Neutrophil Mobilization from the Bone Marrow during Polymicrobial Sepsis Is Dependent on CXCL12 Signaling


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Neutrophils are essential for successful host eradication of bacterial pathogens and for survival to polymicrobial sepsis. During inflammation, the bone marrow provides a large reserve of neutrophils that are released into the peripheral circulation where they traverse to sites of infection. Although neutrophils are essential for survival, few studies have investigated the mechanisms responsible for neutrophil mobilization from the bone marrow during polymicrobial sepsis. Using a cecal ligation and puncture model of polymicrobial sepsis, we demonstrated that neutrophil mobilization from the bone marrow is not dependent on TLR4, MyD88, TRIF, IFNARα/β, or CXCR2 pathway signaling during sepsis. In contrast, we observed that bone marrow CXCL12 mRNA abundance and specific CXCL12 levels are sharply reduced, whereas splenic CXCR4 mRNA and cell surface expression are increased during sepsis. Blocking CXCL12 activity significantly reduced blood neutrophilia by inhibiting bone marrow release of granulocytes during sepsis. However, CXCL12 inhibition had no impact on the expansion of bone marrow neutrophil precursors and hematopoietic progenitors. Bone marrow neutrophil retention by CXCL12 blockade prevented blood neutrophilia, inhibited peritoneal neutrophil accumulation, and increased polymicrobial sepsis mortality. We concluded that changes in the pattern of CXCL12 signaling during sepsis are essential for neutrophil bone marrow mobilization and host survival but have little impact on bone marrow granulopoiesis.

Sepsis is the leading cause of death in the critically ill, with 750,000 cases and 210,000 deaths annually (1, 2). Sepsis mortality has been attributed to derangements in the innate immune system (3). Neutrophils are a fundamental component of innate immunity and essential for bacterial eradication and polymicrobial sepsis survival in humans and animals (4–6). Excessive neutrophil activity during inflammatory states can induce unwanted tissue damage and organ dysfunction (7). Hence, neutrophil production, release, and clearance are tightly regulated. Under normal circumstances, 1–2% of the total bone marrow neutrophil population is released into the circulation (8). During episodes of infection, the bone marrow provides a large neutrophil reserve (9).

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Abbreviations used in this article: CLP, cecal ligation and puncture; CMP, common myeloid progenitor; GMP, granulocyte–monocyte progenitor; GR-1, granulocyte marker 1; LSK, lineage sca-1+c-kit+ hematopoietic stem cell.

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Although cytokines, chemokines, leukotrienes, proteases, integrins, and bacterial products (10) have been implicated in bone marrow neutrophil release, attention has centered on CXC chemokine and TLR signaling during neutrophil release. Evidence suggests that CXCR4 and its ligand, stromal cell-derived factor-1 (CXCL12), and CXCR2 and its ligands, MIP-2 (CXCL2) and KC/GRO-1α (CXCL1), initiate neutrophil bone marrow release under normal conditions. In healthy mice, interactions between CXCR4 and CXCL12 are essential for maintaining bone marrow neutrophil populations (11, 12). CXCR4 blockade disrupts the CXCR4/CXCL12 axis and increases neutrophil release from the bone marrow (13–15). Exogenous administration of CXCL1, CXCL2, and CXCL12 increases neutrophil mobilization from the bone marrow, whereas CXCR2 antagonism inhibits neutrophil bone marrow release (7–10, 16).

TLR signaling impacts neutrophil release directly or via chemokine signaling. TLRs recognize pathogen-associated molecular regions on invading pathogens (17, 18) and initiate a multitude of inflammatory processes. MyD88−/− mice, with TLR signaling deficits, fail to increase CXCL1 and CXCL2 blood concentrations during polymicrobial sepsis (19) and Pseudomonas pneumonia (20). These data suggested that CXCL1 and CXCL2 increases involved in neutrophil bone marrow release depend on intact TLR-signaling pathways. Although a growing body of evidence associates bone marrow neutrophil mobilization with TLR, CXCR4/CXCL12, CXCR2/CXCL1, and CXCL2 signaling during homeostasis, the impact of these mediators on bone marrow neutrophil mobilization during polymicrobial sepsis has received little attention.

