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Sustained and Incomplete Recovery of Naive CD8⁺ T Cell Precursors after Sepsis Contributes to Impaired CD8⁺ T Cell Responses to Infection

Stephanie A. Condotta,* Deepa Rai,* Britnie R. James,† Thomas S. Griffith,†‡§,* and Vladimir P. Badovinac*†§

Patients who survive severe sepsis often display compromised immune function with impairment in innate and adaptive immune responses. These septic patients are highly susceptible to “secondary” infections with intracellular pathogens that are usually controlled by CD8⁺ T cells. It is not known when and if this observed immunoparalysis of CD8⁺ T cell immunity recovers, and the long-term consequences of sepsis on the ability of naive CD8⁺ T cells to respond to subsequent infections are poorly understood. In this study, using the cecal-ligation and puncture mouse model of sepsis, we show that sepsis induces a rapid loss of naive CD8⁺ T cells. However, IL-15–dependent numerical recovery is observed a month after initial septic insult. Numerical recovery is accompanied by IL-15–dependent phenotypic changes where a substantial proportion of naive (Ag-inexperienced) CD8⁺ T cells display a “memory-like” phenotype (CD44 hi/CD11a hi). Importantly, the impairment of naive CD8⁺ T cells to respond to viral and bacterial infection was sustained for month(s) after sepsis induction. Incomplete recovery of naive CD8⁺ T cell precursors was observed in septic mice, suggesting that the availability of naive precursors contributes to the sustained impairment in primary CD8⁺ T cell responses. Thus, sepsis can result in substantial and long-lasting changes in the available CD8⁺ T cell repertoire affecting the capacity of the host to respond to new infections.

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Abbreviations used in this article: Arm, Armstrong strain; CLP, cecal-ligation and puncture; GrzB, granzyme B; iLN, inguinal lymph node; LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; VacV, Vaccinia virus; WT, wild-type.

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CD8⁺ T cells play a critical role in the control and eradication of intracellular pathogens (16). Because of the need to ensure the capacity to respond to the enormous diversity in the microbial universe, naive CD8⁺ T cells that can recognize particular pathogen-derived epitopes (Ags) are infrequent in the total CD8⁺ T cell population (ranging from 10 to 1000 cells in an inbred laboratory mouse) (17–22). Upon recognition of cognate Ag, naive Ag-specific CD8⁺ T cells undergo massive proliferative expansion and differentiate into effector cells able to defend against the invading pathogen. Expansion is followed by a contraction phase whereby the numbers of effector Ag-specific CD8⁺ T cell decrease by ∼95%. The cells that survive the contraction phase initiate the memory Ag-specific CD8⁺ T cell pool (23–26). Importantly, the magnitude of the primary CD8⁺ T cell response generally correlates with the size of the naive CD8⁺ T cell precursor pool specific for a particular Ag (21, 27). Thus, alterations in naive Ag-specific CD8⁺ T cell precursor frequencies may seriously compromise the capacity of the host to mount an effective immune response.

Sepsis induces apoptosis of immune cells leading to depletion of critical components of the immune system (5). This results in a significant loss of myeloid cells and lymphocytes (including CD4⁺ and CD8⁺ T cells), creating a lymphopenic environment (5). Lymphocyte homeostasis is dependent on γ-chain cytokines such as IL-2, IL-7, and IL-15 (28, 29). IL-2 and IL-7 are important for T cell growth and survival, respectively (28, 30, 31), and gene expression of both of these cytokines has been shown to be deficient in human sepsis (29). Therapeutic IL-15 administration has been shown to prevent sepsis-induced apoptosis and immunosuppression, thus improving survival in sepsis (32). In addition, IL-15 has been shown to play an important role in the basal proliferation of memory CD8⁺ T cells, as well as the sustained proliferation and accumulation of naive CD8⁺ T cells within a lymphopenic environment (33, 34).

The majority of research in sepsis focuses on understanding the factors that control early events after sepsis induction. However,
survivors of sepsis have an increased risk for death for nonseptic causes years after the initial septic episode (35–37). Little is known about the long-term immune consequences for an individual who has survived sepsis. In particular, the long-term effect(s) of sepsis on the ability of the host to mount primary CD8+ T cell responses to infections is poorly understood. In this study, we used the cecal-ligation and puncture (CLP) mouse model to address both short- and long-term effects of sepsis on the CD8+ T cell response to viral and bacterial infections.

