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doi: 10.4049/jimmunol.1402473
http://www.jimmunol.org/content/195/1/116

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/05/15/jimmunol.1402473.DCSupplemental

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Polymicrobial Sepsis Increases Susceptibility to Chronic Viral Infection and Exacerbates CD8+ T Cell Exhaustion

Stephanie A. Condotta,* Shaniya H. Khan,† Deepa Rai,* Thomas S. Griffith,‡§‖‖ and Vladimir P. Badovinac*†

Patients who survive sepsis display suppressed immune functions, often manifested as an increased susceptibility to secondary infections. Recently, using a cecal-ligation and puncture (CLP) model of sepsis, we showed that sepsis induces substantial and long-lasting changes in the available naive CD8+ T cell repertoire affecting the capacity of the host to respond to newly encountered acute infections. However, the extent to which sepsis changes the host susceptibility to chronic infection and affects CD8+ T cell responses is currently unknown. In this study, we demonstrate that inbred and outbred mice recovering from a septic event are more susceptible to lymphocytic choriomeningitis virus (LCMV) clone-13 infection exhibited by mortality and viral burden. Primary virus-specific CD8+ T cells in LCMV clone-13-infected septic mice displayed exacerbated CD8+ T cell exhaustion illustrated by increased inhibitory molecule expression (e.g., programmed cell death 1, lymphocyte-activation gene 3, and 2B4) and diminished Ag-driven cytokine production (e.g., IFN-γ, TNF-α) compared with similarly infected sham-treated mice. Importantly, therapeutic inhibitory molecule dual blockade (anti–PD-L1 and anti–lymphocyte-activation gene 3) increased the number of circulating LCMV-specific CD8+ T cells and improved CD8+ T cell function and pathogen control in chronically infected septic mice. Together, these results illustrate that polymicrobial sepsis compromises the overall health of the host leading to increased vulnerability to chronic infection and exacerbated CD8+ T cell exhaustion. Collectively, our findings suggest that septic survivors may be more susceptible and at greater risk for developing exhaustible CD8+ T cells upon encountering a subsequent chronic infection.  The Journal of Immunology, 2015, 195: 116–125.

In the United States, septicemia is the cause of >1.6 million hospital cases with an in-hospital mortality rate of ∼16% (1, 2). A septic event triggers massive apoptosis of immune cells, including T cells, resulting in an initial hyperinflammatory phase followed by a prolonged hypoinflammatory immunosuppressive phase (3–8). Septic patients exhibit immunoparalysis manifested by the inability to control and eradicate infections that are normally cleared with functioning CD8+ T cell–mediated immunity (3, 6, 7, 9). Furthermore, viral reactivation of latent viruses can occur after a septic event (5, 10–13), and sepsis survivors have an increased risk for death from nonseptic causes years after the initial septic insult; for example, increased heart, lung, renal, and liver disease, infection, and hematologic disorders experienced in the preceding year are factors associated with increased risk for death in sepsis survivors (14).

CD8+ T cells play a crucial role in the control and eradication of intracellular pathogens (15). The naive CD8+ T cell repertoire is composed of a small number of unique Ag-specific CD8+ T cell precursors (ranging from 10 to 1000 cells in an inbred laboratory mouse), which enables the host to respond to a wide range of pathogen-derived epitopes (16–21). Upon recognition of cognate Ag (22, 23), naive Ag-specific CD8+ T cells proliferate and differentiate into effector CD8+ T cells capable of eliciting effector functions such as cytolytic (cytolytic perforin and granzyme B molecules) and cytokine production (IFN-γ and TNF-α) that facilitates control and clearance of the invading pathogen. After the effector stage, the expanded Ag-specific CD8+ T cells undergo a contraction phase whereby 90–95% of the responding CD8+ T cells die. The surviving CD8+ T cell population constitutes the primary Ag-specific memory CD8+ T cell pool (24–26).

Lymphocytic choriomeningitis virus (LCMV) (27–29) has been used extensively to study adaptive immune responses to viral infection (22, 23). The Armstrong strain of LCMV (LCMV-Arm) causes an acute system infection, which induces a robust CD8+ T cell response (24) that clears the infection within 8 d (30). A variant of LCMV-Arm, the clone-13 strain (LCMV clone-13), was isolated from the spleen of a mouse infected at birth with LCMV-Arm (31) and differs from the parental LCMV-Arm strain by 2-aa functional changes (one change in the polymerase protein [L: K1079Q] and the other in the viral glycoprotein [GP1: L260F]) (32–35). Although these mutations increase viral replication and change cell tropism that results in a chronic viral infection (30), they do not alter LCMV-specific CD8+ T cell epitopes, allowing for the direct evaluation of CD8+ T cell responses to dominant and subdominant LCMV-specific epitopes (33, 35). As LCMV clone-

*Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, Iowa, 52242; †Interdisciplinary Program in Immunology, University of Iowa Carver College of Medicine, Iowa City, IA 52242; ‡Microbiology, Immunology and Cancer Biology Graduate Program, University of Minnesota Medical School, Minneapolis, MN 55455; Center for Immunology, University of Minnesota Medical School, Minneapolis, MN 55455; §Masonic Cancer Center, University of Minnesota Medical School, Minneapolis, MN 55455; ‖Department of Urology, University of Minnesota Medical School, Minneapolis, MN 55455; and #Minneapolis VA Health Care System, Minneapolis, MN 55417

Received for publication September 26, 2014. Accepted for publication April 21, 2015.

