CXCR4 blockade decreases CD4(+) T cell exhaustion and improves survival in a murine model of polymicrobial sepsis

Kimberly M. Ramonell, Emory University
Wenxiao Zhang, Emory University
Annette Hadley, Emory University
Ching-wen Chen, Emory University
Katherine T. Fay, Emory University
John D. Lyons, Emory University
Nathan J. Klingensmith, Emory University
Kevin McConnell, Emory University
Craig Coopersmith, Emory University
Mandy L Ford, Emory University

Journal Title: PLoS ONE
Volume: Volume 12, Number 12
Publisher: Public Library of Science | 2017-12-12, Pages e0188882-e0188882
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1371/journal.pone.0188882
Permanent URL: https://pid.emory.edu/ark:/25593/s7dd5

Final published version: http://dx.doi.org/10.1371/journal.pone.0188882

Copyright information:
© 2017 Ramonell et al.
This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

Accessed March 19, 2019 9:31 PM EDT
CXCR4 blockade decreases CD4⁺ T cell exhaustion and improves survival in a murine model of polymicrobial sepsis

Kimberly M. Ramonell¹, Wenzhao Zhang¹, Annette Hadley¹, Ching-wen Chen¹, Katherine T. Fay¹, John D. Lyons¹, Nathan J. Klingensmith¹, Kevin W. McConnell¹, Craig M. Coopersmith¹,2☯, Mandy L. Ford¹,3☯*

¹ Department of Surgery, Emory University School of Medicine, Atlanta, Georgia, United States of America, 2 Emory Critical Care Center, Emory University School of Medicine, Atlanta, Georgia, United States of America, 3 Emory Transplant Center, Emory University School of Medicine, Atlanta, Georgia, United States of America

☯ These authors contributed equally to this work.
* mandy.ford@emory.edu

Abstract

Sepsis is a dysregulated systemic response to infection involving many inflammatory pathways and the induction of counter-regulatory anti-inflammatory processes that results in a state of immune incompetence and can lead to multi-organ failure. CXCR4 is a chemokine receptor that, following ligation by CXCL12, directs cells to bone marrow niches and also plays an important role in T cell cosignaling and formation of the immunologic synapse. Here, we investigated the expression and function of CXCR4 in a murine model of polymicrobial sepsis. Results indicate that CXCR4 is selectively upregulated on naïve CD4⁺ and CD8⁺ T cells and CD4⁺ central memory T cells following the induction of sepsis, and that CXCR4 antagonism resulted in a significant decrease in sepsis-induced mortality. We probed the mechanistic basis for these findings and found that CXCR4 antagonism significantly increased the number of peripheral CD4⁺ and CD8⁺ T cells and CD4⁺ central memory T cells following the induction of sepsis, and that CXCR4 antagonism resulted in a significant decrease in sepsis-induced mortality. We probed the mechanistic basis for these findings and found that CXCR4 antagonism significantly increased the number of peripheral CD4⁺ and CD8⁺ T cells following sepsis. Moreover, mice treated with the CXCR4 antagonist contained fewer PD-1⁺ LAG-3⁺ 2B4⁺ cells, suggesting that blockade of CXCR4 mitigates CD4⁺ T cell exhaustion during sepsis. Taken together, these results characterize CXCR4 as an important pathway that modulates immune dysfunction and mortality following sepsis, which may hold promise as a target for future therapeutic intervention in septic patients.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection and is responsible for more than 300,000 deaths annually [1, 2]. With the exception of antibiotics, current therapy is limited to non-specific supportive care and mortality remains at 40% [3, 4]. However, there is increasing appreciation for the central role that immunologic dysfunction plays in driving sepsis mortality. In particular, the immunosuppressive phase of sepsis...
contributes to impaired immune competency, susceptibility to secondary infections and increased mortality in septic patients [5–7]. A number of interacting processes contribute to this state, including apoptosis of immune effector cells, expansion of immunosuppressive T regulatory (T<sub>Reg</sub>) cells, T cell exhaustion, and monocyte deactivation [8, 9].

