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A PEPTIDE-BASED CHECKPOINT IMMUNOMODULATOR ALLEVIATES IMMUNE DYSFUNCTION IN MURINE POLYMICROBIAL SEPSIS

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ABSTRACT—Sepsis-induced immunosuppression involves both innate and adaptive immunity and is associated with the increased expression of checkpoint inhibitors, such as programmed cell-death protein 1 (PD-1). The expression of PD-1 is associated with poor outcomes in septic patients, and in models of sepsis, blocking PD-1 or its ligands with antibodies increased survival and alleviated immune suppression. While inhibitory antibodies are effective, they can lead to immune-related adverse events (irAEs), in part due to continual blockade of the PD-1 pathway, resulting in hyperactivation of the immune response. Peptide-based therapeutics are an alternative drug modality that provide a rapid pharmacokinetic profile, reducing the incidence of precipitating irAEs. We recently reported that the potent, peptide-based PD-1 checkpoint antagonist, LD01, improves T-cell responses. The goal of the current study was to determine whether LD01 treatment improved survival, bacterial clearance, and host immunity in the cecal-ligation and puncture (CLP)-induced murine polymicrobial sepsis model. LD01 treatment of CLP-induced sepsis significantly enhanced survival and decreased bacterial burden. Altered survival was associated with improved macrophage phagocytic activity and T-cell production of interferon-γ. Further, myeloperoxidase levels and esterase-positive cells were significantly reduced in LD01-treated mice. Taken together, these data establish that LD01 modulates host immunity and is a viable therapeutic candidate for alleviating immunosuppression that characterizes sepsis and other infectious diseases.

KEYWORDS—Immune dysfunction, immunotherapy, macrophages, programmed cell-death protein 1, T cells

ABBREVIATIONS—CD3—a-cluster of differentiation; CFU—colony-forming units; CLP—cecal-ligation and puncture; IFN—interferon; IL—interleukin; IP—intraperitoneally; irAEs—immune-related adverse events; LAG3—lymphocyte activation gene 3; mAb—monoclonal antibodies; MCP-1—monocyte chemoattractant protein-1; MPO—myeloperoxidase; PBS—phosphate-buffered saline; PD-1—programmed cell-death protein 1; PD-L1—programmed death-ligand 1; RIIH—Rhode Island Hospital; TNF—tumor necrosis factor-alpha; WUSTL—Washington University in St. Louis

INTRODUCTION

Sepsis is defined as a dysregulated host response to infection that causes life-threatening organ dysfunction, leading to high mortality worldwide (1). The hallmarks of this host response include two divergent states: a hyperinflammatory response with demonstrated fever and exaggerated cytokine production, and suppression or paralysis of the innate and adaptive immune systems. Although early mortality and organ dysfunction are often linked to an initial inflammatory process, the primary source of morbidity and mortality stems from the inability to eradicate the inciting pathogen. This leads to the development of secondary, sometimes resistant or opportunistic, infections due to immune suppression.

A lack of success in sepsis clinical trials for anti-inflammato-ry approaches has led to the emergence of new therapies that restore suppressed host immunity. These therapies include many of the same immunotherapies used in cancer trials including the innate and adaptive immune restorative therapies interleukin 2 (IL-2), IL-7, IL-15, and checkpoint inhibitors (2). These emerging therapies and others target a growing library of evidence that characterizes the paralyzed immune state, namely, high expression of suppressive cytokines such as IL-10, significant lymphocyte apoptosis, and reduced T cell and...
monocyte function that includes impaired cytokine production and surface expression of the antigen presentation molecule HLA-DR.

Increased expression of checkpoint inhibitors such as programmed cell-death protein 1 (PD-1) impairs immune cell function (3, 4), and thus, represents a novel therapeutic target for sepsis. PD-1 is expressed predominantly on T cells; however, other immune cells, including B cells, monocytes/macrophages, and natural killer cells, express PD-1 (5–9). PD-1 binds to its primary ligand, programmed death-ligand 1 (PD-L1), which is expressed as a cell surface transmembrane protein on immune cells as well as a variety of non-hematopoietic cell types (10–13). Activation of the PD-1/PD-L1 axis on T cells suppresses the T-cell receptor and costimulatory signaling, which generates a nonresponsive response referred to as anergy or exhaustion, characterized by impaired proliferation, cytokysis, cytokine production, and increased apoptosis (14–18). Similarly, PD-1 expression on monocytes/macrophages has been shown to mediate cellular dysfunction (8, 19, 20). It has been posited that PD-1 on tumor-infiltrating immune cells drive immune exhaustion, thus limiting the host's ability to attack the tumor cells. Consequently, many cancer therapies have adopted the use of α-PD-1 monoclonal antibodies (mAb) as adjunctive therapy.