This work was supported by National Institutes of Health Grants AI114543 (to V.P.B.), P30CA060682, and T32 AI007485, the U.S. Department of Veterans Affairs Merit Review Award (to T.S.G.), and an award from American Heart Association (to S.A.C.)

Address correspondence and reprint requests to Dr. Vladimir P. Badovinac, University of Iowa, 500 Newton Road, 100 Biomedical Laboratories, Iowa City, IA 52242.

E-mail address: vladimir-badovinac@uiowa.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: CLP, cecal-ligation and puncture; gMFI, geometric mean fluorescence intensity; LAG-3, lymphocyte-activation gene 3; LCMV, lymphocytic choriomeningitis virus; NIH, National Institutes of Health; PD-1, programmed cell death 1.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402473
13 infection persists, CD8+ T cells progress through stages of dysfunction or exhaustion. Certain CD8+ T cell effector functions are lost before others in a stepwise manner (e.g., cytokine production; IL-2 > TNF-α > IFN-γ) (30, 36, 37). This is accompanied by increased expression of inhibitory molecules (e.g., programmed cell death 1 [PD-1], lymphocyte-activation gene 3 [LAG-3], and 2B4) (38–40) and increased viral load (30, 36). Ultimately, deletion of Ag-specific CD8+ T cells occurs that results in an altered CD8+ T cell repertoire and skewed immunodomination hierarchy (30).

Recently, using pMHC class I tetramer-based enrichment technology, we demonstrated that sepsis-induced apoptosis reduces the number of Ag-specific naive CD8+ T cell precursors, which leads to impairment in primary Ag-specific CD8+ T cell responses to acute systemic bacterial and viral infections (41). In this study, we used the cecal-ligation and puncture (CLP) mouse model of sepsis to investigate both the short and the long-term effects of polymicrobial sepsis on primary Ag-specific CD8+ T cell responses to chronic LCMV clone-13 infection. Our data demonstrate that polymicrobial sepsis increases the susceptibility of mice to chronic viral infection and accelerates the path to CD8+ T cell exhaustion.

Materials and Methods

Mice and viral infection

Inbred C57BL/6 mice (wild type, Thy1.2/1.2) were bred at the University of Iowa, and outbred National Institutes of Health (NIH) Swiss mice were purchased from the National Cancer Institute and used at 6–10 wk of age. Thy1.1/1.1 or Thy1.1/1.2 P14 TCR-transgenic (specific for LCMV-derived GP33 epitope) mice were described previously (41–45). LCMV clone-13 (2 × 10^6 PFU/mouse i.v.; nonlethal viral dose) was provided by Dr. Steven M. Varga (Department of Microbiology, University of Iowa) and previously described (31, 46–48). Infected septic mice were housed at the University of Iowa under the appropriate biosafety level. All animal studies were approved by the University of Iowa Institutional Animal Care and Use Committee, and meet the stipulations of the Guide for the Care and Use of Laboratory Animals (NIH).

Cecal-ligation and puncture

Polymicrobial sepsis was induced by CLP (41, 49–51). 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 third was ligated with 4–0 silk sutures. The ligated portion was punc-
tured 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 grafted 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 LCMV clone-13 infection. Polymicrobial sepsis increases the susceptibility of inbred C57BL/6 mice, or CD11a CD8+ T cells were identified by tetramer staining for endogenous GP276- and GP33-specific CD8+ T cells or Thy1.1 staining for P14 TCR-transgenic cells in inbred C57BL/6 mice, or CD11a CD8α staining for CD8+ T cells in outbred NIH Swiss mice (53). Inhibitory molecule expression (PD-1, LAG-3, and 2B4) was determined on virus-specific CD8+ T cells. The frequency of CD8+ T cells producing cytokine after stimulation with indicated peptides was determined using intracellular cytokine staining (IFN-γ, TNF-α) after 5-h incubation at 37˚C in the presence of brefeldin A (BD Biosciences) (54). Cytokine production (IFN-γ) from the peripheral blood was determined after 5-h stimulation at 37˚C with indicated peptides in the presence of brefeldin A (BD Biosciences) and EL4 (10^5) APCs as previously described (47). FlowJo software (Tree Star) was used for analysis of samples acquired on a Canto flow cytometer (BD Biosciences).

In vivo Ab blockade

Anti–PD-L1; 200 μg; clone 10F9G2; BioXCell), anti–LAG-3 (200 μg; clone C9B7W), or rat IgG (200 μg; Sigma) was injected i.p. every third day for 2 wk (total 5 injections) beginning on day 21 post-LCMV clone-13 infection. For dual blockade of anti–PD-L1 and anti–LAG-3, 200 μg was used of each Ab as previously described (39, 55). Control groups were injected with PBS or 200 μg isotype Ab.