Additionally, sepsis triggers extensive apoptosis-induced depletion of innate and adaptive immune cells and some remaining cells are rendered dysfunctional or exhausted, due to the prolonged exposure to excessive pro- and anti-inflammatory cytokines. Phenotypically, immune cell exhaustion is characterized by increased expression of co-inhibitory markers including programmed cell death (PD-1), 2B4, BTLA, and LAG-3 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Signaling through these coinhibitory molecules may limit the ability of T cells to proliferate and produce cytokines and attenuate cytotoxic T cell function [10, 11]. For instance, PD-1 overexpression on circulating T cells from septic patients correlates with decreased T cell proliferative capacity, increased secondary nosocomial infections, and increased mortality. Pharmacologic blockade of T cell coinhibitory pathways such as PD-1, BTLA, and 2B4 has been shown to at least partially reverse the state of immune dysregulation and improve survival in pre-clinical models of sepsis [12–19] and PD-1 blockers are currently under investigation for use in clinical sepsis.

Moreover, emerging evidence shows a correlation between lymphopenia and impaired immune cell function, underscoring the importance of restoring both number and function to both innate and adaptive immune systems when treating sepsis [20]. The chemokine receptor CXCR4 and its ligand CXCL12 are involved in regulating the homeostatic recirculation and retention of myeloid and lymphoid cells in the bone marrow [21–25]. CXCR4 is expressed on B and T lymphocytes, dendritic cells, and monocytes [25] and inhibition of CXCR4/CXCL12 signaling results in the release of these cells into the circulation, increasing peripheral absolute cell counts [25]. Interestingly, a recent study of human septic patients revealed that CXCL12 levels were higher in patients with severe sepsis/septic shock as compared to healthy subjects. Moreover, the same study also found that patients who survived their septic insult possessed lower serum levels of CXCL12 than those who died [26]. Thus, we hypothesized that mitigating the detrimental effects of sepsis-induced immune dysfunction by restoring depleted or dysfunctional immune effector cells with functional cells mobilized from bone marrow stores may be beneficial in sepsis. We sought to test this hypothesis by evaluating the effect of CXCR4 blockade on sepsis-induced mortality and immune dysregulation using plerixafor (AMD3100), a CXCR4-antagonist currently FDA approved for stem cell mobilization prior to autologous bone marrow transplantation that is also being investigated as a treatment for several chronic inflammatory diseases including rheumatoid arthritis and inflammatory bowel disease [27–30].

Materials & methods

Mice

Adult male and female 9–13 week old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in the same facilities and allowed to acclimate at least one week prior to surgery. Experiments were conducted with approval of the Institutional Animal Care and Use Committee of Emory University (protocol number DAR-2003199-071415N).

Cecal ligation and puncture (CLP)

Sepsis was induced using CLP, a murine model of polymicrobial sepsis. Injury was titrated to achieve a ~50% 14-day mortality to mimic the clinical scenario of sepsis [31]. In brief, C57BL/
6 mice were anesthetized using isoflurane and underwent laparotomy, the cecum was exteriorized, ligated distal to the ileocecal valve, and punctured twice with a 25-gauge needle. Sham-operated animals underwent laparotomy and exteriorization of the cecum only. Personnel conducting sham and CLP surgeries received training and competency testing from Emory University Division of Animal Resources veterinary staff. All animals received buprenorphine (0.1mg/kg) preoperatively for pain relief and 1mL of normal saline for intraoperative fluid losses as well as antibiotics (ceftriaxone 25mg/kg and metronidazole 12.5mg/kg) subcutaneously postoperatively. Antibiotics were continued on a q12hr dosing schedule for 48 hours postoperatively. For experiments evaluating CXCR4 blockade, mice designated to the CLP+ Plerixafor group received a 100μl SQ injection of 5mg/kg Plerixafor (AMD3100, Sigma-Aldrich) one hour after abdominal closure. Throughout this manuscript, mice that underwent CLP and were given normal saline as a control injection are referred to as “septic control mice”. Mice were sacrificed by CO₂ asphyxiation. For survival studies, mice were observed daily for 7-days after surgery. Animals were observed every 12 hours during this 7-day period. The following criteria were used as humane endpoints; animals meeting any one of these criteria were considered moribund, counted as deceased in the enumeration of surviving animals, and sacrificed by CO₂ asphyxiation. 1) Loss of 25% of body weight from baseline weight. 2) Major organ failure or medical conditions unresponsive to treatment such as severe respiratory distress, icterus, uremia, intractable diarrhea, or self-mutilation. 3) Surgical complications unresponsive to immediate intervention (bleeding, infection, wound dehiscence). 4) Clinical or behavioral signs unresponsive to appropriate intervention persisting for 24 hours including significant inactivity, labored breathing, sunken eyes, hunched posture, piloerection/matted fur, and abnormal vocalization when handled. Once any animal reached endpoint criteria, the amount of time elapsed before euthanasia was <12 hours. Some animals died before meeting the criteria for euthanasia. A total of 42 animals were used in the survival study, and 26 of them died or met endpoint criteria. The cause of death for all animals was septic shock.