Materials and Methods

Mice

C57BL/6 mice (wild-type [WT], Thy1.2/1.2) were purchased from the National Cancer Institute and used at 6–10 wk of age. Thy1.1/1.1 P14 TCR-transgenic [specific for lymphoproliferative choriomeningitis virus (LCMV)]–derived GP33 epitope mice were provided by Dr. John T. Harty (Department of Microbiology, University of Iowa) and described previously (27, 38–41). IL-15−/− mice were purchased from Taconic (42).

Viruses, bacteria, and infections

Armstrong strain (Arm) of LCMV (LCMV-Arm; 2 × 10^6 PFU/mouse i.p.) and Western Reserve strain of Vaccinia virus (VacV; 1 × 10^6 PFU/mouse i.p.) were previously described (43). Attenuated Listeria monocytogenes–expressing OVA (1 × 10^6 CFU/mouse i.v.) was used as described previously (44, 45). Infected mice were housed at the University of Iowa under the appropriate biosafety level.

Adoptive transfer

Thy1.1/1.1 P14 CD8+ T cells were obtained from spleens or peripheral blood of young naive P14 mice and injected i.v. at indicated numbers into naive WT C57BL/6 (Thy1.2/1.2) recipients.

Cecal-ligation and puncture

Septic insult was induced by CLP (14, 46). In brief, mice were anesthetized and the abdomen was shaved and disinfected. A midline abdominal incision was made, the cecum was identified, and the distal one-third was ligated with 4–0 silk sutures. The ligated portion was punctured once using a 25-gauge needle and a small amount of cecal contents was extruded through the puncture. The cecum was returned into the abdomen and the peritoneum was closed with continuous suture. The skin was glued together with Vetbond tissue adhesive (St. Paul, MN), and 1 ml saline was injected for resuscitation. This level of injury was used to create a chronic septic state characterized by the loss of appetite and body weight, ruffled hair, shivering, diarrhea, and/or periportal exudates, and with 5–10% mortality rate. Sham-treated mice underwent the same procedure excluding cecum ligation and puncture. Bupivicaine was administered at the incision site, and flunixin meglumine was administered twice for postoperative analgesia to all sham and CLP-treated mice.

Peptides

All peptides were synthesized by Bio-Synthesis (Louisville, TX). LCMV-specific peptides were NP396–404 (FPQPONGQFI), GP33–41 (KAVYNFATC) and NP396–404 (FPQPONGQFI) containing D1b MHC class I tetramers. In brief, spleens were harvested for each mouse analyzed, a single-cell suspension was prepared, and allophycocyanin-conjugated GP33 and PE-conjugated NP396 tetramers were added at 1:1000 dilution in Fc block solution (FACS buffer; PBS supplemented with 2% FCS and 0.02% NaN3, 1:50 normal mouse serum and 1:100 anti-CD16/32 mAb). Cells were incubated in the dark at room temperature for 1 h, followed by a 10-min cold FACS buffer wash. The tetramer-stained cells were then resuspended in 200 μl FACS buffer, mixed with 50 μl of both anti-allophycocyanin and anti-PE mAb-conjugated magnetic microbeads (Miltenyi Biotech), and incubated in the dark on ice for 30 min. The cells were washed and resuspended in 3 ml cold FACS buffer and passed over a MACS separation column (Miltenyi Biotech) to enrich for the tetramer-specific cells. Columns were washed three times with 3 ml cold FACS buffer before eluting the bound fraction with 5 ml cold FACS buffer. The resulting enriched fractions were then stained with a mAb mixture: Pacific Blue–conjugated anti-B220, -CD11b, and -CD11c, FITC-conjugated anti-CD3, and PE–conjugated anti-CD44 (BioLegend), and Orange-conjugated anti-CD8 (Invitrogen). Cell numbers for each sample were determined using AccuCheck Counting Beads (Invitrogen). Samples were then analyzed using an LSR II flow cytometer (Becton-Dickinson Biosciences) and FlowJo software (Tree Star, Ashland, OR). The percentage of tetramer+ events was multiplied by the total number of cells in the enriched fraction to calculate the total number of GP33- and NP396-specific CD8+ T cells in the spleen.

Statistical analyses

Data were analyzed with Prism4 GraphPad software and specific tests to determine statistical significance are indicated in figure legends (**p < 0.0001, *p < 0.001, *p < 0.01, *p < 0.05). Data generated as scatter dot plots are presented as mean, and data generated as bar graphs are presented as mean ± SE.