Virus titers

For analysis of virus burden, mice were infected on the indicated days post-CLP or sham surgery with LCMV clone-13 (2 × 10^6 PFU/mouse i.v.), and kidneys, liver, and serum were harvested on indicated days post-LCMV clone-13 infection. Kidneys and livers were homogenized and virus titers were quantified using a Vero cell plaque assay as previously described (31, 46).

Statistical analyses

Data were analyzed with Prism6 GraphPad software, and specific tests to determine statistical significance are indicated in the figure legends (***p < 0.0001, **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 ± SEM.

Results

Polymicrobial sepsis increases the susceptibility of inbred mice to chronic viral infection

To examine the extent to which sepsis impacts the susceptibility of mice to chronic viral infection, we infected CLP- or sham-treated inbred C57BL/6 mice with LCMV clone-13 (2 × 10^6 PFU/mouse i.v.; nonlethal viral dose) early after sepsis induction (day 2 postsurgery). Mice were monitored for morbidity and mortality for 8 d postinfection (Fig. 1A). Two days postsurgery, CLP-treated mice displayed significant weight loss compared with sham-treated mice (Fig. 1B). Upon subsequent infection with LCMV clone-13, CLP-treated mice demonstrated sustained weight loss (~25% weight loss; Fig. 1B) and increased mortality (Fig. 1C) compared with sham-treated infected mice. In all of the experiments in which mice were infected with LCMV clone-13 on day 2 postsurgery, survival rates in sham and CLP C57BL6/6 mice were 100% (47/47) and 81% (72/89), respectively. These results demonstrate that the induction of sepsis compromises the overall health of the host, leading to sustained morbidity and decreased survival with an otherwise nonlethal LCMV clone-13 infection. LCMV clone-13–infected septic mice demonstrate accelerated CD8+ T cell exhaustion

The path to CD8+ T cell exhaustion is a stepwise process resulting in a temporally hierarchical loss in CD8+ T cell functions (30, 36, 37). To investigate the extent to which sepsis impacts CD8+ T cell exhaustion, we infected CLP- or sham-treated inbred C57BL/6 mice with LCMV clone-13 (2 × 10^6 PFU/mouse i.v.) on day 2 postsurgery, and the endogenous GP276- and GP33-specific CD8+ T cell populations were examined in the spleen at the peak of the primary CD8+ T cell response (day 8 postsinfection; Figs. 1, 2,
Supplemental Fig 1A). CLP-treated infected mice had a significant reduction in the frequency (Fig. 1D, 1E) and number (Fig. 1F) of IFN-γ–producing, GP276-specific CD8+ T cells after ex vivo peptide stimulation compared with sham-treated infected mice. This reduction was also observed in the frequency of inhibitory molecule surface expression (as measured by PD-1, LAG-3, and 2B4 geometric mean fluorescence intensity [gMFI]) revealed a significant increase in PD-1, LAG-3, and 2B4 expression on D8 GP276–specific CD8+ T cells from CLP-treated mice compared with sham-treated infected mice. Importantly, analysis of inhibitory molecule surface expression (as measured by PD-1, LAG-3, and 2B4 geometric mean fluorescence intensity [gMFI]) revealed a significant increase in PD-1, LAG-3, and 2B4 expression on D8 GP276–specific CD8+ T cells from CLP-treated mice compared with sham-treated infected mice. 8 d postinfection (Fig. 2A). Similar data were obtained when endogenous D8 GP33-specific CD8+ T cells were analyzed (Supplemental Fig 1A). The surface expression of inhibitory molecules was significantly increased on D8 GP33-specific CD8+ T cells from LCMV clone-13–infected CLP-treated mice compared with sham-treated mice (Supplemental Fig 1B, 1C). A significant reduction was also observed in the amount of IFN-γ (Fig. 2B and Supplemental Fig 1D, 1E) and TNF-α (Fig. 2C, Supplemental Fig 1D–F) produced on a per-cell basis after ex vivo peptide stimulation (as measured by IFN-γ and TNF-α gMFI), suggesting that sepsis not only decreases the overall number of LCMV-specific CD8+ T cells but also affects their functionality.

