

Immunosuppression in Sepsis

Peter A. Ward, MD

ABUNDANT EVIDENCE EXISTS IN BOTH ANIMALS AND humans with sepsis for the appearance of a “cytokine storm,” characterized by high plasma levels of proinflammatory cytokines and chemokines,¹ clinical signs of fever, tachycardia, and tachypnea and followed rapidly by development of shock, multiorgan failure, and death.² Additional evidence indicates that sepsis can be associated with a state of immunosuppression, broadly defined as lymphopenia and loss of immune function, though the timing, incidence, and nature of the immunosuppression remain poorly characterized, especially in humans.³

The study by Boomer and colleagues⁴ in this issue of *JAMA* is perhaps the most authoritative investigation exclusively involving humans and confirming the onset of immunosuppression developing during septic shock or severe sepsis (defined as a systemic response to infection complicated by acute organ dysfunction).⁵ The investigators evaluated 40 patients who had sepsis following bacterial peritonitis or ventilator-associated bacterial pneumonia, using “bedside autopsies” to obtain splenocytes and lung cells immediately after the declaration of death. Trauma patients undergoing splenectomy and brain-dead patients (often following intracranial hemorrhage) who had not had sepsis were included as controls.

The observations in the current study provide compelling evidence for development of immunosuppression in patients with sepsis, albeit with the important caveat that all of the study patients died of sepsis—those who survived may well have had a different immunologic course. Splenocytes obtained from these patients and subsequently stimulated in vitro with bacterial lipopolysaccharide (LPS) or with agonist antibodies revealed markedly suppressed production of cytokines (<10% compared with supernatant fluids from LPS-stimulated splenocytes obtained from sepsis-free donors). The reason for high cytokine blood levels in sepsis is not known but may be due to exposure of macrophages and other cytokine-producing cells to high levels of agonists released from the infecting organisms (bacteria, viruses, etc).

In addition, the data clearly indicate that splenocytes from patients with sepsis showed reduced content of costimulatory molecules such as CD28 on T cells, indicating that these cells had defective immune function. Antigen-presenting cells, largely consisting of macrophages and dendritic cells, were deficient in the costimulatory molecule CD86 and expressed increased levels of HLA-DR and the inhibitory ligand PD-L1 and its receptor, PD-1. Furthermore, there was a buildup of inhibitory cells (regulatory T cells and myeloid-derived suppressor cells) in spleens and in cells isolated from lungs obtained from patients with sepsis. In lung, alveolar epithelial cells and endothelial cells expressed multiple inhibitory receptors and ligands, suggesting that such cells bring about reduced responsiveness of nearby lymphocytes.

Collectively, at least in lung and spleen, the cellular changes essentially reflected that the “stars were aligned” in a manner leading to profound immunosuppression. The reasons for such an undesirable constellation of changes are obscure. Preventing or reversing such outcomes in experimental sepsis may be achieved by use of inhibitors of apoptosis (synthetic inhibitors of caspases or other types of inhibitors⁶), but in human sepsis, lymphopenia and immunosuppression are usually well advanced by the time the patient is admitted to the intensive care unit,^{3,4} raising the question whether such derangements can be reversed over a short period. There have been suggestions that orally administered protease inhibitors of the retroviral class improve survival in the murine model of sepsis induced by cecal ligation and puncture, with reduced plasma levels of proinflammatory cytokines and diminished apoptosis in thymic and splenic tissues.⁷

Another poorly understood outcome in sepsis is the extent to which development of multiorgan failure may be related to the cytokine storm. Furthermore, whether the cytokine storm has any linkage to the development of immunosuppression is also largely unknown. Devices that adsorb cytokines may be useful. For example, use of a hemoperfusion device with polymyxin B improved survival in 64 patients with abdominal sepsis.⁸ Whether the effects of such devices are limited only to removal of LPS

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from blood or might be associated with other effects remains to be determined. Removal of LPS from the blood of patients with abdominal sepsis and gram-negative sepsis makes sense. However, because many cases of adult human sepsis are associated with *Streptococcus pneumoniae* or *Staphylococcus aureus*, both gram-positive organisms, and LPS is often not detected in these patients, a strategy in sepsis patients targeted exclusively toward removal of LPS has serious drawbacks. A further caution related to the role of LPS and its receptor (TLR4) in human sepsis comes from the failed clinical trials in which an antagonist for TLR4 was used.⁹

Boomer and colleagues have presented an informative report documenting immunosuppression in humans with septic shock, along with the broad array of cellular changes that can be linked to the loss of immune competence. A next step might be to determine why during sepsis immune cells switch from a phenotype with proimmune receptors and ligands to a phenotype featuring anti-immune receptors and ligands. Another important research question is whether such derangements in sepsis involving humans can be reversed by treatment with agents such as interleukins 7^{10,11} or 15¹². These agents in some settings may restore immune responsiveness by increasing the number of competent T cells. Such a strategy, if successful, would be a novel approach for reconstituting the T-cell depletion state in sepsis and would be especially encouraging in view of the recent withdrawal of recombinant protein C (Xigris) because of lack of clinical efficacy in sepsis.¹³ This underscores the desperate need for a better understanding of sepsis and the urgent need for new therapeutic strategies.

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Late Sepsis Associated With Immunosuppression

Norra MacReady

December 21, 2011 — Patients who die in the late stages of sepsis show evidence of immunosuppression compared with patients who die of nonseptic causes, a [new study](#) shows.

These findings support the hypothesis that patients evolve from a hyperinflammatory state early in sepsis to a hypoinflammatory, [hypoimmune](#) state [later](#) on, lead author Jonathan S. Boomer, PhD, from the Washington University School of Medicine in St. Louis, Missouri, and colleagues write in the December 21 issue of *JAMA*.

Current [therapies](#) for sepsis focus on blocking immune activation and modulating the [early, hyperinflammatory "cytokine storm,"](#) the authors explain. This has allowed many patients to [survive](#) that [phase](#), only to [succumb](#) to [nosocomial](#) infections from organisms that are [not pathogenic](#) in healthier people, leading some observers to suspect that septic patients eventually [lose immunocompetence](#). The [new](#) findings "may allow for targeted therapeutic immune interventions to restore host immunity," they write.

Dr. Boomer and colleagues performed [rapid, postmortem analyses of lungs and spleens](#) harvested from [40](#) intensive care patients who died of sepsis-related causes and compared them with [spleens](#) from 29 patients who either underwent emergent splenectomy or [died of nonseptic](#) causes, and [lungs](#) from 20 patients who were transplant [donors](#) or underwent lung resection because of cancer. The mean ages of the septic and the control patients were 71.7 and 52.7 years, respectively. The septic patients had been in the intensive care unit for a median of 8 days (range, 1 - 195 days) compared with a median of 4 days (range, 1 - 40 days) for the control patients.

The organs of the septic patients showed evidence of [immunosuppression](#). For example, splenocytes from the septic patients showed significant [reductions](#) in the secretion of [cytokines](#), [tumor necrosis factor](#), [interferon gamma](#), and [interleukins 6 and 10](#) compared with in control patients ($P < .001$ for all comparisons). "[Cytokine](#) secretion in sepsis patients was generally [less than 10%](#) that in controls, [independent](#) of age, [duration](#) of sepsis, [corticosteroid](#) use, and [nutritional](#) status," the authors report.

This reduction in cytokine secretion suggests that the splenocytes from the septic patients had significant [functional impairments](#), they add.

In an [accompanying editorial](#), Peter A. Ward, MD, from the University of Michigan Medical School, Ann Arbor, writes that this study is "perhaps the most authoritative investigation exclusively involving humans and confirming the onset of immunosuppression developing during septic shock or severe sepsis." The observations "provide compelling evidence for development of immunosuppression in patients with sepsis, albeit with the important caveat that [all of the study patients died of sepsis](#) — those who survived may well have had a different immunologic course."

The authors note that the study had several important limitations, including the small sample size and the heterogeneous nature of both the study and the control populations. In addition, many of the [septic](#) patients were [hypoalbuminemic](#). Not only was [poor nutritional](#) status "[undoubtedly](#)" a contributing factor to their demise, but "malnutrition has numerous effects on host immunity that could be responsible for some of the observed immunologic findings," the authors write.

In addition, the study patients all died in intensive care, some after a lengthy bout with sepsis, and findings may differ for patients who recover from sepsis, who die earlier in the course of the illness, or who die in other surroundings.

Finally, the observed phenomena [may have been prodromal](#) events that do not reflect changes in all septic patients, but only those in patients who do [not respond](#) successfully to initial supportive measures.

Nevertheless, the authors conclude that these findings suggest that "immune-enhancing therapy may be a valid approach in selected patients with sepsis."

