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Large Peritoneal Macrophages and Transitional Premonocytes Promote Survival during Abdominal Sepsis

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ABSTRACT

Monocytes and macrophages are early sentinels of infection. The peritoneum contains two resident populations: large and small peritoneal macrophages (LPMs and SPMs). While LPMs self-renew, circulating monocytes enter the peritoneum and differentiate into SPMs. We lack information on the dynamics of monocyte–macrophage trafficking during abdominal sepsis, reflecting an important knowledge gap. In this study, we characterize the presence of LPMs, SPMs, and monocytes in the peritoneum of mice following cecal ligation and puncture (CLP)–induced sepsis and sham surgery. LPMs rapidly disappeared from the peritoneum and were scarce at 18–66 h after CLP or sham surgery. By 14 d, LPMs returned for sham mice, but they remained scarce in CLP mice. Depletion of LPMs from the peritoneum of CD11b-DTR mice greatly increased animal mortality. These data imply that LPMs are critical for sepsis survival. Monocytes rapidly infiltrated the peritoneum and were abundant at 18–66 h after CLP or sham surgery. Surprisingly, SPMs only increased at 14 d post-CLP. Therefore, monocytes may defend hosts from acute sepsis mortality without generating SPMs. More monocytes were present in mice predicted to survive sepsis versus mice predicted to die. However, altering monocyte numbers via CCR2 deficiency or adoptive transfer did not significantly affect animal survival. We reasoned that animals destined to survive sepsis may exhibit a different monocyte phenotype, rather than merely enhanced numbers. Indeed, mice predicted to survive possessed more CD31+, CXCR4hi transitional premonocytes in their abdomen. Inhibition of CXCL12–CXCR4 signaling via AMD3100 exacerbated sepsis. These data imply that recruitment of transitional premonocytes to the abdomen promotes sepsis survival.


INTRODUCTION

With most infections, the immune system successfully eliminates the pathogen from the body, resulting in no symptoms, or a relatively mild and transient illness. In contrast, when infection cannot be resolved, dysregulated immunity may ensue, leading to organ damage: this condition is called sepsis (1). During the early stages of sepsis, the adaptive immune response has not fully developed and the body relies on myeloid cells to clear the infection, in particular neutrophils, monocytes, and macrophages (2).

Monocytes and macrophages are early sentinels of infection and exert both beneficial and detrimental functions during...
sepsis (3, 4). Similar to neutrophils, monocytes phagocytose and kill bacteria, but they retain a longer lifespan. Monocytes may differentiate into macrophages or dendritic cells. Monocytes and their progeny also produce proinflammatory cytokines (such as IL-6) that promote immunity, but can be detrimental at high levels, as well as anti-inflammatory cytokines that regulate the immune response (4, 5). Although it is well known that monocytes infiltrate the peritoneum in response to abdominal infection, the dynamics of their trafficking and differentiation into macrophages have not been extensively investigated in the context of abdominal sepsis, reflecting an important knowledge gap. It is unclear whether the inflammatory response to abdominal infection differs from the response to sterile inflammation, for example following abdominal surgery. Furthermore, it is unclear whether certain patterns of trafficking compose a functional response in animals that survive sepsis, whereas other patterns compose a dysfunctional response in animals that succumb.

Two tissue-resident macrophage populations exist in the peritoneum of mice: large and small peritoneal macrophages (LPMs and SPMs) (6, 7). These subsets are both CD11b⁺, but differ in their expression levels of F4/80 and MHC class II (MHC-II) (6). LPMs express high levels of F4/80 and low levels of MHC-II. In contrast, SPMs express low levels of F4/80 and high levels of MHC-II (6). In a naïve mouse, LPMs comprise ~90% of peritoneal macrophages, whereas SPMs account for only ~10% (6). LPMs are a longer-lived, self-renewing population that derives from a yolk sac progenitor (7–10). In contrast, SPMs are a shorter-lived population that is replenished by monocytes that infiltrate the peritoneum (6–8, 10, 11). This process occurs at low levels in healthy animals to maintain a steady state. During peritoneal inflammation, however, additional monocytes infiltrate this body cavity and have the capacity to differentiate into SPMs, increasing their numbers (6, 7, 11). Simultaneously, LPMs disappear from the peritoneal fluid (6, 7, 10, 11) and have the capacity to migrate to damaged tissues and phagocytose apoptotic cells (12).

In this study, we characterize the dynamic changes in the peritoneal monocyte-macrophage populations during sepsis induced by cecal ligation and puncture (CLP), and following sterile sham surgery. We identify trends in animals that are predicted to live, versus those predicted to die. We test the functional importance of LPMs and monocytes for sepsis survival.

MATERIALS AND METHODS

Animals

For animals and surgical procedures, all experiments were approved by the Institutional Use and Care Committee at Texas Tech University Health Sciences Center El Paso. C57BL/6J [wild-type (WT)], CD11b-DTR, and CCR2-knockout (KO) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in the laboratory animal research facility at Texas Tech University Health Sciences Center El Paso, under specific pathogen-free conditions, to generate offspring for this study. Experimental animals included both male and female mice 6–16 wk of age. Mice were housed in social groups of one to five in nonsterile Optimice cages (Animal Care Systems, Centennial, CO). Animals were provided corncob bedding and SRS extruded PicoLab rodent diet 20 (Lab Diet, St. Louis, MO). A 12-h light/12-h dark cycle was used in the facility. The colony was found to be free from specific pathogens (including Helicobacter pylori and norovirus) via sentinel testing.