The increased expression of PD-1 and PD-L1 is associated with poor outcomes in septic patients (21–24). Moreover, recent studies have demonstrated that PD-1 or PD-L1 inhibitory Abs confer a survival benefit in animal models of sepsis (22, 25–30). While inhibitory Abs can effectively restore immune function therapeutically, the benefit of such Ab-based checkpoint inhibitors must be weighed against the potential risk of immune-related adverse events (irAEs) in critically ill patients. An alternative modality to Abs is peptide-based immunotherapies that provide a shorter pharmacokinetic profile, thus reducing the chance for potential irAEs. Furthermore, peptide-based immunomodulators offer more formulation and delivery options, and have better tissue penetration and rapid synthetic manufacturing (31–34). We recently reported that LD01, a 22-amino acid peptide derived from a Bacillus bacteria, antagonized PD-1, enhanced antigen-specific CD8 T-cell expansion, and promoted the survival of mice in a lethal malaria model (35).

In the current study, we tested the effects of LD01 in a cecal ligation and puncture (CLP)-induced murine polymicrobial sepsis model. Therapeutically, LD01 treatment enhanced survival and decreased the bacterial burden associated with increased macrophage and T-cell function. Moreover, splenic myeloperoxidase (MPO) levels and esterase-positive cells were significantly reduced in LD01-treated mice. Collectively, these data demonstrate that LD01 modulates both innate and adaptive immune responses, and is a viable therapeutic modality for sepsis and other infectious diseases to alleviate immune dysfunction.

MATERIALS AND METHODS

Murine sepsis model of CLP

All procedures were performed in accordance with strict institutional guidelines for animal care and use. Protocols were conducted in accordance with the National Institutes of Health guidelines. Protocol approvals were obtained from the Institutional Animal Care and Use Committee of Rhode Island Hospital (RIH) (No. 5025-17) and Washington University in St. Louis (WUSTL) (Nos. 20180135 and 19086600). Murine models performed at RIH and WUSTL were nearly identical. Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice (8–10 weeks old) were housed in a room at an ambient temperature of 22°C and a 12:12-h light:dark cycle. They were allowed to acclimate in the animal facility for 1 week before their use in experiments. A number of pioneering studies have shown the impact of sex as a variable in experimental mice being subjected to septic challenge, with protective effects of the pre-estrus state (and other members of the estrogen family) seen in mature adult female mice when compared with males (36, 37). Thus to have uniform and predictable responses the use of male mice is preferred. polymicrobial sepsis was induced in the CLP mouse model as previously described (38, 39). Briefly, mice were anesthetized with isoflurane, and a ventral midline incision was made below the diaphragm to expose the cecum. The cecum was ligated with 5-0 silk, punctured twice with a 21-gauge needle (RIH) or 27-gauge needle (WUSTL), and gently compressed to extrude a small amount of fecal content through the punctured holes. The cecum was returned to the abdomen and the incision was closed in layers with 6-0 Ethilon suture (Ethicon Inc, Somerville, NJ). Buprenorphine 0.05 mg/kg (Hospira Inc, Lake Forest, Ill) was used for analgesia at the end of the procedure. No antibiotics were used for this study. Then, all animals were resuscitated with 0.6 mL lactated Ringer’s-saline solution. At RIH, mice were treated at 3 and 9 h post-CLP with 200 µg LD01 injected intraperitoneally (IP) or 200 µg α-PD-1 Ab (RMP1-14 mAb; Bio X Cell, Lebanon, NH) was injected IP once at 3 h post-CLP. For survival studies, mice received two daily injections of LD01 on days 0, 1, and 2 following CLP or once daily injection of α-PD-1 Ab on days 0, 1, 2, 3, and 4 following CLP, and were monitored for 7 days. At WUSTL, LD01 and control peptide were administered at a dose of 200 µg/injection/mouse IP twice daily on days 0, 1, and 2 following CLP with the first dose administered 3 h post-surgery. For survival studies, mice were monitored for 7 days post-CLP.

Sample preparation for blood and peritoneal fluid and cells

At 24 h post-procedure, mice were euthanized by CO2 asphyxiation. Blood was collected by cardiac puncture for bacterial burden. Peritoneal fluids and cells were obtained from mice by lavage of the peritoneal cavity. For bacterial burden lavage, peritoneal fluids were collected after the injection of 1 mL phosphate-buffered saline (PBS) into the peritoneum, clarified by centrifugation (800 g at 4°C for 15 min), used for bacterial count by serial dilution in PBS, and plated on blood agar plates.