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Immunosuppression in Patients Who Die of Sepsis and Multiple Organ Failure

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SEPSIS IS RESPONSIBLE FOR MORE than 225 000 deaths annually in the United States.¹ Developing new therapies for sepsis has been particularly challenging, with more than 25 unsuccessful drug trials.²⁻⁶ Characterized by an initial intense inflammatory response or “cytokine storm,” patients with sepsis may present with fever, shock, altered mental status, and organ dysfunction.⁵⁻⁸ Numerous investigative agents have been directed at down-modulating this initial phase. Improved clinical management algorithms have led to survival of the majority of patients in this early period.⁹⁻¹¹ However, those who survive early sepsis often develop nosocomial infections with organisms not typically pathogenic in immunocompetent hosts and have reactivation of latent viruses.^{9,12,13} These observations have led to the controversial

See also pp 2614 and 2618.



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and questions on p 2625.

Context Severe sepsis is typically characterized by initial cytokine-mediated hyperinflammation. Whether this hyperinflammatory phase is followed by immunosuppression is controversial. Animal studies suggest that multiple immune defects occur in sepsis, but data from humans remain conflicting.

Objectives To determine the association of sepsis with changes in host innate and adaptive immunity and to examine potential mechanisms for putative immunosuppression.

Design, Setting, and Participants Rapid postmortem spleen and lung tissue harvest was performed at the bedside of 40 patients who died in intensive care units (ICUs) of academic medical centers with active severe sepsis to characterize their immune status at the time of death (2009-2011). Control spleens (n=29) were obtained from patients who were declared brain-dead or had emergent splenectomy due to trauma; control lungs (n=20) were obtained from transplant donors or from lung cancer resections.

Main Outcome Measures Cytokine secretion assays and immunophenotyping of cell surface receptor-ligand expression profiles were performed to identify potential mechanisms of immune dysfunction. Immunohistochemical staining was performed to evaluate the loss of immune effector cells.

Results The mean ages of patients with sepsis and controls were 71.7 (SD, 15.9) and 52.7 (SD, 15.0) years, respectively. The median number of ICU days for patients with sepsis was 8 (range, 1-195 days), while control patients were in ICUs for 4 or fewer days. The median duration of sepsis was 4 days (range, 1-40 days). Compared with controls, anti-CD3/anti-CD28-stimulated splenocytes from sepsis patients had significant reductions in cytokine secretion at 5 hours: tumor necrosis factor, 5361 (95% CI, 3327-7485) pg/mL vs 418 (95% CI, 98-738) pg/mL; interferon γ , 1374 (95% CI, 550-2197) pg/mL vs 37.5 (95% CI, -5 to 80) pg/mL; interleukin 6, 3691 (95% CI, 2313-5070) vs 365 (95% CI, 87-642) pg/mL; and interleukin 10, 633 (95% CI, -269 to 1534) vs 58 (95% CI, -39 to 156) pg/mL; ($P < .001$ for all). There were similar reductions in 5-hour lipopolysaccharide-stimulated cytokine secretion. Cytokine secretion in sepsis patients was generally less than 10% that in controls, independent of age, duration of sepsis, corticosteroid use, and nutritional status. Although differences existed between spleen and lung, flow cytometric analysis showed increased expression of selected inhibitory receptors and ligands and expansion of suppressor cell populations in both organs. Unique differences in cellular inhibitory molecule expression existed in immune cells isolated from lungs of sepsis patients vs cancer patients and vs transplant donors. Immunohistochemical staining showed extensive depletion of splenic CD4, CD8, and HLA-DR cells and expression of ligands for inhibitory receptors on lung epithelial cells.

Conclusions Patients who die in the ICU following sepsis compared with patients who die of nonsepsis etiologies have biochemical, flow cytometric, and immunohistochemical findings consistent with immunosuppression. Targeted immune-enhancing therapy may be a valid approach in selected patients with sepsis.

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hypothesis that the early hyperinflammatory state evolves to a subsequent hypoinflammatory state with significant immunosuppression.¹⁴⁻¹⁹ Although animal studies demonstrate progression to an immunosuppressive phase, epidemio-

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logic studies in clinical sepsis are lacking.¹⁴⁻¹⁹ The purpose of this investigation was to assess evidence of immunosuppression in sepsis and to determine mechanisms that might be responsible for the presumed impaired immunity. Cells from spleen and lung were studied to compare and contrast the functional status and phenotype of cells from a lymphoid organ and a peripheral organ that is a frequent site of nosocomial infection.

METHODS

An overview of study design including the purpose of the various immunologic tests is shown in TABLE 1. Methods are described in further detail in the eAppendix (available at <http://www.jama.com>).

Inclusion and Exclusion Criteria

Patients with sepsis who died while undergoing treatment in surgical or medical intensive care units (ICUs) were included. Sepsis was defined using a consensus panel definition: presence

of microbiologically proven, clinically proven, or suspected infection and presence of systemic inflammatory response syndrome (SIRS). The diagnosis of SIRS required at least 2 of the following: hypothermia ($\leq 36^{\circ}\text{C}$) or hyperthermia ($\geq 38^{\circ}\text{C}$); tachycardia ($\geq 90/\text{min}$); tachypnea (≥ 20 breaths/min) and/or arterial PCO_2 32 mm Hg or lower and/or mechanical ventilation; and leukocytosis ($\geq 12\,000/\mu\text{L}$) or leukopenia ($\leq 4000/\mu\text{L}$) and/or left-shifted white blood cell differential count of 10% or higher.²⁰

To try to limit potential confounding effects of other conditions affecting immunity, patients with cancer, chronic viral infections (human immunodeficiency virus, hepatitis B or C), or autoimmune diseases and patients taking high-dose corticosteroids (hydrocortisone, >200 mg/d) or immunosuppressive medications were excluded.

Control Population

Control spleens were obtained from critically ill patients with no sepsis including

those declared dead by neurological criteria and trauma patients who had emergent splenectomy because of splenic injury (see eAppendix for inclusion and exclusion criteria). Patients who met brain death criteria typically had been mechanically ventilated for 48 to 72 hours, required vasopressors to maintain adequate organ perfusion, and had associated organ injuries. Patients requiring mechanical ventilation for more than 4 days were excluded because of the high incidence of ventilator-associated pneumonia. Control lung tissue was obtained from the excess tissue of transplant donor lungs and from the non-tumor-involved tissue of lobectomies performed for cancer (eAppendix).

All studies were approved by the Washington University or St John's Hospital human research protection offices. For tissue obtained by rapid autopsy of patients who died in the ICU, the studies were determined by the Washington University and St John's Hospital human research protection of-

Table 1. Immune System Analysis

Immune Function	Analysis of Cell Populations	Assay Methods	Organ/Tissue	Results
Immune effector cells				
Innate	Dendritic cells, macrophages, natural killer cells, monocytes	Flow cytometry, immunohistochemistry	Spleen and splenocytes Lung and lung cells	Splenocytes: Figure 1, Figure 2, Figure 3 Lung cells: Figure 4 Spleen: Figure 5 Lung: Figure 6, Figure 7
Adaptive	T cells (CD4 and CD8)			
Immune suppressor cells	Regulatory T cells, myeloid-derived suppressor cells	Flow cytometry	Splenocytes: regulatory T cell Lung cells: regulatory T cells and myeloid-derived suppressor cells	Results section of text
Immune cell receptor expression				
Expression of molecules associated with antigen presentation	HLA-DR expression	Flow cytometry, immunohistochemistry	Splenocytes, spleen	Splenocytes: Figure 3 Spleen: Figure 5
Expression of molecules that enhance immune responses	Receptors: CD28, CD69, IL-2 α , IL-7R α Ligands: CD86	Flow cytometry, immunohistochemistry	Splenocytes Lung cells	Splenocytes: Figure 2, Figure 3, Figure 5 Lung cells: Figure 4
Expression of molecules that suppress immune responses	Receptors: BTLA, PD-1, CTLA-4 Ligands: PD-L1, PD-L2, HVEM	Flow cytometry, immunohistochemistry	Splenocytes or lung cells Spleen or lung	Splenocytes: Figure 2, Figure 3 Lung cells: Figure 4 Lung: Figure 6, Figure 7
Immune cell effector function				
Cytokine secretion				
Innate immune response: lipopolysaccharide	Natural killer cells, dendritic cells, macrophages, monocytes	Enzyme-linked immunosorbent assay	Splenocytes	Figure 1
Adaptive immune response: anti-CD3/anti-CD28	T cells			

Abbreviations: BTLA, B- and T-lymphocyte attenuator; CTLA, cytotoxic T-lymphocyte antigen; HVEM, herpes virus entry mediator; IL, interleukin; PD, programmed cell death.

fices not to constitute human subjects research because there was no interaction with the patient prior to death. After the patient had died, written permission to collect postmortem tissue for research purposes was obtained from the patient's next of kin. For lung resection specimens, all participants provided written informed consent prior to surgery. For lung tissue obtained from transplant donors, the tissue was collected after death and provided to the laboratory completely anonymized; therefore, it was also determined not to constitute human subjects research. However, the transplant donation consent signed by next of kin included a provision that tissue may be used for research purposes. Similarly, spleens were obtained from Mid-America Transplant, St Louis, Missouri, from brain-dead organ donors whose next of kin gave written consent for use of tissues for research purposes.