Study approval

All animal studies were approved by the University of Iowa Institutional Animal Care and Use Committee and meet stipulations of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Results

Primary Ag-specific CD8+ T cell responses to viral infections are reduced early after sepsis induction

To induce septic injury, we performed CLP on C57BL/6 mice (14, 46). CLP is considered the gold standard in sepsis research because it mimics human polymicrobial sepsis and provides a better representation of the complexity of human sepsis compared with other animal models (2, 53, 54). Using the CLP mouse model, we recently demonstrated increased susceptibility and reduced primary pathogen-specific CD8+ T cell responses after bacterial infection with L. monocytogenes (14). These data raised the question whether impairment in primary CD8+ T cell responses early after sepsis induction (day 2) was specific for L. monocytogenes or can be generalized to other infections such as LCMV-Arm and the Western Reserve strain of VacV. These viruses have been used routinely to study adaptive immune responses to viral infection and multiple H-2b–restricted CD8+ T cell epitopes have been identified in C57BL/6 mice (47, 48). Two days after CLP or sham surgery, mice were infected with LCMV-Arm, and Ag-specific CD8+ T cell responses were examined at the peak of the primary expansion (day 8 postinfection [p.i.]) using peptide-stimulated intracellular IFN-γ staining (50) (Fig. 1A). A significant reduction in...
the percentage and number of IFN-γ Ag-specific CD8+ T cells was observed in the spleen of CLP mice compared with sham-surgery mice to four immunodominant LCMV epitopes (NP396, GP33, GP276, and NP205) (47) (Fig. 1B–D). This resulted in a 9-fold decrease in the total number of LCMV-specific effector CD8+ T cells (based on the sum of all IFN-γ CD8+ T cell responses for the peptides used for stimulation) in the spleens of CLP mice (Fig. 1E). In addition, we observed a similar reduction (11-fold decrease) in the total number of VacV-specific CD8+ T cells in the spleens of CLP mice compared with sham controls (Fig. 1F, 1G). Together, these results show that sepsis significantly compromises the capacity of the host to mount optimal effector CD8+ T cell responses to systemic viral infections.

Morbidity resolution and numerical recovery of naive CD8+ T cells after sepsis

Most experimental research examines the short-term effects of sepsis (i.e., within the first few days after sepsis induction) on the immune system (8, 55, 56). However, the majority of patients survive the early hyperinflammatory phase of sepsis but present with an increased risk for death due to severe impairment of their adaptive immune system (9, 57). Septic patients are highly susceptible to “secondary” infections that are typically controlled by CD8+ T cells when a normal, functioning immune system is present (5, 13). Viral reactivation of critically ill patients has been reported in the later stages of sepsis, which has been associated with prolonged hospitalization or death (7, 58–60). Furthermore, sepsis survivors have an increased risk for death from nonseptic causes years after hospital discharge (35). Therefore, gaining a further understanding of the long-term effects of sepsis is important and clinically relevant.

Thus, to study the long-term effects of sepsis on naive CD8+ T cells, we induced a mild septic state that most mice survive and that permits longitudinal studies lasting >30 d postsurgery. This mild septic insult was characterized by ruffled fur, hunched back, reduced mobility, and diarrhea, but importantly resulted in <10% mortality rate (Fig. 2A). Although CLP mice exhibited weight loss within the first few days after surgery, they were able to regain their weight to presurgery levels 7 d postsurgery and had weights similar to sham-surgery mice 1 mo later (Fig. 2B). Loss of immune cells by apoptosis, resulting in an overall lymphopenic state, has also been reported in septic patients (61, 62). After mild septic insult, we observed a significant decrease in total cell numbers 2 d postsurgery in the peripheral blood, spleen, and inguinal lymph node (iLN) in CLP mice compared with sham-surgery mice (Fig. 2C). This was a transient decrease in cellularity as total cell numbers in the CLP mice returned to sham-surgery mice levels by 30 d postsurgery (Fig. 2C). Specific evaluation of CD8+ T cell numbers within the peripheral blood, spleen, and iLN of both groups of mice revealed a similar pattern (Fig. 2D). Taken together, these data illustrate that sepsis induces a rapid and transient loss of CD8+ T cells in all organs examined, and that CD8+ T cell numbers return to normal within a month after sepsis induction.

