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Pre-existing malignancy abrogates the beneficial effects of CXCR4 blockade during sepsis

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Abstract

Patients with cancer are at an increased risk of developing and dying from sepsis. We previously reported that blockade of the chemokine receptor CXCR4 resulted in decreased CD4+ T cell exhaustion and improved survival in a model of polymicrobial sepsis in previously healthy mice. Here, we sought to determine whether CXCR4 blockade could improve mortality and immune dysregulation during sepsis complicated with malignancy. Results in animals inoculated with a lung cancer cell line and subjected to CLP three weeks later indicated that CXCR4 was upregulated on naïve and central memory T cells following sepsis. Of note, and in contrast to results in previously healthy mice, CXCR4 blockade failed to improve survival in cancer septic animals; instead, it actually significantly worsened survival. In the setting of cancer, CXCR4 blockade failed to result in T cell egress from the bone marrow, reverse lymphopenia in the spleen,

DISCLOSURE
The authors declare no conflicts of interest.
or reverse T cell exhaustion. Mechanistically, elevated expression of CD69 on naïve T cells in the bone marrow of cancer septic animals was associated with their inability to egress from the bone marrow in the setting of CXCR4 blockade. In conclusion, these results illuminate the differential impact of CXCR4 blockade on sepsis pathophysiology in the setting of cancer and highlight the need for personalized therapy during sepsis.

Introduction

Sepsis is defined as life-threatening organ dysfunction resulting from a dysregulated host response to infection.\textsuperscript{1} Despite of decades of research on the pathophysiology of sepsis, the disease is still the leading cause of death in critically ill patients globally\textsuperscript{2} and WHO is advocating recognizing sepsis as a global health priority.\textsuperscript{3} In addition, there are no specific FDA-approved therapies for sepsis once antibiotics and supportive care fail.

Cancer is the most common co-morbidity associated with septic patients.\textsuperscript{4} Patients with malignancy are at higher risk of developing sepsis than the general population.\textsuperscript{5} Particularly, it has been reported that the ICU and in-hospital mortality rates for cancer patients with sepsis were 42\% and 56\% respectively, frequencies that are much higher than immunocompetent patients.\textsuperscript{6} While the etiology behind the increased mortality observed in septic cancer patients compared to previously healthy patients is multifactorial\textsuperscript{7} (and likely includes exposure to various anti-cancer drugs), we previously demonstrated that the presence of cancer (in the absence of any other treatment) negatively impacts the mortality following sepsis\textsuperscript{8}, and is associated with phenotypic and functional changes in CD4\(^{+}\) T cell responses following sepsis.\textsuperscript{9} Specifically, cancer mice contained more resting memory and activated CD4\(^{+}\) effector cells, exhibited increased frequencies of PD-1\(^{hi}\) cells that failed to make any cytokines, and a distinct 2B4\(^{hi}\) BTLA\(^{hi}\) LAG-3\(^{hi}\) population that secreted more TNF compared to previously healthy (PH) septic controls. This observed heterogeneity in co-inhibitory receptor expression and cytokine secretion demonstrates the complex immunological changes occurring in cancer septic hosts.

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 (BM).\textsuperscript{10} CXCR4 is widely expressed on many different cell lineages and inhibition of CXCR4/CXCL12 signaling results in the release of these cells into the circulation, increasing peripheral absolute cell counts.\textsuperscript{11} Our previously published data demonstrated that CXCR4 was upregulated on T cells in mouse models of sepsis.\textsuperscript{12} Moreover, a previous study of human septic patients also revealed that CXCL12 levels were higher in patients with severe sepsis/septic shock as compared to healthy subjects.\textsuperscript{13} Efron and colleagues have shown that bone marrow CXCL12 mRNA was reduced while splenic CXCR4 expression was increased during sepsis\textsuperscript{14}. By blocking CXCL12 activity, which significantly reduced bone marrow release of granulocytes, they demonstrated that changes in the pattern of CXCL12 signaling are essential for neutrophil bone marrow mobilization during sepsis. In addition, we recently showed that administration of AMD3100 (plerixafor), a CXCR4-antagonist which is approved by the FDA for stem cell mobilization prior to autologous bone marrow transplantation, abrogated the loss of peripheral T cells during sepsis,
mitigated CD4+ T cell exhaustion during sepsis, and resulted in a significant decrease in sepsis-induced mortality in PH mice. These findings suggest that redirecting functional cells out of the BM may be beneficial in sepsis. Although CXCR4 has been extensively studied in many different pathologic conditions, the role of this pathway in the context of sepsis and malignancy has never been explored. This study therefore aimed to elucidate the impact of CXCR4 blockade in septic animals with cancer.