Preparation of peritoneal macrophages for phagocytosis assay

Peritoneal macrophages were obtained from mice at 24 h post-CLP by lavage of the peritoneal cavity. Cells were collected after the injection of 1 mL PBS into the peritoneum and centrifugation (800 g at 4°C for 15 min). A second lavage was performed using 4 mL PBS to collect more cells. The cells were combined from the two lavage collections, resuspended in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum at 1 × 106 cells/mL, plated in 12-well tissue culture plates, and incubated at 37°C for 6 to 12 h. After incubation, nonadherent cells were removed by washing twice with fresh DMEM. As previously described (8), for the phagocytosis assay adherent macrophages were fed with phRodoTM-conjugated Escherichia coli BioParticles (Molecular Probes, Eugene, Ore) at 37°C for 1 h and washed with PBS. Cells were scraped from the plates, stained with α-F4/80 for 30 min, washed and analyzed by flow cytometry. Macrophage phagocytic efficiency was evaluated by the M-Quanti (Quant-Bio, Bergisch Gladbach, Germany). Peritoneal macrophage numbers of double labeled with pHrodo BioParticles and allophycocyanin-α-F4/80 Ab were compared between the groups. In brief, cells were assessed for their extent of fluorescence intensity (mean fluorescence intensity). A region, M1, in which a cell was considered positive for containing fluorescent E. coli BioParticles, was established by comparison with a negative control that was run at 4°C as opposed to the 37°C used in all other cases. The percentage of cells containing fluorescent BioParticles (considered positive) was then determined and used as an index of the “Phagocytic capacity of F4/80+ macrophages.” Data were analyzed with FlowJo software (8, 40, 41).

Bacterial burden

The samples of blood and peritoneal lavage fluids in PBS were plated on Trypticase soy agar with 5% sheep blood plates (BD Biosciences, San Jose, Calif), cultured at 37°C for 24 h. Then colonies on the plates were counted (8). Data is expressed as Log10 CFU/mL peritoneal fluid or blood.
Quantification of cytokines and chemokines in peritoneal fluid

Enzyme-linked immunosorbent assay (ELISA) kits were employed to measure the levels of monocyte chemoattractant protein-1 (MCP-1), IL-6, and tumor necrosis factor-alpha (TNF-α) (Biologend, San Diego, Calif) in clarified peritoneal lavage fluids from mice 24 h post-CLP, as previously described (42–44). The 24 h time point was selected as myeloid cells are known to respond robustly and rapidly to septic stimuli within this time frame.

Assessment of splenic MPO protein and esterase level

MPO levels in the spleen, as an assessment of neutrophil influx in tissue homogenates, were measured 24 h post-CLP by ELISA according to the manufacturer’s instructions (#EMMPO; Thermo Fisher Scientific, Waltham, Mass). Similar to cytokine analysis, the 24 h time point was selected as myeloid cells are known to respond robustly and rapidly to septic stimuli within this time frame. To measure esterase levels, spleen sections were stained with naphthol AS-D chloroacetate esterase (Sigma-Aldrich, St. Louis, Mo) according to the manufacturer’s instructions. The images were collected with the Nikon Eclipse 80i microscope using a ×20 and ×40 objective and Spot RT3 camera. Slides were randomly screened and blindly evaluated with three to six images acquired per specimen (42–44).

ELISA and ELISpot quantitation of T-cell interferon-γ production

Interferon gamma (IFN-γ) production by splenic T cells was measured from mice euthanized on day 2 post-CLP. The 48 h time point was selected to allow LD01 enough time to influence the activation, survival, and stimulation of the T cells in vivo. A shorter timeframe (i.e., 24 h) would have not allowed additional doses of LD01 between 24 and 48 h post-CLP. Spleens were harvested and cell counts were obtained using the Beckman Coulter Vi-Cell counter (Beckman Coulter, Fullerton, Calif). For the quantification of IFN-γ in the supernatant, 5 × 10^6 spleenocytes were incubated overnight with α-cluster of differentiation 3 (CD3)/α-CD28 Abs (α-CD3 at 1 μg/mL and α-CD28 at 5 μg/mL), and supernatants were harvested the following morning. ELISA was performed using IFN-γ Mouse Antibody Pair (Life Technologies, Grand Island, NY), according to the manufacturer’s instructions. The μQuant Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, Vt) was used for analysis. For ELISpot quantification, 96-well ELISpot strip plates and capture/detection antibody kits (EL485) were purchased from R&D Systems (Minneapolis, Minn) and precoated with IFN-γ capture antibody as per the manufacturer’s instructions. To detect IFN-γ, cells were incubated overnight in the presence of α-CD3/α-CD28 Abs at 37°C and 5% CO2. Following incubation, wells were decanted from ELISpot membranes and biotinylated detection antibody was applied, followed by streptavidin-alkaline phosphatase and color substrate. Images were captured and spot numbers were counted using the C.T.L ELISpot Image reader (Cellular Technologies Limited, Cleveland, Ohio).

