IL-7 treatment accelerates neutrophil recruitment through γδ T cell IL-17 production in a murine model of sepsis

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Summary: IL-7 can mediate the cross talk between Th1 and Th17 lymphocytes during sepsis such that neutrophil recruitment and bacterial clearance is improved while early tissue injury is not increased.

Running Title: IL7 improves septic response through gamma-delta cells

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Abbreviations:
AST – Aspartate aminotransferase
Bcl-2 – B cell lymphoma 2
CLP – Cecal ligation and puncture
CXCL1/KC – Chemokine ligand 1
CXCL10/IP-10 – Chemokine ligand 10, Interferon-inducible protein 10
DTH – Delayed-Type Hypersensitivity
IACUC - Institutional Animal Care and Use Committee
IFN-γ – Interferon-gamma
IL – Interleukin
MPO – Myeloperoxidase
rhIL-7 – Human recombinant interleukin-7
TCR – T cell receptor
WT – Wild type
ABSTRACT:
The sepsis syndrome represents an improper immune response to infection and is associated with an unacceptably high rate of mortality and morbidity. The interactions between T cells and the innate immune while combating sepsis are poorly understood. In this report, we observed that treatment with the potent, anti-apoptotic cytokine, IL-7, accelerated neutrophil recruitment and improved bacterial clearance. We first determined that T cells were necessary for the previously observed IL-7-mediated enhanced survival. Next, IL-7 increased Bcl-2 expression in T cells isolated from septic mice as early as 3 hours following treatment. This treatment resulted in increased IFN-γ and IP-10 production within the septic peritoneum together with local and systemic increases of IL-17 in the IL-7 treated mice. We further demonstrate that the increased IL-17 was largely due to increased recruitment and production by γδ T cells, which express CXCR3. Consistent with increased IL-17 production, IL-7 treatment increased CXCL1/KC production, neutrophil recruitment, and bacterial clearance. Significantly, end organ tissue injury was not significantly different between vehicle and IL-7 treated mice. Collectively, these data illustrate that IL-7 can mediate the cross talk between Th1 and Th17 lymphocytes during sepsis such that neutrophil recruitment and bacterial clearance is improved while early tissue injury is not increased. Altogether, these observations may underlay novel potential therapeutic targets to improve the host immune response to sepsis.
INTRODUCTION:

Sepsis entails a complex immune process involving both the innate and adaptive immune systems, which can effect concurrent changes on the host (39, 56). The immune response to infection is typically modeled by phagocytic cells as first responders for the early removal of pathogenic bodies, while the adaptive system becomes involved later for mop-up duty and maintenance of a long-term immune response against the current pathogens. Recent literature suggests an early role for the adaptive immune system in mediating sepsis (30); however, a paucity of effective sepsis therapies suggests a gap in our knowledge concerning crosstalk and the respective roles of the adaptive and innate immune during the host response to sepsis. An increased understanding of the complex interplay of these immune response arms are critical given the annual toll this diagnosis places on both human life and healthcare dollars (2, 41).

\( \gamma \delta \) T cells represent a small subset of cells with a restricted T cell receptor (TCR) repertoire of ligands. These cells are widespread in epithelial-rich tissues such as the skin and intestine, with cytokine production dictated by tissues in which they reside (8, 12, 20, 40, 54). Contradictory results have been published on the role of \( \gamma \delta \) T cells during sepsis, suggesting that these cells become more pathogenic as the severity of sepsis model used increases (6, 10, 13). Our previous report demonstrates that \( \gamma \delta \) T cells play a key role in neutrophil recruitment to the site of infection and control of bacterial load following CLP. Recently, \( \gamma \delta \) T cells have been implicated as the major producer of the cytokine IL-17 during sepsis (13). IL-17A is part of the IL-17 family of cytokines produced by diverse cell populations such as conventional CD4 and CD8 T cells, as well as \( \gamma \delta \) T cells (58). While the protective or pathogenic role of IL-17 in sepsis is not yet clear (13, 16), it is
known that IL-17 mediates recruitment of myeloid cells through downstream production of CXCL1/KC and CXCL2/MIP-2, presumably through the stabilization of mRNA (18). Additionally, IL-17R is necessary for neutrophil recruitment to the site of infection and control of bacterial load following CLP (16). The functions of IL-17 on myeloid cell recruitment, along with its production by T cells during sepsis, make this cytokine an interesting target in mediating the cross-talk that occurs between the innate and adaptive responses to sepsis.