Phenotype of naive (Ag-inexperienced) CD8+ T cells after sepsis

When lymphocyte numbers are reduced below a certain threshold, a lymphopenic environment is created (63, 64). Residual naive CD8+ T cells present in the periphery will undergo lymphopenia-induced, Ag-independent expansion known as homeostatic proliferation to restore T cell homeostasis and replenish the T cell compartment (63, 65). Naive (Ag-inexperienced) CD8+ T cells that undergo homeostatic proliferation express activation markers such as CD44, CD11a, CD122, and Ly6c, thus exhibiting a “memory-like” phenotype and function (66). To determine the extent to which naive (Ag-inexperienced) CD8+ T cells acquire a “memory-like” phenotype, we determined the expression of CD44 and CD11a at various time after sepsis induction (Fig. 3A, 3B). Two days postsurgery, when cell numbers in CLP mice were significantly lower (Fig. 2D), there was no difference in the percentage of CD44hi/CD11ahi CD8+ T cells in CLP- and sham-surgery mice (Fig. 3A). However, 30 d postsurgery, when cell numbers had

**FIGURE 1.** Primary CD8+ T cell responses to viral infection early after sepsis induction. (A) Experimental design. Two days post CLP- or sham-surgery, C57BL/6 (Thy1.2/1.2) mice were infected with LCMV-Arm (2 × 10^6 PFU/mouse i.p.). CD8+ T cell responses were examined on day 8 p.i. (B) Peptide-stimulated intracellular IFN-γ staining of CD8+ Thy1.2+ T cells from the spleen. LCMV peptides used for stimulation: NP396, GP33, GP276, and NP205. Numbers represent the percentage of LCMV-specific CD8+ T cells. Percentage (C) and total number (D) of Ag-specific IFN-γ CD8+ T cell responses in the spleen. (E) Total number of Ag-specific CD8+ T cells (based on the sum of all IFN-γ CD8+ T cell responses in the spleen). (F) Experimental design. Two days after CLP or sham surgery, mice were infected with VacV (1 × 10^6 PFU/mouse i.p.). CD8+ T cell responses were examined on day 7 p.i. (G) Total number of Ag-specific CD8+ T cells (based on the sum of all IFN-γ CD8+ T cell responses in the spleen). VacV peptides used for ex vivo stimulation: B8R, K3L, A47L, A42R, and A19L. Dots represent individual mice. Data analyzed by two-tailed, unpaired Student t test. Data are representative of two to three independent and similar experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
recovered in CLP mice (Fig. 2D), we observed a significant increase in the percentage of naive CD8+ T cells with a CD44hi/CD11ahi phenotype (Fig. 3A, 3B). We observed a similar increase in the percentage of CD8+ T cells with a CD122 and Ly6c phenotype 30 d postsurgery (data not shown). These results demonstrate that a substantial percentage of CD8+ T cells present after CLP surgery exhibit a “memory-like” phenotype suggesting that numerical recovery may be driven by lymphopenia-induced homeostatic proliferation.

We and others have shown that upon Ag stimulation, the increased expression of activation markers (CD44, CD11a) on CD8+ T cells can distinguish naive (Ag-inexperienced) from pathogen-specific effector and memory (Ag-experienced) CD8+ T cells (67–71). Thus, the increase in expression of activation markers on CD8+ T cells may be a consequence of Ag encounter because of the bacteria that are present after CLP surgery (46, 72). To address this, we adoptively transferred naive congenically marked (Thy1.1/1.2) TCR transgenic P14 CD8+ T cells (specific for the LCMV-derived GP33 epitope) (27, 38–41) into naive (Thy1.2/1.2) mice before CLP or sham surgery, and the expression of CD44 and CD11a was examined on CD8+ T cells on the indicated days postsurgery (Fig. 3C). If the acquisition of a “memory-like” phenotype were driven by cognate Ag, CD44 and CD11a would not be up-regulated on the P14 CD8+ T cells, because GP33 is absent from these uninfected mice. However, if the expression of these markers was driven independently of Ag, the naive P14 CD8+ T cells that remain after the sepsis-induced lymphopenia will replenish the T cell compartment and acquire the “memory-like” phenotype. We observed a significant increase in the expression of CD44 and CD11a on the P14 CD8+ T cell population (Fig. 3D), as well as on the endogenous CD8+ T cell population (Fig. 3E), suggesting that the acquisition of a “memory-like” phenotype in CLP-treated mice is not driven by sepsis-induced infection. Although Ag cross-reactivity cannot be completely ruled out in this system, similar results were obtained with naive TCR transgenic OT-I CD8+ T cells (specific for the OVA-257 epitope) (73) (data not shown). Taken together, the data suggest that after sepsis-induced lymphopenia, residual naive CD8+ T cells replenish the CD8+ T cell compartment via lymphopenia-induced, “Ag-independent” homeostatic proliferation, and subsequently acquire a “memory-like” phenotype.

