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Interleukin 7 and anti-programmed cell death 1 antibody have differing effects to reverse sepsis-induced immunosuppression

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Abstract

Sepsis remains a major cause of morbidity and mortality in most intensive care units. Protracted sepsis can evolve into a state of profound immunosuppression characterized by secondary infections, frequently with opportunistic-type pathogens. Immuno-adjuvant therapy is currently being evaluated as a novel treatment for patients with sepsis. Two of the most promising immuno-adjuvants are interleukin-7 (IL-7) and anti-programmed cell death 1 antibody (anti-PD-1). Both IL-7 and anti-PD-1 have been reported to boost host immunity and improve outcomes in patients with viral infections and cancer. The purpose of this study was to define the immunological mechanisms of action of IL-7 and anti-PD-1 in the two-hit sepsis model of cecum ligation and puncture followed by *Candida albicans*. In addition, we examined whether combined treatment with IL-7 and anti-PD-1 provided any additive beneficial effects in reversing immune dysfunction. The present findings demonstrated that IL-7 and anti-PD-1 had differing effects on innate and adaptive immune functions. Compared to anti-PD-1, IL-7 increased lymphocyte proliferation, expression of lymphocyte adhesion molecules, lymphocyte function-associated antigen 1 and very late antigen-4, interferon- γ production, and CD28 expression on splenic CD8+ T cells. In contrast, anti-PD-1 appeared to have a greater effect on major histocompatibility complex class II expression on splenic macrophages and dendritic cells than IL-7. Combined treatment with IL-7 and anti-PD-1 produced additive effects on CD28 expression, lymphocyte proliferation, and splenic secretion of interferon- γ . In conclusion, the present study shows differences in immunomodulatory actions between IL-7 and anti-PD-1, and provides a potential rationale for combining IL-7 and anti-PD-1 in the therapy of sepsis.

Keywords

Candida; endotoxin; innate immunity; adaptive immunity; shock

INTRODUCTION

Sepsis is the major cause of mortality in most intensive care units and results in more than 250,000 deaths annually in the United States (1, 2). Although both pro- and anti-inflammatory responses occur after sepsis onset, there is a shift to a predominant anti-inflammatory and immunosuppressive phase if sepsis persists (3–7). Studies have shown that treatment with immunomodulatory agents that boost host immunity can improve survival in clinically-relevant animal models of sepsis (3, 8). Two of the most promising immunoadjuvants in sepsis are interleukin-7 (IL-7) and anti-programmed cell death 1 antibody (anti-PD-1) (9–12). Work from multiple independent laboratories has demonstrated that both IL-7 and anti-PD-1 can reverse sepsis-induced immunosuppression and improve survival in bacterial and fungal models of sepsis (9–11, 13, 14). Importantly, IL-7 and anti-PD-1 have been effective in augmenting host immunity and improving morbidity and/or mortality in patients with a variety of disorders including various viral infections (3, 15), e.g., HIV (16), hepatitis C (17), hepatitis B (18), progressive multifocal leukoencephalopathy (19), and in cancer (20). In this regard, anti-PD-1 antibody was recently approved by the FDA because of its efficacy in boosting immunity in patients with refractory metastatic melanoma and thereby inducing long term remission (<http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm412802.htm>). Large multi-center trials of anti-PD-1 as an immuno-adjuvant are currently ongoing in other types of cancer and in infectious diseases and similar trials are planned for IL-7 (8, 15, 20).

The purpose of this study was to examine the immunologic mechanisms of action of IL-7 and anti-PD-1 in a clinically relevant animal model of sepsis that reproduces the immunosuppressive phase of the disorder. The two-hit sepsis model of cecum ligation and puncture (CLP) followed by *Candida albicans* was utilized because fungal organisms are now the third to fourth most common cause of bloodstream infections in many intensive care units and because recent studies indicate that immuno-adjuvant therapy in fungal infections may be a viable additional therapeutic strategy in this highly lethal disease (10, 11, 21). IL-7 is a pluripotent cytokine that is essential for T cell development and function (15). It has potent anti-apoptotic properties and increases T cell migration to sites of infection (3, 5). Anti-PD-1 acts to reverse T cell “exhaustion” and can induce T cell proliferation and restore cytokine production (3, 8). Although IL-7 and anti-PD-1 both act to improve host immunity and lead to increased pathogen clearing, they have differing mechanisms of action with distinct effects on innate and adaptive immunity. Furthermore, their exact effects on key sepsis-induced immune defects have not been rigorously defined. A second purpose of the study was to evaluate whether combined treatment with IL-7 and anti-PD-1 in sepsis produced any additive beneficial effects in reversing immunosuppression in sepsis. Combination therapy with different immunoadjuvants is one of the most exciting advances in oncology and may be equally efficacious in infectious disorders (22, 23). Thus, differentiating the effects of IL-7 and anti-PD-1 on key host defense mechanisms will define