Using a cecal ligation and puncture (CLP) model of polymicrobial sepsis, we investigated the role of TLR, CXCL12, and
CXCR2 signaling in neutrophil bone marrow mobilization. The results indicated that bone marrow neutrophil mobilization is not dependent on TLR4, MyD88, TRIF, IFNARα/β, or CXCR2 signaling pathways. Sepsis alters the tissue-expression patterns of CXCL12, inhibiting mRNA abundance and chemokine levels in the bone marrow compared with blood, plasma, and spleen levels. CXCL12 blockade prevented bone marrow neutrophil mobilization, yet had little impact on bone marrow neutrophil and hematopoietic progenitor expansion. The data suggested that in polymicrobial sepsis, CXCL12 inhibition resulted in peritoneal cavity neutropenia, unopposed bacterial invasion, and a 40% increase in polymicrobial sepsis mortality. These findings suggested that CXCL12 signaling patterns are altered in sepsis, are essential for bone marrow neutrophil mobilization, and are necessary for host survival during polymicrobial sepsis.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida or Merck Research Laboratories. Specific pathogen-free C57BL/6, C3H/HeJ (TLR4 receptor mutation), and their control (C3H/HeOu) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the University of Florida College of Medicine and were studied between 8 and 12 wk of age. MyD88−/− mice and TRIF−/− mice on a B6 × 129/F1 background were a kind gift of Dr. Shizuo Akira (Osaka University, Osaka, Japan) to Merck Research Laboratories and were maintained at Merck Research Laboratories. IFN-γR−/−129 mice on the 129Sv/SvEv background (H-2b) and wild-type 129Sv mice were purchased from B & K Universal (Hull, East Yorkshire, U.K.).

Inhibitors

When indicated, mice were injected i.p. with either 500 μg/l anti-CXCL12 or 500 μg/l anti-CXCR2 polyclonal goat antisera beginning 12 h prior to the initiation of sepsis. The anti-CXCL12 and anti-CXCR2 Ab preparations were gifts from R.M.S. Heat-inactivated polyclonal goat serum (Sigma) was used (500 μg/l i.p.) as a control. All inhibitors were injected 12 h before the induction of sepsis.

Cecal ligation and puncture

For induction of polymicrobial sepsis, mice underwent sham laparotomy or CLP induced by ligation of the cecum and a double enterotomy created with either a 23- or 27-gauge needle (21, 22). With the smaller enterotomy (27-gauge needle), mortality was ~10–15%, whereas mortality with the larger enterotomy (23-gauge needle) was 20–30%. In both cases, death occurred predominantly within the first 3 d; thereafter, surviving mice developed abscesses surrounding the devitalized cecum, as previously described (23–26). Survival analyses were performed with the larger enterotomy (23-gauge needle) in the CLP model, with shams serving as surgery controls, without infection, in each experiment. Mice with CLP were pretreated with vehicle control, heat-inactivated goat serum with irrelevant IgG (500 μg/l i.p.), or anti-CXCL12 antisera (n = 20 mice/group) (500 μg/l i.p. anti-CXCL12) beginning 12 h prior to the onset of sepsis and continuing daily for 8 d. The survival analyses were repeated twice.

Flow cytometry

Splenic, bone marrow cells, and whole blood were analyzed by flow cytometry, as previously described (21, 22, 25, 27), in sham and CLP mice created with the smaller enterotomy (27-gauge needle). Abs included anti-granulocyte marker 1 (GR-1) (RB6-8C5) conjugated to PerCP5.5, anti-CD11b (integrin αM, chain Mac-1a chain [M1/70]) conjugated to Pacific Blue, anti-F4/80 Ag (Paw macrophage marker [B93]) conjugated to allophycocyanin, anti-CD31 (MEC 13.3) conjugated to PE, Fc-block (CD16/CD32 Fc g III/II receptor [2.4G2]), lineage cocktail conjugated to biotin (CD3e [145-2C11], CD11b [M1/70], CD45R/B220 [RA3-6B2], Ly6G and Ly6C [RB6-8C5], TER-119 [TER-119]), Sca-1 conjugated to PE or PECy7 (D7), c-KIT conjugated to either FITC or allophycocyanin (2B8), CD34 conjugated to either Alexa Fluor-647 or FITC (RAM34), FcγR conjugated to biotin (CD64/CD16 clone 93), and Sytox Blue. F4/80−/− (CD11b−/−), CD34−, and FcγR-specific Abs were purchased from eBioscience; all other Abs were purchased from BD Pharmingen. Splenes, whole blood, and bone marrow were harvested 12 h after either CLP or sham surgery, and single-cell suspensions were created by passing the cells through cell striainers with 70-μm pores (Falcon). Erythrocytes were then lysed using ammonium chloride lysis buffer and washed two times using PBS without calcium, phenol red, or magnesium. Samples were acquired and analyzed using an LSRII flow cytometer (BD Biosciences). A minimum of 5 × 10^6 live, nondebris cells (Sytox−) were collected for analysis.