Because the TCR on Ag-specific CD8+ T cells is internalized upon peptide recognition in the intracellular cytokine assay used in this study (56), simultaneous detection of LCMV-specific CD8+ T cells using tetramers and peptide-stimulated intracellular cytokine staining is not feasible. However, the functionality of Ag-specific CD8+ T cells can be measured indirectly on a per-cell basis by calculating the percentage of D8 GP276–specific CD8+ T cells capable of producing cytokine (IFN-γ, TNF-α) (30). Most D8 GP276–specific CD8+ T cells were able to produce IFN-γ (∼86% functional upon stimulation; Fig. 2D), and ∼9.2% was capable of producing TNF-α (Fig. 2E) from LCMV clone-13–infected sham-treated mice. In contrast, LCMV clone-13–infected CLP-treated mice had a significant reduction in the ability of D8 GP276–specific CD8+ T cells to produce IFN-γ (∼43% functional upon stimulation; Fig. 2D) and TNF-α (∼2.3% functional upon stimulation; Fig. 2E) compared with sham-treated infected mice. In addition to the decreased number of virus-specific CD8+ T cells, increased inhibitory molecule expression, and loss in Ag-specific CD8+ T cell function, CD8+ T cell exhaustion is associated with an increase in viral burden during chronic infection (30, 36, 38–40). To determine whether the sepsis-induced changes in CD8+ T cell responses to chronic infection led to an increase in viral load, we determined LCMV titers in the kidneys, liver, and serum on day 8 postinfection. Importantly, a significant increase in viral burden was detected in all organs of CLP-treated mice tested compared with sham-treated mice (Fig. 2F). Thus, these results collectively demonstrate that septic insult before chronic viral infection compromises the host to mount optimal primary Ag-specific CD8+ T cell responses, accelerates the path to CD8+ T cell exhaustion, and reduces pathogen control.

Outbred septic animals exhibit increased susceptibility and CD8+ T cell exhaustion post LCMV clone-13 infection

The data presented in Figs. 1 and 2 were generated using inbred C57BL/6 mice and demonstrated that septic mice are more susceptible to chronic viral infection and that CD8+ T cell exhaustion occurs at an accelerated rate. To extend our analysis and verify the results obtained in inbred mice, we performed similar experiments in which endogenous CD8+ T cell responses to chronic LCMV infection were analyzed in sham- and CLP-treated cohorts of outbred NIH Swiss mice. Recently, we demonstrated that all CD8+...
the increased susceptibility in the CLP-treated, infected NIH Swiss mice, CD8+ T cell analysis was performed on day 7 post LCMV clone-13 infection, because the majority of these mice would not have survived to the day 8 postinfection time point. LCMV clone-13-infected, CLP-treated outbred mice displayed decreased frequency (Fig. 3D, 3E) and number (Fig. 3F) of splenic CD11ahi CD8axlo CD8+ T cells compared with sham-treated mice. Furthermore, CLP-treated mice had a significant increase in inhibitory molecule PD-1 (Fig. 3G, 3H) and LAG-3 (Fig. 3G, 3I) surface expression on splenic CD11ahi CD8axlo CD8+ T cells compared with sham-treated, infected controls. Finally, a significant (4-fold) increase in viral burden was detected in the kidneys from LCMV clone-13–infected, CLP-treated NIH Swiss mice compared with sham-treated controls (Fig. 3J). These results demonstrate that a septic episode induced in outbred mice significantly increases their susceptibility to chronic viral infection, compromises the host’s ability to mount optimal CD8+ T cell–mediated immunity, and decreases pathogen control as observed in inbred septic animals. Collectively, our findings suggest that septic individuals may be more vulnerable to chronic infections and at greater risk for developing exhausted CD8+ T cells.

Timing of sepsis induction impacts the rate of CD8+ T cell exhaustion

The data presented in Figs. 1–3 were all generated when CLP-treated mice were infected with LCMV clone-13 on day 2 postsurgery. To examine the extent to which the time of secondary infection after septic insult impacts CD8+ T cell exhaustion, we infected CLP-treated mice with LCMV clone-13 (2 × 106 PFU/mouse i.v.) on various days (CLP 1 = 2 d; CLP 2 = 9 d; CLP 3 = 29 d) postsurgery, and we examined the endogenous GP76-specific CD8+ T cell response in the spleen on day 8 postinfection (Fig. 4A). Notably, although uninfected CLP mice exhibited weight loss within the first week after surgery, they were able to regain their weight to presurgery levels in 1–2 wk and had weights greater than the starting weight at the time of LCMV clone-13 infection (Supplemental Fig. 2) (41). The functionality of Ag-specific CD8+ T cells was determined indirectly on a per-cell basis by calculating the frequency of D8 GP76-specific CD8+ T cells capable of producing cytokine (IFN-γ, TNF-α; Fig. 4B–D). The majority of D8 GP76-specific CD8+ T cells from sham-treated, infected mice were capable of producing IFN-γ (∼97% functional upon stimulation; Fig. 4B, 4C). In contrast, CLP-treated, infected mice from the CLP 1 and CLP 2 sepsis groups had a significant reduction in the ability of D8 GP76-specific CD8+ T cells to produce IFN-γ (∼50% functional upon stimulation) compared with sham-treated mice. Interestingly, the majority of D8 GP76-specific CD8+ T cells from the CLP 3 sepsis group were able to produce IFN-γ (∼94% functional upon stimulation; Fig. 4B, 4C). Similar results were observed when assessing the ability of D8 GP76-specific CD8+ T cells to produce TNF-α from all sepsis groups compared with sham mice (Fig. 4D). Consistent with the functional data, there was a significant (8- and 8.8-fold) increase in viral burden detected in the kidneys from LCMV clone-13–infected, CLP-treated mice from the CLP 1 and CLP 2 sepsis groups compared with sham-treated mice, respectively (Fig. 4E); however, no difference was detected in LCMV clone-13 viral burden from the CLP 3 sepsis group compared with shams (Fig. 4E). Together, these results demonstrate that the early events after sepsis (∼10 d posts insult) have the most impact on CD8+ T cell exhaustion when CD8+ T cell responses are examined at an early time point (on day 8) post LCMV clone-13 infection.