The suggestion of early cross-talk between the innate and adaptive immune responses extends from recent reports indicating the necessity of T cells in bacterial clearance following infection (29, 37, 54, 55). Additionally, T cell activation was newly linked to bacterial clearance through increased neutrophil oxidative burst and phagocytosis (29). These findings provide a bridge to literature demonstrating worsened outcomes in septic T cell deficient mice (26) that are reversed with adoptive transfer of T cells (47). Further, it is known that T cells undergo apoptosis within the first 24 hours after onset of sepsis (25, 27, 57) and the degree of apoptosis correlates with the sepsis severity (24, 32). Additionally, remaining T cells demonstrate reduced functionality illustrated by decreased production of interferon-gamma (IFN-γ) (28). Taken together, these results provide mechanistic insight into how prevention of T cell apoptosis improves survival in a murine model of sepsis (26), and illustrate a therapeutic avenue for reduction of morbidity and mortality associated with this disease process.

The cytokines IL-2, IL-7, and IL-15 are ligands for the cytokine receptor common gamma chain. The IL-7 receptor is mainly expressed on T lymphocytes, with IL-7
playing a key role in T cell differentiation, survival, and proliferation (36). Recently, we observed that IL-7 treatment significantly reduced T cell apoptosis in the spleen and mesenteric lymph nodes following sepsis (55). Additionally, we reported that treatment with rhIL-7 improved the delayed-type hypersensitivity (DTH) response, while partially preventing sepsis-induced decrease in T cell IFN-γ production (55). The attenuation of T cell apoptosis and improved functionality following rhIL-7 treatment was associated with improved survival (55). To build upon our previous study, we undertook investigations to further determine how IL-7 stabilization of T cell numbers and function mediated tissue injury, bacterial load, and the neutrophil response during sepsis. Here, we found that IL-7 treatment significantly accelerated γδ T cell recruitment to the site of infection such that bacterial load was decreased. Significantly, we postulate that the early IL-7 stabilization of the adaptive immune response allows for a more beneficial innate immune response during sepsis.
Materials and Methods

Cecal ligation and puncture

Male C57BL/6 (WT) mice between 6 and 8 weeks of age (20 to 28 grams), TCR-αβ/γδ-deficient (B6.129P2-Tcrb<sup>tm1MOM</sup>Tcrd<sup>tm1MOM</sup>j, Jackson Laboratory, Bar Harbor, ME) and TCR-γδ-deficient (B6.129P2-Tcrd<sup>tm1Mom</sup>J, Jackson Laboratory) mice were utilized. All experiments involving animals were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati. Polymicrobial sepsis was induced as previously described (9). Briefly, cecal ligation and puncture (CLP) operations were always performed between 6 am and 10 am. Normal fed mice were anesthetized to effect by 2.5% isoflurane in oxygen via facemask. After laparotomy, the latter 80% of the cecum was ligated and punctured once on the anti-mesenteric side with a 23 gauge needle. A small amount of bowel content was extruded through the puncture hole to assure full thickness perforation. The cecum was replaced in its original location and the midline incision closed by two-layer suture. Prior to closure of the peritoneum with one figure of eight stitch, 5 µg human recombinant IL-7 (Cytheris, Issy-les-Moulineaux, France) or equivalent volume of vehicle was injected via pipette. Sham-operated animals received midline laparotomy, exteriorization of the cecum with prompt replacement, and closure of incision in two layers. The animals were resuscitated with 1 mL of sterile saline subcutaneously and kept on a heating blanket with additional oxygen supply for 1 hour. In survival studies, animals were given ad libitum access to food and water and followed until death or humane sacrifice per protocol. Animals were evaluated every 12 hours following CLP.
Flow cytometry for surface and intracellular staining

Analyses of cell surface antigen expression and in situ cytokine and Bcl-2 expression were performed as previously described (5) on spleen and peritoneal lavage samples. Flow cytometry data acquisition and analysis were performed on an LSR II using FACS Diva software (BD Biosciences, Mountain View, CA). Antibodies: CD4 (Clone: RM4-5, BioLegend, San Diego, CA), CD8 (Clone: 53-6.7, BD Biosciences, San Diego, CA), CD69 (Clone: H1.2F3, BD Biosciences), CD44 (Clone: Pgp-1,Ly-24, BD Biosciences), CD62L (Clone: MEL-14, Invitrogen, Carlsbad, CA), αβ T cell (Clone: H57-597), γδ T cell (Clone: GL3, BioLegend), CXCR3 (Clone: 49801, R and D, Minneapolis, MN), Bcl-2 (Clone: 3F11, BD Biosciences), IL-17A (Clone: TC11-18H10.1, Biolegend), CD11b (Clone: M1/70, BD Biosciences), and Gr-1/Ly6G (Clone: 1A8, BD Biosciences).