Splenic and Lung Harvesting and Cell Isolation

Spleen samples were obtained from 40 patients with sepsis and 29 patients without sepsis within 30 to 180 minutes of death. Splenocytes were dissociated and resuspended in sterile media.²¹ Splenocytes either were studied acutely or stored at 4°C for subsequent analysis within 12 to 72 hours. Cell counting and viability were determined as described previously.²¹

Lungs samples from patients with sepsis (n=34) or without sepsis (n=20) were immediately processed. Lungs were fixed overnight for immunohistochemistry or single-cell suspensions were prepared, counted, viability determined, and stained for flow cytometry.

Cytokine Production of Splenocytes

Mononuclear cells were prepared by density gradient centrifugation.²¹ Cell viability was greater than 85% to 90%. Cells were stimulated with lipopolysaccharide, anti-CD3/anti-CD28, or phorbol 12-myristate 13-acetate (PMA)/ionomycin. Supernatants were harvested at 5 and 22 hours and cytokines quantitated by enzyme-linked immunosorbent assay.

Flow Cytometric Analysis of Spleen and Lung Cells

Cell suspensions were incubated with isotype control or type-specific antibodies. To determine the percentage of cells positive for each marker, the percentage positive isotype control was subtracted from that within the positive gate of the type-specific marker. The mean fluorescence index (MFI) was determined by subtracting the geometric MFI of the isotype control from the geometric MFI of the type-specific marker.

Immunohistochemistry of Tissues

For spleen tissue, formalin-fixed sections underwent antigen retrieval followed by incubation with primary antibodies or isotype-matched controls. Slides were sequentially incubated with biotinylated antibody and peroxidase-labeled streptavidin and immunoreactive cells were visualized with diaminobenzidine-chromogen. Lung tissue was fixed in paraformaldehyde. Sections underwent endogenous peroxidase quenching and antigen retrieval followed by incubation with primary antibodies or isotype-matched controls.²² Slides were incubated in horseradish peroxidase (HRP)-conjugated antibodies followed by visualization with HRP-AB-C substrate.

Evaluation of Tissue Slides

Slides were evaluated in blinded fashion and scored as described previously.²³

Statistical Analysis

Differences in sepsis vs nonsepsis cytokine production and phenotypic expression were analyzed by 2-tailed nonparametric *t* test (Mann-Whitney *U* test) using the statistical program GraphPad Prism, version 5.0 (GraphPad Software). Statistical significance was set at $P \leq .05$.

RESULTS

Patient Characteristics

The most common etiologies of sepsis were ventilator-associated pneumonia and peritonitis (TABLE 2 and eTable 1). Other causes included necrotizing fasciitis, retroperitoneal abscess, in-

fecting intravascular catheters, urinary tract infections, intrapelvic abscess, and osteomyelitis. The nonsepsis patient population for control spleen tissues consisted of 20 patients who met brain death criteria and 9 patients who had splenectomies because of traumatic injury (Table 2 and eTable 2). Control populations for lung consisted of specimens from lung transplant donors (n=10) or lobectomy for lung cancer (n=10).

The mean ages of patients with sepsis and controls were 71.7 (SD, 15.9) and 52.7 (SD, 15.0) years, respectively. The median number of ICU days for sepsis patients was 8 (range, 1-195), while the median duration of sepsis was 4 days, with a range of 1 to more than 40 days (Table 2). Control patients were in ICUs for 4 or fewer days. Patients with sepsis had numerous comorbidities; in contrast, comorbidities were much less frequent in organ donor controls. The mean serum albumin level for sepsis patients was 2.4 (SD, 0.62) g/dL, with a range of 1.0 to 3.9 g/dL (normal hospital laboratory range for albumin is 3.6-5.0 g/dL) (eAppendix and eFigure 4).

Cytokine Production in Splenocytes

Compared with controls, splenocytes from sepsis patients produced significantly fewer cytokines (tumor necrosis factor [TNF], interferon [IFN- γ], and interleukins [IL] 6 and 10) at 5 and 22 hours (FIGURE 1, eFigure 1, eTable 3, and eTable 4). Decreased cytokine production was profound at 5 hours, when selected sepsis tissues produced minimal amounts of cytokines regardless of stimulus. For example, cytokine production stimulated with anti-CD3/anti-CD28 at 5 hours from controls vs sepsis patients, respectively, resulted in TNF, 5361 (95% CI, 3327-7485) pg/mL vs 418 (95% CI, 98-738) pg/mL; IFN- γ , 1374 (95% CI, 550-2197) pg/mL vs 37.5 (95% CI, -5 to 80) pg/mL; IL-6, 3691 (95% CI, 2313-5070) pg/mL vs 365 (95% CI, 87-642) pg/mL; and IL-10, 633 (95% CI, -269 to 1534) pg/mL vs 58 (95% CI, -39 to 156) pg/mL ($P < .001$ for all). There

were similar reductions in 5-hour lipopolysaccharide- and PMA/ionomycin-stimulated cytokine secretion (Figure 1, eFigure 1, eTable 3, and eTable 4).

By 22 hours, some splenocytes from sepsis patients exhibited partial recovery of cytokine production. Collectively, secretion of cytokines by stimulated sepsis splenocytes at 5 hours was less than 10% of that of controls. However, by 22 hours, secretion had increased to approximately one-third that of controls and, for selected sepsis patients, cytokine production was similar to that of controls (Figure 1). Cell viability at 5 and 22 hours was 93% and 86%, respectively, for controls vs 81% and 60%, respectively, for sepsis patients.

To determine if the age differential between controls and sepsis patients was associated with changes in stimulated cytokine production, comparison of data from sepsis patients aged 52 years or younger (n=5) vs older than 52 years (n=21) was performed and revealed no statistical differences between these 2 groups (eFigure 2). Furthermore, for virtually all cytokines, sepsis patients in both age groups were statistically different from controls. To determine if duration of sepsis affected cytokine secretion, data from patients who had sepsis for 4 or fewer days were compared with that of patients who had sepsis for more than 4 days. No significant difference was seen between these 2 groups (eFigure 3). Likewise, because of potential confounding effects of low-dose corticosteroids on cytokine production, comparisons were also made between sepsis patients receiving corticosteroids (n=9) and not receiving corticosteroids (n=15). Data from both of these groups were statistically different from that of controls but were not different from that of each other (eTable 4). To determine if the patient nutritional state (as reflected by serum albumin) had an effect on cytokine secretion in sepsis patients, TNF and IL-6 secretion were plotted against patient serum albumin (eFigure 4). No correlation was observed.

Analysis of Immune Cell Populations and Receptor Profiles

To assess possible etiologies for the markedly depressed cytokine secretion, we performed flow cytometric analysis and examined expression of cell surface receptors important in cellular activation (FIGURE 2, FIGURE 3, and eTable 5). The splenic T cells of sepsis patients had increased expression of activation marker CD69 on both CD4 and CD8 subsets and of IL-2R α (CD25) on CD4 T cells, when analyzed both as percentage of positive cells and as cellular expression (as determined by geometric fluorescence intensity [MFI]). Expression of the percentage positive cells and MFI of the potent positive costimulatory receptor CD28 was decreased on CD4 T cells in sepsis; MFI for CD28 was also decreased on CD8 T cells. Inhibitory members of the CD28 family programmed cell death 1 (PD-1) (CD279) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (CD152) were also examined. Sepsis patients had significantly increased percentages of CD4 T cells expressing PD-1 and increased CTLA-4 positive CD8 T cells. Both MFI and the percentage of cells expressing the IL-7 receptor α (IL-7R α , CD127), which is critical for cell survival and is decreased in cell exhaustion, were reduced on CD4 and CD8 T cells in sepsis spleen.

CD28 and PD-1 are engaged by their ligands (CD80/CD86 for CD28, PD-L1/PD-L2 for PD-1), which are expressed primarily on antigen-presenting cells (APCs), including dendritic cells and macrophages. Similar to findings on T cells, the costimulatory ligand CD86 was decreased and the inhibitory ligand, PD-L1, was increased on both macrophages and other APCs (Figure 2 and Figure 3). HLA-DR expression was highly significantly decreased on these APCs.