Data analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, La Jolla, Calif) for survival data and SAS Version 9.4 for Windows (SAS Institute, Cary, NC) for other analyses. For the survival study, groups were compared by Kaplan–Meier survival curves with the log-rank test used to compute the P value. Bacterial burden, macrophage function, MPO levels, number of esterase-positive cells, and IFN-γ production among the different groups were compared using Kruskal–Wallis analyses using SAS/STAT NPAR1WAY software. Adjusted pairwise comparisons following significant overall tests were done with the Dwsaw, Steel, Critchlow-Fligner analysis. Data are expressed as the mean ± SEM and P < 0.05 was considered statistically significant.

RESULTS

LD01 reduces bacterial burden during sepsis

To determine whether LD01 reduced sepsis, C57BL/6 mice were subjected to CLP surgery and treated with LD01 at two independent laboratories, RIH and WUSTL, as detailed in the Methods. Then their survival was monitored. At RIH, LD01 was administered at a dose of 200 μg/injection/mouse IP twice daily on days 0, 1, and 2 following CLP (Fig. 1A). α-PD-1 mAb was administered once daily on days 0, 1, 2, 3, and 4 following CLP at 200 μg/injection/mouse (Fig. 1A). At WUSTL, the α-PD-1 mAb was not tested; rather, a control peptide, which is a derivative of LD01, was included. Control peptide and LD01 were administered at a dose of 200 μg/injection/mouse IP twice daily on days 0, 1, and 2 following CLP (Fig. 1B). Note that at both RIH and WUSTL the first doses were given 3 h post-surgery. As shown in Figure 1A and B, the survival rate in CLP-only vehicle-treated or control peptide-treated mice was 47% (14 of 30 mice) and 43% (13 of 30 mice), respectively, at 7 days post-CLP. However, the survival rate increased to 70% (21 of 30 mice; Fig. 1A) and 76% (23 of 30 mice; Fig. 1B) when mice were treated with LD01. Of note, a survival rate of 60% (18 of 30 mice) was observed in mice treated with α-PD-1 mAb (Fig. 1A). The overall test of differences among the survival curves in Figure 1A was not significant, log-rank P = 0.18. While the change in survival at RIH was not significant between LD01 and CLP-only vehicle-treated mice, an increased trend in survival was revealed. At WUSTL, LD01 treatment relative to control peptide did significantly increase the survival rate (P = 0.008, log-rank Mantel-Cox test). Taken together, these results argue that LD01 treatment improves survival in the CLP model.

To elucidate whether the trends in increased survival after LD01 treatment resulted from altered bacterial clearance, bacteria levels in the peritoneal cavity and blood were measured (Fig. 1, C and D). At 24 h post-induction of sepsis, CLP-only mice had significantly higher peritoneal cavity bacteria levels than mice treated with LD01 (CLP-only = 6.8 ± 0.08 SEM CFU/mL vs. LD01 = 6.5 ± 0.07 SEM CFU/mL; Fig. 1C) with the difference significant after adjustment for multiple comparisons (P < 0.01). Bacterial loads also decreased with α-PD-1 mAb treatment (6.6 ± 0.10 SEM CFU/mL), although it was not significant and was slightly higher than that in the LD01 group (Fig. 1C). Comparison of blood bacterial loads revealed similar trends with reduced CFU counts in LD01- and α-PD-1 mAb-treated mice relative to the CLP-only group; however, differences were not significant (LD01 = 2.5 ± 0.14 SEM CFU/mL, α-PD-1 mAb = 2.6 ± 0.12 SEM CFU/mL, CLP-only = 2.8 ± 0.10 SEM CFU/mL; Fig. 1D). Hence, treatment with LD01 leads to bacterial control in the peritoneal cavity and potentially in blood.