Leukocyte sub-sets were defined as follows: CD4+ Naïve (CD62L<sup>Hi</sup>/CD44<sup>Lo</sup>), CD4+ Central Memory (CD62L<sup>Hi</sup>/CD44<sup>Hi</sup>), CD4+ Effector Memory (CD62L<sup>Lo</sup>/CD44<sup>Hi</sup>), CD8+ Naïve (CD62L<sup>Hi</sup>/ CD44<sup>Lo</sup>), CD8+ Central Memory (CD62L<sup>Hi</sup>/CD44<sup>Hi</sup>), CD8+ Effector Memory (CD62L<sup>Lo</sup>/ CD44<sup>Hi</sup>), neutrophils (Gr-1<sup>high</sup>, CD11b<sup>+</sup>).

Bacterial load determination

Bacterial load determination was performed with blood harvested aseptically by cardiac puncture as previously described (54). Samples were serially diluted in sterile saline and cultured on soy agar pour plates. Plates were incubated at 37°C for 24 hours and colony enumeration was performed.

Cytokine and chemokine measurement by ELISA

Whole blood was harvested by sterile cardiac punch and serum isolated using serum
separator tubes. Peritoneal lavage and cells were obtained by injection of 9 ml 0.9% normal saline intraperitoneally and removal via syringe. IL-6 (Invitrogen), IFN-γ (Invitrogen), CXCL1/KC (R and D), IL-17 (eBioscience, San Diego, CA) and CXCL10/IP-10 (Peprotech, Rocky Hill, NJ) levels were analyzed by ELISA according to manufacturer’s protocol.

Phagocytosis Determination

Cells from peritoneal lavage were harvested 24 h after CLP and mixed with opsonized Alexa Fluor 488-labeled E. coli (K.12 strain, Invitrogen). This suspension was incubated at 37°C for 5 minutes. This time point was chosen based upon previous optimization experiments. Following the incubation period, unattached extracellular bacteria were removed by washing three times. Samples were run on a Becton Dickinson LSR II to determine the mean fluorescence intensity (MFI), a measure of the number of bacteria taken up per cell. To quench the fluorescence of adherent bacteria, trypan blue was added 1 minute before the second acquisition. Quenching with trypan blue reduced the FITC fluorescence of adherent bacteria by excitation energy transfer (21).

Neutrophil oxidative burst assay

Oxidative burst was determined as previously described (48). Briefly, polystyrene tissue culture dishes were coated with 1µg/well fibronectin for two hours at 37°C and 5% CO₂ and then washed. One hundred µl of reaction mixture (10mM scopoletin, 1mg/mL horseradish peroxidase, 4mM NaN₃ in KRPG) and 20 µl neutrophils resuspended in KRPG at 7.5x10⁵ cells/mL were allowed to incubate 10 minutes at 37°C in the wells prior to stimulation with either buffer or fMLP (100 nM). Fluorescence was measured...
immediately and at ten minute intervals for 60 minutes. H$_2$O$_2$ production was calculated from the decrease in fluorescence due to oxidation of scopoletin. The data are expressed as nanomoles of H$_2$O$_2$ produced per 1.5x10$^4$ PMNs.

**Tissue injury determination**

For general tissue damage assessment, serum samples were obtained as described above, diluted 1:4, and run for AST on Siemens Dimension Xpand Plus Integrated Chemistry System (Siemens Healthcare Diagnostics, Deerfield, IL). Lung tissue (100 mg) was homogenized in 2 mL buffer A (3.4 mM KH$_2$HPO$_4$ and 16 mM Na$_2$HPO$_4$, pH 7.4). After centrifugation for 20 min at 10,000 g, the pellet was resuspended in 10 vol buffer B (43.2 mM KH$_2$HPO$_4$, 6.5 mM Na$_2$HPO$_4$, 10 mM EDTA, and 0.5% hexadecyltrimethylammonium, pH 6.0) and was sonicated for 10 s. After heating for 2 h at 60°C, the supernatant was reacted with 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO, USA), and OD was determined at 655 nm. Two centimeter sections of intestine were harvested and placed on previously weighed trays. Samples were incubated at 55°C for 72 hours and reweighed. They were incubated for another 24 hours at 55°C and reweighed to ensure complete fluid evaporation. The wet weight to dry weight ratio was then calculated.