Cytokine production

At days 0, 1, 3, and 5 after sham or CLP procedure, whole blood was harvested by cardiac puncture and immediately centrifuged at 14,000 rpm for 10 min. The plasma supernatant then was carefully isolated and stored at −80°C until the time of analysis. The plasma samples were analyzed for cytokines using Luminex technology with reagents obtained from Upstate Cell Signaling Solutions (Beadlyte Mouse Multi-Cytokine Detection System) (Temecula, CA).

Real-time RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase (1 U/l; Invitrogen) at room temperature for 15 min. The RNA was reverse-transcribed using SuperScript II First-Strand cDNA Synthesis (Invitrogen). Real-time PCR was performed with an Opticon 2 continuous fluorescence detector (MJ Research, Waltham, MA) using SYBR Green core reagents and AmpliTaq gold DNA polymerase from Perkin-Elmer Applied Biosystems (Foster City, CA). Gene expression was normalized to 18S rRNA (Ambion). Primers were designed using the Primer3 output program (Whitehead Institute for Biomedical Research, Cambridge, MA) and were as follows: CXCR4 (NM_009011), forward: 5′-CACGGCTGTAGAGCGAGTT-3′, reverse: 5′-TCCGCACTATGCGTCAGTCAG-3′; CXCL12 (NM_001012477), forward: 5′-AGGGCCAAAGGCTGTTTCCAG-3′, reverse: 5′-ACAAGGCGATCTGTGCGAGG-3′; CXCR2 (NM_001012477), forward: 5′-GGGAACTCTTGTTGATGCT-3′, reverse: 5′-AGTAGCCTTCACTGTCG-3′; CXCL2 (NM_0010491), forward: 5′-ACCTCATGGAACGCCCTTAC-3′, and CXCL12 (NM_0010491), forward: 5′-AGCTTCAAAAGCCCTCTTAC-3′, reverse: 5′-GGGACATCGGATCGTCACA-3′. Optimal reaction concentrations were determined over four logs of linear amplification. Forty-five cycles of PCR were performed in duplicate for each primer. The fold change in gene transcript quantity compared with 18S-rRNA was measured using the comparative (2−DDCt) method, where Ct equals the cycle threshold.

Statistics

Continuous variables were first tested for normality and equality of variances. Differences among groups in flow cytometric data were evaluated by ANOVA for multiple groups and by the Student t test for two groups. Post hoc comparisons were performed using Student–Newman–Keuls multiple range tests. Statistical analysis for survival was performed using the Fisher exact test of significance. In all cases, significance was designated at the 95% confidence level using a two-tailed test.

Results

Acute polymicrobial sepsis induces bone marrow granulocyte mobilization

During acute infection and sepsis, neutrophils are rapidly mobilized from the bone marrow into the peripheral circulation where they traverse to sites of inflammation. Using a modestly lethal CLP (LD50) model of polymicrobial sepsis, we investigated several of the mediators that may be responsible for mobilizing neutrophils from the bone marrow into the peripheral circulation. At serial time points from 3 to 9 h after the induction of sepsis, a dramatic difference was observed in the relative percentage and absolute number of total neutrophils in the femurs and blood between sham and CLP-treated animals (Fig. 1A, 1B). The decrease in neutrophils in bone marrow and the increase in neutrophils in blood was most apparent at 12 h after CLP, with a 50% reduction in the percentage of total bone marrow GR-1−CD11b+ cells and a concomitant 3-fold increase in the percentage of total blood neutrophils compared with sham and healthy naive control animals (Fig. 1C, 1D). The bone marrow myeloid compartment consists of a large, heterogeneous population of cells that possesses both mature and
immature monocyte, macrophage, dendritic cell, erythroid, and neutrophil phenotypes (28). During episodes of inflammatory stress and acute infection, the vast majority of cells released from the bone marrow consists of mature neutrophils, with lymphocytes and monocytes making up the minority of cells (data not shown) (9, 10, 28). Immune phenotyping with the cell surface markers F4/80, a marker of monocytes and macrophages, and CD31, a marker of immature cells, demonstrated that <1% of the total bone marrow neutrophils expressed F4/80, and <5% are CD31+ 12 h after sepsis initiation (Fig. 1E, 1F). As neutrophils mature, Ly6G and CD11b cell surface expression becomes more dense. Cell surface analysis revealed that, at 12 h after sepsis induction, the bone neutrophils had a high cell surface density of both Ly6G and CD11b compared with the remaining immature bone marrow neutrophils (data not shown). The immune phenotype of the circulating GR-1+CD11b+ population is consistent with that of more mature granulocytes.