The results in Fig. 4 suggested that LCMV clone-13 infection on day 29 postsurgery (CLP 3 sepsis group) had little impact on

T cells responding to pathogen-derived Ag could be detected using a surrogate activation marker approach (upregulation of CD11a and downregulation of CD8α surface expression on CD8+ T cells), which enabled identification and analysis of polyclonal CD8+ T cell responses without a priori knowledge of specific epitopes or MHC restriction elements (53, 57–59). CLP- or sham-treated NIH Swiss mice were infected with LCMV clone-13 (2 × 106 PFU/mouse i.v.) on day 2 postsurgery, and morbidity and mortality were monitored for 7 d postinfection (Fig. 3A). CLP-treated mice were highly susceptible to chronic LCMV clone-13 infection as demonstrated by substantial weight loss (∼28% weight loss; Fig. 3B) and increased mortality (∼55% survival rate; Fig. 3C) compared with sham-treated infected mice (∼10% weight loss and 100% survival rate; Fig. 3B, 3C, respectively). Because of

FIGURE 2. CD8+ T cell exhaustion is exacerbated in LCMV clone-13–infected septic mice. Inhibitory molecule expression of splenocytes on day 8 post LCMV clone-13 infection. (A) Summary data of gMFI of PD-1+, LAG-3+, and 2B4+ expression on tetramer+ GP276-specific CD8+ T cells. Ex vivo GP276-peptide stimulation and staining of splenocytes on day 8 post LCMV clone-13 infection. Summary data of gMFI of PD-1 (B), IFN-γ, and TNF-α expression. Summary data of the percentage of IFN-γ– and TNF-α–producing CD8+ T cells of tetramer+ GP276-specific CD8+ T cells. (F) Viral titers were determined from the kidney, liver, and serum at day 8 post LCMV clone-13 infection. Dots represent individual mice. Data were analyzed by two-tailed, unpaired Student t test (4–5 mice/group). Data are representative of two to three independent and similar experiments. *p < 0.05, **p < 0.01, ***p < 0.0001.
CD8⁺ T cell exhaustion compared with sham-treated controls when CD8⁺ T cell responses were examined early post-LCMV chronic infection. However, this snapshot analysis performed during the expansion phase of virus-specific CD8⁺ T cell responses does not exclude the possibility that “the path” or kinetics of exhaustion differs in septic and control groups of mice post chronic LCMV infection. To investigate whether CD8⁺ T cell exhaustion progressed at different rates, we infected CLP-treated mice with LCMV clone-13 \((2 \times 10^6 \text{ PFU/mouse i.v.})\) on day 29 postsurgery, and the endogenous Db GP276-specific CD8⁺ T cell response was examined in the spleen ∼2 mo postinfection (Fig. 5A). As expected, the ability of Db GP276–specific CD8⁺ T cells to produce IFN-γ after peptide stimulation was reduced in sham-treated mice when responses at days 8 and 57 post LCMV infection were compared (∼94 and 72%, respectively; Figs. 4B, 4C, 5B, 5C). Interestingly, CLP-treated, infected mice had a significant reduction in the ability of Db GP276–specific CD8⁺ T cells capable of producing TNF-α (Fig. 5D). Finally, a significant (5.6-fold) increase in LCMV clone-13 viral burden was detected in the kidneys from CLP-treated mice 2 mo post LCMV infection compared sham-treated mice (Fig. 5E). These results demonstrate that exacerbated CD8⁺ T cell exhaustion is delayed late after sepsis induction but still occurs at an accelerated rate when CD8⁺ T cell responses are examined at a later time point post LCMV clone-13 infection. Collectively, these results suggest that the initial septic insult has sustained negative effects on CD8 T cell functionality and indicates that sepsis survivors may be at a greater risk for developing exhausted CD8⁺ T cells upon encountering a subsequent chronic infection. Notably, the CLP-treated mice were able to regain their weight to presurgery levels in 1–2 wk after surgery (Supplemental Fig. 2) (41), suggesting that the increased T cell exhaustion post LCMV clone-13 infection was not simply due to overall poor health of the septic mice, but instead represent long-term effects on CD8 T cell biology.