LD01 diminishes peritoneal macrophage dysfunction in septic mice

To determine whether the reduced bacterial burden in LD01-treated mice was associated with the relief of sepsis-induced macrophage dysfunction, peritoneal macrophages were isolated 24 h post-CLP and their ex vivo phagocytic capacity was assessed in an in vitro assay system in which fluorescein-conjugated E coli were incubated with the macrophages. As previously reported (8), CLP-induced sepsis significantly reduced the activity of peritoneal macrophages, decreasing their phagocytic capacity to 50 ± 4.5% SEM relative to the 82 ± 4.0% SEM observed in sham-treated mice (Fig. 2A). The difference between sham and CLP-only mice was significant after adjustment for multiple comparisons (P < 0.01). Macrophages derived from LD01-treated mice showed greater phagocytic activity (65 ± 4.4% SEM) compared with CLP-only-derived macrophages (Fig. 2A); however, the
difference was not significant after adjustment for multiple comparisons ($P < 0.20$) though it was significant before adjustment ($P < 0.05$). These data suggest that LD01 treatment, similar to PD-1 deficiency (8), alleviated sepsis-induced macrophage dysfunction. Another characteristic of macrophage dysfunction observed in sepsis is the reduced capacity to secrete cytokines (8, 45, 46). To investigate whether LD01 treatment altered the production of cytokines in vivo, we measured levels of MCP-1, IL-6, and TNF-$\alpha$ in the peritoneal cavity 24 h post-CLP. As anticipated, the peritoneal washes from septic mice had considerably more MCP-1, IL-6, and TNF-$\alpha$ relative to sham mice (Fig. 2, B–D). The levels of TNF-$\alpha$ and IL-6 in the LD01-treated mice were comparable to those of CLP-only mice. In contrast, peritoneal washes from the LD01-treated mice showed increased MCP-1 levels (Fig. 2B) relative to CLP-only mice; however, the difference was not significant after adjustment for multiple comparisons ($P < 0.10$) though it was significant before adjustment ($P < 0.05$).

**LD01 reduces splenic MPO levels and esterase-positive cells in septic mice**

Excessive neutrophil activity during hyperinflammatory states, including sepsis, can induce unwanted tissue damage and/or organ dysfunction caused by leukocyte-specific esterase. To determine whether LD01 treatment alters neutrophil recruitment and/or activity, levels of tissue-derived MPO were measured by ELISA 24 h post-CLP (47). Three tissues were

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**Fig. 1.** Improved survival and bacterial reduction in septic mice following treatment with LD01. A, Mice were subjected to CLP and treated with vehicle (0.1 M sodium acetate solution; $n = 30$), LD01 ($n = 30$), or $\alpha$-PD-1 mAb ($n = 30$) and survival was monitored for 7 days. LD01 was administered at 200 $\mu$g/injection/mouse IP twice daily on days 0, 1, and 2 following CLP. $\alpha$-PD-1 mAb was administered once daily on days 0, 1, 2, 3, and 4 following CLP at 200 $\mu$g/injection/mouse. Data were pooled from six independent experiments. Groups were compared by Kaplan–Meier survival curves with the log-rank test to compute a $P$ value = 0.18.

B, Mice were subjected to CLP and treated with a control peptide ($n = 30$) or LD01 ($n = 30$) and survival was monitored for 7 days. The control peptide and LD01 were administered at a dose of 200 $\mu$g/injection/mouse IP twice daily on days 0, 1, and 2 following CLP. Data were pooled from three independent experiments. Groups were compared by Kaplan–Meier survival curves with the log-rank test to compute a $P$ value = 0.008.