**Immunophenotyping by flow cytometry**

Groups of mice were sacrificed at 24 hours following surgery. Spleens were removed aseptically, placed in a 10mL culture dish containing 5mL of PBS (Mediatech, Herndon, VA), and disrupted using the rubber end of a sterile 3mL syringe. Cells were passed through a 70μm mesh filter (BD Falcon), and single cell suspensions were centrifuged and resuspended in 10mL of PBS. Whole blood was harvested by cardiac puncture, 100μL per sample was aliquoted into 12 x 75mm flow tubes and incubated in 2mL of HYL solution (Thermo Fisher Scientific, Waltham, MA, USA) for 15 minutes followed by centrifugation and resuspension in 1mL PBS. For splenic and blood samples, cells were counted by trypan blue exclusion staining, and suspensions adjusted to a concentration of 1x10⁷ cells/mL. Aliquots containing 200μL were apportioned into FACS tubes then centrifuged and resuspended in 100μL FACS Buffer (PBS + 2% FCS + 0.1% Sodium Azide). Samples were pre-incubated with anti-CD16/CD32 mAb (BD Bioscience) for 15m, followed by 30m incubation with the following fluorochrome-conjugated mAbs: CD3 (Biolegend, 17A2), CD4 (BD Biosciences, RM4-5), CD8 (Invitrogen, MCD0830), B220 (BD Biosciences, RA3-6B2), CD44 (BD Biosciences, IM7), CD62L (BD Biosciences, MEL-14), PD-1 (Biolegend, 29F.1A12), LAG-3 (Biolegend, C9B7W), and 2B4 (BD Biosciences, 2B4). Accucheck Counting beads (Invitrogen) were added prior to data collection, per manufacturer’s protocols, and samples were analyzed on a BD LSRII Cytometer. Flow cytometric data were analyzed using FlowJo Software (Treestar, Ashland, OR). Absolute cell counts (per sample) were determined by factoring in the absolute number of cells per spleen enumerated at the time of harvest according to previously published protocols [32].
Intracellular cytokine staining

2×10⁶ splenocytes from each sample were plated in a 96-well plate. After centrifugation, cells were resuspended and incubated in culture medium (R10) consisting of RPMI 1640 containing 10% FBS (Mediatech, Herndon, VA), 2 mM L-glutamine, 0.01 M HEPES buffer, 100 mg/ml gentamicin (Mediatech), and 5×10⁻⁵ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). To test intracellular cytokine, cells were stimulated with 30 mg/ml PMA and 400 ng/ml ionomycin in the presence of GolgiStop (BD Pharmingen) for 4 hours at 37°C.

After incubation and stimulation, cells were surface-stained with anti-CD3-PB (BD), anti-CD4-PerCP (BD), anti-CD8-PO (Biolegend). Then cells were permeabilized using fixation and permeabilization solution (BD). We used anti-IL-2-FITC (BD), anti-TNF-PE-Cy7 (Biolegend) and anti-IFN-γ-Alexa 700 (BD) for intracellular cytokine staining. Samples were analyzed on an LSRII flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Cytokine quantification

Sham, CLP, and CLP + Plerixafor mice were sacrificed at 24h following surgery and whole blood was aspirated via cardiac puncture. Following 30min of incubation, samples were centrifuged (1000g x 10min) and supernatant (serum) was apportioned into 100μL aliquots and stored at -80°C until use. Serum cytokines were evaluated using BioPlex suspension array system and BioPlex Mouse Cytokine 11-Plex Panel according to the manufacturer’s instructions (both Bio-Rad, Marnes-La-Coquette France). Cytokine assays included antibodies for: IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFNγ, MIP-1b, and TNF. Results were analyzed using Bio-Plex Manager™ 3.0 software with 5PL curve fitting for determination of serum concentrations (pg/mL) of individual cytokines per sample.