**IL-15 controls numerical recovery and phenotypic changes of naive CD8+ T cells after sepsis**

IL-15 is an important cytokine for the basal proliferation of memory CD8+ T cells, but it also plays a role in the sustained proliferation and accumulation of naive CD8+ T cells within a lymphopenic environment (33, 34, 63). Because our results suggested that CD8+ T cell numerical recovery was driven by lymphopenia-induced homeostatic proliferation, we tested the hypothesis that IL-15 drives numerical recovery and the acquisition of a “memory-like” phenotype of naive CD8+ T cells after sepsis. CLP and sham surgery were performed on WT or il-15−/− mice, and numerical recovery and phenotype of naive CD8+ T cells was determined at late time points postsurgery (Fig. 4A). Strikingly, we observed that numerical recovery was severely blunted in il-15−/− mice after sepsis induction (Fig. 4B). Furthermore, as opposed to WT hosts, the number of CD44hi/CD11ahi CD8+ T cells was not significantly increased in il-15−/− mice (Fig. 4C). These data show mechanistically that IL-15 is important for the restoration of CD8+ T cells after sepsis-induced lymphopenia and the acquisition of a “memory-like” phenotype.

**Sepsis-induced reduction of Ag-specific CD8+ T cell responses to viral and bacterial infection is sustained long after the initial septic insult**

Viral reactivation in critically ill patients has been reported in the later stages of sepsis, and sepsis survivors have an increased risk for death from nonseptic causes years after the initial septic episode (7, 35, 58–60). Thus, it is important to determine the long-term consequences of sepsis on the ability of naive CD8+ T cells to respond to pathogenic challenge. Primary CD8+ T cell responses are dependent on the environment in which naive CD8+ T cells recognize pathogen-derived Ags, and the magnitude of the response correlates with the size of the naive Ag-specific CD8+ T cell precursor pool.

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**FIGURE 2.** Survival, morbidity, and numerical recovery of CD8+ T cells after sepsis. Survival (A) and morbidity (B) were analyzed on indicated days after CLP or sham surgery. Data analyzed by two-tailed, unpaired Student’s t test (24–25 mice/group). Total number of lymphocytes (C) and CD8+ T cells (D) in blood, spleen, and iLN at 2 and 30 d postsurgery. Data are presented as mean ± SEM (4–5 mice/group) and analyzed by one-way ANOVA with Tukey’s posttest of multiple comparisons. Data are representative of three to four independent experiments. *p < 0.05, **p < 0.001. n.s., Not significant.
Because we observed a significant reduction in Ag-specific CD8+ T cell responses to infections early after sepsis (Fig. 1), next we examined to what extent primary CD8+ T cell expansion was impaired at later time points after sepsis induction.

In a preliminary experiment, we adoptively transferred physiological numbers (500 cells/mouse) of naive congenically marked P14 CD8+ T cells (Thy1.1/1.1) into naive (Thy1.2/1.2) mice before CLP or sham surgery. Mice were infected with LCMV-Arm 30 d postsurgery, and P14 CD8+ T cell numbers were determined at the peak of CD8+ T cell expansion (day 8 p.i.; Fig. 5A). Interestingly, a significant, 3-fold decrease in the magnitude of expansion of P14 CD8+ T cells was observed in CLP mice compared with sham-surgery mice (Fig. 5B, 5C). In a similar experiment, CLP- or sham-surgery mice were infected with LCMV-Arm 30 d postsurgery, and endogenous Ag-specific CD8+ T cell responses (based on cytokine production [IFN-γ, TNF-α, IL-2] after ex vivo peptide stimulation) were examined on day 8 p.i. (Fig. 6A, 6B). Despite delaying the infection to a time when CD8+ T cell numbers had recovered from the sepsis-induced lymphopenia, we still observed a significant reduction in the endogenous CD8+ T cell responses to four immunodominant LCMV epitopes (NP396, GP33, GP276, and NP205) in CLP mice compared with sham-surgery mice (Fig. 6C, 6D). This resulted in a significant decrease in the total number of Ag-specific CD8+ T cells (based on the sum of all IFN-γ, CD8+ T cell responses) in the spleens of CLP mice (Fig. 6E). We also observed a similar decrease in the total number of TNF-α* and IL-2* CD8+ T cells in the spleen of CLP mice (Fig. 6F, 6G). In addition, we examined the cytotoxic potential (based on GrzB expression after ex vivo peptide stimulation) of CD8+ T cells from CLP mice compared with sham-surgery mice (Fig. 6H, 6I). On a per-cell basis, we observed that CD8+ T cells from either CLP or sham-surgery mice produced similar levels of GrzB (Fig. 6H). However, the septic event significantly reduced the total number of GrzB+ CD8+ T cells in the spleens of CLP mice (Fig. 6I). We observed similar results when measuring surface expression of CD107a (to indicate degranulation potential) on CD8+ T cells after ex vivo peptide stimulation (Fig. 6J, 6K). We extended these studies and examined whether primary CD8+ T cell responses were also impaired to bacterial challenge (attenuated L. monocytogenes–expressing OVA infection) at late time points after sepsis (Supplemental Fig. 1A). We observed a similar decrease in the total number of Ag-specific CD8+ T cells (based on the sum of all IFN-γ, TNF-α, or IL-2* CD8+ T cell responses after ex vivo stimulation; Supplemental Fig. 1F, 1G) in the...
spleens of CLP mice compared with sham-surgery mice. Taken together, these data suggest that sepsis induces long-lasting changes in the host that lead to impaired primary CD8+ T cell responses to new bacterial and viral infections.