**Materials and Methods**

**Mice**

All experiments were approved by the Institutional Animal Care and Use Committee at the Emory University (Protocol: DAR-2003199–071418BN). Both male and female C57BL/6 mice aged between 8–12 weeks were used. All mice were purchased from The Jackson Laboratory and maintained at Emory University Division of Animal Resources according to Emory IACUC guidelines.

**Cancer model**

Lewis lung carcinoma cells (LLC1) was subcutaneously injected in the right inner thigh to induce murine carcinoma. LLC1 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin-streptomycin and 1% 4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid (HEPES). Each mouse received 50,000 LLC1 cells suspended in 0.1mL of PBS and tumors were allowed to grow for 3 weeks. Beginning from eighth day after LLC tumor cell inoculation, tumor size was measured daily. Mice were held in hand for measuring the tumor length from the left edge to the right edge using a digital caliper with 0.01mm precision (Mitutoyo). The length was measured along its longest linear dimension on the skin as described. Only animals with tumor size ranging from 1.5 cm to 2.0 cm on day 21 post-tumor inoculation were eligible for the experiment. Eligible mice were then subjected to either CLP or sham surgery 3 weeks post injection.

**Sepsis model**

Sepsis was established through cecal ligation and puncture (CLP). Sham animals underwent laparotomy alone. Per previously published work, a 1cm incision was made in the midline of the abdomen and cecum was ligated at 1cm from the end with 4–0 surgical suture. The ligated cecum was perforated by a through-and-through puncture (generating two individual punctures) with a 25-gauge needle and stool was extruded. After the incision was closed, mice were resuscitated with 1ml saline, given buprenorphine (0.1 mg/kg, Reckitt Benckiser Healthcare, Hull, UK) for pain control, and monitored continuously as they awoke from anesthesia following each operation. Ceftriaxone (50mg per kg body weight, Sigma-Aldrich, St. Louis, MO, USA) and metronidazole (35mg per kg body weight, Sigma-Aldrich) were also administered immediately after CLP. For survival studies, eligible cancer mice were subjected to CLP and either treated with AMD3100 (n = 19) or same volume PBS (n = 15) as septic control 1h after abdominal closure. Antibiotics were continued on a q12hr dosing schedule for 48 hours postoperatively. Mice were observed every 12 hours during the 7-day period and survival rates were recorded. All procedures follow the recommendations of the
international expert consensus initiative for minimum quality threshold in pre-clinical sepsis studies (MQTiPSS).\textsuperscript{16}

**AMD3100 administration**

One hour after sepsis induction, mice were subcutaneously injected with 5mg/kg AMD3100 (Sigma-Aldrich) suspended in 100ul phosphate buffered saline (PBS). The same volume of PBS was used as a control.

**Flow cytometry and reagents**

Twenty-four hours after surgery, the spleen and BM in sham and CLP mice were collected and analyzed through flow cytometry as previously described. All tissue cells were passed through cell strainers with 70-mm pores (Falcon) to create single cell suspensions. Cells were surface-stained with anti-CD3-AF700, anti-CD4-PB, and anti-CD62L-FITC (all from BD Biosciences, San Jose, CA, USA), anti-CD19-PerCP and anti-CD8-PO, anti-2B4-APC, anti-PD-1-APC-Cy7, anti-CXCR4-APC, anti-TIGIT-PE, anti-CD69-PE (all from Biolegend, San Diego, CA, USA).