C and D, Mice were subjected to CLP and treated with vehicle (0.1 M sodium acetate solution; CLP only) or LD01 at 3 and 9 h post-CLP. $\alpha$-PD-1 mAb was administered once at 3 h following CLP at 200 $\mu$g/injection/mouse. At 24 h post-CLP bacteria levels were assessed and expressed as Log$_{10}$ CFU/mL peritoneal fluid (C) or Log$_{10}$ CFU/mL blood (D). Data were pooled from five to seven independent studies ($n = 14–20$). Data are expressed as the mean ± SEM. Difference between CLP-only and LD01-treated mice in (C) was significant after adjustment for multiple comparisons ($P < 0.01$). CLP indicates cecal-ligation and puncture; IP, intraperitoneally.
assessed: liver, kidney, and spleen. For the liver and kidney, the levels of MPO detected among sham, CLP-only, and LD01-treated mice were similar (liver: sham = 949 ± 85 SEM ng/mL, CLP only = 1,002 ± 59 SEM ng/mL, LD01 = 1,020 ± 174 SEM ng/mL; kidney: sham = 1,828 ± 164 SEM ng/mL, CLP only = 2,082 ± 308 SEM ng/mL, LD01 = 2,078 ± 236 SEM ng/mL). By contrast, in the spleen, considerably more MPO was detected in the CLP-only mice relative to sham mice (CLP only = 255 ± 36 SEM pg/mL vs. sham = 128 ± 10 SEM pg/mL; Fig. 3A) with the difference significant after adjustment for multiple comparisons (P < 0.05). LD01-treated mice showed a significant decrease in MPO levels compared with CLP-only mice (Fig. 3A), with levels returning to that of the sham mice. The difference between CLP-only and LD01-treated mice was significant after adjustment for multiple comparisons (P < 0.05). These data suggest that LD01 inhibits splenic neutrophil influx and/or increased activation during sepsis.
FIG. 3. **LD01 treatment reduces MPO and esterase levels in septic mice.** Mice were subjected to CLP and treated with vehicle (0.1 M sodium acetate solution; CLP only) or 200 μg of LD01 at 3 and 9 h post-CLP. A, MPO levels in the spleen were measured at 24 h post-CLP by ELISA. B, To measure esterase levels, spleen sections at 24 h post-CLP were stained with naphthol AS-D chloroacetate esterase. Images were collected and slides were randomly screened and blindly evaluated with three to six images acquired per specimen. Data were pooled from two independent studies (n = 6). Data are expressed as the mean ± SEM. The difference between sham and CLP-only mice for (A) and (B) was significant after adjustment for multiple comparisons (*P < 0.05). Difference between CLP-only and LD01-treated mice for (A) was significant after adjustment for multiple comparisons (*P < 0.05). The difference between CLP-only and LD01-treated mice for (B) was not significant after adjustment for multiple comparisons (P < 0.10) though it was significant before adjustment (P < 0.05). C to H, Spleen sections at 24 h post-CLP were stained with naphthol AS-D chloroacetate esterase. Representative images from sham (C and D), CLP-only (E and F), and LD01-treated (G and H) mice are shown. Esterase-positive cells stained maroon. Photomicrographs are presented at a magnification of ×20 (C, E, and G) and ×40 (D, F, and H). The dashed-box in (C, E, and G) designate the area of the ×40 magnification. The scale bar is 50 μm. CLP indicates cecal-ligation and puncture; ELISA, enzyme-linked immunoassay; MPO, myeloperoxidase.
Esterase-positive cells are generally granulocytic leukocytes, including neutrophils. Thus, in addition to measuring MPO levels, the number of esterase-positive cells in the spleen was quantified via histology (Fig. 3B) and considered a marker of neutrophil influx. Similar to MPO analysis, the number of splenic esterase-positive cells was significantly greater in the CLP-only mice relative to sham mice (CLP only = 270 ± 73 SEM vs. sham = 65 ± 4 SEM; Fig. 3B) with the difference significant after adjustment for multiple comparisons (*P < 0.05). A decrease in splenic esterase-positive cells was observed in LD01-treated mice relative to CLP-only mice (Fig. 3B); however, the difference was not significant after adjustment for multiple comparisons (P < 0.10) though it was significant before adjustment (P < 0.05). These data further support altered septic-induced neutrophil recruitment to the spleen in LD01-treated mice. Figure 3C to H shows representative photomicrographs of the naphthol AS-D chloroacetate esterase staining for each group, with esterase-positive cells staining maroon.

**LD01 relieves T-cell suppression in septic mice**

To determine whether LD01 treatment also reduced septic-induced T-cell suppression, splenocytes were harvested and stimulated, and IFN-γ was measured by ELISA and ELISPOT. For these studies, control peptide or LD01 were administered at a dose of 200 μg/injection/mouse IP twice daily on days 0 and 1 following CLP. Mice were euthanized and their spleens harvested on day 2 post-CLP. Splenocytes were prepared and stimulated with α-CD3/α-CD28 overnight and supernatants were harvested for IFN-γ quantification via ELISA (Fig. 4A), while the relative number of IFN-γ-secreting T cells were assessed via ELISPOT (Fig. 4B). As previously described (39), CLP-induced sepsis significantly reduced the production of IFN-γ by α-CD3/α-CD28 stimulated splenic T cells, decreasing IFN-γ secretion from 6,698 ± 381 SEM pg/mL in naïve splenocytes to 1,975 ± 398 SEM pg/mL in the control peptide-treated splenocytes (Fig. 4A). However, splenocytes derived from LD01-treated mice showed significantly greater IFN-γ production (3,539 ± 584 SEM pg/mL) compared with control peptide-treated splenocytes (Fig. 4A). Difference between control peptide- and LD01-treated mice for (A) was significant after adjustment for multiple comparisons (*P < 0.05). CLP indicates cecal ligation and puncture; ELISA, enzyme-linked immunoassay; IFN, interferon.