Bone marrow neutrophil release is independent of CXCR2 signaling during polymicrobial sepsis

Increases in the expression of chemokines CXCL1 and CXCL2 are used as indicators of acute inflammation and have been associated with peripheral recruitment of neutrophils to sites of local infection (29). To understand and characterize the comprehensive impact of polymicrobial sepsis on bone marrow neutrophil release, we first characterized the expression of CXCL1, CXCL2, and CXCR2 mRNA in total bone marrow, blood, and spleen cells 12 h after CLP. The data demonstrated a dramatic elevation in both the total bone marrow and splenocyte mRNA abundance of CXCL1 at 12 h. Plasma CXCL1 concentrations peaked at 24 h after the initiation of sepsis (Fig. 2A–C). Contrary to CXCL1, CXCL2 mRNA abundances actually declined in the bone marrow after sepsis, whereas mRNA abundances increased in the spleen. Likewise, CXCR2 expression changed in a similar fashion as CXCL2 expression, with decreases in the abundance of mRNA in the bone marrow and increases in the spleen (Fig. 2D). The data suggested that elevations in peripheral CXCL1 and CXCL2 mRNA and chemokine levels coincide temporally with blood neutrophilia and may be involved in bone marrow neutrophil release during polymicrobial sepsis.

Based on our prior results, we sought to determine the impact of CXCR2 blockade on neutrophil bone marrow release during polymicrobial sepsis. Incorporating an anti-CXCR2 Ab that blocks ligand binding to CXCR2, we investigated the implications of CXCR2 blockade on bone marrow neutrophil release during polymicrobial infection. Mice were injected i.p. with anti-CXCR2 Abs or vehicle control 12 h before CLP. Mouse bone marrow, blood, and spleen cells were harvested 12 h after the induction of sepsis and analyzed for the total percentages of GR-1+CD11b+ cells. Sepsis produced a 50% reduction in the bone marrow granulocyte population at 12 h (Fig. 2E). However, CXCR2 blockade had no significant effect on the reduction in the numbers or percentages of GR-1+CD11b+ cells in the bone marrow. Concomitantly, sepsis produced >2-fold increase in the percentages of total blood granulocytes in the septic mice compared with mice undergoing sham procedure (Fig. 2F), and CXCR2 blockade had no effect on this response.

**Bone marrow neutrophil release is also independent of TLR signaling**

Interruption of TLR signaling during acute infection has been associated with significant reductions in peripheral CXCL1 and CXCL2 levels (19, 20), which may inhibit neutrophil bone marrow mobilization and peripheral recruitment. Although our experiments demonstrated that neutrophil bone marrow mobilization is not dependent on CXCR2 signaling, other reports indicated that peripheral neutrophil recruitment requires intact TLR signaling (30). Because TLR signaling occurs through MyD88 and TRIF dependent pathways and may also involve type I IFN signaling, the CLP model of polymicrobial sepsis was performed in MyD88−/−, TRIF−/−, IFN-α/βR−/−, and C3H/HeJ (TLR4 mutant) mice. As shown in Fig. 3A and 3B, mice devoid of MyD88 signaling demonstrated no deficit in neutrophil mobilization from the bone marrow into the peripheral circulation. We next induced polymicrobial sepsis in TRIF−/−, IFN-α/βR−/−, and C3H/HeJ mice and found no attenuation in neutrophil reduction in the bone marrow (Fig. 3C–E) or elevation in the blood (data not shown).

**CXCL12 expression changes in the bone marrow and spleen during acute infection**

Considering the previous results, we hypothesized that neutrophil bone marrow egression may be dependent on CXCL12 signaling during polymicrobial sepsis. To understand the impact of CLP-induced sepsis on the fluctuation of CXCL12, we first...
characterized the mRNA abundance of CXCL12 and CXCR4 in total bone marrow and spleen cells at 12 h after sepsis induction. The results demonstrated a reciprocal change between the expression of CXCL12 in the bone marrow and spleen after sepsis, with the abundance and protein levels of CXCL12 mRNA in total bone marrow cells significantly reduced, whereas levels in the spleen remained constant or were modestly elevated (Fig. 4A, 4B).

