexpression of Bcl-2 in T or B cells prevents lymphocyte apoptosis in sepsis (17, 20) is also consistent with a mitochondrial-mediated death pathway in the disorder.

Other potential mechanisms of immunosuppression in sepsis

Although we speculate that sepsis-induced lymphocyte apoptosis is a central pathogenic event, it is possible that other mechanisms are involved in the immunosuppression that characterizes the disorder (7, 37). A large body of studies indicates that critically ill patients with trauma are anergic and have multiple defects in immune function (11, 37–41). Pellegrini et al. (39) demonstrated that T cells from trauma patients underwent apoptosis at an accelerated rate compared with normal volunteers. However, T cell anergy did not appear to correlate with lymphocyte apoptosis (39). Lederer et al. (38) and Wichman et al. (40) have shown that major injury and sepsis induce increased production of IL-10, a counterinflammatory cytokine that impairs resistance to infection and/or decreases the ability to combat infection. Puyana et al. (41) reported that both Th1- and Th2-type lymphokines are depressed in posttrauma anergy. Lyons et al. (42) noted that major injury induced increased production of IL-10 and thereby impaired resistance to infection. Thus, lymphocyte apoptosis may be only one of the many factors that are involved in compromising host defense.

Limitations and alternative hypotheses

The major focus of the present study was to determine the effect of sepsis on the various lymphocyte phenotypes. The best way to determine whether sepsis is having an effect is to compare spleens from a "normal" population. Because it is not possible to obtain spleens from healthy normal persons, we elected to use spleens removed acutely from patients who had no comorbidities but who had trauma to the spleen necessitating a splenectomy. Other than the fact that their spleens were acutely fractured and bleeding, the cellular composition and architecture should be normal (except for the area of injury).

A possible concern is whether the relatively longer delay in spleen fixation in septic patients vs trauma patients (15-min to 6-h delay in septic patients vs 5- to 30-min delay in trauma patients; see *Materials and Methods*) could be responsible for some of the experimental differences. Data suggest that the differences in time of spleen fixation did not play a role in the results. Previous work from our laboratory did compare spleens of septic patients to spleens obtained from a group of patients after sudden cardiovascular death and whose tissues were not obtained until 3–12 h postmortem (10). Microscopic examination of H&E tissue sections from these control spleens showed normal splenic architecture, no lymphocyte depletion, and little evidence of apoptosis (10). In a previous study, we showed that immunohistochemical staining for active caspase-3, a key cell death protease, was also significantly less in spleens from control vs septic patients (10). In the present study, we demonstrated that active caspase-9 was not effected by delay in tissue fixation (see *Results* on caspase-9). Also, in the present study, spleens removed intraoperatively from three septic patients (and formalin fixed immediately) exhibited some of the most remarkable loss of lymphocytes (Fig. 1*F*). Finally, the changes in lymphocyte phenotypes observed in spleens from septic

patients were not observed in critically ill nonseptic patients even though the delay in spleen fixation was longer in the latter group.

A final limitation concerns interpretation of the effect of lymphocyte apoptosis on host survival in septic patients. Although we speculate that loss of lymphocytes is detrimental to survival in septic patients because of the resultant immunosuppression, it is possible that lymphocyte apoptosis may have beneficial effects (12). Apoptosis of lymphocytes may lead to decreased production of proinflammatory cytokines, which induce or contribute to the systemic inflammatory response syndrome and organ injury in sepsis. A recent study of *Pseudomonas aeruginosa* pneumonia showed that apoptosis of lung epithelial cells (lymphocyte apoptosis was not evaluated) helped to limit the spread of the infection systemically and was essential for survival (43).

The present results show that sepsis rather than inducing lymphocyte proliferation causes a profound and progressive decrease in B cells and CD4 T cells. It is possible that this loss in B cells and CD4 T lymphocytes impairs the ability of the patient to eradicate the infection and predisposes to other invading pathogens. If immunosuppression resulting from loss of lymphocytes is determined to be a key factor in patient survival in sepsis, therapy with caspase inhibitors (which have shown remarkable success in clinically relevant animal models of sepsis) (17, 30) may represent a novel approach in the treatment of this highly lethal disorder.

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