**DISCUSSION**

While mAbs targeting checkpoint receptors have been groundbreaking in cancer treatment and have shown early promise in treating sepsis (48), their irAEs may prevent utilization for the treatment of sepsis. Our peptide-based biologic, LD01, has a shorter circulating pharmacokinetic half-life than a mAb-based therapeutic (data not shown) suggesting that it acts in a pulsatile rather than sustained manner, which may result in overall decreased irAEs. The data herein demonstrate that LD01, a linear 22-amino acid peptide, when dosed following CLP-induced sepsis, improved survival and decreased bacterial burden, in accordance with previous studies indicating that PD-1 inhibition is beneficial in experimental sepsis. Moreover, our data validate the use of a peptide-based therapeutic to target the PD-1 pathway in sepsis, as a recent study demonstrated that an α-PD-L1 peptide was efficacious in a distinct second-hit fungal sepsis model (49). Notably, the LD01 dosage and treatment schedule used in the CLP studies is likely nonoptimized as the optimal dose and regimen have yet to be fully explored. Importantly, while LD01 has a shorter half-life relative to a mAb-based therapeutic (data not shown), the peptide was nonetheless as potent in this model, which may be attributable to the peptide’s unique mode of action. Specifically, we have evidence that LD01 binds and acts through a novel allosteric site (data not shown), whereas anti-PD-1 mAbs function by

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**Fig. 4.** **LD01 treatment increases IFN-γ production by T cells.** Mice were subjected to CLP and treated with a control peptide or LD01. Control peptide and LD01 were administered at a dose of 200 μg/injection/mouse IP twice daily on days 0 and 1 following CLP. Splenocytes harvested on day 2 post-CLP or from naïve mice were prepared and stimulated with α-CD3/α-CD28 overnight. Supernatants were harvested for IFN-γ quantification via ELISA (A) and the relative number of IFN-γ-secreting T cells was assessed via ELISPOT assay (B). Data were pooled from two independent studies (n ≥ 4). Data are expressed as the mean ± SEM. Difference between control peptide- and LD01-treated mice for (A) was significant after adjustment for multiple comparisons (*P < 0.05). CLP indicates cecal ligation and puncture; ELISA, enzyme-linked immunoassay; IFN, interferon.
inhibiting the binding of the PD-L1 to the receptor. We computationally mapped this novel allosteric site on several members of the CD28 family receptors including cytotoxic T-lymphocyte-associated protein 4, CD28, and inducible T-cell costimulatory (data not shown).

Sepsis affects both innate and adaptive immune systems and T-cell exhaustion is generally considered the hallmark of immunosuppression in sepsis. The enhanced survival seen when LD01 was administered to septic mice was associated with increased supernatant levels of IFN-γ by T cells. Similar numbers of IFN-γ-producing cells were detected in control peptide- versus LD01-treated splenocytes via ELISpot, indicating that the administration of LD01 peptide restores function to T cells experiencing exhaustion due to sepsis, but does not initiate the activation of quiescent or nascent cells. In addition to reversing T-cell suppression, α-PD-1 therapy reduces sepsis-induced lymphocyte apoptosis (30). However, we did not conduct studies to evaluate whether LD01 treatment resulted in decreased T-cell apoptosis; rather, future studies are planned to assess this as well as to evaluate the effects of LD01 on T-cell proliferation.

There is a growing body of literature demonstrating that PD-1 is expressed on a wide range of immune cells, including macrophages that alter their function. Indeed, we showed improved macrophage phagocytosis following LD01 treatment, suggesting that the peptide also alleviates aspects of phagocyte immune dysfunction. Our data corroborate work in PD-1-deficient mice in which PD-1 on macrophages was shown to play a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis (8). Furthermore, the absence of PD-1 in Kupffer cells (resident liver macrophages) has shown increased phagocytic activity and cytokine release in a sepsis model (50). Accordingly, we found that MCP-1 levels from peritoneal washes were greater in LD01-treated mice, suggesting that treatment may prevent peritoneal macrophage dysfunction, as macrocytes/macrophages are a major source of MCP-1 (51). Notably, MCP-1 levels detected in the serum of LD01-treated mice relative to the CLP-only mice were similar (data not shown), suggesting that the effect of LD01 was restricted to peritoneal macrophages and did not cause systemic macrophage activation. While levels of TNF-α and IL-6 in the peritoneal washes of LD01-treated mice were unaltered at 24 h post-CLP, these cytokines may be reduced at later time points, which warrants further investigation. Additionally, we found decreased levels of MPO, an indicator of neutrophil recruitment, and a lower number of splenic esterase-positive cells following LD01 treatment. The decrease in MPO suggests that PD-1 inhibition alters the migration of granulocytic leukocytes into the spleen, thus limiting excessive tissue damage and/or organ dysfunction. Previous studies have shown that the emigration of hepatic invariant natural killer T cells to the peritoneum following sepsis is dependent upon PD-1, which appears to not result from changes in chemokine receptor expression (52, 53). In future efforts, we plan to identify the specific cell(s) that may be reduced in the spleen, as well as other tissues, following LD01 treatment of CLP-induced septic mice.