For Intracellular cytokine staining, $2 \times 10^6$ splenocytes from each sample were plated in a 96-well plate. After centrifugation, cells were re-suspended and incubated in RPMI 1640 culture medium containing 10% FBS (Mediatech, Herndon, VA, USA), 2mM L-glutamine, 0.01 M HEPES buffer, 100mg/ml gentamicin (Mediatech), and $5 \times 10^{-5}$M 2-mercaptoethanol (Sigma-Aldrich). Then cells were stimulated with 30 mg/ml PMA and 400 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Pharningen) for 4 hours at 37 °C. After incubation and stimulation, cells were surface-stained with anti-CD4-PB (BD Biosciences), anti-CD44-PerCP and anti-CD3-APC-Cy7 (Biolegend). Then cells were permeabilized by using fixation and permeabilization solution (BD Biosciences). We used anti-IL-2-FITC (BD Biosciences), anti-TNF-PE-Cy7 (Biolegend) and anti-IFN-γ-Alexa 700 (BD Biosciences) for intracellular cytokine staining. Samples were acquired and analyzed using an LSR II flow cytometer (BD Biosciences). A minimum of $3 \times 10^6$ live cells were collected, and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

Bone marrow was harvested by removing mouse femurs and spinning at 10,000g for 30s. The pellet was re-suspended in 100μL of PBS and centrifuged once again. Bone marrow supernatants were frozen at −80 °C for further analysis. CXCL12 was measured by ELISA (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s instructions.

**Lung histology, weights and myeloperoxidase (MPO) activity**

H&E-stained lung sections were evaluated for the presence of histopathology by an observer blinded to tissue identity\textsuperscript{17}. In a separate set of animals, the left lung was excised and weighed to obtain a “wet” weight. Lungs were then dried for 16 hours in an oven at 115°C and reweighed to establish a dry weight. To quantify MPO, lungs were homogenized and proteins resolved by SDS-PAGE. MPO was detected by immunoblot using rabbit anti-MPO (Sigma) using enhanced chemiluminescence reagent (GE Healthcare, Pittsburgh, PA). Blots

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were imaged and quantified using a ChemiDoc XRS+ Molecular Imaging System (BioRad) and analyzed using Image Lab 2.0.1 software.\textsuperscript{18}

**Statistics**

All data were analyzed using GraphPad Prism 7.0 software (San Diego, CA, USA). Continuous variables were expressed as mean $\pm$ SD and were compared via Student’s t test or Mann-Whitney test where appropriate. Multi-group differences in flow cytometric data were evaluated using ANOVA and Tukey’s test. Survival data were analyzed by log-rank test. P values less than 0.05 were considered significant.

**Results**

**CXCR4 expression increases on CD4$^+$ and CD8$^+$ T cells in cancer mice following CLP**

Given our previous results demonstrating the upregulation of CXCR4 on immune cells in (PH) animals following sepsis,\textsuperscript{12} we hypothesized that CLP would also induce CXCR4 upregulation in mice with pre-existing malignancy. To test this hypothesis, we utilized our previously published model\textsuperscript{9} in which naïve B6 mice are injected with a lung cancer cell line in the thigh and develop malignancy over the course of a 3-week period. As shown in Figure 1A–B, CXCR4 expression was elevated significantly on CD4$^+$ and CD8$^+$ T cells after CLP ($p=0.043$ and 0.0079 respectively) compared with sham cancer mice. Furthermore, CXCR4 expression mainly upregulated on naïve T cells (T$_N$, $p = 0.01$ for both CD4$^+$ and CD8$^+$ T cells) and central memory T cells (T$_{CM}$, $p = 0.003$ for both CD4$^+$ and CD8$^+$ T cells) but did not increase on effector memory T cells (T$_{EM}$, $p = 0.069$ and 0.149 for CD4$^+$ and CD8$^+$ T cells respectively, Fig. 1C). In our previous study in PH mice, the increase in frequency of CXCR4-expressing CD4$^+$ T cells was also limited to T$_N$ and T$_{CM}$\textsuperscript{12}. That study also reported that although there was no difference in the frequency of CXCR4 expression on total CD8$^+$ T cells in PH septic mice compared to sham mice, the frequency of CXCR4$^+$ in CD8$^+$ T$_N$ cells was significantly increased post CLP.\textsuperscript{12} These results were consistent with the current study in cancer mice, indicating that CLP-induced CXCR4 upregulation was specific to less differentiated T cell subsets, and is independent of the presence of a pre-existing malignancy, since a similar level of CXCR4 upregulation was observed in PH septic mice and cancer septic mice relative to sham controls in both conditions.