Cells from lung tissue were extensively characterized for activating and inhibitory receptors (eAppendix), and data are presented for markers in which we detected meaningful differences (FIGURE 4 and eTable 6). The majority of T cells from lungs of all groups expressed

Table 2. Clinical Characteristics

Characteristics	No. of Patients ^a
Patients with sepsis (n = 40)	
Age, mean (SD) [range], y	71.7 (15.9) [29-94]
Sex	
Male	20
Female	20
Site of infection	
Intrapelvic abscess	1
Intravascular catheters	2
Necrotizing fasciitis	1
Osteomyelitis	1
Pneumonia	22
Peritonitis	21
Retroperitoneal abscess	1
Urinary tract infection	3
Days in hospital, median (range)	11 (1-195)
Days in intensive care unit, median (range)	8 (1-195)
Days of sepsis, median (range) ^b	4 (1->40)
Comorbidities	
Diabetes	10
Heart disease	18
Morbid obesity	3
Neurologic	6
Renal disease	3
Respiratory	10
Liver	2
Organ failure	
Circulatory, vasopressors	36
Hepatic	12
Renal	28
Respiratory	35
Microbe	
Gram-positive	18
Gram-negative	25
Fungal	4
Control spleen donor patients (n = 29)	
Age, mean (SD) [range], y	52.7 (15.0) [24-84]
Sex	
Male	17
Female	12
Admission diagnosis	
Motor vehicle collision, head trauma	1
Motor vehicle collision, abdominal trauma	2
Gunshot wound, head	2
Gunshot wound, abdominal	2
Abdominal trauma ruptured spleen	5
Blunt head trauma	1
Anoxic brain injury	3
Cerebrovascular accident with internal hemorrhage	13

^aData are expressed as No. of patients unless otherwise indicated.

^bThe number of days that the patient was septic was frequently difficult to know with certainty; some patients may have been home with sepsis for several days prior to hospital admission.

PD-1. However, the MFI of PD-1 was higher on CD4 cells from sepsis patients compared with both transplant and lung cancer controls. Analysis of B- and T-lymphocyte attenuator (BTLA) (CD272), another T-cell inhibitory ligand, revealed that BTLA was also expressed on the majority of T cells but was increased primarily on cells from lung cancer patients. Interestingly, both PD-L1 and PD-L2 expression were markedly increased on plasmacytoid dendritic cells of sepsis patients, presenting opportunity for potent inhibitory receptor-ligand interactions. In

contrast to plasmacytoid dendritic cells, PD-L2 was not detected on myeloid dendritic cells, and PD-L1 was expressed at low levels that were not statistically different between sepsis patients and controls.

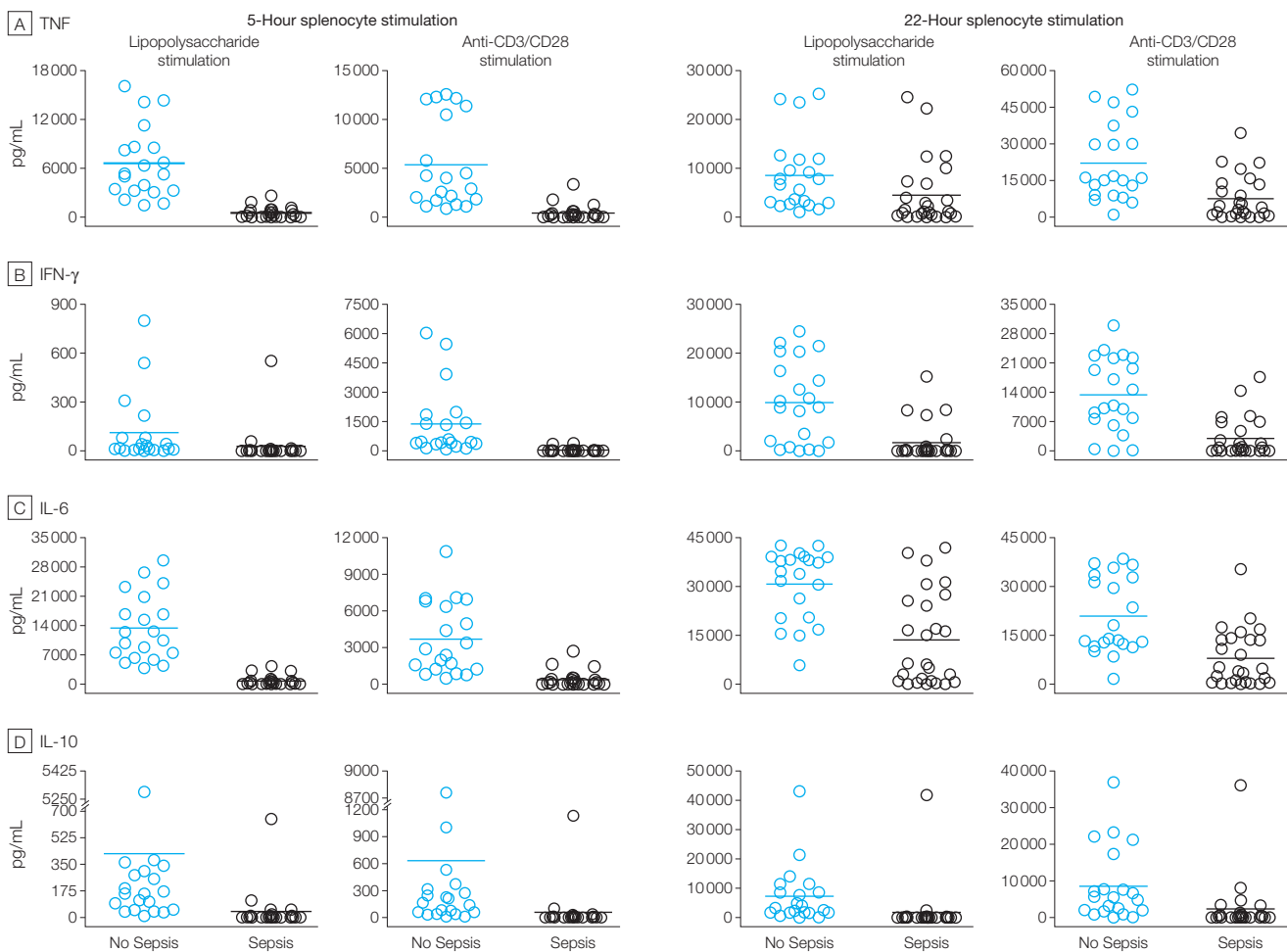
Expansion of suppressive cells, including regulatory T cells and myeloid-derived suppressor cells (MDSCs), has been reported in sepsis and provides another plausible mechanism for immunosuppression.¹⁰ In spleen, regulatory T cells were increased approximately 2-fold in sepsis vs control patients (mean, 8.5% [SD, 0.9%] vs 4.6% [SD,

0.6%], respectively; $P < .05$). In contrast, in lung, no increase in regulatory T cells was detected, but there were increased cells consistent with an MDSC phenotype, defined by Lin1-/HLA-DR low/CD33/CD11b high staining, isolated from sepsis vs control tissue (47.9% vs 15.7%; $P \leq .01$).

Spleen Immunohistochemistry

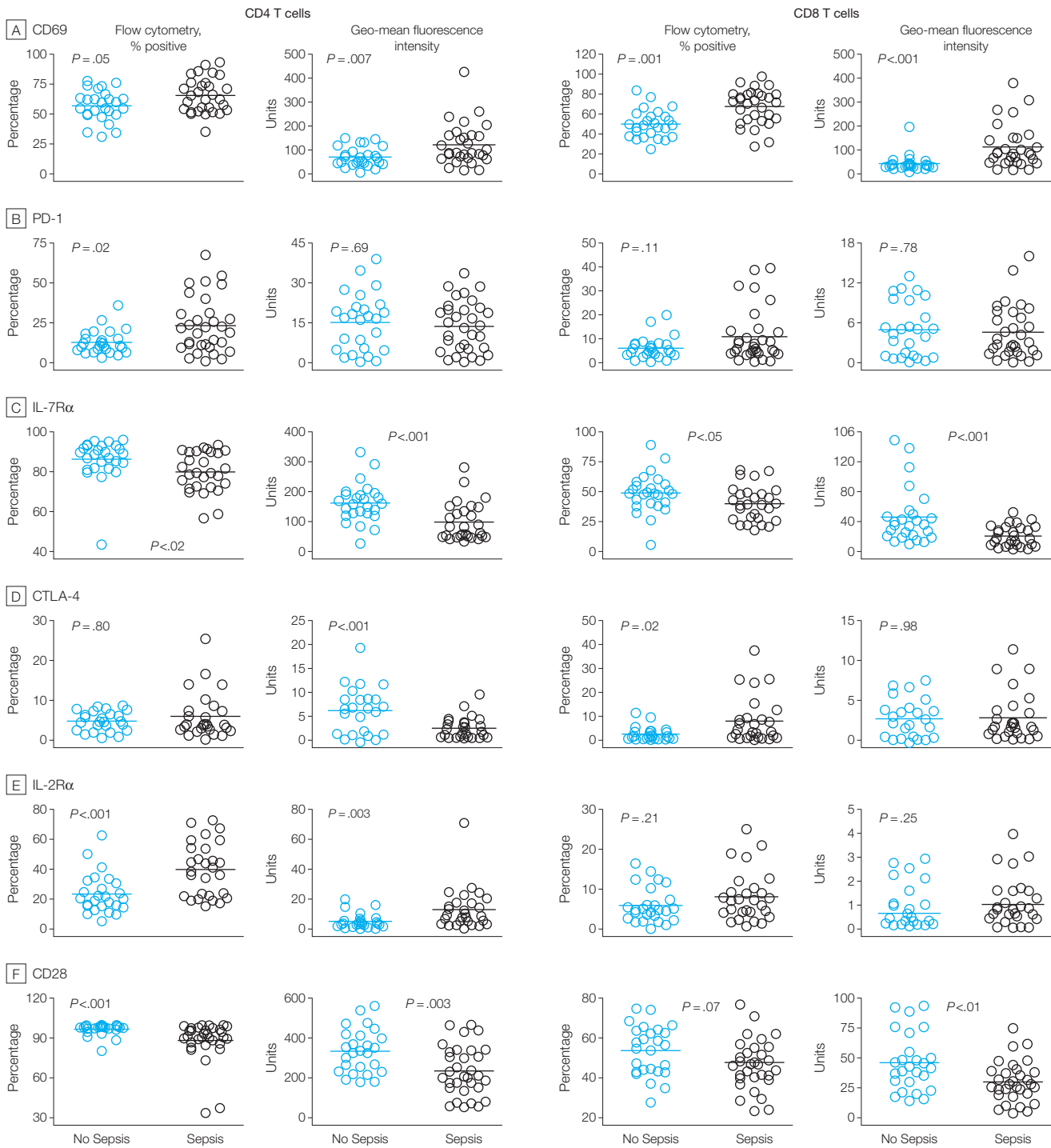
Evaluation of spleen tissue demonstrated cellular loss in periarteriolar lymphoid sheath (PALS) and diminished number and size of splenic follicles in sepsis patients, as previously reported.²⁴