Simultaneous PD-L1 and LAG-3 blockade improves CD8⁺ T cell function in chronically infected septic mice

All the data generated thus far have demonstrated that a prior septic event increases the susceptibility of mice to subsequent chronic viral infection and exacerbates CD8⁺ T cell exhaustion. Therapeutic dual blockade of inhibitory molecule pathways with anti-PD-L1 and anti–LAG-3 blocking Abs has a synergistic effect in
improving CD8+ T cell function in the setting of chronic infections (39, 55). To determine the extent to which CD8+ T cell function could be improved in LCMV clone-13-infected septic mice, we used a therapeutic inhibitory molecule dual-blockade approach. Naive P14 CD8+ T cells (Thy1.1/1.1; TCR-transgenic CD8+ T cells specific for the GP33 epitope of LCMV) were adoptively transferred into naive C57BL/6 (Thy1.2/1.2) recipients before surgery. Because Thy1.1 expression on T cells is stable and is not influenced during infection and/or Ag recognition, the functionality (ability to produce effector cytokines upon cognate Ag encounter) on Thy1.1+ virus-specific CD8+ T cells can be assessed directly. One day after adoptive transfer, CLP or sham surgery was performed, and mice were subsequently infected with LCMV clone-13 (2 × 10^6 PFU/mouse i.v.) on day 2 postsurgery. Dual-blockade therapy (anti–PD-L1 + anti–LAG-3) commenced on day 21 postinfection (a time when chronic infection is established), mice were treated every 3 d for 2 wk (total of five treatments), and CD8+ T cell analyses were performed in the spleen 2 d after the last treatment (day 35 postinfection; Fig. 6A). Before dual-blockade therapy, PD-1 expression and P14 CD8+ T cell function (IFN-γ production) were evaluated in the peripheral blood on day 14 postinfection (Supplemental Fig. 3). P14 CD8+ T cells from LCMV clone-13–infected, CLP-treated mice had a significant increase in PD-1 expression (Supplemental Fig. 3A) compared with sham-treated mice. Importantly, CLP-treated mice had a significant reduction in the frequency (Supplemental Fig. 3B) and total number (Supplemental Fig. 3C) of P14 CD8+ T cells after ex vivo peptide stimulation compared with sham-treated mice. In addition, the capacity of P14 CD8 T cells to produce IFN-γ was also reduced in CLP-treated mice (Supplemental Fig. 3D). These data indicate that similar to the experiments when endogenous GP276-specific CD8+ T cell responses were analyzed, CD8+ T cell exhaustion also occurs in GP33-specific, TCR-transgenic CD8+ T cells from septic mice.

After dual-blockade therapy (anti–PD-L1 + anti–LAG-3), the frequency (Fig. 6B) and number (Fig. 6C) of P14 CD8+ T cells in the peripheral blood increased in infected CLP-treated mice (16- and 4.7-fold, respectively), compared with infected CLP-treated mice that received isotype control Ab. Upon ex vivo GP33-peptide stimulation, P14 CD8+ T cell function improved in infected CLP-treated mice that received dual blockade exhibited by an increase in frequency (~60% functional upon stimulation; Fig. 6D, 6E) and amount (Fig. 6F) of IFN-γ produced compared with infected CLP-treated mice that received control Ab. Notably, infected CLP-treated mice that did not receive dual-blockade therapy displayed a decline in frequency (Fig. 6B) and number (Fig. 6C) of P14 CD8+ T cells in the peripheral blood (14- and 4.5-fold, respectively), as well as a decrease in frequency (~45% functional upon stimulation; Fig. 6D, 6E) and amount (Fig. 6F) of IFN-γ produced after ex vivo GP33-peptide stimulation in the spleen compared with infected sham-treated mice. Finally, a significant (3.4-fold) decrease in LCMV clone-13 viral burden was detected in the kidneys from infected CLP-treated mice that received dual-blockade therapy compared with infected CLP-treated mice that received isotype control Ab. These results demonstrate

**FIGURE 4.** CD8+ T cell exhaustion is pronounced early after septic insult. (A) Experimental model. Two (CLP 1), 9 (CLP 2), or 29 d (CLP 3) before LCMV clone-13 infection (2 × 10^6 PFU/mouse i.v.), CLP or sham surgery was performed on inbred C57BL/6 (Thy1.2/1.2) mice. On day 8 post LCMV clone-13 infection, splenocytes and kidneys were harvested for analysis and viral titers, respectively. (B) Representative flow-cytometry plots illustrating expression of tetramer+ GP276-specific CD8+ T cells (left panels) and IFN-γ+ (right panels) of CD8+ T cells. Numbers within plots represent the frequency of tetramer+ GP276-specific CD8+ T cells and IFN-γ+ of CD8+ T cells, respectively. Numbers right of plots represent the percentage of tetramer+ GP276-specific CD8+ T cells able to produce cytokine (IFN-γ). Summary data of the percentage of (C) IFN-γ– and (D) TNF-α–producing CD8+ T cells of tetramer+ GP276-specific CD8+ T cells. (E) Viral titers were determined from the kidneys from day 8 post LCMV clone-13 infection. Dots represent individual mice. Data were analyzed by two-tailed, unpaired Student t test (3–5 mice/group). Data are representative of two independent and similar experiments. **p < 0.01.
that inhibitory molecule dual-blockade therapy in chronically infected septic animals increases the number of circulating CD8+ T cells, improves CD8+ T cell function and pathogen control, and highlights a potential therapeutic strategy to improve disease outcome.