**Statistical analyses**

Statistical comparisons were performed using Kaplan Meier LogRank (survival), Student t Test (two groups), or one way ANOVA with Holm-Sidak post-hoc test (more than two groups) using StatView 3.5 (SAS Institute, Cary, NC) and GraphPad Prism 5.0 (Graphpad Software, La Jolla, CA). The mean and standard error of the mean were calculated in experiments containing multiple data points. A value of P ≤ 0.05 was considered statistically significant.
RESULTS:

Previously, we demonstrated enhanced survival in septic wild type mice treated with recombinant human IL-7 at the time of CLP (54). To confirm the requirement for αβ and γδ T cells for the rhIL-7 mediated survival advantage during sepsis, we subjected mice deficient in these cells treated with either rhIL-7 or vehicle and then monitored for survival. Here, we observed no differences in survival or median survival time (Figure 1). Altogether, these data implicate the necessity of αβ and/or γδ T cells for the IL-7-induced increase in survival of septic wild type mice.

A signature hallmark of sepsis is the profound and rapid depletion of lymphocytes (23). T cell apoptosis has been reported as the underlying cause of decreased lymphocyte numbers following sepsis (27, 60). Previously, we observed that splenic T cells isolated from rhIL-7 treated mice had increased Bcl-2 expression as well as reduced T cell apoptosis 24 hours after CLP. As increased expression of Bcl-2 24 hours after CLP may represent selection bias of surviving lymphocytes, we sought to elucidate the effect of rhIL-7 on T cell Bcl-2 expression 3 hours after sepsis induction when cells have not yet undergone significant depletion. Here, we determined that IL-7 treatment resulted in increased Bcl-2 expression in naive CD4 and CD8 T cells as well as effector memory CD4 T cells compared to T cells isolated from vehicle-treated septic mice (Figure 2a-d). Thus, rhIL-7 treatment can begin to enhance T cell survival quite early after treatment.

IL-7 treatment enhances IFN-γ and IP-10 production during sepsis
IFN-γ is a key cytokine in the host immune response to sepsis as evidenced by decreased survival in IFN-γ knock-out mice subjected to CLP when compared to WT mice (37). Here, we observed increased peritoneal IFN-γ concentrations in rhIL-7 treated mice almost double the level found in vehicle-treated animals (Figure 3). Sham levels were undetectable, as were serum levels of IFN-γ at this time point (data not shown). Of the many roles IFN-γ can play during the immune response, one involves stimulating the release of CXCL10/IP-10 by macrophages (52). CXCL10/IP-10 is one ligand for the CXCR3. It has been previously reported that γδ T cells can express CXCR3 and this receptor can mediate recruitment (1). Therefore, we evaluated CXCR3 ligand in WT mice following CLP and observed its expression upon 89% of peritoneal γδ T cells (Figure 4a). Additionally, we demonstrated an approximate 2-fold increase in CXCL10/IP-10 in the rhIL-7 treated versus vehicle treated mice, while sham mice had no appreciable levels of this ligand (Figure 4b). Thus, rhIL-7 treatment, possibly through prevention of T cell apoptosis, enhances peritoneal levels of IFN-γ with subsequent downstream increase in CXCL10/IP-10 production.

Neutrophil recruitment, but not activation or functionality, is accelerated by rhIL-7 treatment

Neutrophils clear bacterial pathogens during sepsis as part of a complex process involving recruitment to the site of infection, activation, cytokine secretion, phagocytosis and oxidative burst killing of bacteria. Here, we evaluated the enumeration, activation and functionality of neutrophils found at the site of infection at multiple time points following CLP. Treatment with rhIL-7 caused an accelerated recruitment to the...
peritoneum at 6 hours following CLP compared to vehicle-treated mice, with no
difference between groups seen at 24 hours (Figure 5a). CD11b expression was used as a
surrogate marker for neutrophil activation and showed no difference between WT and
rhIL-7-treated mice at 6 or 24 hours following CLP (Figure 5b) or in sham animals
treated with rhIL-7 versus PBS (data not shown). Lastly, we evaluated the phagocytosis
and oxidative burst potential of neutrophils obtained from the site of infection at 6h and
24h following CLP in WT and rhIL-7-treated mice. Interestingly, we found no
differences in either phagocytosis (Figure 5c) or oxidative burst (Figure 5d) between the
two groups at either time point. These findings suggest that rhIL-7 plays a singular role
in neutrophil recruitment to the site of infection during sepsis.