Statistical analysis

Data were analyzed using the statistical software Prism V; all data are reported as mean +/- SEM. For comparison of absolute cell counts and frequencies of exhausted immune cells across two groups, the Mann Whitney non-parametric test was used. For comparison of three groups, One-way ANOVA and Tukey’s post-test were used. Survival studies were analyzed by Mantel-Cox analysis. For all data, a p-value of ≤ 0.05 was used to determine significance.

Results

Sepsis increases the frequency of CXCR4⁺ cells within CD4⁺ T naïve and T_CM and CD8⁺ T naïve subsets

To determine the expression profile of CXCR4 in the setting of sepsis, mice underwent CLP following by sacrifice and splenic harvest for immunophenotyping of CD4⁺ and CD8⁺ T cells at 24-hours post-sepsis induction (Fig 1A and 1B). The frequency of CXCR4⁺ cells among total CD4⁺ T cells was increased in septic mice compared to sham mice in the spleen (22% vs 17.8%; p = 0.002; Fig 1C). When the CD4⁺ T cell compartment was further analyzed, it was noted that the increase in frequency of CXCR4-expressing CD4⁺ T cells was limited to naïve (CD4⁺CD62L⁺) CD4⁺ T cells (21.7% vs. 15.6%; p = 0.001; Fig 1D) and central memory (T_CM; CD4⁺CD62L⁺) CD4⁺ T cells (24.1% vs. 16.5%; p = 0.0002; Fig 1E). In contrast, there was no difference in the frequency of CXCR4-expressing effector memory (T_EM CD4⁺CD62L⁻) CD4⁺ T cells in septic mice compared to sham mice (32% vs. 31.8%; p = 0.86; Fig 1F) suggesting that sepsis induces an upregulation of CXCR4 on less differentiated or antigen experienced CD4⁺ T cells. We then turned our attention toward the CD8⁺ T
We found no difference in the frequency of CXCR4+ total CD8+ T cells in septic mice compared to sham mice (28.8% vs. 27.9%; p = 0.4136; Fig 1G). However, the frequency of CXCR4+ naive CD8+ T cells was significantly increased in septic mice compared to sham mice (22% vs. 16.6%; p = 0.0003; Fig 1H) but there were no differences in the frequencies of CXCR4+ central memory CD8+ T cells (17.8% vs. 15.7%; p = 0.115; Fig 1I) or effector memory CD8+ T cells (50.8% vs. 50.9%; p = 0.970; Fig 1J).

Plerixafor administration improved 7-day survival in murine model of polymicrobial sepsis

Given the finding that CXCR4 is upregulated on CD4+ and CD8+ T cells during sepsis, we hypothesized that blocking these signals using a CXCR4 blocking agent could improve survival and immune dysregulation in a murine model of polymicrobial sepsis. To test this, groups of mice were treated with plerixafor as described in materials and methods or were left untreated, and all animals underwent CLP. Results indicated that mice treated with plerixafor one-hour