their precise mechanism of action and shed light on whether combination therapy with IL-7 and anti-PD-1 might be efficacious in sepsis.

MATERIAL AND METHODS

Mice

Eight- to ten-week-old male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

Sepsis model with secondary *Candida* blood stream infection: Two-hit model of sepsis

The two-hit sepsis model of CLP induced polymicrobial peritonitis followed by *Candida albicans* has been developed so that it reflects the impaired immune status of patients with protracted sepsis who have secondary nosocomial fungal infection (24). Our laboratory has characterized the timing and mechanisms of the immunosuppressive state in this prolonged model of serious infection (24). For CLP, mice were anesthetized with isoflurane and a midline incision performed (see Fig. 1). The cecum was ligated, punctured, and the abdomen closed. One ml of 0.9% normal saline mixed with 0.05 mg/kg bodyweight buprenorphine was administered immediately postoperatively. Imipenem (25 mg/kg) was administered subcutaneously 4 hours postoperatively.

Candida albicans (ATCC MYA-2430) was grown overnight in Difco™ Sabouraud dextrose broth medium. Cells were harvested, washed, and suspended in saline to obtain an optical density of 0.3A₆₀₀ as previously described (11). Three days post-CLP, surviving mice were injected intravenously with ~30 µl of 0.3 A₆₀₀ *Candida albicans* suspension. Note that over 90% of mice survived the CLP in this study, and our previous study has shown that surviving mice at three days post-CLP when they are challenged with the secondary fungal infection were in an immunosuppressive phase (24). A single dose of fluconazole (200 µg/mouse) was intraperitoneally administered at day 5 and 6 post-CLP.

Treatment by IL-7 and anti-PD-1 antibody

Recombinant human IL-7 was provided by Cytheris (Rockville, MD) and prepared as described previously (25). 2.5 µg of IL-7 in 100 µl of normal saline was administered subcutaneously on 5 consecutive days beginning at day 4 post-CLP (24 hours after *C. albicans* injection) (see Fig. 1). Mice in the control group received saline diluent. Anti-mouse PD-1 antibody was purchased from Bio X Cell (West Lebanon, NH) and was diluted with sterile 1X phosphate buffered saline to a final concentration of 1 mg/ml. Mice received 200 µg anti-PD-1 antibody intraperitoneally on day 4 and day 8 post-CLP (Fig. 1). Mice in the control group received saline diluent.

Combination therapy with IL-7 and anti-PD-1 antibody was examined to evaluate for potential additive effects of these 2 agents. The concentrations of IL-7 and anti-PD-1, method, and timing of administration of IL-7 and anti-PD-1 antibody are as previously described (10, 11). For the control group, 100 µl of saline was administered subcutaneously

on 5 consecutive days from day 4 post-CLP (within 24 hours after *C. albicans* injection) through day 8 post-CLP.

Spleens and peripheral lymph nodes (axillary, cervical, and inguinal) were harvested from naive and septic animals at day 9 post-CLP (6 days after *C. albicans* injection), cells were isolated and underwent immunostaining followed by flow cytometric analysis. The survival proportion at day 9 was 83.3% (25/30) in saline treated mice; 82.8 % (24/29) in IL-7; 92.9% (26/28) in anti-PD-1; and 93.1% (27/29) in combination of IL-7 and anti-PD-1.