Figure 1. Cytokine Secretion in Stimulated Splenocytes



Spleens were harvested from patients who died of sepsis (n=24-26) or nonsepsis etiologies (n=20-21). Cells were dissociated and washed and viability determined by trypan blue exclusion. Viable splenocytes (1×10^7) were stimulated with lipopolysaccharide or anti-CD3/anti-CD28 antibody. Supernatants were harvested at 5 and 22 hours and tumor necrosis factor (TNF), interferon γ (IFN- γ), and interleukins (IL) 6 and 10 were measured by enzyme-linked immunosorbent assay. There was a marked decrease in cytokine secretion in sepsis patients vs nonsepsis controls. Data were analyzed by 2-tailed nonparametric *t* test (Mann-Whitney *U* test). Each data marker represents an individual patient. Horizontal lines represent mean values. $P < .001$ for all plots, except $P < .01$ for TNF with lipopolysaccharide stimulation at 22 hours.

Figure 2. Expression of Cell Surface Receptors on Splenic CD4 and CD8 T Cells



See Figure 3 legend for explanation of geo-mean fluorescence intensity units, laboratory methods and statistical analysis. Compared with nonsepsis controls (n=24-26), sepsis patients (n=28-31) had activated T cells (increased CD69 in CD4 and CD8 T cells as well as increased CD25 [interleukin (IL) 2 receptor α] in CD4 T cells). Despite an activation phenotype, sepsis induced down-regulation of positive costimulatory receptors (CD28 in CD4 and CD8 T cells) as well as increased inhibitory receptors (programmed cell death 1 [PD-1] for CD4 T cells and cytotoxic T-lymphocyte antigen 4 [CTLA-4] for CD8 T cells). The IL-7 receptor α chain (CD127) was decreased in CD4 and CD8 T cells in sepsis.

Significantly reduced numbers of CD4, CD8, and HLA-DR cells (dendritic cells, macrophages, and B cells) typified sepsis splenic tissue (FIGURE 5), though a small subset of sepsis tissue retained a “normal” complement of these cell populations (eTable 7). Nineteen of 22 sepsis patients (but no controls [n=12]) had either decreased HLA-DR positivity, depletion of HLA-DR cells, or both. Conversely, in red pulp, increased expression of HLA-DR on sinusoidal endothelial lining cells was noted in 16 of 26 sepsis patients (Figure 5) but not in any controls. PD-1 was noted in follicular dendritic cells but generally not in T-cell zone dendritic elements. In contrast, PD-L1 was more typically seen in T-cell zone dendritic cells. In sepsis spleen tissue with severe cellular deple-

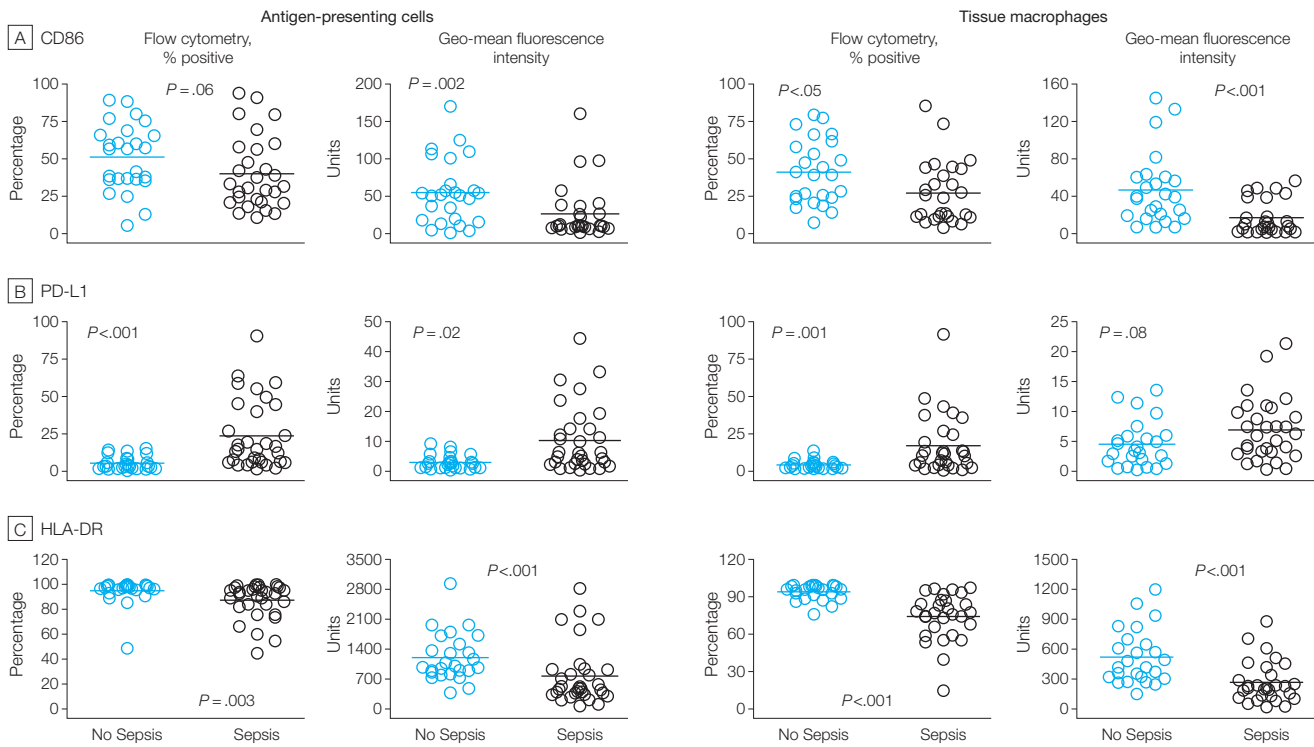
tion, the dominant PD-L1–positive PALS population was capillary endothelium, and this was increased relative to controls (eFigure 5). Macrophages were consistently PD-L1 positive in all cell zones and were increased in PALS and red pulp. PD-L1 was uniformly present in arteriolar and sinusoidal endothelium in both sepsis and control spleen. PD-1 reactivity was unevenly expressed in arteriolar endothelium and absent on sinusoidal linings. Both B and T lymphocytes were variably positive for PD-1 and PD-L1 in both sepsis and control patients.

Ligands for T-Cell Inhibitory Receptors in Lung Parenchymal Cells

Signaling through inhibitory receptors on T cells requires engagement by their cog-

nate ligand. While typically expressed by APCs, these may be induced on nonimmune cells by inflammatory cytokines, perhaps serving to locally inhibit T cells and dampen inflammation. Immunohistochemistry showed intense airway epithelial staining for herpes virus entry mediator (HVEM), the receptor for BTLA, on lung isolated from the majority of sepsis patients but not controls (FIGURE 6 and eTable 8). This was true for patients both with and without pneumonia. Herpes virus entry mediator was expressed on macrophages from both groups. We also stained for PD-L1 and PD-L2, the ligands for PD-1 (FIGURE 7). PD-L1 and PD-L2 appeared to be expressed in sepsis lung and lung resections to a greater degree compared with transplant donor lung.