https://doi.org/10.1371/journal.pone.0188882.g001

Fig 1. CXCR4 was upregulated on less differentiated T cell subsets during sepsis. Representative histograms demonstrating the expression of CXCR4 on (A) CD4+ T cells and (B) CD8+ T cells. (C) The frequency of CXCR4 expression was increased in septic mice compared to sham mice on total CD4+ T cells in the spleen (22% vs. 17.8%; p = 0.002). When the CD4+ T cell compartment was further analyzed, the increase in CXCR4 expression on CD4+ T cells was limited to (D) naive CD4+ T cells (21.7% vs. 15.6%; p = 0.001) and (E) central memory CD4+ T cells (24.1% vs. 16.5%; p = 0.0002). (F) There was no difference in the frequency of CXCR4 expression on effector memory CD4+ T cells in septic mice compared to sham mice (32% vs. 31.8%; p = 0.86). (G) There was no difference in the frequency of CXCR4+ total CD8+ T cells in septic mice compared to sham mice (28.8% vs. 27.9%; p = 0.4136). However, the frequency of CXCR4+ naive CD8+ T cells (H) was significantly increased in septic mice compared to sham mice (22% vs. 16.6%; p = 0.0003) but there were no differences in the frequency of CXCR4+ central memory CD8+ T cells (I; 17.8% vs. 15.7%; p = 0.115) or effector memory CD8+ T cells (J; 50.8% vs. 50.9%; p = 0.970). Data shown are n = 5/group, representative of a total of 3 independent experiments with a total of n = 15/group.
post-sepsis induction had significantly improved 7-day survival compared to septic mice treated with isotype control. Survival was improved from 20% to 65% (Fig 2).

**Plerixafor administration abrogated the loss of peripheral T cells during sepsis**

To determine the effect of CXCR4 blockade on the number of peripheral T cells during sepsis, spleens were harvested at 24h from sham, septic control mice (CLP), and septic mice treated with plerixafor (CLP+Plerixafor), and absolute counts of CD4⁺ T cells and CD8⁺ T cells were analyzed. Sepsis resulted in a decrease in the absolute count of splenic CD4⁺ T cells at 24 hours post sepsis compared to sham mice (p = 0.0012). In contrast, spleens from septic mice treated with plerixafor contained numbers of CD4⁺ T cells that were not significantly different from sham animals, and exhibited a trend toward an increase in the absolute number of CD4⁺ T cells relative to untreated CLP controls (p = 0.055, Fig 3A). Similarly, septic mice exhibited a decrease in the absolute number of splenic CD8⁺ T cells compared to sham mice (p<0.0004), but septic mice treated with plerixafor demonstrated a trend toward an increase in CD8⁺ T cells in the spleen compared to untreated septic animals (p = 0.054, Fig 3B). These effects of plerixafor on peripheral T cell counts were not the result of diminished T cell apoptosis, as frequencies of AnnexinV⁺ 7-AAD⁺ T cells in splenocytes were not different between untreated and plerixafor-treated septic animals (data not shown).

**CXCR4-blockade decreased the percentage of PD-1 expressing T cells during sepsis**

Given the results that plerixafor treatment increased the number of peripheral CD4⁺ and CD8⁺ T cells during sepsis, we sought to interrogate the immunophenotypic characteristics of

---

**Fig 2. Plerixafor administration improved 7-day survival in murine sepsis.** Animals were subjected to CLP and were treated with plerixafor at one hour post-CLP as described in Materials and Methods. Plerixafor administration increased survival from 20% to 65% (n = 21/group; p = 0.0039).

https://doi.org/10.1371/journal.pone.0188882.g002
these two cell populations to determine the effect of plerixafor administration on co-inhibitory marker expression, specifically the expression of PD-1. As expected, the frequency of PD-1^+ CD4^+ T cells was significantly increased in septic mice as compared to sham mice (Fig 4A and 4C; 28.3% vs. 16.8%; p = 0.002). When septic mice were treated with plerixafor, the frequency of PD-1^+ cells among CD4^+ T cells was significantly decreased compared to septic control mice (21.1% vs. 28.3%; p = 0.0156; Fig 4A and 4C). Additionally, in septic mice treated with plerixafor, the level of PD-1 expression on a per-cell basis on CD4^+ T cells, as measured by MFI (median fluorescence intensity), was significantly decreased as compared to septic control mice (66.1 vs. 76.8; p = 0.033; Fig 4D). Analysis of the effect of plerixafor administration on PD-1 expressing CD8^+ T cells (Fig 4B) revealed a trend toward a decreased percentage and MFI of PD-1^+ among CD8^+ T cells as compared to septic control mice (Fig 4E and 4F) but these results did not reach statistical significance.