Flow cytometric analyses for cell surface markers

Antibodies were purchased as follows: BioLegend (San Diego, CA): anti-CD3-FITC (Cat. # 100306), anti-Ly6G-PE (Cat. # 127615), anti-Interferon- γ -PE (Cat. # 505808), anti-Ki-67-PE (Cat. # 652404), anti-I-A/I-E-PerCP/Cy5.5 (Cat. # 107626), anti-CD8-PerCP/Cy5.5 (Cat. # 100734), anti-CD11c-APC (Cat. # 117310), and anti-CD4-APC (Cat. # 100516); eBioscience (San Diego, CA, USA): anti-DX5-FITC (Cat. # 11-5971-85), anti-CD11a-FITC (Cat. # 11-0111-85), anti-CD49d-PE (Cat. # 12-0492-83), and anti-CD28-PE (Cat. # 12-0281-81); and Caltag Laboratories (Buckingham, UK): anti-F4/80-FITC (Cat. # MF48020).

Flow cytometric analysis was performed on FACScan (Becton Dickinson, San Jose, CA) and Cell Quest Pro software (BD Pharmingen, San Diego, CA) was utilized to analyze data. Major histocompatibility complex class II (MHC II) expression was quantitated on macrophages (F4/80+) and dendritic cells (CD11c+). CD4 and CD8 T cell expression of the positive co-stimulatory molecule CD28 was quantitated. Lymphocyte adhesion molecule expression for lymphocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4) were quantitated on CD4 and CD8 T cells. Splenocytes and cells in peripheral lymph nodes were prepared and were stained with the following combinations of fluorochrome-conjugated antibodies: F4/80-FITC, Ly6G-PE, I-A/I-E (MHC II)-PerCP-Cy5.5, and CD11c-APC; CD11a (LFA-1)-FITC, CD49d (VLA-4)-PE, CD8-PerCP-Cy5.5, and CD4-APC; and CD3-FITC, CD28-PE, CD8-PerCP-Cy5.5, and CD4-APC.

Determination of cell proliferation: Ki-67 staining

Ki-67 staining was done according to the manufacturer's protocol for intracellular (nuclear) proteins for flow cytometry provided by eBioscience (San Diego, CA).

Quantification of interferon gamma (IFN- γ) - intracellular staining

Intracellular staining for cytoplasmic IFN- γ was performed using the manufacturer's recommendations and as previously described (10).

Quantitation of supernatant IFN- γ via ELISA

Splenocytes (5×10^6 cells) were incubated overnight with anti-CD3 and anti-CD28 antibodies. The following morning, supernatant of cell suspension was harvested. ELISA was performed using IFN- γ Mouse Antibody Pair (Life Technologies, Grand Island, NY), according to the manufacturer's instruction. The μ Quant Scanning Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT) was used for analysis (10).

Statistical analysis

Data were analyzed by non-parametric ANOVA with Kruskal-Wallis test among the following four treatment groups: saline (control), IL-7, anti-PD-1, and combination with IL-7 and anti-PD-1. The statistical software Prism (GraphPad, San Diego, CA, USA) was used. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Effects of anti-PD-1 and IL-7 on MHC II expression

MHC II molecules present antigens derived from extracellular pathogens to CD4 and CD8 T cells and thereby help initiate and coordinate the host immune response to infection. Monocyte and macrophage MHC II expression is typically decreased in sepsis (26, 27). We quantitated effects of IL-7 and anti-PD-1 on MHC II expression on macrophages and dendritic cells from spleen and peripheral lymph nodes. Sepsis caused a decrease in the percentage of macrophages and dendritic cells that were positive for MHC II expression (Fig. 2). Compared to septic mice treated with saline, anti-PD-1 treated mice had similar percentage of MHC II positivity in splenic macrophages and dendritic cells (Fig. 2B). The MHC II positivity tended to be lower in mice treated with IL-7 and combination of IL-7 and anti-PD-1 than in those treated with saline.

Splenic macrophage and dendritic cell mean fluorescence intensities (MFIs), a reflection of the degree of expression of MHC II on a per cell basis, were likely to be higher in both the IL-7 and anti-PD-1 treated septic mice compared to saline-treated septic mice (Fig. 2A and B). Combination therapy with IL-7 and anti-PD-1 showed a similar but non-statistically significant effect to increase MHC II MFI on splenic macrophages and dendritic cells.

In contrast to the spleen, there seemed to be no effect of either IL-7 or anti-PD-1 to increase the percentage of peripheral lymph node macrophages or dendritic cells that were positive for MHC II in sepsis (Fig. 2C). Mice that received treatment with IL-7 alone and combination of IL-7 and anti-PD-1 had lower MHC II MFI compared to septic mice that were treated with saline (Fig. 2C).