Sepsis model and animal monitoring

Mice underwent CLP to induce sepsis (1-cm ligation, 1× 21G puncture), per our prior study (13). All animals received buprenorphine SR (sustained release) analgesia prior to surgery and at 48- to 66-h intervals, 1 ml of lactated Ringer’s solution (Covetrus, Chicago, IL) s.c. postoperative, and imipenem/cilastatin antibiotics (Primaxin, Merck, Kenilworth, NJ) i.p. at 12-h intervals for 5 d, starting at 2–6 h post-CLP. In sham surgeries, the cecum was not tied off or punctured; these animals also received buprenorphine SR, fluids, and antibiotics. Animal survival was monitored using a humane endpoint: mice that were unable to regain sternal recumbence when placed on their side were considered moribund. These animals were recorded as “dead” at the subsequent time point. We observed a 9–50% mortality rate in this model, depending on the operator and seasonal effects, similar to our prior studies (13–15). We adjusted the timing of the first dose of Primaxin (from 2 to 6 h postsurgery) to attenuate these seasonal effects. Mouse disease scores were quantified as follows: 0, alert; 1, slightly lethargic; 2, lethargic; 3, very lethargic; 4, dead. Surface temperature was measured with an infrared thermometer on the animal’s sternum. Our model follows recent guidelines on minimum thresholds for preclinical sepsis models (16).

Injection of mice with 4% thioglycollate or CS

Animals were injected with 1.5 ml of 4% thioglycollate solution or cecal slurry (CS) at a dose of 1.5 mg of cecal content per gram of body weight, using a stock solution of 60 mg/ml CS in 5% dextrose, per our prior study (17).

Bleeds and serum preparation

The retro-orbital bleed technique is described in detail in our prior studies (17, 18). Blood was collected into a gold-top BD Microtainer with clot activator and serum separator additive (Becton Dickinson, Franklin Lakes, NJ) and spun at 12,700 rpm for 2 min. Serum was collected above the clot separator interface and stored at −80°C.

Harvesting peritoneal lavage and cell staining

The procedure to harvest peritoneal lavage is described in detail in our prior study (18). Lavage cells were diluted with trypan blue and counted on a hemacytometer slide. Peritoneal lavage cells (1 × 10⁶) were aliquoted per tube and suspended in

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45 μl of staining buffer (2% FBS in PBS). We used a modified version of the staining protocol described in our prior study (18). One microliter of Fe Block (MS CD16/CD32; clone 2.4G2; Tonbo Biosciences, San Diego, CA) was added to each tube, and the tubes were incubated for 5 min on ice. Subsequently, 5 μl of working Ab mix was added to each tube (this mix contained fluorescently labeled Abs at 10× the final concentration) and the tubes were incubated on ice for 1 h. Subsequently, 500 μl of staining buffer was added to each tube to wash the cells, and the tubes were spun at 2400 rpm for 2 min. The supernatant was removed and cells were resuspended in 250 μl of staining buffer and transferred to 12×75-mm polypropylene tubes for FACS analysis. To assess cell viability, 5 μl of 7-aminoactinomycin D (7-AAD) (Tonbo Biosciences, San Diego, CA) was added and the tubes were incubated at room temperature for 10 min. Fluorescence data were collected with a BD FACS Canto II (BD Biosciences, San Jose, CA) flow cytometer and analyzed with FlowJo software (FlowJo, Ashland, OR).

To identify LPMs and SPMs, we used the following Abs: Pacific Blue anti-mouse CD45.2, clone c104; allophycocyanin anti-mouse CD11b, clone M1/70; PE-Cy7 anti-mouse F4/80, clone BM8 (all from BioLegend, San Diego, CA); PerCP-Cy5.5 anti-mouse Ly6G, clone 1A8; PerCP-Cy5.5 anti-mouse CD3e, clone 145-2C11; PerCP-Cy5.5 anti-mouse CD11c, clone HL3; PerCP-Cy5.5 anti-mouse Siglec-F, clone E50-2440 (all from BD Biosciences, San Jose, CA); PerCP-Cy5.5 anti-mouse CD19, clone I3D (Tonbo Biosciences, San Diego, CA); and FITC anti-mouse MHC-II (I-Ab), clone E50-2440 (Thermo Fisher Scientific, Waltham, MA). The gating strategy is illustrated in Fig. 1. Leukocytes were gated according to their forward light scatter (FSC) area versus side light scatter area profile, after which singlets were identified according to their FSC height versus FSC area profile. We used a dump gate to exclude cells that were Ly6G+ (neutrophils), CD3+ (T cells), CD19+ (B cells), Siglec-F+ (eosinophils), and CD11c+ (dendritic cells), as well as dead cells that took up the 7-AAD dye. CD45.2+ leukocytes were gated, followed by CD11b+ myeloid cells. LPMs were then identified as F4/80hi, MHC-IIhi cells and SPMs as F4/80lo, MHC-IIhi cells. To identify monocytes, we used the same Abs listed above for LPM and SPM identification, but substituted FITC anti-mouse Ly6C, clone HKL4 (BioLegend, San Diego, CA) for the MHC Ab. We used the strategy described above to gate CD11b+ myeloid cells, and then monocytes were identified as Ly6C− cells, as illustrated in Fig. 1.

To identify CD11b+ and CD31− monocytes and neutrophils, we used the following Abs: allophycocyanin anti-mouse CD11b, clone M1/70; PerCP anti-mouse Ly6G, clone 1A8; Pacific Blue anti-mouse Ly-6C, clone HKL4 (all from BioLegend, San Diego, CA); and PE anti-mouse CD31, clone MEC 13.3 (BD Biosciences, San Jose, CA). In this case, due to limited availability of FACS channels, we omitted the use of CD45, the dump gate Abs, and 7-AAD (our analyses indicated that virtually all singlets were CD45+ and 7-AAD+, and the dump gate Abs were dispensable to distinguish monocytes, with the exception of Ly6G). We gated singlet cells as described above and then gated CD11b+ cells, after which we gated monocytes according to their Ly6C+, Ly6Glo profile and neutrophils according to their Ly6C−, Ly6Ghi profile. Subsequently, CD31+ and CD31− cells were distinguished within the monocyte gate.

To quantify CXCR4 expression within CD31+ and CD31− monocytes, we used the following Abs: allophycocyanin anti-mouse CD11b, clone M1/70; PerCP anti-mouse Ly6G, clone 1A8; Pacific Blue anti-mouse Ly-6C, clone HKL4 (all from BioLegend, San Diego, CA); PE-Cy7 anti-mouse CD31, clone 390; and PE anti-CD184/CXCR4, clone 2B11 (BD Biosciences, San Jose, CA). We gated CD31+ and CD31− monocytes as described above, and then quantified CXCR4 mean fluorescence intensity within each population.