CXCR4-blockade decreased the percentage of LAG-3 and 2B4 expressing CD4^+ T cells during sepsis

In light of the decrease in PD-1 expressing CD4^+ T cells observed in septic animals treated with plerixafor, we next evaluated the effect of CXCR4 blockade on the expression of additional coinhibitory markers on CD4^+ T cells, including LAG-3 and 2B4. As expected, septic mice exhibited a significant increase in the frequency of LAG-3^+ CD4^+ T cells compared to sham mice (17.7% vs. 8.5%; p = 0.046; Fig 5A and 5B). When septic mice were treated with plerixafor, the frequency of LAG-3^+ CD4^+ T cells was significantly decreased compared to septic control mice (6.9% vs. 17.7%; p = 0.0173; Fig 5A and 5B). Additionally, septic mice demonstrated a significant increase in the expression of LAG-3 on CD4^+ T cells on a per-cell basis as compared to sham mice, as measured by the MFI (104.4 vs. 64.6; p = 0.025; Fig 5C), and septic
mice treated with plerixafor exhibited a significant decrease in the expression of LAG-3 on CD4+ T cells as compared to septic control mice (71.9 vs. 104.4; p = 0.0461; Fig 5C).

Examination of the expression of 2B4 on CD4+ T cells revealed similar results. Septic mice exhibited a significant increase in frequency of 2B4+ CD4+ T cells as compared to sham mice (7.79% vs. 4.3%; p = 0.0421; Fig 6A and 6B). However, when septic mice were treated with plerixafor, the frequency of 2B4+ CD4+ T cells was significantly decreased compared to septic control mice (3.4% vs. 7.79%; p = 0.0108; Fig 6A and 6B). Likewise, septic control mice demonstrated an increase in the per-cell expression of 2B4 on CD4+ T cells compared to sham mice, as measured by MFI (101.4 vs. 80.9; p = 0.068; Fig 6C); septic mice treated with plerixafor displayed a significant decrease in the expression of 2B4 on CD4+ T cells compared to untreated septic animals (80.6 vs. 101.4; p = 0.05; Fig 6C).

Plerixafor treatment did not affect frequencies of cytokine-producing T cells or the levels of circulating pro- or anti-inflammatory cytokines

To determine the effect of CXCR4 blockade on T cell functionality during sepsis, we harvested splenocytes at 24 hours post-CLP from either untreated mice or mice treated with plerixafor as described above, and restimulated them with PMA/ionomycin for 4 hours in vitro to assess...
their ability to produce the effector cytokines IL-2, IFN-γ, and TNF. We observed that frequencies of IL-2-, IFN-g, and TNF-secreting both CD4+ and CD8+ T cells were not different between untreated and plerixafor-treated groups (Fig 7A–7F). Next, to determine the effect of CXCR4 antagonism on the systemic cytokine environment during sepsis, we studied the levels of multiple circulating pro-inflammatory (Fig 8A) and anti-inflammatory cytokines (Fig 8B).
known to be elevated in sepsis in sham controls, CLP animals, and CLP animals treated with plerixafor. While all cytokines analyzed were significantly higher in both septic groups as compared to sham controls, no statistically significant differences in levels of cytokines between untreated CLP mice and and plerixafor-treated CLP animals at 24 hours post-surgery were identified, although there was a trend toward decreased IL-4 and IL-10 in plerixafor-treated septic animals.

**Fig 6. Plerixafor decreased the frequency of 2B4^+ CD4^+ T cells in septic mice.** (A) Representative flow plots (gated on CD3^+ cells) demonstrating 2B4 expression on CD4^+ T cells. (B) Septic mice exhibited a significant increase in frequency of 2B4^+ CD4^+ T cells compared to sham mice (7.79% vs. 4.3%; p = 0.0421). When septic mice were treated with plerixafor, the frequency of 2B4^+ CD4^+ T cells was significantly decreased compared to septic control mice (3.4% vs. 7.79%; p = 0.0108). (C) Septic control mice exhibited an increase in the per-cell expression of 2B4 on CD4^+ T cells compared to sham mice, as measured by the MFI (101.4 vs. 80.9; p = 0.068). Septic mice treated with plerixafor exhibited a significant decrease in the expression of 2B4 on CD4^+ T cells compared to septic control mice (80.6 vs. 101.4; p = 0.05). N = 3–5 mice/group. Representative of 3 independent experiments with a total of 9–15 mice/group. [https://doi.org/10.1371/journal.pone.0188882.g006](https://doi.org/10.1371/journal.pone.0188882.g006)
Fig 7. Cytokine production by CD4+ and CD8+ T cells is similar in plerixafor-treated septic animals as compared to control septic animals. Control or plerixafor-treated septic animals were sacrificed at 24h post-CLP and splenocytes were restimulated ex vivo with PMA/ionomycin for 4 h. Cells were fixed, permeabilized, and frequencies of IL-2 (A-B), IFN-γ (C-D), and TNF (E-F) secreting CD4+ (A, C, E) and CD8+ (B, D, F) T cells were assessed by flow cytometry. Data shown are cumulative from two independent experiments (n = 4-8/group).