**CXCR4 blockade fails to improve (and actually diminishes) mortality in cancer septic animals**

As discussed above, we have previously published that CXCR4 antagonism with AMD3100 is an effective treatment to reduce mortality following CLP in PH C57BL/6 mice. Specifically, we found that 5mg/kg AMD3100 administered at 1h post CLP resulted in survival improvement from 20% to 65%.\textsuperscript{12} To explore the possibility that sepsis survival in cancer hosts could be improved by targeting CXCR4, AMD3100 was given at 1h post CLP and the same volume of PBS was given to another group of cancer septic mice as a control. However, contrary to what was observed in PH animals, AMD3100 treatment following CLP in cancer mice actually decreased 7-day survival from 20% to 0%, $p = 0.023$ (Fig. 1D). This worsened mortality was not associated with altered lung histology (Supplemental Fig. 1A–C), lung wet to dry ratio (Supplemental Fig. 1D), or protein levels of pulmonary

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myeloperoxidase (MPO) (Supplemental Fig. 1E) levels in AMD3100-treated cancer septic mice relative to untreated cancer septic mice. Moreover, no differences were observed in the bacterial load (log CFU) in the peritoneal fluid of cancer septic animals treated with AMD3100 as compared to untreated cancer septic controls (Supplemental Fig. 1F). Taken together, these results demonstrated that pre-existing malignancy not only abrogated the beneficial effect of CXCR4 blockade during sepsis, but actually led to significantly worsened survival.

**AMD3100 treatment of cancer septic mice fails to reverse splenic T cell loss**

Next, we aimed to elucidate the impact of AMD3100 on sepsis induced T cell depletion. Because our prior published data in PH mice found that AMD3100-induced increase in sepsis survival was associated with a significant inhibition of T cell loss in the spleen, we speculated that AMD3100 might be failing to inhibit the loss of splenic T cell numbers in the context of cancer. Spleens were collected from mice in sham and CLP groups treated with either PBS or AMD3100 and T cell numbers in the spleen were assessed. As shown in Figure 2A–B, splenic CD4⁺ T cells and CD8⁺ T cell numbers were significantly reduced in CLP compared with sham animals, but importantly were not restored by AMD3100 administration (p = 0.18 and 0.38 for CD4⁺ and CD8⁺ T cells, respectively). These data were in direct contrast to our previously published findings in PH septic mice, which showed an increase in the absolute number of CD4⁺ T cells and CD8⁺ T cells. Moreover, analysis of subsets of CD4⁺ T cells and CD8⁺ T cells revealed that neither T_N, T_CMV, nor T_EM subsets were increased in cancer septic animals treated with AMD3100 as compared to cancer septic untreated controls (Figures 2A–B).

**AMD3100 treatment failed to inhibit expression of T cell exhaustion markers in cancer septic mice**

Co-inhibitory receptors represent an emerging and promising target for sepsis immunotherapy. Our previous data in PH septic mice demonstrated that T cell expression of several of these co-inhibitory molecules, which are one of the major characteristics of T cell exhaustion in sepsis, were significantly downregulated following AMD3100 administration. Specifically, we showed that the frequency of PD-1⁺ CD4⁺ T cells was significantly decreased, as well as LAG3⁺ and 2B4⁺ CD4⁺ T cells. In cancer septic mice, PD-1, 2B4 and TIGIT were upregulated on CD4⁺ T cells and 2B4 was upregulated on CD8⁺ T cells (p = 0.027, 0.027, 0.009 and 0.049 respectively) following CLP. However, AMD3100 administration in cancer septic animals failed to reduce T cell expression of PD-1, 2B4, and TIGIT (Figure 3A–B).