To quantify intracellular arginase-1 expression within CD31+ and CD31− monocytes, we used the same Abs listed above, but substituted PE anti-human/mouse arginase-1, clone A6exF5 (Thermo Fisher Scientific, Waltham, MA). After staining with cell surface Abs (as described above), cells were fixed with 200 μl of IC (intracellular) fixation buffer (Thermo Fisher Scientific, Waltham, MA) for 10 min on ice followed by two washes (500 μl each) with permeabilization buffer (Thermo Fisher Scientific, Waltham, MA) and then spun as described above and resuspended in 45 μl of permeabilization buffer. Cells were then stained with PE anti-human/mouse arginase-1 on ice for 30 min. Cells were washed with permeabilization buffer again and resuspended in staining buffer. Identification of CD31+ cells followed the procedure described in the previous paragraph.Arginase-1 mean fluorescence intensity was calculated within the CD31+ and CD31− cell gates.

**Depletion of LPMs in CD11b-DTR mice**

To study the role of LPMs in CLP-induced sepsis, male and female CD11b-DTR transgenic mice were used. Animals were injected i.p. with diphtheria toxin (DT; 25 ng per gram of body weight from a stock of 2 mg/ml, Sigma-Aldrich, St Louis, MO) or the same volume of PBS vehicle. To determine the effect on monocytes and macrophages, animals were euthanized at 18 h after DT injection, peritoneal lavage was harvested, and lavage cells were stained for LPMs, SPMs, and monocytes as described above. To study the effect of LPM depletion on sepsis survival, animals were injected with DT or PBS vehicle 18 h prior to CLP.

**Monocyte adoptive transfer**

For adoptive transfer of monocytes, C57BL/6J mice were used as donors. The mouse femurs and tibias were harvested and the marrow was flushed out as described previously (14, 15). Monocytes were enriched via magnetic selection using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) per the manufacturer’s protocol. Monocyte purity was determined to be >90% via FACS analysis. Monocytes (3 × 10⁶, 200 μl of PBS) were injected into the peritoneum of recipient C57BL/6J mice 1 h after they underwent CLP.
**AMD3100 treatment**

AMD was suspended in PBS at 0.4 mg/ml for experimental use. Animals were treated with 5 mg of AMD3100 per kilogram of body weight, delivered via i.p. injection, while controls received the same volume of PBS. The drug was administered immediately postsurgery and then twice daily (at 9:00 AM and 5:00 PM) for 4 d. To determine the effect on monocytes and neutrophils, animals were euthanized at 18 h post-CLP, peritoneal lavage was harvested, and lavage cells were stained to identify monocytes, neutrophils, and CD31, as described above.

**Statistical analyses**

Graphs show pooled data from at least three experimental repeats, except where noted. GraphPad Prism 6 software (GraphPad Software, San Diego, CA) was used to perform statistical analyses. The Kaplan–Meier log-rank test was used for survival comparisons. Two-way ANOVA was used to compare animal disease scores. A Mann–Whitney U test was used for pairwise comparisons of cells populations and cytokine levels. A $p$ value $\leq 0.05$ was considered significant.

**RESULTS**

**Abdominal sepsis and sterile surgery induce dramatic changes in the monocyte-macrophage populations present in the abdomen**

Monocyte and macrophage populations were quantified in the peritoneum of mice at 0 h, 18 h, 66 h, and 14 d after CLP (to induce sepsis) or sham surgery. We used flow cytometry to identify LPMs (CD11b$^+$, F4/80$^{hi}$, MHC-II$^{lo}$), SPMs (CD11b$^+$, F4/80$^{lo}$, MHC-II$^{hi}$), and monocytes (CD11b$^+$, Ly6C$^{hi}$) within the singlet, CD45$^+$ leukocyte gate, after excluding cells of non-interest, as described in the Materials and Methods (Fig. 1). We found that total leukocyte numbers were increased in the peritoneum of mice at 18 h, 66 h, and 14 d post-CLP, and at 66 h and 14 d after sham surgery (Fig. 2A). Additionally, we observed a dramatic shift in the peritoneal monocyte-macrophage populations following surgery, both in the presence and absence of infection. In naive mice, we observed that LPMs were the dominant monocyte-macrophage population present, exhibiting a high number (Fig. 2B) and representing a significant percentage of the CD45$^+$ leukocytes (Fig. 2E). However, LPM numbers and percentages were dramatically reduced in the peritoneal cavity at 18 and 66 h after CLP or sham surgery (Fig. 2B, 2E). CLP mice had a significantly lower LPM percentage in comparison with sham mice at 18 h (Fig. 2E); however, LPM numbers were similar (Fig. 2B). By 14 d postsurgery, LPMs had returned to the peritoneum of most sham-operated mice (Fig. 2B, 2E). In contrast, most CLP mice still exhibited a deficit in LPMs at 14 d (Fig. 2B, 2E).

Monocytes were virtually absent from the peritoneal cavity of naive mice (Fig. 2C, 2F). However, at 18 and 66 h after CLP or sham surgery, we observed high numbers of monocytes (Fig. 2C), and they represented a major percentage of the CD45$^+$ leukocytes present (Fig. 2F). The monocyte number and percentage were significantly increased in CLP mice versus naive controls at 18 and 66 h postsurgery, and similar effects...
were observed for sham mice (Fig. 2C, 2F). Furthermore, monocyte numbers were significantly higher in CLP mice versus sham controls at 18 and 66 h (Fig. 2C). Conversely, the monocyte percentage was significantly lower in CLP mice versus sham controls at 18 h post-CLP (Fig. 2F). Monocytes remained significantly elevated at 14 d after CLP or sham surgery (Fig. 2C, 2F). The monocyte percentage was significantly higher in CLP mice versus sham controls at 14 d, but the absolute numbers were similar (Fig. 2C, 2F).