Figure 3. Expression of Cell Surface Receptors on Splenic Antigen-Presenting Cells and Tissue Macrophages



Splenocytes (2×10^6) were stained with fluorescently conjugated antibodies or isotype-matched control antibodies and analyzed by flow cytometry (eAppendix). A positive gate was established based on isotype control staining. The percentage positive for each marker was defined by subtracting the percentage within the positive gate in the isotype control from the percentage within the positive gate in the specific stain. The geo-mean fluorescence intensity was determined by subtraction of the nonspecific fluorescence of the isotype control antibody from the fluorescence of the specific conjugated antibody and is expressed in units, which are an average of fluorescence intensity of the data collected within the selected gate after subtracting fluorescence intensity of isotope control. Antigen-presenting cells, ie, dendritic cells and macrophages/monocytes, as well as tissue-specific macrophages, showed an immunosuppressive phenotype in sepsis as evidenced by decreased expression of CD86 and HLA-DR. In addition, antigen-presenting cells from sepsis patients had increased expression of PD-L1, the ligand for the inhibitory receptor programmed cell death 1 PD-1 on T cells. Each data marker represents an individual patient. Statistical analysis was performed by 2-tailed nonparametric *t* test (Mann-Whitney *U* test). Horizontal lines represent mean values.

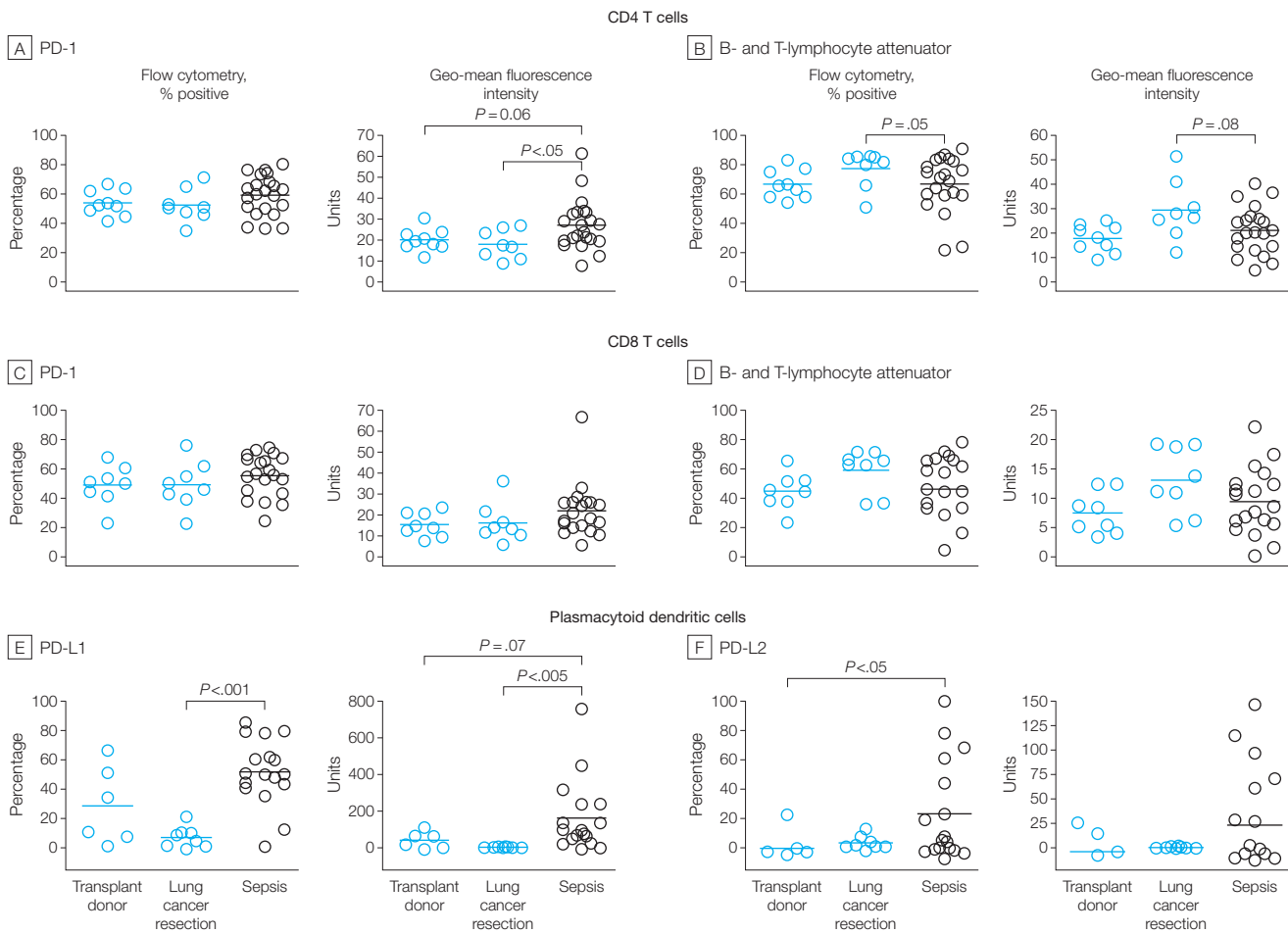
COMMENT

Early sepsis is characterized by excessive inflammation in what is often termed a “cytokine storm.”²⁻⁸ As sepsis persists, patients often have reactivation of endogenous viruses and develop nosocomial infections with opportunistic pathogens.^{9,12,13} Investigators have argued that these findings suggest that patients with sepsis enter an immunosuppressive state, but this is highly controversial and most potential therapy for sepsis remains fo-

cused on blocking immune activation.^{14,25-28} While several potential abnormalities have been identified, a comprehensive analysis of the immune status of patients who die of sepsis has not been conducted and mechanistic explanations remain speculative.²⁵⁻²⁹ The present study shows that splenocytes from sepsis patients had highly significant functional impairments as evidenced by major reductions in cytokine secretion. Multiple inhibitory

mechanisms were identified, including dominance of inhibitory over activating receptors, expansion of suppressive cell types, and induction of inhibitory ligands on both APCs and tissue parenchymal cells. These findings are present in the setting of apoptosis-induced depletion of immune cells (Figure 5).^{23,24} Both proinflammatory and anti-inflammatory cytokines were impaired. These spleen data are consistent with multiple previous studies examining

Figure 4. Expression of Cell Surface Receptors on Cells Isolated from Lung Tissue



Lung tissue was digested via collagenase followed by isolation of single cells. Lung cells (3×10^6) were stained with fluorescently conjugated antibodies or isotype-matched control antibodies and analyzed via flow cytometry (eAppendix). Lung CD4 and CD8 T cells from sepsis patients had increased programmed cell death 1 (PD-1), as determined by geo-mean fluorescence intensity (MFI), compared with nonsepsis controls. Data for MFI are expressed in units, which are an average of fluorescence intensity of the data collected within the selected gate after subtracting fluorescence intensity of isotope control. The majority of T cells from both sepsis and nonsepsis lung also expressed PD-1 (>50%) and B- and T-lymphocyte attenuator (BTLA) (>60%). Lung CD4 T cells from resection controls had elevated BTLA expression (in percentage and MFI) compared with sepsis patients. Plasmacytoid dendritic cells, a subset of dendritic cells, isolated from sepsis lungs had increased PD-L1 (in percentage and MFI) and PD-L2 (in percentage), ligands for PD-1 expressed on lung T cells, compared with both nonsepsis control groups. For PD-1 analysis, n=22 (CD4) and n=20 (CD8) for sepsis patients, n=9 (CD4) and n=8 (CD8) for transplant donor controls, and n=8 (both CD4 and CD8) for lung resection controls. For BTLA analysis, n=21 (CD4) and n=19 (CD8) for sepsis patients, n=9 (CD4) and n=8 (CD8) for transplant donor controls, and n=8 (both CD4 and CD8) for lung resection controls. For plasmacytoid dendritic cell analysis, n=17 for sepsis patients, n=6 for transplant donor controls, and n=8 for lung resection controls. Statistical analysis was performed using a non-parametric 2-tailed Mann-Whitney U test. Each data marker represents an individual patient. Horizontal lines represent mean values.