We next assessed cytokine-secreting functionality of splenic T cells isolated from AMD3100-treated cancer septic animals following ex vivo stimulation with PMA/ionomycin. As shown in Figure 4, the frequencies of both IFNγ⁺ and IFNγ⁺ IL-2⁺ cells among CD4⁺ T cells were significantly lower in CLP group than sham group (p = 0.039 and 0.015 respectively). For memory CD4⁺ T cells, the frequencies of IFNγ⁺, IL-2⁺, and IFNγ⁺ IL-2⁺ cells were decreased (p = 0.038, 0.020 and 0.01 respectively). Total CD8⁺ T cells isolated from CLP mice also exhibited reduced frequencies of cytokine producers, including IFNγ⁺, IL-2⁺, and IFNγ⁺ IL-2⁺ (p = 0.028, 0.033 and 0.017). IFNγ⁺, IL-2⁺, IFNγ⁺ IL-2⁺
and TNF$^+$ cells were all present at reduced frequencies among CD8$^+$ CD44$^{hi}$ memory T cells ($p < 0.0001$, $p = 0.0003$, 0.0009 and 0.014 respectively). However, no differences in cytokine production were detected in either the CD4$^+$ or CD8$^+$ T cell populations in cancer septic animals treated with AMD3100 relative to untreated cancer septic controls (Figure 4B). We also examined the frequencies of IL-10-, IL-4, and IL-17 secreting CD4$^+$ CD44$^{hi}$ T cells in AMD3100-treated cancer septic animals compared to untreated cancer septic controls, and found no differences in the frequencies of any of these populations (Supplemental Fig. 2). Of note, previous work in PH animals also demonstrated no differences in cytokine production in CD4$^+$ or CD8$^+$ T cells isolated from AMD3100-treated septic animals as compared to untreated septic controls (not shown).

**AMD3100 fails to promote effective T cell egress from the BM in cancer septic mice**

As a stem cell mobilizing drug, AMD3100 promotes the egress of stem cells as well as mature T lymphocytes from the BM and blocks T cell homing back to the BM.\textsuperscript{19} Thus, to further interrogate the impact of AMD3100 in the setting of cancer and sepsis, we first assessed the impact of sepsis alone on accumulation of mature T cells in the BM. Interestingly, results in PH septic mice showed that mature CD4$^+$ T cell and CD8$^+$ T cell numbers were significantly elevated in BM after CLP ($p < 0.0001$, $p = 0.0001$). AMD3100 treatment resulted in a reduction in CD4$^+$ and CD8$^+$ T cell numbers in the BM ($p = 0.004$ and 0.0005 respectively, Fig. 5A–B), which is consistent with its pharmaceutical effects in lymphocyte migration. CLP also induced an increase in the number of mature CD4$^+$ T cell and CD8$^+$ T cell numbers in the BM of cancer mice ($p = 0.002$ and 0.009 respectively, Fig. 5A–B) (albeit not to the same extent as in PH mice (not shown)). However, AMD3100 treatment failed to impact T cell counts in BM of cancer mice after CLP ($p = 0.69$ and 0.99 respectively). Absolute numbers of CD4$^+$ Foxp3$^+$ T cells were also not impacted by AMD3100 treatment in either PH or CA septic mice, in either the bone marrow or the spleen (Supplemental Figure 3A–B).

To further elucidate why AMD3100 failed to redirect T cells from the BM, we compared the CXCR4 expression on T cells from the spleen and BM between PH mice and cancer mice after CLP. As shown in Figure 5C, there were no significant differences in CXCR4 expression on T cells isolated from PH vs. CA septic mice in either the spleen or BM. In addition, we also examined the CXCL12 concentration in BM extracellular fluid (Figure 5D). Compared with PH septic mice, cancer septic mice had less CXCL12 in the BM. Furthermore, we found that cancer mice possessed fewer CD4$^+$ and CD8$^+$ T cells in the BM ($p = 0.0002$ for both, Figure 5E) relative to PH mice in either the sham or CLP group. This result indicated that the failure of AMD3100 to promote T cell egress from the BM was related to the cancer condition.