A significant reduction in primary virus-specific CD8+ T cell responses observed at late time points after sepsis induction might be controlled by several factors such as the environment in which CD8+ T cells recognize Ag and/or the availability of naive CD8+ T cell precursors at the time of infection. To examine whether the postseptic environment affects the ability of the host to mount CD8+ T cell responses to infection, low numbers of naive congenically marked TCR transgenic P14 CD8+ T cells (Thy1.1/1.1) were transferred into naive (Thy1.2/1.2) mice before CLP or sham surgery. More than 30 d postsurgery, mice were infected with LCMV-Arm and the magnitude of the primary P14 CD8+ T cell expansion was examined in the peripheral blood (PBL) on day 8 p.i. The frequency (B) and total number (C) of P14 CD8+ T cells were measured at the peak of expansion. Data are presented as mean + SEM (five mice/group) and analyzed by two-tailed, unpaired Student t test. Data are representative of two to three independent and similar experiments. *p < 0.05.

**FIGURE 5.** The magnitude of CD8+ T cell expansion is reduced after sepsis. (A) Experimental design. Physiological numbers (5 × 10^5 cells/mouse) of naive P14 CD8+ T cells (Thy1.1/1.1) were adoptively transferred into naive (Thy1.2/1.2) mice before CLP or sham surgery. More than 30 d postsurgery, mice were infected with LCMV-Arm and the magnitude of the primary P14 CD8+ T cell expansion was examined in the peripheral blood (PBL) on day 8 p.i. The frequency (B) and total number (C) of P14 CD8+ T cells were measured at the peak of expansion. Data are presented as mean + SEM (five mice/group) and analyzed by two-tailed, unpaired Student t test. Data are representative of two to three independent and similar experiments. *p < 0.05.

Sepsis induces changes in composition of naive CD8+ T cell compartment leading to potential “holes” in the CD8+ T cell repertoire

Our data presented so far suggest that CD8 T cell intrinsic factors (e.g., availability of naive CD8+ T cell precursors) might contribute to the impairment of primary CD8+ T cell responses after sepsis. In a mouse model of influenza virus, it has been demonstrated that an age-associated alteration in the naive CD8+ T cell pool diversity can be particularly profound for subdominant responses (low naive precursor frequencies), resulting in potential “holes” in the CD8+ T cell repertoire and compromised immunity (75). Therefore, to test the extent to which sepsis-induced alterations in the naive CD8+ T cell pool might lead to the inability of the host to mount CD8+ T cell responses directed to subdominant epitopes, we used the LCMV infection model where Ag specificity of the majority of effector CD8+ T cells has been determined (47).

CLP or sham surgery was performed on mice and >30 d postsurgery, mice were infected with LCMV-Arm (Fig. 7A). However, before LCMV infection, the phenotype of the naive (Ag-inexperienced) CD8+ T cells (based on expression of CD44 and CD11a) was examined. We observed variability in expression of the “memory-like” phenotype among the CLP mice and grouped them according to low CD44hi/CD11ahi expression (CLPlow) or high CD44hi/CD11ahi expression (CLPhigh) (Fig. 7B). We wanted to determine whether there was a correlation in the reduction of Ag-specific CD8+ T cell responses with CLPlow or CLPhigh mice, thus providing clues to which individual was most affected by the initial septic insult. CD8+ T cell responses to the subdominant NP235 epitope was examined via direct ex vivo peptide stimulation on day 8 p.i. (Fig. 7A). Interestingly, we observed no significant difference in the percentage and number of NP235-specific CD8+ T cells in the spleens of CLPlow mice compared with sham-surgery mice (Fig. 7C, 7D). In contrast, there was no detectable NP235-specific CD8+ T cell response in any of the CLPhigh mice (Fig. 7C, 7D).