**Discussion**

Annually, >1.6 million hospital cases in the United States are the result of sepsis (1, 2). Sepsis survivors experience long-term negative effects postsepsis and are at greater risk for acquiring secondary infections that are normally controlled with a functioning immune system (3, 6, 7, 9). Experimental “two-hit” mouse models of sepsis have focused on immune dysfunction postsepsis to opportunistic pathogens such as *Aspergillus, Streptococcus, Pseudomonas,* and *Candida* (60–63). Despite these models, there remains a knowledge gap in understanding the immune response to viral infections postsepsis. In particular, the impact of sepsis on CD8+ T cell responses to chronic viral infection is currently poorly understood. In this study, we used a mouse model of polymicrobial sepsis to investigate the primary Ag-specific CD8+ T cell response postsepsis to chronic viral infection. Our data demonstrate that mice are more susceptible to chronic viral infection after a septic event and CD8+ T cell exhaustion occurs at an accelerated rate in LCMV clone-13–infected septic animals.

The CLP mouse model of sepsis is considered the gold standard in experimental sepsis research; however, the septic injury induced varies among research groups, making data comparisons challenging. Despite the variation of sepsis induction, the majority of studies to date demonstrate that a prior septic event increases the susceptibility of mice to secondary infections (60–63). For instance, Muenzer et al. (61) demonstrated that CLP-treated mice had increased mortality when subsequently infected with *Streptococcus pneumonia* or *Pseudomonas aeruginosa* (95 and 85% mortality rate, respectively) on day 3 post surgery compared with *S. pneumonia* or *P. aeruginosa* alone (20% mortality rate for both pathogens). Davis et al. (63) demonstrated that CLP-treated mice had increased susceptibility to *Candida albicans* infection when infected on day 2 post surgery compared with *C. albicans* infection alone (91 versus 11% mortality rate, respectively). Finally, Benjamim et al. (60) also demonstrated that CLP-treated mice infected on day 3 post surgery succumbed to *Aspergillus fumigatus* infection compared with sham-treated infected mice (100 versus 0% mortality rate, respectively). Our findings showed that both inbred C57BL/6 and outbred NIH Swiss CLP-treated mice infected on day 2 post surgery were more susceptible to LCMV clone-13 infection compared with infected sham-treated mice. In particular, outbred NIH Swiss septic mice were highly vulnerable to LCMV clone-13 infection with 55% survival rate by day 7 postinfection. We have also observed that septic animals infected on day 2 or 30 post surgery are highly susceptible to malaria with CLP-treated mice succumbing to *Plasmodium yoelii* infection compared with *P. yoelii* infection alone (S.A. Condotta et al., unpublished observations). Together, these data demonstrate that despite the variability in sepsis induction, sepsis compromises the overall health of the host leading to increased vulnerability to subsequent infections. However, the potential mechanism(s) for how CLP induction is altering host susceptibility and CD8+ T cell responses to chronic viral infection is currently unknown. Interestingly, early treatment with a high-dose antibiotic Primaxin ameliorates, but does not prevent, observed exacerbation of CD8+ T cell exhaustion after polymicrobial sepsis induction (S.H. Khan and V.P. Badovinac, unpublished observations), suggesting that decreasing the duration and/or severity of bacterial infection might represent a useful approach in deciphering the factors that control overall susceptibility of a septic host to secondary (unrelated) bacterial or viral infections.

Chronic infections such as hepatitis B virus, hepatitis C virus, HIV, and protozoan infections (e.g., malaria) affect ∼10% of the world’s population (64–67). Because infection persists during chronic infections, CD8+ T cells progress through stages of dysfunction with a temporal hierarchical loss of functional properties (30, 36, 37, 55). T cell exhaustion has been reported in humans with chronic infections of hepatitis B virus, hepatitis C virus, HIV, and malaria (55, 68–71). In mouse models of chronic infection, LCMV clone-13 has been extensively used as a model pathogen to study CD8+ T cell exhaustion. Wherry et al. (30) demonstrated that during LCMV clone-13 infection, the subdominant GP276-
from the peripheral blood before and after dual mAb blockade. (IFN-γ) infection. Representative flow-cytometry plots illustrating expression of GP33-peptide stimulation of splenocytes on day 35 post LCMV clone-13 (Thy1.1+) CD8+ T cells from the peripheral blood on day 35 post LCMV clone-13 infection. Dots represent individual mice. Data were analyzed by two-tailed, unpaired Student t test (3–4 mice/group). Data are representative of three independent and similar experiments. *p < 0.05.