**Bone marrow cells in cancer septic mice have higher CD69 expression during sepsis**

Sphingosine-1-phosphate (S1P) and its receptor, sphingosine-1-phosphate receptor 1 (S1P1) is another important regulator of lymphocyte migration. As S1P concentrations are high in blood and lymph compared with the interstitial fluid of lymphoid organs, S1P1 expression is critical for lymphocyte egress from the thymus, secondary lymphoid organs, as well as BM.\textsuperscript{20,21} One key post-translational regulator of S1P1 is the activation marker CD69, which can

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form a complex with S1P1, and promote S1P1 receptor internalization and degradation, thus inhibiting lymphocyte egress from secondary lymphoid organs.22 Recently, it has been demonstrated that targeting of CD69 induces the rapid mobilization of immature leukocytes in an S1P-dependent manner.23 As such, we assessed CD69 expression on the T cells in the BM. Our flow cytometry data revealed that although there were no differences in CD69 expression on total T cells and memory subsets, CD4+ and CD8+ T_N isolated from cancer mice (as gated in Figure 6A) contained higher frequencies of CD69+ cells relative to their PH counterparts (Figure 6B–C), which may inhibit T cell mobilization induced by AMD3100.

Discussion

Lymphopenia is one of the main characteristics of immunosuppression during sepsis and many therapies exist aimed at restoring immune cell numbers and function. They include cell death inhibition,24 cell proliferation improvement25 and cell migration manipulation.26 In this study we explored T cell migration between the BM and second lymphoid organs during polymicrobial sepsis in the context of a cancer model. The results showed that while CXCR4 expression was significantly elevated on T cells isolated from cancer septic hosts. CXCR4 blockade failed to reverse T cell accumulation in BM niche, reinvigorate exhausted T cells, or improve sepsis survival. This may be due to the fact that compared with PH mice, cancer mice had reduced levels of serum CXCL12 and increased expression of CD69 on BM T cells. We posit that AMD3100 failed to result in T cell egress from the BM because increased T cell expression of CD69 sequestered cells in the BM.

CXCR4 is expressed on B and T lymphocytes, dendritic cells, and monocytes and plays a pivotal role in lymphocyte homing and recruitment into inflammatory sites.27 For T lymphocytes, CXCR4 is expressed globally on T_N, T_CM and T_EM cells. Following sepsis, CXCR4 was mainly upregulated on CD62L hi T_N and T_CM, with no significant differences on CD62L lo T_EM, a finding which is consistent with our previous data in PH mice.12 This may be due to the fact that, as reported by Ziqinang et al.,28 ligation of CD62L increased CXCR4 expression and also inhibited CXCL12-induced CXCR4 internalization, which in turn enhanced CXCR4 expression on surface. Further corroborating these results, a report by Mazo et al. indicated that T_N and T_CM, but not T_EM, migrated toward a CXCL12 gradient to the BM.29 Another study also demonstrated that CD8+ T_N and T_CM cells of CXCR4 knockout mice failed to home to the BM efficiently.30 Interestingly, our study showed that only T_N and T_CM numbers were elevated in the BM in CLP mice. It has previously been shown that the BM can function as a T_N priming site for the initiation of systemic immune control of blood-borne pathogens 31. As such, we posit that CD62L ligation results in CXCR4-mediated homing of naïve and central memory T cells to the BM during sepsis, which provides a refuge from the peripheral cytokine storm. Actually, it has been proposed that one of physiological importance of preventing naïve T cell homing to the BM is to ensure efficient immune surveillance and induction of immune response.19 This notion is supported by our data showing that survivals of PH septic mice were improved by redirecting accumulated T cells out of the BM.12