Collectively, these results suggest that sepsis induces changes in the composition of the naive CD8+ T cell compartment, potentially leading to “holes” in the CD8+ T cell repertoire, thus contributing to the reduction in primary CD8+ T cell responses to new infections. In addition, the variability observed among the CLP mice suggest that each individual mouse is uniquely affected from the initial septic insult, and that the extent of upregulation of the “memory-like” CD44hi/CD11ahi phenotype on naive CD8+ T cells may be used to predict the severity of impairment.

Reduction and incomplete recovery of endogenous Ag-specific naive CD8+ T cell precursors after sepsis

Given that our results suggested a sepsis-associated loss of Ag-specific naive CD8+ T cell precursors, we wanted to formally evaluate this by quantitating endogenous naive Ag-specific CD8+ T cell precursors after sepsis using p:MHC class I tetramer-based enrichment (19). Tetramer-based enrichment has allowed for the direct enumeration of rare naive Ag-specific T cell precursors (19–21). For example, ~150 and 280 naive CD8+ T cell precursors specific for 2 immunodominant LCMV-derived NP396 and GP33 epitopes, respectively, can be found in a C57BL/6 mouse (20, 47). Spleens were harvested on days 2 and 30 postsurgery, and the number of NP396- and GP33-specific CD8+ T cell precursors was determined (Supplemental Fig. 3). Two days postsurgery, we observed a significant reduction in the number of NP396- and GP33-specific naive CD8+ T cell precursors in CLP mice compared with sham-surgery mice (Fig. 8A, 8B). Importantly, on day 30 postsurgery, when cell numbers had recovered in CLP mice (Fig. 2D), we still observed a significant reduction in the number of naive CD8+ T cell precursors specific for LCMV-derived NP396 and GP33 epitopes in CLP mice compared with sham-surgery mice (Fig. 8A, 8B). These data demonstrate that naive Ag-specific CD8+ T cell precursors are significantly reduced early after sepsis induction. In addition, these results also show that despite numerical recovery, there is incomplete recovery of naive CD8+ T cell precursor numbers (at least for the two epitope specificities examined). Taken together, the results suggest that the reduction in primary CD8+ T cell responses observed at late time points after sepsis could be, at least in part, attributed to changes in the naive Ag-specific CD8+ T cell precursor pool at the time of infection.

**CD8+ T CELL RESPONSES AFTER SEPSIS**
Discussion

Most epidemiology studies and experimental models of sepsis focus on short-term outcomes, thus providing the view that sepsis is a deadly acute syndrome related to the initial hyperinflammatory state that develops. However, as sepsis progresses, an anti-inflammatory response predominates. Septic patients exhibit an immunosuppressive state or “immunoparalysis” that is manifested by the inability to clear infections that would otherwise be eradicated in a host with normally functioning CD8+ T cell immunity (5, 8, 9, 13). Moreover, it is during this later immunosuppressive phase that viral reactivation and “secondary” infections can occur, events that have been associated with prolonged hospitalization or death (7, 59, 60). Sepsis survivors have an increased risk for death from nonseptic causes years after the initial septic incident that has been associated with advanced stages of comorbidities, reducing the mean remaining life span from a predicted 7.66 y to 2.5 y for septic patients after hospital discharge (35–37). Therefore, the time it takes for the immune system to recover from sepsis may be a critical contributing factor to the increased morbidity and mortality associated with “secondary” infections. Currently, little is known about the long-term effects of sepsis on the functional capacity of naive CD8+ T cells to respond to new infections. In this study, we provide direct evidence that sepsis has profound and sustained detrimental effects on the ability of the host to mount primary pathogen-specific effector CD8+ T cell responses. Our results show that reduction in primary CD8+ T cell responses to infection after sepsis is a consequence of sepsis-associated changes in the CD8+ T cell compartment and/or loss of naive CD8+ T cell precursors.