SPMs (a population of macrophages that differentiate from infiltrating monocytes) represented a minor population of cells in the peritoneal cavity of naive mice, in terms of their absolute numbers (Fig. 2D) and percentage (Fig. 2G). Furthermore, SPMs showed only a slight increase at 18 and 66 h after CLP or sham surgery (Fig. 2C, 2F). The monocyte percentage was significantly higher in CLP mice versus sham controls at 14 d, but the absolute numbers were similar (Fig. 2C, 2F).

Prior studies found that during peritoneal inflammation, monocytes infiltrate the peritoneal cavity and rapidly differentiate into SPMs, substantially increasing their number (6, 7, 11), contrasting with the aforementioned results. We employed additional models of peritoneal inflammation to resolve this discrepancy. In mice injected with CS or thioglycollate, we observed high leukocyte numbers in the peritoneum of mice at 18 and 66 h postinjection (Supplemental Fig. 1A). LPMs were absent from the peritoneal wash at both 18 and 66 h postinjection of CS or thioglycollate (Supplemental Fig. 1B, 1E). We observed monocytes in the peritoneal cavity of mice at 18 h postinjection of CS or thioglycollate, and these cells remained abundant at 66 h (Supplemental Fig. 1C, 1F). In thioglycollate-injected mice, we observed a substantial increase in SPMs at 66 h postinjection (Supplemental Fig. 1D, 1G). In contrast, SPMs remained rare in the mice injected with CS at 66 h (Supplemental Fig. 1D, 1G). In summary, our results indicate that chemical inflammation (thioglycollate) induced monocyte infiltration, followed by rapid SPM differentiation, whereas sepsis in the presence or absence of surgery (CLP, CS), or sham surgery alone, induced monocyte infiltration with limited SPM differentiation during the first few days (Fig. 2, Supplemental Fig. 1).

**LPMs are critical for sepsis survival**

To determine the functional role of LPMs during sepsis, we employed CD11b-DTR mice. We found that this strain could be selectively depleted of LPM via i.p. DT treatment. At 18 h after injection of DT, peritoneal LPMs were virtually absent, whereas they remained abundant in PBS-injected mice (Fig. 3A). SPMs showed minimal changes following DT injection (Fig. 3A). Blood monocytes remained intact in CD11b-DTR mice treated with DT (Fig. 3A), and a small number of monocytes were recruited into the peritoneal cavity after DT treatment (Fig. 3A).

To determine how depletion of LPMs affected sepsis survival, CD11b-DTR mice were depleted of LPMs via i.p. DT injection, or administered PBS (control group) 18 h prior to...
sepsis induction via CLP. Animals depleted of LPMs exhibited a substantially worse outcome from sepsis versus PBS-treated controls, exhibiting significantly greater mortality (Fig. 3B) and higher disease score (Fig. 3C). Furthermore, the mice depleted of LPMs exhibited significantly higher levels of serum cytokines at 18 h post-CLP, including IL-6 (Fig. 3D), IL-12p40 (Fig. 3E), and MCP-1 (Fig. 3F).

Mice predicted to survive sepsis have more peritoneal monocytes versus mice predicted to die
We next sought to investigate cellular differences in animals that are destined to survive sepsis versus those destined to succumb. A prior study showed that serum IL-6 levels predict sepsis outcome in mice (19). To validate this biomarker, we analyzed data from a cohort of n = 26 WT C57BL/6 mice subjected to CLP (with 38.4% mortality), described in our prior study (14). All animals that survived sepsis exhibited serum IL-6 levels <5 ng/ml, whereas all animals that succumbed acutely (day 0–3) demonstrated serum IL-6 levels ≥5 ng/ml (Fig. 4). For the few animals that exhibited late mortality (day 4+), the IL-6 levels at 18 h post-CLP were variable (Fig. 4). Therefore, a serum IL-6 cutoff of 5 ng/ml at 18 h postsurgery segregates mice into groups predicted to live (P-live) or die acutely after CLP (P-die), akin to the prior study (19).

We performed CLP on a separate cohort of mice and measured serum IL-6 at 18 h postsurgery to segregate the animals predicted to live (P-live; IL-6 < 5 ng/ml) versus those predicted to die (P-die; IL-6 > 5 ng/ml; Fig. 5A). In tandem, we euthanized these animals at 18 h postsurgery and quantified cells in their peritoneal cavity. We observed that mice predicted to live had a higher number (Fig. 5B) and percentage (Fig. 5C) of monocytes in their peritoneal cavity versus mice predicted to die acutely. In contrast, the numbers of LPMs and SPMs were similar (data not shown).
Altering monocyte numbers via CCR2 deficiency or adoptive transfer did not significantly affect animal survival

The CCR2 chemokine receptor plays a major role in emigration of mature monocytes from the bone marrow into the blood and subsequently to the local site of inflammation (20, 21). We therefore used CCR2-KO mice to examine the effect of infiltrating monocytes on sepsis survival. We confirmed that CCR2-KO mice have fewer monocytes in their peritoneal cavity 18 h after CLP, relative to WT counterparts, in terms of their number (Fig. 6A) and percentage (Fig. 6B). Contrary to our expectations, however, CCR2-KO mice exhibited a nonsignificant trend toward improved survival (Fig. 6C) and disease score (Fig. 6D) after CLP, relative to WT counterparts. We observed similar levels of IL-6 (Fig. 6E), IL-12p40 (Fig. 6F), and MCP-1 (Fig. 6G) in the sera of CCR-2 KO mice and WT mice at 18 h postsurgery.

As a complementary approach, we examined whether supplementing monocytes via adoptive transfer attenuated sepsis pathogenesis. Mice that received 3 x 10^6 monocytes i.p. 1 h after CLP had similar survival to controls that received no monocytes (Fig. 7A), as well as similar disease score (Fig. 7B) and body temperature (Fig. 7C). Furthermore, serum levels of IL-6 (Fig. 7D), IL-12p40 (Fig. 7E), and MCP-1 (Fig. 7F) were similar. In summary, our experimental manipulations of monocyte numbers via CCR2 deficiency or adoptive transfer had little effect on sepsis pathogenesis.