The significant reduction in bone marrow CXCL12 mRNA and protein expression during sepsis is not surprising given the dramatic mobilization of neutrophils from bone marrow to the peripheral circulation. The decrease in total bone marrow CXCL12 transcripts and protein expression during sepsis is not surprising given the dramatic mobilization of neutrophils from bone marrow to the peripheral circulation. The decrease in total bone marrow CXCL12 transcripts and protein levels agrees with the work of Semerad et al. (12), who demonstrated that sterile inflammation also induced a decrease in the bone marrow expression of CXCL12.

Considering the splenic accumulation of immature myeloid cells that occurs after sepsis, it is not surprising that splenic CXCL12 mRNA and protein levels are modestly elevated in septic animals compared with sham controls.

The changes in CXCR4 expression, which are dramatically reduced in the bone marrow and elevated in the spleen during sepsis, are similar to the reciprocal changes in CXCL12 mRNA abundance in the bone marrow and spleen. The increased expression of CXCR4 in the spleen was confirmed at the protein level by cell surface expression analysis (data not shown). Although the total number of splenic GR-1+CD11b+ granulocytes was reduced early after sepsis (21, 22), a greater number of the remaining splenic granulocytes expressed 6-fold more CXCR4 on their cell surface compared with sham controls (Fig. 4C, 4D).

Using an anti-CXCL12 antisera, we investigated the impact of CXCL12 blockade on bone marrow neutrophil release during

**FIGURE 2.** Neutrophil egress from the bone marrow does not depend on CXCR2 signaling during sepsis. A, Relative transcription level of CXCL1 in the bone marrow and spleen at 12 h after the induction of sepsis. B, Increase in CXCL1 levels in blood plasma extends through day 5 compared with sham levels. C, Absolute transcriptional level of CXCL2 in the bone marrow and spleen in sham and CLP-treated mice 12 h after sepsis. D, Relative transcriptional level of CXCR2 in the bone marrow and spleen of sham and CLP-treated mice 12 h after sepsis. E, Representative contour plots and bar graphs of GR-1+CD11b+ levels in the bone marrow of mice receiving CXCR2 blockade or vehicle control. F, Representative contour plots and bar graphs of GR-1+CD11b+ levels in the blood of mice receiving CXCR2 blockade or vehicle control. Data in A–D represent the mean and SE of five to seven animals/group from two independent experiments. Data in E and F represent the mean and SE of five animals/group from three independent experiments. A, C, D–F, *p < 0.01, sham versus CLP groups, Student t test. B, *p < 0.05, each time point compared with time point 0, ANOVA and post hoc Dunn test of significance.

**FIGURE 3.** Effects of transgenic mice on bone marrow neutrophil egression during polymicrobial sepsis. A and B, Changes in the percentage of total neutrophils (GR-1+CD11b+ cells) in the bone marrow and blood of MyD88−/− and wild-type control C57BL/6 or B6.129 mice 12 h after either sham or sepsis induction. MyD88−/− animals demonstrated similar bone marrow neutrophil mobilization into the circulation compared with wild-type control mice. Bone marrow release of neutrophils 12 h after either sepsis or sham procedure in IFNα/βR−/− (C) TRIF−/− (D), or C3H/HeJ (TLR4 mutant) (E) transgenic mice. There was no attenuation in the decrease of bone marrow neutrophils in any of these transgenic animals. Data represent the mean and SE of five to seven animals/group from three independent experiments. *p < 0.01, Student t test.
polymicrobial infection. In healthy mice, such treatments would be expected to result in bone marrow neutropenia and marked blood neutrophilia (13–15) by disrupting the CXCR4/CXCL12 interaction, culminating in bone marrow neutrophil mobilization (11). G-CSF induces stem cell mobilization by decreasing bone marrow stromal cell-derived factor-1 and upregulating CXCR4 (11). CXCR4 desensitization is associated with tissue localization of hematopoietic progenitor cells (31); however, their effects in sepsis are unknown. Mice were injected i.p. with either anti-CXCL12 or control antisera 12 h before the onset of sepsis. Mouse bone marrow, blood, and spleen were harvested 12 h after CLP induction in the total percentages of bone marrow GR-1+CD11b+ cells. Expectedly, sepsis resulted in a 50% reduction in the bone marrow CXCL12 blockade had no significant effect on the bone marrow LSK, CMP, and GMP populations, suggesting that CXCL12 does not regulate neutrophil or neutrophil precursor development during acute polymicrobial infection.