https://doi.org/10.1371/journal.pone.0188882.g007
Discussion

In this series of investigations, we found that CXCR4 blockade improved survival in murine polymicrobial sepsis, increased the absolute number of circulating CD4+ and CD8+ T cells, and mitigated sepsis-induced T cell exhaustion phenotypes. Specifically, plerixafor treatment following CLP significantly decreased the percentage of PD-1, LAG-3, and 2B4-expressing CD4+ T cells in septic mice. Given these findings, one potential explanation for the improved survival observed following CXCR4 blockade is that mobilizing bone marrow stores of...
functional immune cells replenishes circulating populations of T cells that have been diminished in the periphery due to sepsis-induced apoptosis or migration to the site of inflammation. This hypothesis is supported by our findings of increased numbers of CD4$^+$ and CD8$^+$ T cells in the periphery of plerixafor-treated septic mice as compared to control septic mice, and is consistent with the known mechanism of action of the drug [33]. Recruitment of “fresh” T cell populations from bone marrow stores may replenish splenic T cell populations with cells that have been less affected by the inflammatory milieu of the circulation. As such, we speculate that plerixafor treatment results in a decrease in PD-1$^{hi}$ cells not because of a down-regulation in PD-1 on existing PD-1$^{hi}$ cells, but because the population of PD-1$^{hi}$ cells may be “diluted out” by the influx of non-exhausted T cells mobilized from the bone marrow, thereby mitigating T cell exhaustion and immune-incompetence that occurs during sepsis.

Alternatively, CXCR4 antagonism could be having a direct effect on T cell activation and/or expansion independently of any effect of bone marrow niche mobilization. Indeed, CXCR4 has been shown to associate with the TCR complex, and CXCL12/CXCR4 signaling is necessary for TCR-initiated immune synapse formation, enhanced phosphorylation of early signaling molecules, and thymic β selection [34–39]. A recent study also showed that upon ligation of TCR, the TCR associates with and trans-activates CXCR4 in order to activate a PREX1-Rac1 signaling pathway that stabilizes IL-2, IL-4, and IL-10 mRNA transcripts [40]. Thus, it is possible that increased CXCR4 expression during sepsis may amplify this pathway and result in enhanced IL-2, IL-4, and/or IL-10 secretion. Consistent with this possibility, our data indicated that CXCR4 antagonism with plerixafor resulted in a trend toward reduced serum concentrations of IL-4 and IL-10 in septic animals (Fig 7).

As discussed above, our study shows that CXCR4 is upregulated on naïve CD4$^+$ and CD8$^+$ T cells, as well as CD4$^+$ T$_{CM}$, during sepsis. Interestingly, two pathways of CXCR4 upregulation on T cells have been described. One is mediated via ligation of common gamma chain cytokine receptors including IL-2, IL-4, IL-7, IL-15, and IL-21 [41], which are not the classic inflammatory cytokines thought to be induced following CLP. An alternative pathway of CXCR4 upregulation has also been described, driven by NFKB binding to the Cxcr4 promoter [42]. This pathway depends on CD154/CD40 and CD134/CD134L interactions, and is independent of TCR ligation [43]. Intriguingly, CD40/CD134-dependent CXCR4 ligation was actually inhibited in the presence of CD3-mediated signals. Taken together with our results, these data suggest that bystander activation of T cells in an inflammatory environment in which CD40 and/or CD134 signals are present in the absence of cognate antigen may drive CXCR4 upregulation during sepsis.