FIGURE 5. Sepsis survival is associated with increased numbers of peritoneal monocytes.
Mice were subject to CLP. Eighteen hours later, serum and peritoneal lavage cells were obtained. (A) Animals were stratified into those predicted to survive (P-live; IL-6 < 5 ng/ml) versus those predicted to die (P-die; IL-6 > 5ng/ml). (B) Absolute numbers and (C) percentages of monocytes in P-live versus P-die mice at 18 h post-CLP. Graph shows individual values, as well as the 5 ng/ml cutoff for IL-6, and the median for monocyte numbers and percentage.

Altering monocyte numbers via CCR2 deficiency or adoptive transfer did not significantly affect animal survival

FIGURE 6. CCR2 deficiency does not exacerbate sepsis.
CCR2-KO and WT (C57BL/6J) mice were subject to CLP. (A) Absolute numbers and (B) percentages of monocytes in the peritoneum of CCR2-KO mice versus WT mice were quantified at 18 h post-CLP. (C–G) In a second cohort of animals we monitored (C) survival, (D) disease score, and quantified serum cytokines, including (E) IL-6, (F) IL-12p40, and (G) MCP-1. Graphs show individual values and the median for monocyte number and percentage as well as cytokines, Kaplan–Meier plots for animal survival, and line graphs with the mean and SE for disease score.

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Animals predicted to survive sepsis possess more transitional premonocytes in their abdomen versus animals predicted to die

Based on the data above, we reasoned that the additional monocytes present in mice predicted to survive sepsis may include cells with different characteristics, rather than merely increased numbers. We therefore examined cellular markers associated with immaturity (CD31 and CXCR4). We found that mice predicted to live showed a higher percentage of monocytes that were CD31$^+$ versus mice predicted to die (Fig. 8A). Moreover, the absolute number of CD31$^+$ monocytes was significantly higher in mice predicted to live versus mice predicted to die (Fig. 8B), whereas the number of CD31$^{-}$ monocytes was similar (Fig. 8C). Furthermore, the CD31$^+$ monocytes expressed higher levels of CXCR4 versus their CD31$^{-}$ counterparts (Fig. 8D). The Ly6C$^{hi}$, CD31$^+$, CXCR4$^{hi}$ phenotype identifies these cells as transitional premonocytes (the immediate precursor of mature monocytes), as described in a prior study (22). We also analyzed expression of arginase-1, an enzyme associated with the myeloid-derived suppressor cell (MDSC) phenotype. We found that the CD31$^+$ monocytes expressed higher levels of intracellular arginase-1 in comparison with the CD31$^{-}$ monocytes (Fig. 8E).

The CXCL12/CXCR4 axis promotes sepsis survival in mice

We reasoned that migration of transitional premonocytes from the bone marrow to the peritoneum may promote acute sepsis survival. A prior study showed that transitional premonocytes express high levels of CXCR4 but low levels of CCR2 (22). We therefore theorized that their trafficking is controlled by CXCL12 (CXCR4 ligand) instead of MCP-1 (CCR2 ligand). To test the effects of CXCL12-mediated CXCR4$^+$ antagonist on cell mobilization, we treated septic mice with the CXCL12 antagonist AMD3100 via i.p. injection, whereas control animals received PBS. Animals that received AMD3100 after CLP exhibited significantly fewer monocytes in their peritoneum at 18 h postsurgery, relative to controls that received PBS (Fig. 9A), and significantly fewer CD31$^+$ monocytes (transitional premonocytes; Fig. 9B). We also observed fewer neutrophils in animals that received AMD3100 after CLP, in comparison with PBS controls, although this difference was not statistically significant (Fig. 9C). We then used a separate cohort of mice to test the effects of AMD3100 on sepsis pathogenesis. Animals that received AMD3100 after CLP exhibited significantly worse survival than did PBS controls (Fig. 9D), as well as a higher disease score (Fig. 9E) and more severe hypothermia (Fig. 9F). The animals treated with AMD3100 also exhibited higher levels of serum cytokines, including IL-6 (Fig. 9G), IL-12$p40$ (Fig. 9H), and MCP-1 (Fig. 9I). Taken together, these data support the notion that recruitment of transitional premonocytes to the peritoneal cavity, via the CXCL12/CXCR4 chemokine axis, promotes sepsis survival.

DISCUSSION

Before this study, we lacked a comprehensive understanding of how monocyte and macrophage populations change in the peritoneal cavity during abdominal sepsis, reflecting an important knowledge gap. In this study, we determined the monocyte and macrophage populations present in the peritoneum of mice...
after CLP to induce sepsis, or sterile surgery. We observed substantial changes in these populations and identified cell populations that support survival during abdominal sepsis.

LPMs (CD11b\(^{+}\), F4/80\(^{hi}\), MHC-II\(^{lo}\)) are a hematogenous macrophage population in the abdomen of healthy mice, representing ~90% of resident macrophages (6, 7). They disappear from the peritoneal wash, however, after inflammatory insults to the peritoneum, or after organ injury (6, 7, 11, 12). In line with these findings, we observed that LPMs disappeared from abdominal wash within 18 h of CLP or sham surgery (Fig. 2B, 2E). A prior study indicated that the disappearance of LPMs is not caused by cell death, but rather by reversible migration and cell adhesion: in a model of sterile liver injury, LPMs migrated to the site of injury, adhered to this tissue, and dismantled necrotic cells (12). We predicted that LPMs could be relocating to the surgical wound in animals that have undergone surgery, as well as the punctured cecum in CLP mice. By 14 d after sham surgery, LPMs can once again be observed in the non-adherent lavage fraction of most sham mice, but have not yet returned for most CLP mice (Fig. 2B, 2E). We predicted that LPMs might be relocating to the surgical wound in animals that have undergone surgery, as well as the punctured cecum in CLP mice. By 14 d after sham surgery, LPMs can once again be observed in the non-adherent lavage fraction of most sham mice, but have not yet returned for most CLP mice (Fig. 2B, 2E). We predicted that by 14 d after sham surgery, the surgical wound has healed enough to release the LPMs. By 14 d post-CLP, we observed that an adhesion forms around the necrotic cecum tissue (data not shown). We predicted that LPMs may linger at this adhesive focus. However, this likely does not reflect the entire story, as LPMs also disappear by 18 h after injection of CS or thioglycollate (Supplemental Fig. 1B, 1E), where little tissue injury is expected, and others obtained similar findings with LPS, thioglycollate, and zymosan (6, 10–12). Okabe and Medzhitov (10) found that mice injected with LPS showed LPM relocation to the omentum, identifying another potential destination for these cells. In future studies, it will be important to determine the destination or fate of LPM following abdominal sepsis or sterile surgery.