Differential effect of IL-7 and anti-PD-1 on CD28 in splenic and peripheral node T cells

CD28 is a co-stimulatory molecule present on T cells that is required for optimal T cell activation when antigens are presented by APCs. Loss in T cell CD28 expression has been associated with impaired immune response to pathogens (28). Fig. 3 shows comparison of the effect of IL-7, anti-PD-1, or combination therapy on CD4 and CD8 T cell expression of CD28. Approximately 90% of splenic CD4 T cells were positive for CD28 in all groups tested, i.e., naïve, septic, and septic mice treated with the three immuno-adjuvant therapies (Fig. 3). Sepsis caused increases in MFIs for CD28 on splenic CD4 and CD8 T cells which were not further increased by IL-7, anti-PD-1, or combination therapy. In contrast to splenic CD4 T cells, only ~ 40% of splenic CD8 T cells from naïve mice were positive for CD28 expression. Although sepsis had no effect on the percentage of CD8 T cells that were positive for CD28, the percentage of splenic CD8 T cells from septic mice treated with IL-7 increased to ~ 60% and this was statistically different than septic mice which were treated

with anti-PD-1 alone; ($p < 0.05$) (Fig. 3A and C). Combination therapy with IL-7 and anti-PD-1 did not have any additive effect on CD28 expression compared to IL-7 alone.

Baseline expression of CD28 was considerably lower on peripheral lymph node CD4 and CD8 positive T cells; the percentage of CD4 and CD8 T cells that were positive for CD28 was on average between 10–20% (Fig. 3D). In contrast to the effect observed in the spleen, anti-PD-1 but not IL-7 had a statistically significant effect to increase the percentage of CD4 T cells that were positive for CD28. Combination therapy with IL-7 and anti-PD-1 had a greater effect to increase CD28 expression on CD4 and CD8 T cells compared to therapy with IL-7 or anti-PD-1 alone (Fig. 3B and D). Anti-PD-1, but not IL-7, increased the MFI for CD28 on CD4 and CD8 T cells (Fig. 3B and D).

IL-7 increases T cell proliferation compared to anti-PD-1

Because of the profound apoptosis-induced loss of lymphocytes in sepsis (6, 29), therapies that induce T cell proliferation may significantly improve the immunologic response to sepsis (3). Ki-67 is a nuclear protein that is used as a marker of increased cellular proliferation (30). Fig. 4 demonstrates the effects of IL-7 and anti-PD-1 on T cell proliferation. Compared to naïve, i.e., non-operated, uninfected mice, splenic CD4 T cells Ki-67 positivity was increased in septic mice (Fig. 4B). Splenic CD4 T cell Ki-67 positivity tended to be higher in septic mice treated with IL-7 than in those treated with anti-PD-1. The effects of combination therapy with IL-7 and anti-PD-1 were similar to those of IL-7 monotherapy but these effects on proliferation on splenic CD4 and CD8 T cells did not reach statistical significance (p values equaled 0.066 and 0.066, respectively).

A similar trend in Ki-67 positivity was seen in peripheral lymph node CD4 and CD8 T cells. Compared to septic mice that did not receive immuno-adjuvant therapy, the mean percentage of Ki-67 positivity in CD4 T cells was significantly higher in mice treated with combination therapy (IL-7 plus anti-PD-1) ($p = 0.004$), and those treated with IL-7 also showed the higher percentage (Figs. 4A and C). Anti-PD-1 showed a lesser effect to increase Ki-67 in peripheral node CD4 or CD8 T cells than IL-7 or combination of IL-7 and anti-PD-1.

IL-7 but not anti-PD-1 increases key lymphocyte trafficking molecules: LFA-1 and VLA-4

LFA-1 and VLA-4 are important adhesion molecules that are expressed on T cells and help guide lymphocytes to sites of infection. The importance of these adhesion molecules has been demonstrated in studies showing that mice deficient in LFA-1 have increased lethality in *Streptococcus pneumoniae* induced sepsis (31). In spleens, the mean values of LFA-1 positivity on CD4 and CD8 T cells were greater than 95% in all five groups (Fig. 5C). IL-7 and combination of IL-7 and anti-PD-1 increased LFA-1 MFIs of splenic CD4 and CD8 T cells compared to septic mice treated with anti-PD-1. Furthermore, IL-7 and combination of IL-7 and anti-PD-1 increased the percentage of CD4 T cells positive for VLA-4 and VLA-4 MFI compared with anti-PD-1 treated group (Fig. 5C). There were no significant differences in VLA-4 percent positivity and the MFI in CD8 T cells in the septic groups, i.e., septic mice not receiving immunotherapy, septic mice treated with either IL-7, anti-PD-1, or combination therapy.