The CXCR4/CXCL12 signaling axis also regulates B lymphopoiesis and myelopoiesis by confining these precursors within the supportive fetal liver and bone marrow microenvironments for further maturation (15). In response to noninfectious stimuli, bone marrow B lymphocytes and mature granulocytes are mobilized to the peripheral circulation through a CXCL12-associated mechanism (34). Because our prior data demonstrated that CXCL12 inhibition during polymicrobial sepsis attenuated the release and ultimate reduction in bone marrow neutrophils (Fig. 4), we surmised that CXCL12 blockade should alleviate the decrease in bone marrow B lymphocytes during sepsis. CXCL12 blockade during sepsis attenuated the inflammation-associated mobilization of CD19+AA4.1highB220intermediate immature B lymphocytes compared with sepsis alone (Fig. 5D). These results underscore the

Given that CXCR4/CXCL12 signaling has been implicated in hematopoietic stem cell release from the bone marrow (11), as well as in hematopoietic stem and progenitor cell proliferation and differentiation (32, 33), we sought to determine whether CXCL12 blockade imposed any upstream effects on neutrophil precursors in the bone marrow during CLP-induced sepsis. Twelve hours after the initiation of polymicrobial sepsis, total bone marrow cells were harvested, and the major myeloid lineage precursor populations were evaluated in the presence or absence of CXCL12 blockade. Because granulocyte production begins with lineage-sca-1+CD34+ hematopoietic stem cells (LSKs), which generate lineage-sca-1+CD34+FcγRhigh common myeloid progenitors (CMPs) that further differentiate into lineage-sca-1+CD34+FcγRhigh granulocyte–monocyte progenitors (GMPs) that ultimately develop into immature neutrophils (34), we chose to evaluate the bone marrow LSK, CMP, and GMP populations after the induction of sepsis in the presence or absence of CXCL12 blockade (Fig. 5A–C). The results demonstrated that CXCL12 blockade had no significant effect on the bone marrow LSK, CMP, and GMP populations, suggesting that CXCL12 does not regulate neutrophil or neutrophil precursor development during acute polymicrobial infection.

FIGURE 4. CXCL12 blockade inhibits neutrophil egression from the bone marrow into the systemic circulation. A, Relative transcription level of CXCL12 in total bone marrow and spleen cells harvested at 12 h after the induction of either sham procedure or CLP sepsis. B, CXCL12 chemokine level in total splenocytes and bone marrow aspirates at 12 h after sham or sepsis. C, Relative expression of the chemokine receptor CXCR4 in total bone marrow and spleen cells in sham and CLP-treated mice 12 h after sepsis. D, Percentage of GR-1+CD11b+ cells that express CXCR4 in the spleen at 12 h after sham procedure or CLP induction. E, Fluctuations in the percentage of total GR-1+CD11b+ neutrophils in the bone marrow in the presence or absence of CXCL12 inhibition 12 h after sham procedure or CLP sepsis. CXCL12 blockade inhibited neutrophil release from the bone marrow. F, Neutrophil elevation in the blood was inhibited by CXCL12 blockade. Data in A–C represent the mean and SE of five to seven animals/group from three independent experiments. Data in D and E represent the mean and SE of five to seven animals/group from four independent experiments. Dashed line represents neutrophils observed in healthy naive wild-type C57BL/6 mice. *p < 0.01, sham versus CLP groups, Student t test.
FIGURE 5. CXCL12 blockade attenuates B lymphocyte efflux from the bone marrow during polymicrobial sepsis. CXCL12 or CXCR2 blockade has no impact on the bone marrow levels of hematopoietic stem cell (A), CMP cell (B), or GMP cell (C) levels in the bone marrow during polymicrobial sepsis. D, Immature B lymphocyte CD19^AA4.1^high, B220^intermediate, B^high bone marrow efflux is attenuated by CXCL12 blockade and not CXCR2 inhibition during polymicrobial sepsis. Changes in the B lymphocyte bone marrow levels are evident from the representative contour plot for each group. Data represent the mean and SE of five to seven animals/group from three independent experiments. *p < 0.05, compared with sham animals, ANOVA and post hoc Dunn test of significance.

codependence of granulocyte and B lymphocyte bone marrow egression on CXCL12 signaling during polymicrobial sepsis.