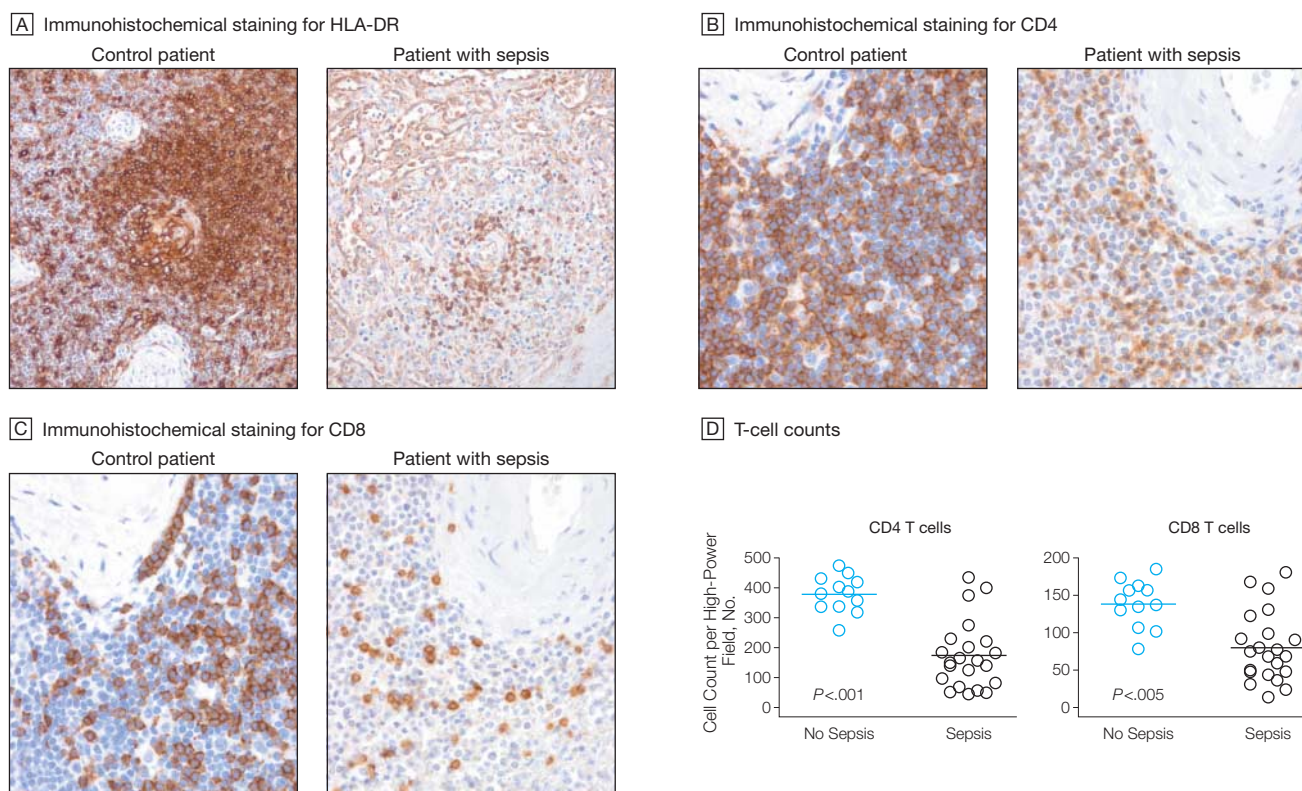
blood that showed similar degrees of cytokine suppression in sepsis.^{25,30-32} Thus, the severely reduced cytokine production observed in spleen is consistent with a systemic abnormality. In a subset of sepsis patients, cytokine production at 22 hours was comparable with controls without sepsis, suggesting that in some patients, defective cytokine secretion may be reversible if cells are removed from the sepsis milieu.

Of particular interest is the expression of ligands for T-cell inhibitory receptors on tissue parenchymal cells. Compared with controls, splenic capillary endothelial cells of sepsis patients had increased expression of the inhibitory ligand PD-L1 (eFigure 5). Similarly,

lung cells from sepsis patients, both with and without pneumonia, demonstrated intense airway epithelial staining for HVEM, a key regulator of host inflammatory response in autoimmunity and infection (Figure 6).³³ Significantly, the majority of lung T cells expressed BTLA, one of several ligands for HVEM and another regulator of host inflammation.³³ Thus, the required elements for activation of this immunosuppressive pathway are present in sepsis. Collectively, these data demonstrate that parenchymal cells of spleen and lung express important immunoregulatory proteins and that HVEM appears to be specifically induced in lungs of sepsis patients.

The PD-1 pathway has emerged as an important mechanism that inhibits T-cell function. Expression of PD-L1 and PD-L2 by dendritic cells promotes a tolerogenic phenotype, resulting in T-cell suppression.³⁴⁻³⁶ In lung from sepsis patients, both PD-L1 and PD-L2 were detected on resident dendritic cells and airway epithelial cells. Thus, these pathways might be engaged on T cells trafficking through lung, resulting in localized inhibition of T cells and thereby predisposing to infection at this site. In spleen, PD-1 expression was detected on CD4 and CD8 T cells, and both PD-L1 and PD-L2 are known to be expressed on endothelial cells (eFigure 5) and impair CD8 T-cell cytolytic properties.³⁷

Figure 5. Immune Effector Cells in Spleen Tissue



Spleen from sepsis patients (n=22) or nonsepsis controls (n=12) was stained for HLA-DR, CD4, or CD8 and examined by an investigator (P.E.S.) blinded to sample identity (eAppendix). 3,3'-diaminobenzidine 4-HCl was used as a chromogen to stain the cells of interest (brown), and a hematoxylin counterstain (blue) was used for background staining. A (HLA-DR immunostain; 200×). In a representative control sample, HLA-DR immunoreactivity is robust in all periarteriolar T- and B-cell zones, consistent with major histocompatibility complex (MHC) II pattern 2A (see reference 42 and eTable 7). There is marked loss of HLA-DR reactivity in B and T lymphocytes typical of nearly subtotal depletion of HLA-DR-reactive elements in sepsis (MHC II pattern 4). Note that sinusoidal endothelium staining is pronounced, a change seen only in sepsis. B (CD4 immunostain; 400×), Periarteriolar CD4 cells are quantitatively decreased in sepsis (right panel) relative to control (left panel). C (CD8 immunostain; 400×), Periarteriolar CD8 cells are also quantitatively decreased in sepsis (right panel) relative to controls (left panel). D, The dot plots are cell counts for CD4 and CD8 T cells, obtained by counting number of cells per field in periarteriolar lymphoid sheaths. Two fields were counted per slide and averaged. Statistical analysis was performed using a non-parametric 2-tailed Mann-Whitney U test. Each data marker represents an individual patient. Horizontal bars represent mean values.

A long-standing question in sepsis is whether immunologic changes are organ specific.³⁸ Although differences existed, both spleen and lung shared common immunosuppressive mechanisms. CD86 and HLA-DR expression were decreased in cells isolated from both spleen and lung (Figure 1). In contrast, regulatory T cells were increased in spleen but not in lung. T cells isolated from both organs of sepsis patients had increased expression of the major inhibitory receptor PD-1. Relative to spleen, there were twice as many PD-1-expressing cells in lung, suggesting the possibility of selective recruitment of PD-1-expressing cells to lung. In both organs, expression of ligands for major inhibitory receptors was detected on APCs and parenchymal cells.

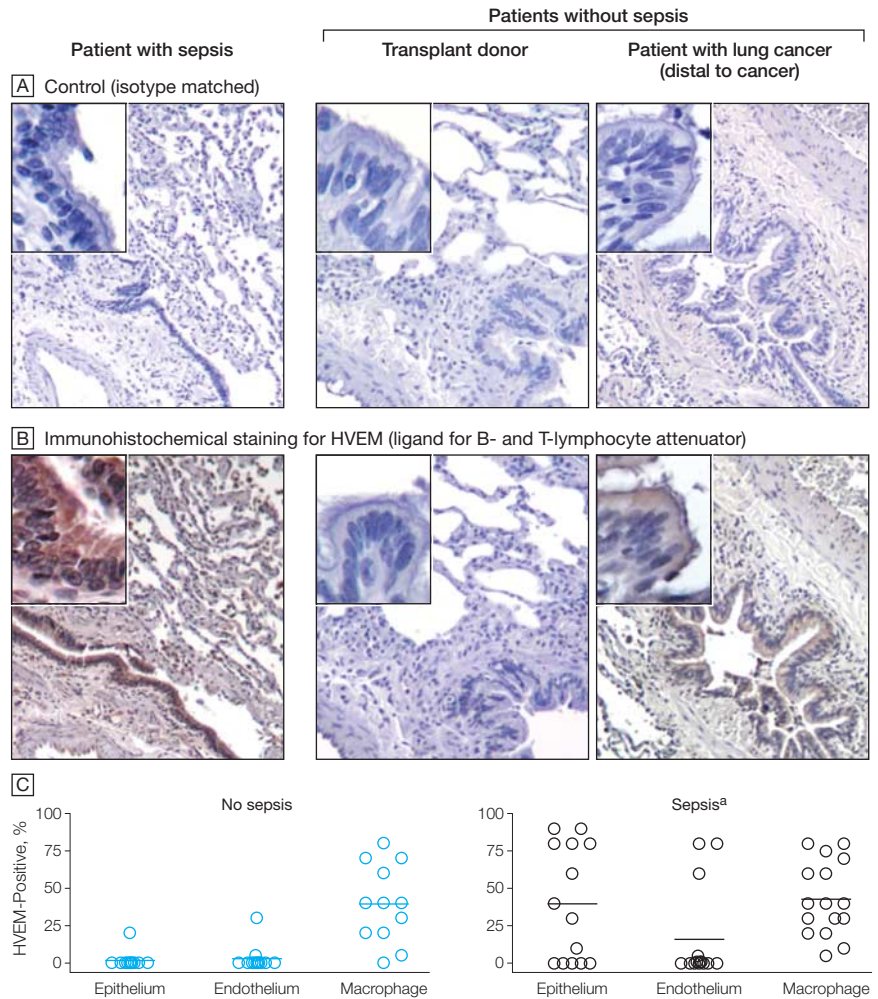
Functional unresponsiveness of T cells during chronic viral infections has long been recognized and recently mechanistically described as a state of cell “exhaustion.”³⁹⁻⁴¹ Driven by persistent antigen exposure, exhaustion is typified by progressive loss of cell function.³⁹⁻⁴¹ Phenotypically, exhaustion is characterized by persistent expression of multiple inhibitory receptors, including PD-1, TIM-3, and LAG-3, along with decreased expression of CD127 (IL-7R α) and CD62L. The functional and phenotypic characteristics described for cell exhaustion are similar to the present findings in sepsis, a state that is highly likely to result in protracted antigen exposure and cell stimulation.³⁹ As noted in the present study, splenic T cells demonstrated profound reductions in cytokine production as well as increased expression of PD-1 and CD69 and decreased CD127. Collectively, these findings suggest that T-cell exhaustion may be an important immunosuppressive mechanism in sepsis.