The effect of IL-7 and combination therapy with IL-7 and anti-PD-1 on the MFI of LFA on peripheral node CD4 and CD8 T cells was similar to that occurring in spleen (Fig. 5C and D). Likewise, anti-PD-1 had no effect on LFA-1 MFI or percent positivity of VLA-4 in peripheral node CD4 or CD8 T cells. Interestingly, there was a trend toward an additive effect of IL-7 and anti-PD-1 to increase the MFI for LFA-1 and percent positivity of VLA-4 in CD4 T cells in septic mice that received combination therapy with both IL-7 and anti-PD-1 compared to monotherapy with IL-7 alone (Fig. 5D).

IL-7 and combination of IL-7 and anti-PD-1 increase IFN- γ production

IFN- γ is produced by CD4 T, CD8 T, and NK cells and is a potent activator of monocytes and macrophages which play key roles in phagocytosis and pathogen killing in sepsis (32). IL-7 and anti-PD-1 have been reported to increase cellular IFN- γ production (10, 11). Both intracellular IFN- γ production and stimulated splenocyte secretion of IFN- γ were quantitated to evaluate the percentage of cells producing IFN- γ and the relative quantity of IFN- γ produced. Results showed that compared to cells from septic mice that received no immuno-adjuvant therapy, IL-7 significantly increased the percentage of CD8 T cells that were positive for IFN- γ , and the percentages of both CD4 and CD8 T cells were significantly increased by combination therapy with IL-7 and anti-PD-1 (Fig. 6A and B). There was a trend toward increased IFN- γ production in mice treated with anti-PD-1 alone, although statistical significance was not reached. There was no effect of IL-7 or anti-PD-1 on NK cell IFN- γ . Data from stimulated splenocytes showed that combination therapy with IL-7 and anti-PD-1 significantly boosted IFN- γ production compared to septic mice that were not treated with either IL-7 or anti-PD-1 and those treated with anti-PD-1 alone.

DISCUSSION

The purpose of this study was to compare and contrast the effects of IL-7 and anti-PD-1 on potential beneficial immunologic mechanisms in a clinically relevant two-hit model of sepsis. IL-7 and anti-PD-1 are potent immuno-adjuvants that have shown efficacy in numerous infectious disorders both in animal models and in patients. Their effects to improve morbidity and mortality have occurred in infections due to bacterial, fungal, and viral microorganisms. Combination therapy with immuno-adjuvants is now at the forefront in oncology and may represent a new therapeutic approach in infectious disorders as well. Consequently, we undertook this investigation to determine if IL-7 and anti-PD-1 were working by complementary or more disparate mechanisms and whether combination therapy had any additive or antagonistic effects. MHC II expression, CD28 expression, lymphocyte adhesion marker expression, cell proliferation, and IFN- γ production were quantitated in mice following therapy with IL-7 or anti-PD-1 either alone or in combination.

Sub-lethal peritonitis followed by *C. albicans* sepsis was used because our group has extensively characterized the immune defects that occur during this infection and previously reported that IL-7 and anti-PD-1 improve survival in this model (10, 11, 24). Despite excellent anti-fungal therapy directed against most *Candida* infections, mortality remains >30–40% (33) suggesting that a defect in host immunity may be contributing to mortality by these pathogens. Recently, immunotherapy with IFN- γ has been reported to

have beneficial effects to restore immune function in patients with fungal sepsis and a clinical trial is ongoing (34). IL-7 and anti-PD-1 are potent immuno-adjuvants which may also be useful in the therapy of fungal sepsis. Thus, the present study helps to define their potential role in this lethal disorder.