We reasoned that LPMs may assist the host in surviving sepsis, and therefore performed mechanistic experiments to investigate this. We determined that CD11b-DTR mice were a suitable model to test this hypothesis. In our hands, administration of DT to CD11b-DTR mice specifically depleted LPMs from the peritoneal cavity within 18 h, with little effect on SPMs, and without depleting other populations of CD11b\(^{+}\) cells, including blood monocytes (Fig. 3A; in fact, moderate numbers of monocytes infiltrated the peritoneal cavity in response to the toxin, even in the absence of surgery or infection). It is perhaps surprising that DT specifically depleted LPMs in our mice, whereas other cell types that express CD11b remained intact. Notably, we found that CD11b expression levels were highest in the LPM population relative to other myeloid populations (this can be visualized in Fig. 1). We expect that the levels of DT expression driven by the CD11b promoter were likewise highest in LPMs, resulting in their specific depletion after DT treatment. It is possible that administering higher doses of DT (or a more potent preparation of DT) would deplete other subsets, including monocytes, neutrophils, and NK cells. Notably, our results differed from the original study that generated the

FIGURE 8. Sepsis survival is associated with increased numbers of CD31\(^{+}\) transitional premonocytes, which express high levels of arginase-1 and CXCR4.

Mice were subject to CLP. Eighteen hours later, serum and peritoneal lavage cells were obtained, and then animals were stratified into those predicted to survive (P-live; IL-6 < 5 ng/ml) versus those predicted to die (P-die; IL-6 > 5 ng/ml). (A) Percentage of CD31\(^{+}\) cells among monocytes, (B) absolute number of CD31\(^{+}\) monocytes, and (C) absolute number of CD31\(^{+}\) monocytes in P-live versus P-die mice at 18 h post-CLP. (D) CXCR4 and (E) Arginase-1 expression levels in CD31\(^{+}\) versus CD31\(^{-}\) peritoneal monocytes. Graphs show individual values and the median.

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CD11b-DTR mice, where DT treatment ablated both peritoneal macrophages and circulating monocytes (23). Potency of the DT preparation likely explains the discrepancy between our results and those of this prior study (23).

We were therefore able to use CD11b-DTR mice to evaluate how mice respond to sepsis in the absence of LPMs. CD11b-DTR mice were injected with DT or PBS and subject to CLP 18 h later. We found that depletion of LPMs via DT dramatically worsened sepsis (Fig. 3B–F). These results are consistent with the notion that LPMs play an essential, protective role in sepsis survival. We speculate that LPMs may assist in sealing the punctured cecum, to limit escape of commensal bacteria; however, this remains to be tested. In the future, it will also be interesting to determine whether LPMs play a role in healing the surgical incision. Despite the protective role of LPMs, we did not observe differences in LPM numbers between animals predicted to live and those predicted to die. Other populations, including transitional premonocytes, are likely needed to assure survival, as discussed below.

We found that monocytes infiltrate the peritoneal cavity within 18 h of CLP or sham surgery (Fig. 2C, 2F). Monocytes remain abundant in the peritoneal cavity of CLP and sham mice at 66 h postsurgery, and some mice still possess monocytes at 14 d (Fig. 2C, 2F). These data suggest that the monocyte response to peritoneal surgery and infection is long-lived. In the future it will be interesting to determine whether the monocytes present at later time points represent cells that have persisted over time, or new cells that continue to infiltrate.

SPMs (CD11b<sup>+</sup>, F4/80<sup>lo</sup>, MHC-II<sup>hi</sup>) represent ~10% of resident macrophages in naive mice (6, 7). These cells differentiate from circulating monocytes, and this process proceeds at low levels to maintain the steady state in healthy animals (8, 24). Prior studies found that this process is upregulated during inflammation, as large numbers of monocytes infiltrate the peritoneum and rapidly differentiate into SPMs following injection of LPS (6, 8), thioglycollate (6), or zymosan (11) into this compartment. In our model of sepsis, however, SPMs showed only

FIGURE 9. Administration of CXCL12 inhibitor to the local site of infection exacerbates sepsis.

WT mice were subject to CLP and then treated with AMD3100 (CXCL12 inhibitor) or PBS vehicle postoperatively and then twice daily for 4 d. (A–C) Absolute numbers of (A) monocytes, (B) CD31<sup>+</sup> monocytes, and (C) neutrophils were quantified in the peritoneum at 18 h post-CLP. (D–I) In a second cohort of animals we monitored (D) survival, (E) disease score, (F) surface temperature, and serum cytokines, including (G) IL-6, (H) IL-12p40, and (I) MCP-1. Graphs show individual values and the median for cell numbers, Kaplan–Meier plots for animal survival, line graphs with mean and SE for disease score and cytokines.
a slight increase at 18 and 66 h after CLP or sham surgery (Fig. 2D, 2G). Only at 14 d post-CLP did we observe a more profound increase in SPMs (Fig. 2D, 2G). To further explore the changes induced by inflammation, infection, and surgery, we also tested the cell populations present in mice after thioglycollate or CS injection, relative to our results with CLP and sham surgery. We found that mice injected with thioglycollate exhibited a notable increase in SPMs within 66 h postinjection (Supplemental Fig. 1D, 1G). In contrast, the CS group (infection only), CLP group (infection plus surgery), and sham group (surgery only) exhibited monocyte infiltration without rapid SPM differentiation (few SPMs at 18 and 66 h; Fig. 2C, 2D, 2F, 2G, Supplemental Fig. 1C, 1D, 1F, 1G). These data suggest that monocyte differentiation is limited in the context of polymicrobial infection induced by gut commensals, or tissue injury. We predict that certain pathogen-associated molecular patterns are released during bacterial infection and damage-associated molecular patterns are induced by surgical tissue injury, which inhibit monocyte differentiation into SPMs, or else cause the monocytes to die before maturing. We hope to test this in future studies. Notably, Cassado et al. (11) showed that injection of *Trypanosoma cruzi* into the peritoneal cavity of mice increases SPMs within 2 d, and therefore protozoan infection does not induce the same effect as abdominal sepsis.