CXCL12 blockade reduces bone marrow neutrophil mobilization and peritoneal accumulation, leading to bacterial expansion during polymicrobial sepsis

Our studies suggested that CXCL12 signaling is vital for bone marrow mobilization of neutrophils during the early response to sepsis. We next determined whether CXCL12 blockade affected neutrophil mobilization during later phases of sepsis. Using an anti-CXCL12 Ab, we investigated the impact of CXCL12 blockade on bone marrow neutrophil release during polymicrobial sepsis over a 96-h period. Mice were treated with either anti-CXCL12 or control antisera 12 h before the onset of sepsis. Mouse bone marrow, blood, and peritoneal washings were harvested at 3–96 h after sham surgery or CLP, and the relative percentages and absolute numbers of GR-1^CD11b^ cells were determined. There was a significant reduction in the bone marrow neutrophil population in the CLP group compared with the sham group, while CXCL12 blockade prevented this reduction (Fig. 6A, 6B). Moreover, although the sham and CLP-treated animals exhibited dramatic elevations in blood neutrophil numbers, the CLP mice pretreated with CXCL12-blocking Ab displayed only a modest elevation in blood neutrophil numbers (Fig. 6C).

Because neutrophils are essential for host survival during sepsis, we examined whether CXCL12 blockade also affects neutrophil recruitment to the peritoneum. Peritoneal lavage from mice injected i.p. with anti-CXCL12 12 h prior to the onset of CLP revealed significant reductions in the absolute number of peritoneal neutrophils beginning at 6 h and extending through 96 h compared with CLP treatment alone (Fig. 6D). The reduction in blood and peritoneal neutrophil numbers in anti-CXCL12–treated septic mice coincided with significantly diminished bacterial clearance in these animals (Fig. 6E). These data suggested that CXCL12 inhibition prohibits bone marrow neutrophil mobilization, which results in blood and peritoneal neutropenia and unopposed bacterial invasion.

CXCL12 blockade reduces survival during polymicrobial sepsis

Neutrophils are a fundamental component of the innate immune system and are essential for successful host eradication of bacterial pathogens. During episodes of sepsis or bacterial challenge, the bone marrow provides a large reserve of neutrophils that is released into the peripheral circulation and homes to sites of infection (9). Numerous reports demonstrated that survival to polymicrobial sepsis is dependent on neutrophil-mediated microbe eradication at local sites of infection (4, 5). To determine the significance of CXCL12 blockade on neutrophil bone marrow egression and polymicrobial sepsis survival, we compared survival of animals receiving CXCL12 inhibition for 7 d with mice receiving vehicle control alone after the induction of (LD₃₀) polymicrobial sepsis. The absolute survival of mice receiving CXCL12 blockade was reduced by 40%, such that 70% of the CXCL12 antisera-treated mice died (Fig. 7).

Discussion

Survival to polymicrobial sepsis requires the mobilization of functional neutrophils to local sites of infection (4, 5). However, few studies have investigated the mechanisms involved in bone marrow mobilization of neutrophils in response to a polymicrobial sepsis challenge. Although previous studies identified several mediators capable of bone marrow neutrophil mobilization (19, 20), few studies have focused on the mechanisms of neutrophil release from the bone marrow during a microbial challenge. Although CSFs can induce bone marrow neutrophil mobilization, in part through downregulating CXCR4 (35–37), deletion of G-CSF does not affect neutrophil mobilization in response to Candida albicans infection (35). During episodes of acute streptococcal infection, release of neutrophils into the blood is accelerated; however, the specific mechanisms mediating that release were not investigated (35, 38–40).