The results indicated that IL-7 and anti-PD-1 do have differential effects on several key immune mediators. Although both anti-PD-1 and IL-7 tended to increase the MFI of MHC II expression for splenic macrophages, anti-PD-1 appeared to have a greater effect compared to IL-7 (Fig. 2). In contrast, IL-7 increased splenic and peripheral node T cell proliferation while anti-PD-1 had no effect on cellular proliferation (Fig. 4). Moreover, IL-7 increased expression of LFA-1 and VLA-4, leukocyte adhesion molecules, on CD4 and CD8 T cells while anti-PD-1 had minimal effects (Fig. 5). IL-7 also boosted both the percentage of IFN- γ producing cells and splenocyte secretion of IFN- γ ; there was a trend toward increased IFN- γ production in anti-PD-1 treated mice but, it did not reach statistical significance (Fig. 6). While IL-7, but not anti-PD-1, increased CD28 expression on splenic CD8 T cells, anti-PD-1 increased peripheral node CD4 and CD8 expression of CD28.

While IL-7 and anti-PD-1 antibody act on common signaling pathways, they also have unique effects which are not shared. IL-7 directly and anti-PD-1 indirectly act to increase phosphoinositide 3-kinase (PI3K) (15, 35). PI3K activates AKT which leads to increased IFN- γ production, upregulation of anti-apoptotic Bcl-2 family members, and downregulation pro-apoptotic Bcl-2 family members (15, 36, 37). IL-7, but not anti-PD-1 antibody, also activates STAT5 and CDC25A (cell division 25 homologue A) which increases T cell proliferation (15). One unique effect of anti-PD-1 may be its ability to increase macrophage activation. Although few studies have been performed, it is speculated that interaction of T cells expressing PD-1 with PD-L1 expressing macrophages results in an inhibitory signal to the macrophage that suppresses its function (38, 39). Therefore, blocking PD-1 interaction with PD-L1 by anti-PD-1 may lead to macrophage activation, improved pathogen killing (13, 40), and its enhanced chemotaxis through increased monocyte chemotactic protein 1 (41).

Another potential etiology for the contrasting effects of IL-7 and anti-PD-1 on immune cells may be related to differences in expression of IL-7 and PD-1 receptors. IL-7 receptors are expressed predominantly on naïve and central memory T cells while PD-1 receptor is expressed on effector T cells. It is likely that differences in the relative concentrations of T cell subsets of naïve, memory, and effector T cells in spleen and peripheral lymph nodes may be an additional reason for differential effects of IL-7 and anti-PD-1 antibody on immune mediators in spleen and peripheral lymph node cells.

In conclusion, the present study provides a potential rationale for combining IL-7 and anti-PD-1 antibody in the therapy of sepsis. The present findings demonstrate that IL-7 and anti-PD-1 have differing effects on key immunologic mediators and cell function. Therefore, use of dual therapy with IL-7 and anti-PD-1 might lead to additional effects that would not occur with either agent alone. Further supporting the concept of joint therapy with IL-7 and anti-PD-1 are the data showing that there was an additive effect on CD28 expression, lymphocyte proliferation, and IFN- γ production when mice received combination therapy

versus single agent therapy treatment. In this regard, it is significant that a clinical trial in patients with advanced or metastatic tumors is planned and will consist of combination therapy with IL-21, a closely related family member of IL-7, and anti-PD-1 (<http://clinicaltrials.gov/ct2/results?term=IL-21+and+anti-PD-1&Search=Search>).

It is likely that once advances occur in the ability to immunophenotype patients with sepsis, i.e., determine if patients are in the immunosuppressive phase of sepsis and identify particular defects that are present in patient innate and/or adaptive immunity, that a variety of immuno-adjuvants will be employed concurrently. Based upon the results in the present study as well as a large body of supporting literature, we speculate that IL-7 and anti-PD-1 will be an integral part of that therapy.

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FIG 1. Experimental design

Mice underwent cecal ligation and puncture (CLP) on day 0. One mg of imipenem was subcutaneously administered 4 hours after CLP. Three days post-CLP, ~30 µl of 0.3 A₆₀₀ *Candida albicans* suspension was injected intravenously. IL-7-treated mice received 2.5 µg of IL-7 for 5 consecutive days post-CLP. In mice treated with anti-PD-1 antibody, 200 µg of antibody, were intraperitoneally administered on days 4 and 8. Control group mice received 100 µl saline subcutaneously on consecutive 5 days from day 4 post-CLP. Nine days post-CLP, mice were sacrificed and spleens and peripheral lymph nodes were harvested. Anti-PD-1, anti-programmed cell death 1 antibody; IL-7, interleukin 7; CLP, cecum ligation and puncture.