**rhIL-7 treatment increased γδ T cell numbers, IL-17 production and CXCL1/KC production**

Neutrophil recruitment can be mediated by IL-17 and subsequent production
of CXCL1/KC. During sepsis, γδ T cells have been reported to be significant producers
of IL-17 (13) and can play a critical role in increasing peritoneal neutrophil accumulation
(54). In pilot experiments, we were unable to observe significant numbers of peritoneal
γδ T cells in sham animals. Following CLP, we determined that rhIL-7 treatment resulted
both in increased the numbers of IL-17-producing γδ T cells as well as enhancing IL-17
production (Figures 6a-b). To confirm the necessity of γδ T cells for IL-7-induced
enhancement of IL-17 during sepsis, we subjected wild type and γδ T cell deficient mice
to CLP ± rhIL-7 treatment. Evaluation of the peritoneal lavage 6 hours following injury
demonstrated significantly elevated IL-17 only in WT mice treated with rhIL-7 compared
to all other treatment groups (Figure 6c). Lastly, a positive correlation between neutrophil recruitment and CXCL1/KC expression has been reported (17, 34, 44, 51, 59).

As IL-7 treatment increased neutrophil numbers at the site of infection, we next evaluated local levels of CXCL1/KC and observed significantly increased CXCL1/KC in the peritoneal lavage of rhIL-7-treated compared to WT mice (Figure 6d). Thus, treatment with rhIL-7 rapidly increases both the number of peritoneal γδ T cells present following sepsis, the production of IL-17 by these cells, and increased CXCL1/KC accumulation.

rhIL-7 treatment increases inflammation and bacterial clearance without changing end-organ tissue injury

Properly regulated neutrophils play an important role in bacterial clearance (33), however, decreased apoptosis and increased half-life may result in increased tissue injury during sepsis (22). During sepsis, an optimal balance between early pathogen clearance, associated inflammation, and end-organ tissue damage will likely allow for reduction of host morbidity and mortality. As IL-6 has previously been utilized as a surrogate marker for inflammation (42), we evaluated serum levels of this cytokine at 6 and 24 hours. Mice treated with rhIL-7 had significantly higher serum IL-6 levels at 6 hours versus WT mice, with no difference existing at 24 hours (Figure 7a). Evaluation of bacteremia at 24h following CLP demonstrated a significantly lower bacterial load in those animals treated with rhIL-7 versus vehicle (Figure 7b), consistent with previous findings. We next evaluated general, lung, and intestine tissue injury. We observed that serum aspartate aminotransferase (AST) (Figure 7c), lung myeloperoxidase (MPO) (Figure 7d) as well as BAL protein content (data not shown), and intestinal wet/dry ratios (Figure 7e)
were not significantly different between vehicle treated WT mice and those treated with rhIL-7. Experiments conducted with sham-operated mice did not demonstrate detectable levels of IL-6, bacteremia, or tissue injury. Altogether, mice treated with rhIL-7 had diminished bacteremia, early increased, but not sustained, inflammation, but not worsened tissue damage compared to vehicle-treated mice.
DISCUSSION:

In this study we tested the hypothesis that IL-7, a potent T cell anti-apoptotic cytokine, would mediate the early innate immune response to sepsis. First, we demonstrated the necessity of αβ and/or γδ T cells for the IL-7-induced increase in survival of septic wild type mice. Additionally, we show upregulation of Bcl-2 expression on CD4 T cells as early as 3 hours following CLP in mice treated with rhIL-7. Here, mice treated with rhIL-7 were shown to have increased neutrophil recruitment to the peritoneum, without increased end organ tissue damage when compared to WT controls. Elevated neutrophil recruitment at the site of infection was correlated with elevated local and systemic production of CXCL1/KC and IL-17. We determined that following rhIL-7 treatment, septic mice had increased numbers of γδ T cells in the peritoneum producing elevated amounts of IL-17. Additionally, γδ T cells were necessary for elevated peritoneal IL-17 concentration following IL-7 treatment. Previously, we demonstrated that T cells isolated from IL-7 treated mice had a reversal of the sepsis-induced defective production of IFN-γ (55). Here, we built upon these findings by demonstrating an IL-7-induced increase in peritoneal IFN-γ levels. Increased IFN-γ production has been shown to promote macrophage or endothelial CXCL10/IP-10 expression (3, 19, 31), a ligand for the CXCR3 receptor. We show this receptor is expressed upon gamma-delta T cells. These results suggest that IL-7 treatment improves the host response to sepsis through a rapid recruitment of γδ T cells to the site of infection coupled with accelerated γδ T cell-mediated recruitment of neutrophils to the site of infection. Altogether, these data are summarized in Figure 8.
The work detailing that rhIL-7 treatment improves the murine host response to sepsis has recently been reported (55), although its effects on lymphocyte apoptosis and proliferation in human diseases such as hepatitis C and cancer stem from ongoing clinical trials (46, 49). At 24 hours following CLP, IL-7 treatment significantly increased Bcl-2 expression in splenic CD4 and CD8 T cells subsets versus wild type animals and relative change of mRNA abundance in CD4 T cells (55). Additionally, rhIL-7 blunted both splenic and mesenteric lymph node T cell apoptosis 24 hours following CLP, resulting in significantly elevated numbers of CD4 and CD8 T cells in treated versus WT mice at this time point (55). Clinical findings of decreased Bcl-2 gene expression in septic human patients at 12 and 24 hours following diagnosis were previously noted to correlate highly with decreased absolute numbers of CD4 lymphocytes (4, 24). Furthermore, prevention of apoptosis with overexpression of Bcl-2 is known to prevent T cell apoptosis, providing protection from sepsis-related mortality (26). Here, we show naive CD4, CD8 and effector memory CD4 cells from rhIL-7 treated animals have significantly elevated Bcl-2 protein expression at 3 hours following CLP, suggesting apoptotic signaling in T cells takes place early after induction of sepsis. One key effect of early T cell apoptosis in sepsis is reduced immunocompetence through decreased IFN-γ production. The production of IFN-γ by CD4 T cells during sepsis is associated with improved survival, decreased bacterial load, and increased neutrophil functionality (37, 53). Interestingly, we show that IL-7 treatment significantly increases peritoneal concentration of IFN-γ as early as 3 hours following CLP. These data suggest that rhIL-7 treatment upregulates Bcl-2 expression very early after treatment such that T cell apoptosis can be reduced and IFN-γ production preserved.
The enhanced production of IFN-γ in rhIL-7-treated mice relative to WT controls exerts downstream effects on both adaptive and innate cells. Interferon-gamma-inducible protein 10 (CXCL10/IP-10) is a chemokine secreted by endothelial cells, macrophages and neutrophils upon stimulation with IFN-γ, and serves as a chemoattractant for T lymphocytes (7, 35, 61). It has been suggested that CXCL10/IP-10 plays a distinct role in the recruitment of T cells expressing CXCR3 to sites of inflammation (7), consistent with findings that TCR γδ T cells express CXCR3 and transmigrate upon stimulation with IP-10 (11, 45). Here, we demonstrate that a large percentage of γδ T cells and neutrophils present at the site of infection early following CLP express CXCR3. Further, septic mice treated with IL-7 had significantly higher amounts of CXCL10/IP-10 at the peritoneum. Consistent with these findings, we observed that rhIL-7 treatment resulted in an elevated number of γδ T cells at the site of infection. Altogether, we postulate that IL-7-treatment enhanced T cell IFN-γ production such that CXCL10/IP-10 was increased and γδ T cells entered the site of infection.