To mimic clinical sepsis, our CLP model is designed so that some animals succumb to sepsis while other animals survive. Prior studies showed that IL-6 is a biomarker of sepsis outcome, with higher levels of IL-6 present in the sera of animals that die and lower levels present in animals that survive (19). In our own laboratory, a cutoff of 5 ng/ml IL-6 at 18 h post-CLP was established to effectively segregate animals that live versus those that die acutely (Fig. 4). Using lavage and serum samples obtained from CLP mice 18 h after surgery, we identified that mice predicted to survive sepsis possess a higher number and percentage of monocytes in their peritoneal cavity versus animals predicted to die (Fig. 5). These findings corroborate a prior study by Kuehle et al. (25) that also found that mice predicted to survive sepsis possessed more peritoneal monocytes than did animals predicted to die.

We next performed mechanistic experiments to determine whether altering monocyte numbers affects sepsis pathogenesis, using two complementary strategies. First, we used mice lacking the CCR2 chemokine receptor, which is known to play a major role in recruitment of Ly6C^hi^ monocytes from the bone marrow to the blood and tissues in response to infection (20, 21). CCR2-KO mice showed a significant reduction in peritoneal monocytes (Fig. 6A, 6B) relative to WT mice at 18 h post-CLP. Notably, the number and percentage of monocytes in CCR2-KO mice (<1 × 10^6^ monocytes, representing <8% of the cells in the peritoneum) were even lower than those observed in the WT mice predicted to die acutely (Fig. 6A, 6B). Despite this, sepsis survival was not significantly worsened by CCR2 deficiency. In fact, there was a trend toward improved survival and disease score in CCR2-KO mice versus WT counterparts (Fig. 6C, 6D). Others have investigated the effects of CCR2 in mouse models of sepsis, with mixed results. A study by Souto et al. (26) found that CCR2-KO mice, as well as animals treated with a CCR2 antagonist, were protected from CLP sepsis and showed reduced neutrophil infiltration. Likewise, a recent study by Cebinelli et al. (27) found that CCR2-KO mice showed improved survival after CLP, paired with reduced recruitment of inflammatory monocytes and increased recruitment of neutrophils. In contrast, another study by Feterowski et al. (28) found that Ab blockade of CCR2 reduced monocyte and neutrophil recruitment, impaired bacterial clearance, and exacerbated kidney injury in a mouse model of sepsis induced by colon ascendens stent peritonitis. Our data agree with those of Souto et al. and Cebinelli et al. and support the notion that CCR2 reduces animal survival during abdominal sepsis. Most likely the different results obtained by Feterowski et al. result from methodological differences (e.g., use of the CASP model and CCR2 blockade).

As a complementary strategy, we adoptively transferred 3 × 10^6^ monocytes into the peritoneal cavity of mice 1 h after CLP. However, this did not improve sepsis survival, disease score, or cytokine levels relative to animals that received an injection of PBS vehicle (Fig. 7). One limitation of this approach, however, is that the monocytes used for adoptive transfer were obtained from the bone marrow and have not undergone the numerous changes that occur during the transmigration process, which can alter their immune function. In summary, our experimental manipulations of peritoneal monocyte number did not affect animal survival in this CLP model. We expect that more significant changes in monocyte number (e.g., complete ablation of monocytes, or adoptive transfer of mature monocytes) would influence animal survival, as these cells play an important role in host defense.

We considered that the mice that survive sepsis may possess monocytes with a different character, rather than merely enhanced numbers. Because these mice exhibit lower levels of inflammatory cytokines (including IL-6), we considered that these cells may have a less inflammatory character, and perhaps represent a population of MDSCs. MDSCs are immature cells of the granulocytic and monocytic lineage that exhibit immunosuppressive functions. These cells arise in mice bearing tumors (29), and it is now well established that they play important roles during sepsis (30, 31). MDSCs are CD11b^+^ and GR-1^+^ (the GR-1 Ab clone detects an epitope common to Ly6G and Ly6C) and are somewhat difficult to distinguish from mature monocytes and neutrophils. Prior studies show that immunosuppressive MDSCs express CD31 (31, 32), a marker of immaturity, as well as the intracellular enzyme arginase-1 (31, 33). MDSCs may play a beneficial role to suppress acute inflammation through the production of arginase-1, among other mechanisms (30, 31, 33–40). Their persistence, however, may prevent the host from returning to homeostasis (30, 31, 39). Although it is clear that MDSCs play important roles in sepsis, much remains to be learned about the identity and function of these cells.
Our data indicate that mice destined to survive sepsis mobilize more CD31+ monocytes to the peritoneal cavity by 18 h post-CLP, relative to mice destined to succumb (Fig. 8A, 8B), whereas the number of CD31+ cells does not significantly differ (Fig. 8C). We performed further cell staining to investigate the identity of these immature cells. The CD31+ peritoneal monocytes present in septic mice expressed higher levels of CXCR4 relative to the CD31+ monocytes (Fig. 8D). The phenotype of these cells (CD11b+, Ly6C+, Ly6G−, CXCR4hi) is consistent with the “transitional premonocytes” that were recently described by Chong et al. (22). Transitional premonocytes represent the immediate precursor of mature monocytes (22). Whereas Chong et al. found that transitional premonocytes remain in the bone marrow, both in naive mice and during fatal LPS endotoxemia (22), we show, to our knowledge for the first time, that these cells are mobilized to the site of infection. The difference between our results and the prior study (22) likely stems from the severity of our inflammatory models. Chong et al. used a high dose of LPS that was 100% lethal (22), whereas our CLP model was designed to induce 10–50% mortality. Indeed, we observed few CD31+ monocytes in the peritoneum of mice predicted to succumb to sepsis, especially among the sickest mice, whereas these cells were prevalent in the peritoneum of mice predicted to survive (Fig. 8B). We also found that the CD31+ transitional premonocytes in the abdomen of septic mice express high levels of arginase-1 (Fig. 8E), consistent with an immunosuppressive MDSC phenotype. In future studies, we hope to elucidate the immunophenotype of these cells in more detail and perform more mechanistic experiments to examine how these cells contribute to sepsis survival.