The present study has a number of important therapeutic implications. Most investigative agents in sepsis have been directed at blocking inflammation and immune activation. Although such therapies may be successful if applied early, they may be harmful if applied later in the immunosuppressive phase. As supportive therapies of

sepsis have improved, early deaths have decreased and most patients enter a more protracted phase, with evidence of impaired immunity made manifest by infections with relatively avirulent organisms.^{9,14,15} An important part of implementing more targeted therapies will be to accurately determine the immune status of individual patients

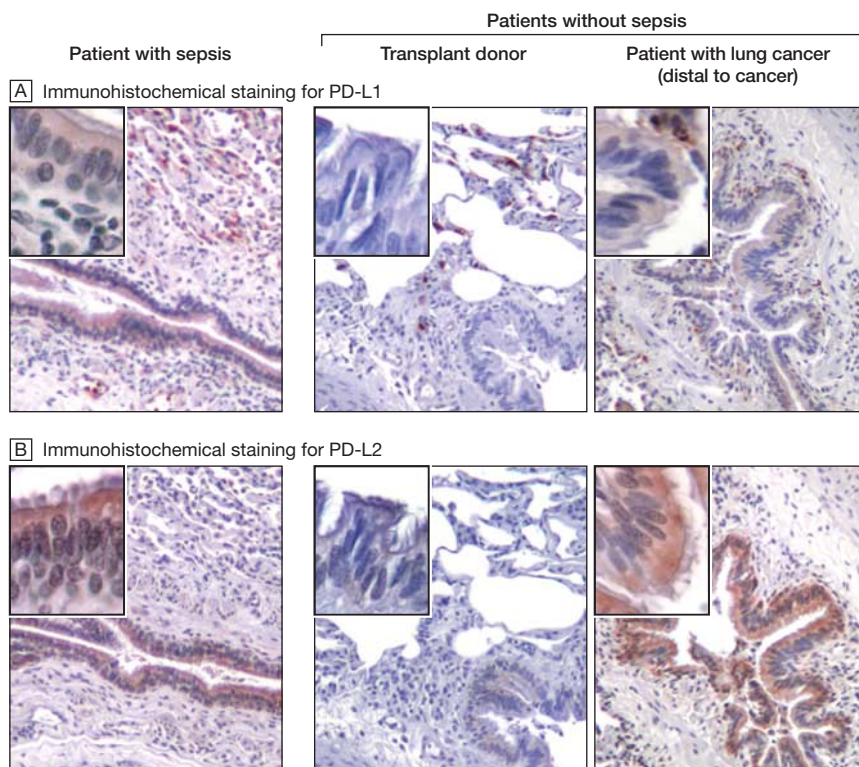
during their disease. By using a combination of functional assays and flow cytometry to characterize immune cell phenotypic changes, it may be possible to identify patients with more severe immune compromise, for which targeted immune-enhancing therapies may be beneficial. A recent study that treated sepsis patients with granu-

Figure 6. Expression of HVEM in Lung Tissue



Sepsis or nonsepsis lung tissue was fixed in 4% paraformaldehyde and paraffin-embedded sections prepared (eAppendix). Lung sections were incubated with isotype-matched controls (A) or primary antibodies to anti-herpes virus entry mediator (HVEM) (B) followed by visualization of brown staining. Sepsis lung tissue stained positive for HVEM, the ligand for B- and T-lymphocyte attenuator. Lung resections, in particular of the same airway, were photographed using the 200 \times and 400 \times (inset) objectives for each antibody stain. C, Slides presented in (B) were evaluated in a blinded fashion and scored (in percentage) based on their positive staining for HVEM. Data were graphed as percentage positive HVEM staining in lung tissue (epithelium, endothelium, and macrophages) of sepsis patients and nonsepsis controls. Data presented in (C) are as follows: n=16 sepsis patients, n=7 transplant donor controls, and n=5 lung resection controls. Each data marker represents an individual patient. Horizontal bars represent mean values. 3,3'-diaminobenzidine 4-HCl was used as a chromogen to stain the cells of interest (brown), and a hematoxylin counterstain (blue) was used for background staining.

^a Comparison of HVEM-positive epithelium greater in sepsis patients vs nonsepsis controls: $P = .01$.

Figure 7. Expression of PD-L1 and PD-L2 in Lung Tissue

Sepsis or nonsepsis lung tissue was fixed in 4% paraformaldehyde and paraffin-embedded sections prepared (eAppendix). Lung sections were incubated with isotype-matched controls (see Figure 6) or primary antibodies to anti-programmed cell death ligand 1 (PD-L1) (A), or anti-PD-L2 (B), followed by visualization of brown staining. Lung resections, in particular of the same airway, were photographed using the 200 \times and 400 \times (inset) objectives for each antibody stain. 3,3'-diaminobenzidine 4-HCl was used as a chromogen to stain the cells of interest (brown), and a hematoxylin counterstain (blue) was used for background staining. Sepsis lung tissue stained positive for PD-L1 and PD-L2, the ligands for PD-1, in lung epithelium compared with transplant donor lung tissue. Lung resection (normal-appearing lung tissue distal to cancerous tissue) stained positive in airway epithelium for PD-L2, like sepsis lung tissue, compared with transplant donor lung tissue.

locyte-macrophage colony-stimulating factor based on monocyte HLA-DR expression suggests that such therapies might be successful.⁴² Our findings suggest additional targets. Interleukin 7 has an excellent clinical safety record and has been used to enhance immunity in patients with cancer, human immunodeficiency virus type 1, and hepatitis C. Interleukin 7 restored immunity in patients with persistent viral infections and has improved viral clearance and survival in animal models of chronic viral disease and sepsis.^{21,43-45} The decreased IL-7R α expression documented in the present study could be causative in T-cell loss in sepsis, and IL-7 administration might ameliorate this problem. Another potential

therapeutic approach is to interfere with inhibitory receptor signaling on T cells. We have demonstrated up-regulation of multiple inhibitory receptors and their ligands, including PD-1 and BTLA. Blockade of PD-1/PD-L1 interactions in chronic viral infection has been shown to reverse the functional unresponsiveness of T cells and to enhance viral clearance.⁴⁰ Blockade of PD-1 improves survival in animal models of fungal infection and sepsis.⁴⁶⁻⁴⁸

This study has several important limitations of the ability to generalize the findings to sepsis at large. These limitations include the relatively small sample size and the heterogeneous nature of both sepsis and control patients. In this regard, the control groups

were markedly different from the sepsis patients in many aspects; for example, nutritional status, degree of comorbidities, and length of illness. Although the hypoalbuminemia noted in the sepsis patients could be due to a number of causes such as increased vascular permeability, poor nutritional status was undoubtedly a major contributing factor, and malnutrition has numerous effects on host immunity that could be responsible for some of the observed immunologic findings.⁴⁹ Also, the study was confined to sepsis patients who died in the ICU, some after a considerable duration of sepsis. Thus, the process may be different for sepsis patients who die sooner of sepsis, who do not die in the ICU, or who make a good recovery, as well as for a broader case mix in general. Finally, it is possible that findings in sepsis patients were prodromal events and not reflective of patients with sepsis in general but of patients with sepsis who had unsuccessful responses to supportive measures. It should be emphasized that deaths within the first 72 hours of sepsis in previously healthy patients with infections of highly virulent organisms are associated with extremely elevated proinflammatory cytokines, and these deaths are likely secondary to an overexuberant immunoinflammatory response.^{7,8,14} Thus, the present study serves as a bridge between preclinical and early clinical findings and must be viewed cautiously.

In conclusion, these data provide a unique insight into the status of the immune system during sepsis, not only in a lymphoid organ but in peripheral tissue. Identification of potential receptor-ligand interactions and signaling pathways leading to immunosuppression may allow for targeted therapeutic interventions to restore host immunity.

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Author Contributions: Dr Hotchkiss had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Boomer, Chang, Walton, Swanson, Green, Hotchkiss.

Drafting of the manuscript: Jarman, Green, Hotchkiss. **Critical revision of the manuscript for important intellectual content:** Boomer, To, Chang, Takasu, Osborne, Walton, Bricker, Kreisel, Krupnick, Srivastava, Swanson, Green, Hotchkiss.

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