After exposure to CXCL10/IP-10, γδ T cells can transmigrate and mediate inflammatory processes in tissue compartments such as the lung and peritoneum. The inflammatory effects of γδ T cells result, in part, from production of the cytokine IL-17 in response to early and sustained stimulation by IL-1β, TLR-2 and IL-23 (1, 38, 43, 50). IL-17 acts upon mesothelial cells to produce CXCL1/KC and CXCL2/MIP-2 (15), and has been shown necessary for promotion of granulopoiesis and neutrophil recruitment in peripheral tissues (14, 62). Here, we show that local and systemic levels of IL-17 are elevated with rhIL-7 treatment following CLP with a subsequent and complimentary
increase in CXCL1/KC. Further, elevated IL-17 following rhIL-7 treatment in sepsis is dependent upon the presence of γδ T cells. This demonstrates a sequential and mechanistic pathway through which rhIL-7-mediated attenuation of T cell dysfunction results in increased neutrophil recruitment to the site of infection following CLP. The elevated number of neutrophils early in the septic course is likely responsible for the decreased bacterial load in rhIL-7-treated mice, even in the absence of neutrophil functionality changes. The rapid return of neutrophil numbers to WT levels at 24 hours may explain the comparable levels of end organ tissue damage seen in these groups, consistent with previous findings in this model assessing the role of neutrophils in sepsis (22). The lack of functional effect on neutrophils following IL-7 treatment may further explain the consonant end organ results in these studies. Altogether, we speculated that the ability of rhIL-7 to maintain numbers and functionality of CD4 T cells following sepsis resulted in accelerated γδ T cell recruitment and functionality at the site of infection, ultimately aiding bacterial clearance without worsening tissue damage.

Our reported here data demonstrate that rhIL-7 treatment increased peritoneal IFN-γ levels, IP-10 levels and CXCR3-expressing γδ T cell accumulation. However, one limitation to this report is a lack of data verifying that IP-10, through CXCR3, recruits γδ T cells to the peritoneum. While trying to address this, we subjected CXCR3-deficient mice to CLP and found that these mice had a significantly increased peritoneal bacterial load as compared to WT mice. In addition, neutrophils isolated from these mice demonstrated a decreased phagocytic capacity. Finally, the CXCR3 KO had increased numbers of neutrophils and macrophages at the site of infection compared to controls. We speculate that the CXCR3 mediated defect in phagocytosis resulted in sustained
leukocyte chemotaxis that ultimately confounded the experimental results. Thus, the use of more specific reagents will be needed to fully address this potentially important mechanism.

Sepsis involves a complex interplay between the innate and adaptive immune systems that occurs very early after the onset of disease, not in a delayed fashion as previously accepted. γδ T cells, through production of IL-17, appear to play a central role in immune response cross-talk between the two arms of the immune response. In addition, the existence of cross-talk between these two responses suggests multiple targets for future therapeutic intervention may be available. One such therapeutic is the cytokine IL-7, shown to play a direct role in prevention of T cell apoptosis with subsequent downstream effects resulting in an enhanced innate response to sepsis. Going forward, these insights may play a role in the ultimate production of a viable sepsis therapeutic that reduces the health care burden imposed by this disease process.

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**FIGURE LEGENDS:**

**Figure 1** – Effects of IL-7 on survival following sepsis are mediated by αβ TCR or γδ TCR cells. Combined αβ / γδ TCR-deficient mice were treated with 5 µg human recombinant IL-7 or vehicle at time of CLP, then monitored for survival. Sample size was 10-11/group. Data presented as Kaplan-Meier survival curve.

**Figure 2** - IL-7 treatment elevates CD4 and CD8 lymphocyte Bcl-2 expression as early as 3 hours after CLP. Sham- and CLP-operated WT mice were treated with 5 µg human recombinant IL-7 or vehicle at time of CLP. Splenocytes obtained 3 hours after CLP were directly analyzed by flow cytometry for Bcl-2 expression on A) Naive CD4+, B) Effector memory (EM) CD4+, C) Naive CD8+, and D) EM CD8+ T cell sub-sets. Naive T cells had CD62L^Hi^CD44^Lo^ expression while EM T cells had CD62L^Lo^CD44^Hi^ expression. Samples size was 4/group. Data are expressed as the mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Figure 3** – IL-7 treatment enhances IFN-γ levels in the septic peritoneum. CLP-operated WT mice were treated with 5 µg human recombinant IL-7 or vehicle at time of CLP. IFN-γ was measured by ELISA in peritoneal samples obtained 3 hours following CLP. Sample size was 8/group. Data are expressed as the mean ± SEM. *, p < 0.05.