FIGURE 6. Anti–PD-L1 and anti–LAG-3 dual mAb blockade improves CD8+ T cell function and pathogen control in chronically infected septic mice. (A) Experimental model. Naive P14 CD8+ T cells (Thy1.1/1.1 or Thy1.1/1.2) were adoptively transferred (10^5 cells/mouse) into naive inbred C57BL/6 (Thy1.2/1.2) mice before surgery. Two days before LCMV clone-13 infection (2 × 10^6 PFU/mouse i.v.), CLP or sham surgery was performed. On day 21 post LCMV clone-13 infection, dual mAb blockade commenced (200 μg anti–PD-L1 + 200 μg anti–LAG-3/mouse i.p. or PBS/ rIgG for untreated controls) and mice were treated every third day for 2 wk (total of five treatments). Two days after the last treatment, splenocytes and kidneys were harvested for analysis and viral titers, respectively. (B) Summary data of the number of P14 CD8+ T cells 5-fold in the peripheral blood and spleen, specific CD8+ T cell response becomes the dominant population. They showed that on day 8 postinfection, the majority of GP276-specific CD8+ T cells from LCMV clone-13–infected mice were able to make IFN-γ upon peptide stimulation (∼97% functional). However, the capacity to produce IFN-γ upon peptide stimulation was diminished on day 30 post LCMV clone-13 infection. Using the established mouse model of CD8+ T cell exhaustion, our data demonstrated that as early as day 8 postinfection GP276-specific CD8+ T cells from LCMV clone-13–infected septic mice lost the ability to produce IFN-γ upon peptide stimulation compared with infected sham-treated mice (∼43–50 versus ∼86–97% functional, respectively). In addition, even when septic mice were infected at a time point of sepsis resolution (day 29 postsurgery), CD8+ T cell exhaustion was still accelerated in chronically infected septic mice compared with sham-infected controls (50 versus 72% functional, respectively). Our findings suggest that not only does sepsis increase the susceptibility of mice to chronic viral infection, but it also exacerbates CD8+ T cell exhaustion. It is important to note that this type of analysis has not been done in the setting of sepsis before, and our data indicate that sepsis survivors may be at greater risk for developing highly exhaustible CD8+ T cells upon subsequent chronic infection.

Although direct secondary viral infection after sepsis is rare, there is a considerable amount of literature describing viral reactivation (e.g., CMV, EBV, or HSV) in the wake of a septic event. The recent publication by Walton et al. (13) presented data showing a strong correlation between viral reactivation and susceptibility to secondary opportunistic bacterial or fungal infections. It is tempting to speculate that the increased susceptibility to these unrelated secondary opportunistic infections is related to the CD8+ T cell exhaustion resulting from chronic viral infection. Recently, we reported that sepsis has long-lasting consequences on the ability of naive (Ag nonexperienced) and memory CD8 T cells to respond to their cognate Ag delivered in the context of newly encountered or repeated infections (41, 59). Thus, a clear and rigorous investigation of the consequences of viral infection after a septic event on the immune system (specifically the ability of nonvirus specific naive and/or memory CD8 T cell responses) becomes quite important, and studies will be designed to experimentally address this concept in the future.

Simultaneous inhibitory molecule blockade therapy has been successful in improving CD8+ T cell function in a setting of chronic infection (39, 55). Blackburn et al. (39) demonstrated that cblockade with anti–PD-L1 and anti–LAG-3 blocking Abs in vivo worked synergistically, increasing the number of GP276-specific CD8+ T cells 5-fold in the peripheral blood and spleen, improving CD8+ T cell function (∼80% functional upon stimulation) and reducing viral burden in LCMV clone-13 chronically infected mice compared with isotype control–treated mice. Butler et al. (55) demonstrated in a mouse model of malaria that dual blockade with anti–PD-L1 and anti–LAG-3 resulted in immediate alleviation (glut) and reducing viral parasite burden, accelerated parasite clearance, and improved T cell responses. Our data showed that coadministration of anti–PD-L1 and anti–LAG-3 blocking Abs improved CD8+ T cell function (∼60% functional upon stimulation) and reduced LCMV clone-13 viral burden in chronically infected septic animals compared with isotype control–treated septic mice. These data suggest that exhausted CD8+ T cell function can be improved with therapeutic coadministration of anti–PD-L1 and anti–LAG-3 blocking Abs in chronically infected septic animals.
Our findings also illustrate a potential therapeutic strategy to improve CD8+ T cell function in sepsis survivors who have developed exhausted CD8+ T cells from subsequent chronic infections. Finally, our data are in agreement with recent data obtained in human and mouse models of polymicrobial sepsis (72–74) that showed that PD-1 blockade increased survival and lymphocyte function, further illustrating the therapeutic potential of inhibitory molecule blockade treatments in septic survivors in the presence or absence of secondary infection.

In summary, we provide evidence that a prior septic event compromises the overall susceptibility of the host to chronic viral infection leading to sustained morbidity, decreased survival, and reduced pathogen control. Furthermore, our findings demonstrate that CD8+ T cell exhaustion occurs at an accelerated rate post chronic infection in septic animals. Together, our data suggest that sepsis patients and/or sepsis survivors may be more vulnerable and predisposed to developing exhausted CD8+ T cells when encountering chronic infections. An increased understanding of the immunological consequences of sepsis will help design therapies to improve postseptic patient outcome.

Acknowledgments

We thank Stacey Hartwig and Dr. Steven M. Varga for LCMV clone-13 virus, and Leica Epping and Dr. John T. Harty for anti–LAG-3 Ab. We also thank members of the Badovinac Lab and Dr. Martin J. Richer for helpful discussion.

Disclosures

The authors have no financial conflicts of interest.

References