Moreover, the effect of sepsis on CXCR4 expression was T cell subset-specific, in that expression was not upregulated on CD8$^+$ T$_{CM}$, or on T$_{EM}$ in either the CD4$^+$ or CD8$^+$ compartments. Our data showing that CXCR4 was most highly upregulated on naïve and T$_{CM}$ cells following sepsis are consistent with findings from human studies which show that the highest expression of CXCR4 is on naïve T cells, with minimal expression on highly differentiated T$_{EM}$ or effector memory-RA (TEMRA) cells [44]. Given what is known about the mechanisms that control CXCR4 expression on T cells, it is possible that this differential expression results from differential expression of common gamma chain cytokine receptors, TNF family members CD40 and/or CD134, or epigenetic remodeling of the Cxcr4 locus in more highly differentiated T cell subsets that render the promoter inaccessible to NFkB.

Our results indicate a strong survival advantage in animals treated with the CXCR4 antagonist plerixafor. In support of this, a recent study of human septic patients revealed that CXCL12 levels were higher in patients with severe sepsis/septic shock as compared to healthy subjects. Moreover, the same study also found that patients who survived their septic insult
possessed lower serum levels of CXCL12 than those who died [26]. The data from our CLP model together with these clinical findings therefore lead us to conclude that engagement of the CXCR4/CXCL12 pathway is deleterious during sepsis. These results are seemingly inconsistent with earlier findings from Efron’s group, which showed that blockade of the CXCR4 ligand CXCL12 resulted in increased mortality in murine models of CLP and Pseudomonas infection [45]. The finding that blocking the receptor (CXCR4) yields a result distinct from that observed when blocking the ligand (CXCL12) may suggest that one or more alternate binding partners is available and capable of modulating the response during sepsis. Adding to the complexity, administration of plerixafor in a model of LPS-induced endotoxemia resulted in enhanced serum levels of TNF, IFN-γ and NO levels and overall worsened outcomes [46]. These results highlight differences between models of LPS-induced endotoxemia and septic shock induced by a live, replicating organism.

Our study is limited by the fact that the data presented here represent only a single time point post-sepsis, and by the focus on analyses of the T cell compartment in plerixafor-treated animals. CXCR4 is expressed on many immune cell types including monocytes and DC, and our preliminary data suggest that CXCR4 may also be upregulated on these lineages during CLP (data not shown). Thus, identification of the effects of plerixafor on the T cell compartment during sepsis does not preclude the possibility that CXCR4 antagonism could affect other immune cell types during sepsis. Additional analyses are warranted in this regard. In sum, our study identifies an important pathway that modulates immune dysfunction and mortality following sepsis, which may hold promise as a target for future therapeutic intervention in septic patients.

Acknowledgments

This work was supported by funding from the National Institutes of Health grants GM104323, GM109779, and GM113228 (to MLF and CMC), GM072808 and GM095442 (to CMC), GM117895 (to JDL), GM110537 (to KWM) and Shock Society Research Fellowship for Early Career Investigators (to KWM).

Author Contributions

**Conceptualization:** Kevin W. McConnell, Craig M. Coopersmith, Mandy L. Ford.

**Formal analysis:** Kimberly M. Ramonell, Wenxiao Zhang, Kevin W. McConnell, Mandy L. Ford.

**Funding acquisition:** Kevin W. McConnell, Craig M. Coopersmith, Mandy L. Ford.

**Investigation:** Kimberly M. Ramonell, Wenxiao Zhang, Annette Hadley, Ching-wen Chen, Katherine T. Fay, John D. Lyons, Nathan J. Klingensmith.

**Methodology:** Kevin W. McConnell, Craig M. Coopersmith, Mandy L. Ford.

**Project administration:** Kevin W. McConnell, Craig M. Coopersmith, Mandy L. Ford.

**Supervision:** Craig M. Coopersmith, Mandy L. Ford.

**Writing – original draft:** Kimberly M. Ramonell, Mandy L. Ford.

**Writing – review & editing:** John D. Lyons, Nathan J. Klingensmith, Craig M. Coopersmith, Mandy L. Ford.
References