FIG 2. Effects of anti-PD-1 and IL-7 on MHC class II molecule expression

(A) Representative histograms of MHC II expression on splenic macrophages in each treatment group are shown. Gray line represents saline (control); pink, IL-7; blue, anti-PD-1; and green, combination of IL-7 and anti-PD-1. Compared to saline-treated mice, the average mean fluorescent intensity of macrophages for MHC II was increased in mice treated with IL-7, anti-PD-1, or combination therapy of IL-7 and anti-PD-1.

(B) Dot plots display positivity of MHC II expression on splenic macrophages and dendritic cells (DCs). Results represent combined findings from three separate studies. The MFIs of macrophages and dendritic cells in mice treated with IL-7 and anti-PD-1 were higher than saline-treated mice. The percentage of MHC II positive cells in anti-PD-1-treated mice was higher than IL-7.

(C) Dot plots display positivity of MHC II expression for peripheral lymph node macrophages and DCs. Results represent combined findings from two separate experiments. Compared with the saline-treated mice, treatment with IL-7, anti-PD-1, or combination therapy of IL-7 plus anti-PD-1 did not increase MHC II percentages or MHC II MFIs on macrophages and the percentages on DCs. The MFI of DCs were lower in mice treated with IL-7 and combination of IL-7 and anti-PD-1 compared to those treated with saline. MHC II, major histocompatibility complex class II; IL-7, interleukin 7; aPD-1, anti-programmed cell death 1 antibody; and MFI, mean fluorescent intensity. Macrophages were identified by positivity for F4/80+ and characteristic forward and side scatter properties. Dendritic cells were identified by positivity for CD11c+ and characteristic forward and side

scatter properties. Horizontal lines in dot charts represent mean values of the treatment groups.

FIG 3. Differential effect of IL-7 and anti-PD-1 on CD28 in splenic and peripheral node T cells
 (A) Representative flow cytometric findings for CD28 expression on splenic CD3+CD8+ T cells. The percentage of CD3+CD8+ splenocytes that were positive for CD28 expression was greater in septic mice treated with IL-7 and combination of IL-7 and anti-PD-1 than mice treated with saline and anti-PD-1.
 (B) Representative flow cytometry findings for CD28 expression on peripheral lymph node CD3+CD4+ T cells. The percentage of CD3+CD4+ peripheral node T cells was increased in septic mice treated with PD-1 and combination of IL-7 and anti-PD-1 compared to mice treated with saline or IL-7.

(C) These dot plots show percent positivity of CD28 expression on splenic CD3+CD4+ and CD3+CD8+ T cells. Values represent results from two separate experiments. The mean value of CD28 positivity on CD3+CD4+ T cells was more than 90% in all groups. IL-7 and combination of IL-7 and anti-PD-1 increased percentage of CD28 expression on CD3+CD8+ T cells compared with anti-PD-1.

(D) These dot plots show percent positivity of CD28 expression on peripheral lymph node CD3+CD4+ and CD3+CD8+ T cells. Values represent results from two separate experiments. Anti-PD-1 increased CD28 expression on CD3+CD4+ and IL-7 increased that on CD3+CD8+ T cells more frequently than saline. In particular, the additive effect of combination of IL-7 and anti-PD-1 was found in CD3+CD4+ and CD3+CD8+ T cells. IL-7, interleukin 7; aPD-1, anti-programmed cell death 1 antibody; MFI, mean fluorescent intensity. Horizontal lines in dot plots represent mean values of the treatment groups.

FIG 4. IL-7 increases T cell proliferation compared to anti-PD-1

(A) Representative flow cytometric findings for intracellular Ki-67 of peripheral lymph node CD3+CD4+ T cells. Compared to the saline-treated group, mice treated with combination of IL-7 and anti-PD-1 had greater percentages of cell positive for Ki-67.

(B) These dot plots display percent positivity for Ki-67 in splenic CD3+CD4+ and CD3+CD8+ T cells. Values represent from two separate experiments. The mean percentages of Ki-67 positivity in CD3+CD4+ T cells in mice treated with IL-7 and combination of IL-7 and anti-PD-1 was higher than in mice treated with saline or anti-PD-1. The trends between CD3+CD4+ and CD3+CD8+ T cells were similar.