**Figure 4** – CXCR3 expression on peritoneal TCRγδ cells. Flow cytometric analysis of WT A) peritoneal TCRγδ cells obtained 3 hours following CLP evaluated CXCR3 expression. Sample size was 4/group. B) Sham and CLP-operated WT mice were treated with 5 µg human recombinant IL-7 or vehicle at time of CLP. Peritoneal lavage obtained 3 hours after CLP was analyzed by ELISA for CXCL10/IP-10. Sample size was 4/group.
Data are expressed as the mean ± SEM. ^, p < 0.01 versus sham groups; &, p < 0.01 versus all groups.

**Figure 5 – IL-7 treatment enhances neutrophil recruitment, but not activation or functionality during sepsis.** CLP-operated WT mice were treated with 5 µg human recombinant IL-7 or vehicle at time of CLP. Neutrophils at the site of infection were analyzed by flow cytometry to A) enumerate and B) determine CD11b expression at the indicated time following CLP. Sample size was 9-13/group at 6h and 12-13/group at 24h. Neutrophil functionality was evaluated using C) phagocytosis and D) oxidative burst at indicated time points. Sample size was 6/group at 3h and 4/group at 6h. Data are expressed as the mean ± SEM. **, p < 0.01.

**Figure 6 – IL-7 treatment increases the number of, and IL-17 production by, peritoneal γδ T cells following CLP.** CLP-operated WT mice were treated with 5 µg human recombinant IL-7 or vehicle at time of CLP. Flow cytometric analysis was used to enumerate the A) number of peritoneal γδ T cells producing IL-17 and B) intensity of IL-17 expression by peritoneal γδ T cells 3 hours after CLP. Sample size was 4/group. C) Peritoneal lavage obtained 6 hours after CLP in WT and TCRγδ-deficient mice treated with IL-7 or vehicle was analyzed by ELISA for IL-17. Sample size was 5/group. D) Peritoneal lavage obtained 6 hours after CLP was analyzed by ELISA for CXCL1/KC. Sample size was 11-14/group. Data are expressed as the mean ± SEM. MFI - Mean fluorescent intensity. *, p < 0.05; &, p < 0.01 versus all groups; ^, p < 0.01 versus sham groups.
Figure 7 - IL-7 treatment mediates serum IL-6 levels, decreases bacteremia, and does not alter tissue injury after CLP. WT mice were subjected to CLP as indicated in materials and methods and treated with 5 µg human recombinant IL-7 or vehicle at time of CLP. A) Serum obtained at indicated time points was analyzed by ELISA for IL-6. B) Serum bacterial load was measured 24 hours after induction of sepsis. C) Serum AST was measured 24 hours after induction of sepsis. D) Lungs were harvested at the indicated time after CLP and MPO was measured. E) 2 centimeter sections of terminal ileum were obtained at the indicated time following CLP, weighed and dried as described in materials and methods. Sample sizes were 8-11/group. Data are expressed as the mean ± SEM. *, p < 0.05; ***, p < 0.001.

Figure 8 – Model for IL-7’s role in modulating neutrophil recruitment in sepsis. This figure represents a schematic hypothesis demonstrating how rhIL-7 treatment, through maintenance of appropriate T cell immunocompetence, results in enhanced neutrophil recruitment to the site of infection.
Figure 1

Figure 2

A

B

C

D

Figure 2

A

B

C

D

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Figure 5

A

WT + IL7 6h CLP 24h CLP

**

B

CD11b of Peritoneal Neutrophils (x10^6)

C

CD11b of Peritoneal Neutrophils (x10^3)

D

H2O2 Produced (nMol)

Figure 6

A

Peritoneal IL-17+ γδ T cells (x10^4)

B

Peritoneal γδ T cell IL17 (MFI x10^3)

C

Peritoneal γδ T cell IL17 (MFI x10^3)

D

Peritoneal γδ T cell IL17 (MFI x10^3)
Figure 7

A

Serum IL6 (ng/ml)

WT +IL7

6hr 24hr

***

B

Bacteremia CFU (Log)

WT +IL7

6h CLP 24h CLP

*

C

Lung MPO (u MPO/mg protein)

WT +IL7

6h CLP 24h CLP

D

Intestinal Wet/Dry Ratio

Serum AST (units/L)

WT +IL7

Figure 8

rhIL-7

T cell

Bcl-2 expression

↓ apoptosis

IFN-γ

Macrophage

IP10

γδ T cell

↑ IL17 production

Neutrophil

↑ CXCR3-cell recruitment

↑ CXCL1/KC

Endothelial Cell

CXCL1/KC

Neutrophil