In addition to PD-1, other checkpoint receptors, including cytotoxic T-lymphocyte antigen 4 and lymphocyte activation gene 3 (LAG3), have been associated with poor outcomes in septic patients and experimental models (30, 54–57). For example, recently Lou et al. (57) showed that targeting LAG3 improved survival and bacterial clearance in septic mice, which paralleled attenuated lymphocyte apoptosis and immunosuppression. Because several checkpoint receptors can be expressed simultaneously, combination treatment of checkpoint inhibitors in some septic patients may result in a better outcome (58, 59). Thus, we have developed LAG3 peptide-based antagonists and plan to evaluate their therapeutic efficacy alone and in combination with LD01 in the CLP sepsis model. Moreover, we have linked LD01 with our lead LAG3 antagonist peptide via a polyethylene glycol linker to efficiently deliver both simultaneously as a bispecific.

In addition to checkpoint inhibitors, other immune-adjuvant therapies may be required to optimally boost patient immunity. IL-7 and OX40L, both of which target primarily T cells, were shown to effectively restore T-cell function in peripheral blood mononuclear cells from patients with multidrug-resistant bacterial sepsis (60). IL-7 treatment has also been shown to clinically raise low absolute lymphocyte counts in patients with sepsis (61), while mediating enhanced survival and stimulating increased T-cell viability and function in models of sepsis (62, 63). Thus, the combined blockade of immune checkpoints and/or costimulatory immunomodulatory therapeutics offer a novel therapeutic direction for sepsis delivering a one-two punch in counteracting immune exhaustion and simultaneously directly enhancing T-cell responses. In this regard, we constructed a DNA vector that encodes both LD01 and IL-7, which can be rapidly and cost effectively manufactured and delivered to patients while allowing more persistent nonevasive administration.

There are limitations to these current studies. The mouse CLP model of sepsis can give variable results depending upon a number of factors. The microbial flora in the cecum of mice varies depending upon diet, bedding, housing facility, etc. If the gut microbial flora are different, the particular type of bacterial strains and the virulence of the various bacteria that induce sepsis in the CLP mice will be different between studies. This microbial difference will impact the host immune response and the experimental findings. A second factor relates to the efficiency of the mouse in walling off the intra-abdominal abscess. The location of the cecum within the abdominal cavity varies in mice. In some mice, the cecum is located in the right lower quadrant. In other mice, the cecum is more mid-line or to the left lower quadrant. When the cecum is ligated and punctured, the body responds by walling off the abscess. The variable position of the cecum within the abdomen makes the efficiency of walling off the abscess different in each mouse. Thus, the severity of the injury can vary. All these factors can make for differences in the type and robustness of the accompanying immune response. We also recognize that while CLP is at present the most commonly applied experimental model of sepsis in mice, it is possible that the results might be different with another septic model. Finally, the mouse immune system has a number of differences compared with the human immune system (64). Therefore, the present findings may not necessarily occur in patients with sepsis.
Taken together, these data establish that LD01 is a novel peptide-based drug that modulates both innate and adaptive immune responses and is a viable therapeutic candidate for sepsis by alleviating immune suppression. Akin to sepsis, a wide variety of pathogens including viruses also exploit checkpoint receptors to dysregulate pathogen-specific immunity that can mediate chronic, or in some cases, lethal disease. For example, T cells from patients infected with SARS-CoV-2 show elevated levels of checkpoint receptors, including PD-L1, and reduced functional T-cell diversity (65, 66), suggesting that T-cell exhaustion contributes to the lack of antiviral immunity. Interestingly, patients with COVID-19 share several immunological similarities with bacterial sepsis (67). Consequently, the treatment of COVID-19 patients with checkpoint inhibitors may be an emerging therapeutic modality with two COVID-19 fast-tracked clinical trials using an anti-PD-1 mAb presently underway (ChiCTR2000029806; NCT04268537). Currently, anti-PD-1 mAbs are delivered intravenously, limiting their rapid deployment against COVID-19. Accordingly, we are testing the therapeutic utility of LD01 in COVID-19 animal models as LD01 can be administered readily (e.g., subcutaneously), cost effectively, and safely.

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