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We previously showed that CXCR4 blockade mitigated cell exhaustion in PH mice by reducing expression of PD-1, LAG3 and 2B4 expression on CD4\(^+\) T cell populations. We posit that this reduction in the frequency of cells expressing co-inhibitory receptors was the result of a “diluting effect” of newly recruited T cell populations from the BM niche that may have been less affected by the inflammatory milieu in the circulation. The findings that AMD3100 failed to promote T cell egress from the BM in cancer septic mice, and also failed to result in the reduction in the frequency of T cells expressing co-inhibitory receptors, are consistent with this hypothesis. Alternatively, CXCR4 antagonism could be having a direct effect on T cell activation and/or expansion independently of any effect of the BM niche mobilization. Indeed, CXCL12 has been shown to induces the close, physical association of CXCR4 and the TCR, leading to prolonged ERK activation and inducing AP-1 dependent gene transcription and the expression of CD69, IL-2, and IL-10.\(^{32}\)

The effect of non-hematologic cancer on host bone marrow hematologic stem cells remains incompletely understood. A recent publication showed that late stage cancer could induce anemia and immune deficiency which was attributed to accumulation of erythroid progenitor cells in spleen, bone marrow, liver, and blood\(^ {33}\). Another report showed that despite defective erythropoiesis, melanoma growth resulted in expansion of myeloid lineages such as myeloid derived suppressor cells (MDSCs), macrophages and dendritic cells along with a reduction in platelets\(^ {34}\). One might also speculate that lymphocyte infiltration into tumors could result in a dearth of peripheral lymphocytes, resulting in altered lymphopoiesis in the setting of cancer. However, this does not seem likely because we have observed that only \(~1–2\%\) of the host’s T cells are infiltrating the tumor in this model.

Because we detected no significant differences in CXCR4 expression between PH and cancer mice, the disparate effects on mortality are unlikely to be an effect of differential target saturation following AMD3100 treatment. Why then do T cells fail to egress from the BM efficiently in AMD3100-treated cancer septic mice? One possible explanation might be that cancer mice have fewer T cells to mobilize in the BM compartment compared to PH mice (Figure 5E). Alternatively, the SP1/S1P1 axis has been indicated to play an important role in regulating lymphocyte egress from the BM\(^ {21}\). Despite many other influencing factors, one key post-translational regulator of S1P1 is the activation marker CD69, which physically associates with and inhibits the function of S1P1\(^ {22}\). Moreover, Juarez et al. observed reduced hematopoietic progenitor cell (HPC) mobilization after administration of AMD3100 in S1P1\(^ {−/−}\) mice or mice treated with the S1P1 antagonist FTY720 as compared to WT or untreated controls\(^ {35}\). These results suggest S1P1 is essential in AMD3100-induced HPC mobilization. Importantly, we found that the S1P1 inhibitor CD69 was expressed on a significantly higher frequency of T cells in the bone marrow of cancer septic mice as compared to PH septic controls. Thus, we speculate that this increased CD69 expression on T cells in the BM of cancer septic mice compared to PH septic mice may have inhibited S1P1 function, thereby contributing to the inhibition of T cell egress in the context of AMD3100.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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Abbreviations:

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<th>Abbreviation</th>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CD62L</td>
<td>L-selectin</td>
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<td>CLP</td>
<td>cecal ligation and puncture</td>
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<td>CXCL12</td>
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References:


Figure 1. CXCR4 expression increases on T cells in cancer mice after CLP.
Cancer mice were subjected either to CLP (n = 6) or sham surgery (n = 5). They were sacrificed, and spleen were harvested 24h post CLP. (A) Representative flow cytometry plots of CXCR4 expression on CD4+ T cells and CD8+ T cells. (B) Summary data of CXCR4 frequency and MFI demonstrated significant upregulation on CD4+ T cells and CD8+ T cells. Data were pooled from 2 independent experiments. (C) CXCR4 is differentially expressed on T cell subsets, representative of 2 independent experiments. Gating of CD4+ and CD8+ T cells based on CD62L and CD44 expression are used to define T cell subsets. (D) Another group of cancer mice were subjected to CLP and either treated with AMD3100 (n = 19) or PBS (n = 15) 1h post abdominal closure as described in the materials and methods. Mice were monitored every 12 hours and 7-day survival was recorded. T_N, CD62LhiCD44lo; T_CM, CD62LhiCD44hi; T_EM, CD62LloCD44hi. *P ≤ 0.05, **P ≤ 0.01.
Figure 2. AMD3100 has no effect on splenic T cell depletion in septic cancer mice.
Cancer mice were subjected to CLP and either treated with AMD3100 or the same volume of PBS (n = 6 mice/group) 1h after abdominal closure with shams serving as surgery controls (n = 5). They were sacrificed, and spleens were harvested 24h post CLP. Data were pooled from 2 independent experiments. (A) Representative flow cytometry plots depict CD4+ and CD8+ T cell gating strategy, as well as memory subsets which are defined according to CD62L and CD44 expression on cell surface. (B) Summary data showed that AMD3100 treatment did not change CD4+ or CD8+ total T cell counts or subset counts. T_N, CD62L^hiCD44^lo; T_CM, CD62L^hiCD44^hi; T_EM, CD62L^loCD44^hi. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
Figure 3. AMD3100 treatment failed to reverse co-inhibitory receptor upregulation on T cells in cancer septic mice. Cancer mice were subjected to CLP and either treated with AMD3100 or the same volume of PBS (n = 6 mice/group) 1h after abdominal closure. Controls were subjected to sham surgery (n = 5). They were sacrificed, and spleens were harvested 24h post CLP. Data were pooled from 2–3 independent experiments. (A) Representative flow cytometry graphs of PD-1, 2B4 and TIGIT expression on CD4\(^+\) T cells and CD8\(^+\) T cells. (B) Summary data showed percentages of PD-1\(^+\), 2B4\(^+\) and TIGIT\(^+\) cells in the CD4\(^+\) and CD8\(^+\) T cell compartments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
Figure 4. AMD3100 treatment failed to improve T cell function in cancer septic mice.
Cancer mice were subjected to CLP and either treated with AMD3100 or same volume PBS 1h after abdominal closure with shams serving as surgery controls (n = 5 mice/group). Single cell suspension of splenocytes harvested 24h post CLP were stimulated with PMA and ionomycin at 37 °C for 4 hours. Data were derived from 1 single experiment. (A) Representative flow cytometry depicts IL-2 and IFNγ expression on CD4+ and CD8+ T cells. (B) Percentages of IFNγ+ and IL-2+ T cells in CD4+ and CD8+ T cells and memory T cells were compared among the three different groups. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
Figure 5. CXCR4 blockade results in T cell egress from the BM in previously healthy mice (PH) post CLP but not in cancer (CA) septic mice. Both PH and CA mice were subjected to CLP and either treated with AMD3100 (n = 5) or same volume PBS (n = 6) 1h after abdominal closure. Controls were subjected to sham surgery. Mice were sacrificed, and BM was harvested 24h post CLP. Data were pooled from 2–3 independent experiments. (A) Representative flow cytometry plots of CD4+ and CD8+ T cells gating were shown. (B) Summary data of CD4+ and CD8+ T cells numbers in either PH or CA mice. (C) CXCR4 expression on T cells in the BM and spleen were compared between PH septic mice or CA septic mice. (D) CXCL12 concentration of BM was compared between PH CLP mice and CA CLP mice. (E) CA mice contained many fewer T cells in the BM compared with PH mice regardless of sepsis. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
Figure 6. Increased frequencies of CD69+ naïve T cells in the BM of CA septic mice compared to PH mice.

PH mice or CA mice were subjected to CLP or sham surgery (n = 5–7 mice/group) and were sacrificed to harvest the BM and spleen at 24h post CLP. Data were pooled from 3 independent experiments. (A) Representative flow plots of CD4+ and CD8+ T cell subset gating in bone marrow based upon CD62L and CD44 expression were shown. (B) Representative flow plots of CD69 expression in naïve T cells of either PH mice or CA mice were displayed. (C) Percentages of CD69+ cells in total T cell and memory T cell subsets were compared between PH and CA mice. **P ≤ 0.01, ***P ≤ 0.001.