In this study, we used a global approach to identify signaling pathways involved in polymicrobial sepsis–induced neutrophil mobilization. The results indicated that bone marrow neutrophil mobilization into the circulation is not dependent on TLR4, MyD88, TRIF, IFNAR/β, or CXCR2 signaling pathways. In contrast, CXCL12 blockade prevented the release of neutrophils from the bone marrow during sepsis, resulting in a failure to increase blood and peritoneal neutrophil counts. CXCL12 blockade during sepsis was accompanied by a 5-fold increase in peritoneal bacteria CFU compared with CLP treatment alone. This failure to clear bacteria led to a 40% increase in sepsis mortality in anti-CXCL12–treated mice. These findings are in striking contrast to the role that CXCR4/CXCL12 plays in neutrophil release from bone marrow in the healthy animal. In this case, local bone marrow signaling through CXCR4/CXCL12 seems to be important in retention of neutrophils and immature B cells in the bone marrow. Blocking the CXCR4/CXCL12 axis causes neutrophilia in the
otherwise healthy animal (13–15). However, unlike the healthy animal, there are marked alterations in the expression of CXCR4 and CXCL12 in polymicrobial sepsis that can explain the differential effects of blockade. Although CXCR4/CXCL12 signaling plays an essential role in bone marrow retention of neutrophils in healthy naive animals, changes in the expression of CXCL12 in peripheral tissues may establish gradients that promote neutrophil emigration from the bone marrow during sepsis. During steady-state exogenous, CXCL12 administration has a modest effect on bone marrow neutrophil mobilization, but during malarial infection, CXCL12 levels are dramatically increased in the periphery (41, 42). Moreover, exogenous CXCL12 administration during malarial infection results in a reduction in malarial loads (42). Although this evidence suggests that CXCR4/CXCL12 signaling may be necessary for host defense to infection, the role of peripheral CXCL12 elevation on neutrophil mobilization is still unclear. Our data indicated that polymicrobial sepsis decreases CXCR4 and CXCL12 expression in the bone marrow but increases the expression of CXCR4 and CXCL12 in the spleen. These data supported the hypothesis that a CXCL12 gradient may exist between the bone marrow and the periphery and may be pivotal for neutrophil release during infection. Similarly, this may explain the difference in the effects of CXCR4 blockade in healthy animals associated with neutrophil release from the bone marrow, whereas CXCL12 blockade during sepsis or inflammatory conditions impeded bone marrow neutrophil release by prohibiting peripheral CXCL12 gradients, as mentioned above, and as observed in pathologic conditions like acute myelogenous leukemia (43). Although CXCL12 blockade also inhibited immature B cell efflux from the bone marrow, as would be expected based on the data of Ueda et al. (34), it is not unreasonable to mention that the anti-CXCL12 effect may stem from an antiapoptotic property that inhibits B cell apoptosis that occurs during sepsis (44).

Much to our surprise, disruption of either TLR signaling or CXCR2 pathways has no significant effect on the neutrophil response to polymicrobial sepsis. Although increases in CXCL1 and CXCL2 levels have been the hallmark of inflammation in murine models of polymicrobial sepsis, their relevance on neutrophil bone marrow mobilization has been rarely observed. This notion is supported by recent findings by Link and colleagues (16), who suggested that CXCR2 signaling is a second chemokine axis that interacts antagonistically with CXCR4 during neutrophil release from the bone marrow. Holzmann and colleagues (19) demonstrated that the production of CXCL1 and CXCL2 during polymicrobial sepsis is dependent on intact TLR signaling through the MyD88 pathway. Further work by Ayala and colleagues (29) demonstrated that Ab inhibition of CXCL2, but not CXCL1, resulted in reduced neutrophil accumulation in the lung following polymicrobial sepsis. Our initial findings that CXCL1, CXCL2, and CXCR2 levels increased in response to infection provided early evidence that neutrophil emigration from the bone marrow may depend on those increases in chemokines during polymicrobial sepsis. Furthermore, coupled with the evidence provided by Holzmann and colleagues (19) implicating TLR signaling in general, and MyD88 signaling in particular, as paramount for increases in CXCL1 and CXCL2 during sepsis, we were optimistic that neutrophil bone

**FIGURE 6.** CXCL12 blockade inhibits peritoneal neutrophil accumulation and allows bacterial proliferation following sepsis. Anti-CXCL12 or anti-CXCR2 was administered 12 h before the onset of sepsis and once daily for the next 4 d. A–D, Effect of CXCL12 blockade, over a 96-h period following sham or CLP sepsis, on the relative percentage and absolute number of bone marrow, blood, and peritoneal cavity neutrophils. E, CXCL12 blockade enhanced i.p. bacterial proliferation following CLP sepsis. Data represent the mean and SE of five to seven animals/group from three independent experiments. A–D, p < 0.05, CLP compared with sham animals, ANOVA and post hoc Dunn test of significance. *p < 0.01, CLP–anti-CXCL12 compared with CLP alone. E, *p < 0.01, CLP–antiCXCL12 compared with CLP alone, Student t test.

**FIGURE 7.** CXCL12 blockade reduces survival to polymicrobial sepsis. Anti-CXCL12 or anti-CXCR2 was administered 12 h before the onset of sepsis and once daily for the next 7 d. CXCL12 blockade produced a significant reduction (40%) in survival following the initiation of polymicrobial sepsis. The survival analysis was repeated two times, with n = 20 mice/group in each experiment. p = 0.025, Fisher exact test of significance.