(C) These dot plots display the percentage of cells positive for Ki-67 in peripheral lymph node CD3+CD4+ and CD3+CD8+ T cells. Results represent findings from one experiment. The effect of IL-7 to increase Ki-67 positivity was more evident in peripheral lymph nodes than in splenocytes. Combination of IL-7 and anti-PD-1 increased CD3+CD4+ T cell Ki-67 positivity more significantly than saline and anti-PD-1 alone.

IL-7, interleukin 7; aPD-1, anti-programmed cell death 1 antibody; and MFI, mean fluorescent intensity. Horizontal lines in dot charts represent mean values of the treatment groups.

FIG 5. IL-7 but not anti-PD-1 increases lymphocyte trafficking molecules: LFA-1 and VLA-4

(A) Representative flow cytometric findings for VLA-4 in different treatment groups on peripheral lymph node CD4+ T cells. Results show an increased expression of VLA-4 in mice treated with IL-7 and combination of IL-7 and anti-PD-1 compared to mice treated with saline or anti-PD-1 alone.

(B) Representative flow cytometry histograms demonstrating LFA-1 expression on peripheral lymph node CD4+ T cells. Compared to saline-or anti-PD-1-treated mice, there was an increase in the mean fluorescence intensity (X axis) in cells from mice treated with IL-7 or combination therapy of IL-7 and anti-PD-1.

(C) Dot plots display percentage positivity for LFA-1 and VLA-4 expression on splenic CD4⁺ and CD8⁺ T cells. Results represent findings from two separate experiments. The mean values of LFA-1 positivity on CD4⁺ and CD8⁺ T cells were more than 95% in all groups. IL-7 and combination of IL-7 and anti-PD-1 increased LFA-1 mean fluorescent intensities of CD4⁺ and CD8⁺ T cells compared with saline and anti-PD-1. Regarding VLA-4 expression, IL-7 increased the percentage of CD4⁺ T cells compared to saline- and anti-PD-1-treated mice. The MFI of CD4⁺ T cells in mice treated with combination of IL-7 and anti-PD-1 was higher than anti-PD-1-treated mice.

(D) Dot plots display percentage positivity of LFA-1 and VLA-4 expression on peripheral lymph node CD4⁺ and CD8⁺ T cells. Results represent findings from two separate experiments. The trend for LFA-1 was similar to that observed in splenocytes. Results for VLA-4, showed that IL-7 and combination of IL-7 and anti-PD-1 increased the percent positivity on CD4⁺ T cells more effectively than anti-PD-1 alone. The VLA-4 MFI of CD4⁺ T cells was higher in anti-PD-1 treated mice than in IL-7 and combination of IL-7 and anti-PD-1.

IL-7, interleukin 7; aPD-1, anti-programmed cell death 1 antibody; LFA-1, lymphocyte function-associated antigen 1; VLA-4, very late antigen-4; MFI, mean fluorescent intensity. CD11a positive cells was identified as LFA-1 positive cells. CD49d positive cells was identified as VLA-4 positive cells. Horizontal lines represent mean values of the treatment groups.

FIG 6. IL-7 and combination of IL-7 and anti-PD-1 increase IFN- γ production

(A) Representative flow cytometry dot plots demonstrating intracellular IFN- γ production in splenic CD8+ T cells. The percentage of IFN- γ positive cells in three active treatment groups was increased compared to saline-treated mice. In particular, IL-7 and combination of IL-7 and anti-PD-1 increased IFN- γ production to the greatest degree.

(B) Dot plots display the percentage positivity for intracellular IFN- γ in splenic NK cells, CD4+, and CD8+ T cells. Results represent findings from two separate experiments. IL-7 and combination of IL-7 and anti-PD-1 significantly increased percentage of IFN- γ in CD8+ T cells compared to saline treatment. Similar trend was observed in CD4+ T cells. There was no effect of IL-7 or anti-PD-1 on NK cell IFN- γ .

(C) Dot plots display results for IFN- γ production in incubated stimulated splenocyte suspensions. Splenocyte suspension supernatants were obtained and IFN- γ quantitated by ELISA. There was an increase in IFN- γ production in splenocytes from mice treated with combination therapy of IL-7 and anti-PD-1 compared to saline- and anti-PD-1-treated mice. IFN- γ , Interferon- γ ; IL-7, interleukin 7; aPD-1, anti-programmed cell death 1 antibody; and MFI, mean fluorescent intensity. DX5+ cells was identified as NK cells. Vertical lines in dot charts represent mean values of the treatment groups.