CXCR4 is the receptor for the CXCL12 chemokine, which plays an important role in regulating the trafficking of myeloid cells from the bone marrow to the periphery (41, 42). To determine the functional importance of the CXCR4hi transitional premonocytes, we treated septic mice with AMD3100, a small molecule drug that blocks the interaction of the CXCR4 receptor with its ligand CXCL12, administered to the peritoneal cavity. AMD3100 (plerixafor) was originally tested as a drug to treat HIV, and it is currently used to mobilize stem cells and progenitor cells for transplantation (43). We confirmed that administration of AMD3100 to the peritoneum of mice after CLP significantly reduced the number of peritoneal monocytes, and specifically the number of CD31+ transitional premonocytes (Fig. 9A, 9B). AMD3100 treatment substantially decreased sepsis survival, disease score, and hypothermia and increased serum cytokine levels, relative to PBS vehicle (Fig. 9). These data are consistent with the notion that mobilization of transitional premonocytes to the peritoneal cavity prevents sepsis mortality.

Interestingly, prior studies have tested how the CXCL12/CXCR4 axis affects sepsis with mixed results. Some showed that this chemokine interaction protects mice from sepsis (42, 44), akin to our own study, whereas others showed that it makes mice more susceptible (22, 45). However, a more detailed analysis of the literature reveals that CXCL12 exhibits tissue-specific effects, which likely explains these findings. During health, a population of bone marrow–derived mesenchymal stem cells avidly express CXCL12, the so-called CXCL12 abundant reticular cells (46). CXCL12 interacts with CXCR4 to retain hematopoietic stem cells and mature immune cells in the bone marrow (41). During sepsis, CXCL12 is downregulated in the bone marrow and upregulated in the periphery (42). Administration of CXCL12 antagonist, or CXCL12 neutralizing Ab, to the peritoneal cavity exacerbated abdominal sepsis (42, 44), whereas s.c. administration of CXCL12 antagonist improved abdominal sepsis (45). Furthermore, specific deletion of CXCR4 in the myeloid lineage improves sepsis (22). These data are consistent with the notion that CXCL12 affects sepsis through its production in multiple body sites, and when the gradient favors migration of CXCR4hi cells to the local infection site, this exerts a protective effect.

Mature monocytes and transitional premonocytes exhibit differential expression of chemokine receptors. Mature monocytes express high levels of CCR2 and low levels of CXCR4 (22). The chemokine MCP-1 disrupts the CXCL12–CXCR4 interaction and allows mature monocytes to exit the bone marrow during inflammation, regardless of the CXCL12 gradient (20, 21). In contrast, transitional premonocytes express high levels of CXCL12 and low levels of CCR2 (22); therefore, their trafficking is likely to be predominantly controlled by the CXCL12 gradient, and our data support this notion (Fig. 9B). In a related finding, Delano et al. (42) also showed that the gradient of CXCL12 controls neutrophil mobilization from the bone marrow to the abdomen during sepsis. Therefore, multiple CXCR4hi populations, including transitional premonocytes and neutrophils, likely work together to promote acute sepsis survival. In future studies it will be important to examine in more detail how AMD3100 affects the migration of immature monocytes versus neutrophils, and the downstream effects on sepsis survival.

In summary, this study found that abdominal sepsis induced dramatic alterations in the composition of monocyte-macrophage cells within the peritoneum (exit of LPMs and infiltration of immature and mature monocytes). We would like to highlight what we perceive to be the novelty of this study in relationship to the prior literature. Prior studies demonstrated that monocytes infiltrate the peritoneum during abdominal inflammation and rapidly differentiate into SPMs (6–8, 11). In contrast, we found that differentiation of monocytes into SPMs occurs much more slowly during abdominal sepsis, and this process has not been completed during the time period when acute mortality is observed (>3 d). Our study shows, to our knowledge for the first time, that animals predicted to survive sepsis possess a large number of immature monocytes in their peritoneal cavity, in contrast to mice predicted to die, specifically, transitional premonocytes. To our knowledge, this is the first report suggesting that transitional premonocytes traffic to a local site of infection, as a prior study suggested that they remain in the bone marrow (22). Additionally, our study...
provides novel evidence suggesting that LPMs and transitional premonocytes promote sepsis survival.

Finally, we consider the relevance of this research for the treatment of septic patients. Although our research does show that LPMs are critical for sepsis survival, LPMs are abundant in all mice prior to surgery and are likely not a factor that varies between animals that survive sepsis versus those that succumb. In contrast, the infiltration of transitional premonocytes did vary, being more abundant in mice that survive sepsis. Therefore, transitional premonocytes are a cell population whose trafficking could potentially be modulated for therapeutic benefit. For example, in septic patients showing signs of overwhelming inflammation, administration of CXCL12 i.v. or to the local site of infection could potentially mobilize transitional premonocytes from the bone marrow to the periphery, to attenuate inflammation. Additionally, future studies should explore whether the frequency of transitional premonocytes in the bloodstream may serve as a biomarker of sepsis, or predict disease prognosis.

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DISCLOSURES

The authors have no financial conflicts of interest.

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Figure S1. Quantification of leukocytes, LPM, monocytes and SPM within the peritoneum after injection of thioglycollate or cecal slurry. Cohorts of mice were injected with thioglycollate or cecal slurry and peritoneal lavage was collected 18 or 66h later. (A) Leukocytes were enumerated in the peritoneal lavage. (B + E) LPM, (C + F) monocytes and (D + G) SPM were identified as shown in Figure 1, and expressed in terms of their absolute numbers (B-D) and their percentages (E-G). Graphs show individual values and the median.