Apoptosis of lymphoid (including CD4+ and CD8+ T cells) and myeloid cells and their decreased functionality during early stages of sepsis combine to increase susceptibility to subsequent infections (5, 12, 76). The degree of sepsis-induced lymphocyte apoptosis observed in human and animal studies correlates with disease se-
verity contributing to sepsis-associated immunoparalysis (13, 61, 62). As expected, we observed a significant decrease in CD8+ T cell numbers early after sepsis induction. The decrease in lymphocyte numbers creates a lymphopenic environment, and adoptive transfer of naive CD8+ T cells into a lymphopenic host results in the homeostatic proliferation of these cells to fill the “empty space” generated (66, 77–79). During the homeostatic proliferation process, the naive CD8+ T cells acquire activation markers (e.g., CD44, CD11a, CD122, Ly6c) and exhibit a “memory-like” phenotype (66). Lymphocyte homeostasis is dependent on cytokines such as IL-2, IL-7, and IL-15 (28, 29). IL-15, a cytokine needed for basal proliferation of memory CD8+ T cells, has also been shown to play a role in the sustained accumulation of naive CD8+ T cells within a lymphopenic environment (33, 34, 63). In addition, therapeutic IL-15 administration prevents apoptosis and immunosuppression, and improves survival in sepsis (32). Although we did not observe any long-lasting postseptic environmental changes, we did observe a sepsis-associated loss of a subdominant LCMV-specific CD8+ T cell response. The generation of “holes” in the CD8+ T cell repertoire after sepsis-induced lymphopenia may be one possible contributing factor to this immunological impairment. Using p:MHC I tetramer-based enrichment, we confirmed that after sepsis there is a reduction in naive LCMV-specific CD8+ T cell precursors (at least for the two epitope specificities examined) demonstrating that the naive CD8+ T cell precursor pool may be altered at the time of infection. Our data are in parallel with aging studies, where an age-associated loss of naive Ag-specific CD8+ T cell precursors correlated with the loss of specific CD8+ T cell responses and resulted in compromised immunity to influenza (75). These findings have public health implications suggesting that septic patients may not be able to mount an appropriate immune response to new infections because certain specificity may be lost after sepsis.

Our results indicate that the availability of naive Ag-specific CD8+ T cell precursors after sepsis contributes to the sustained response to a bacterial infection (14), and in this article, we demonstrated that expansion and accumulation of primary effector CD8+ T cells after viral infection(s) are also impaired early after sepsis induction. More importantly, we observed that the impairment of naive CD8+ T cells responding to viral and bacterial infection was sustained long after the initial septic insult. These results were unexpected because infection was delayed to a time point when CD8+ T cell numbers had completely recovered from the initial sepsis-induced lymphopenia. Multiple factors could contribute to this impairment after sepsis, such as alterations in the environment in which CD8+ T cells recognize Ag and/or the number of available naive Ag-specific CD8+ T cell precursors at the time of infection. Although we did not observe any long-lasting postseptic environmental changes, we did observe a sepsis-associated loss of a subdominant LCMV-specific CD8+ T cell response. The generation of “holes” in the CD8+ T cell repertoire after sepsis-induced lymphopenia may be one possible contributing factor to this immunological impairment. Using p:MHC I tetramer-based enrichment, we confirmed that after sepsis there is a reduction in naive LCMV-specific CD8+ T cell precursors (at least for the two epitope specificities examined) demonstrating that the naive CD8+ T cell precursor pool may be altered at the time of infection. Our data are in parallel with aging studies, where an age-associated loss of naive precursors correlated with the loss of specific CD8+ T cell responses and resulted in compromised immunity to influenza (75). These findings have public health implications suggesting that septic patients may not be able to mount an appropriate immune response to new infections because certain specificity may be lost after sepsis.

Our results indicate that the availability of naive Ag-specific CD8+ T cell precursors after sepsis contributes to the sustained
impairment in CD8⁺ T cell responses and resultant sepsis-induced immunosuppression. We demonstrate that the loss of Ag-specific CD8⁺ T cell responses results from a significant reduction in numbers and incomplete recovery of naive Ag-specific CD8⁺ T cell precursors after sepsis, indicating that alterations in the naive Ag-specific CD8⁺ T cell precursor frequencies may seriously compromise host immunity. This, in turn, might suggest that survivors of sepsis that encounter a new pathogen later in life may not be able to elicit a proper immune response based on a low number of available naive Ag-specific CD8⁺ T cell precursors at the time of infection. It appears likely that the sepsis-induced deletion event occurs stochastically, suggesting that the severity of the “immunopauparalysis” caused by CD8⁺ T cell precursor deletion may vary from patient to patient.

In summary, our results provide new insight into the long-term immunological consequences of sepsis. Increasing our understanding of the sustained sepsis effects has important clinical implications, which may help design immune-based therapies needed to improve postseptic patient outcome.

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Disclosures

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