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Sepsis Induces Early Alterations in Innate Immunity That Impact Mortality to Secondary Infection

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Sepsis, the systemic inflammatory response to microbial infection, induces changes in both innate and adaptive immunity that presumably lead to increased susceptibility to secondary infections, multiorgan failure, and death. Using a model of murine polymicrobial sepsis whose severity approximates human sepsis, we examined outcomes and defined requirements for survival after secondary Pseudomonas aeruginosa pneumonia or disseminated Listeria monocytogenes infection. We demonstrate that early after sepsis neutrophil numbers and function are decreased, whereas monocyte recruitment through the CCR2/MCP-1 pathway and function are enhanced. Consequently, lethality to Pseudomonas pneumonia is increased early but not late after induction of sepsis. In contrast, lethality to listeriosis, whose eradication is dependent upon monocyte/macrophage phagocytosis, is actually decreased both early and late after sepsis. Adaptive immunity plays little role in these secondary infectious responses. This study demonstrates that sepsis promotes selective early, impaired innate immune responses, primarily in neutrophils, that lead to a pathogen-specific, increased susceptibility to secondary infections. The Journal of Immunology, 2011, 186: 000–000.

Sepsis is the leading cause of death in the critically ill population with >750,000 cases per year that result in 210,000 deaths annually in the United States (1, 2). Early studies suggested that the mortality and organ injury associated with severe sepsis were primarily due to an exaggerated innate immune and inflammatory response (3). However, despite the association of inflammation with mortality, anti-inflammatory therapies have for the most part not resulted in significant improvements in clinical outcome (4). This suggests that other mechanisms also contribute to the morbidity and mortality of sepsis. More recently, a number of alterations in adaptive immunity, including increased T cell apoptosis (5), decreased Th1 cell function (6), increased suppressor cell activity (7), and reduced TCR function (6), have been implicated in early mortality from severe sepsis.

Due to improvements in resuscitation and supportive care, most patients survive early sepsis. Currently, mortality is more frequently attributed to subsequent secondary nosocomial infections and multiorgan system failure (8). Recently, emphasis has shifted to understanding how sepsis impacts host immunity as well as host recognition and response to secondary, mostly opportunistic infections. More investigation has focused on phagocytic cells, key effectors of innate immunity that mediate both the direct clearance of pathogens as well as the presentation of Ags, leading to eradication of bacterial pathogens (9). Several reports indicate that sepsis alters myelopoiesis and neutrophil function, and these perturbations contribute to impaired resolution of secondary infections after sepsis (10, 11). Furthermore, sepsis also has been demonstrated to reduce monocyte HLA-DR expression (12, 13) and Ag presentation (6), to reduce the expression of cytokines and other proinflammatory mediators (14), and to increase IL-10 production (15), thus demonstrating a disruption in the bridge between innate and adaptive immunity.

The current report investigates the role of innate and adaptive immunity in the response to secondary opportunistic infections in a septic host. With a survivable, cecal ligation and puncture (CLP) model of polymicrobial sepsis, the roles of neutrophils, monocytes, and adaptive immunity in a secondary Pseudomonas pneumonia or Listeria monocytogenes infection were explored. The findings demonstrate that polymicrobial sepsis produced a transient increase in susceptibility to Pseudomonas pneumonia but not to L. monocytogenes infection. This transient period of susceptibility was associated with reduced neutrophil numbers and function but did not require an intact adaptive immune system. The findings emphasize the requirement for myeloid cells and innate immunity in the host response to secondary opportunistic infections during sepsis.

Materials and Methods

Mice

All of the experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine. Specific pathogen-free C57BL/6, NCF-1 (p47(-/-)), MCP-1(-/-), CCR2(-/-), and Rag(-/-) (RAG-1 deficient, homozygous) mice were purchased from The Jack-
son Laboratory (Bar Harbor, ME). All of the mice were maintained at the University of Florida College of Medicine and were studied between 8 and 12 wk of age.

**Cecal ligation and puncture**

For induction of polymicrobial sepsis, mice underwent sham laparotomy (laparotomy followed by extracorporeal cecum mobilization and i.p. replacement) or CLP induced by ligation of the cecum and a double enterotomy created with a 27 gauge needle with a mortality of ~10–15% at 10 d. Death occurred predominantly within the first 3 d; thereafter, surviving mice developed abscesses surrounding the devitalized cecum as described previously (16).

**Bacteria**

*P. aeruginosa* (PAK, a wild-type strain) was grown overnight in Luria-Bertani (LB) broth, then transferred to fresh medium and grown for 4–5 h to midlog phase. The cultures were centrifuged at 4000 × g for 15 min, and the cell pellets were washed twice with PBS. The bacterial cell dilution was diluted in its original volume, and the OD at 600 nm (A600; SmartSpec 3000 spectrophotometer; Bio-Rad, Hercules, CA) was adjusted to give the approximate desired inoculum. The inoculum was verified by serial 10-fold dilutions of the bacterial suspensions and plating on LB agar.

*L. monocytogenes* was grown to a logarithmic phase in broth heart infusion (HBI) medium (Sigma-Aldrich, St. Louis, MO) diluted in PBS and injected i.v. into the retro-orbital space. Prior to experimentation, the LD$_{50}$ and LD$_{100}$ at 10 d after listeriosis induction in healthy naive C57BL/6 mice were determined to be 1 × 10$^3$ (LD$_{50}$) and 1 × 10$^7$ (LD$_{100}$) bacteria, respectively (data not shown). To measure bacterial titers in the spleen and liver, organs were harvested and dissociated through 70-μm pore sized cell strainers (Falcon, Bedford, MA). Serial dilutions were performed in HBI medium, and 100 μl was plated onto HBI media plates incubated at 37°C overnight.

**Pneumonia induction**

Mice were anesthetized with inhaled isoflurane. The mice then were held supine, and 50 μl of the bacterial suspension was pushed gently down a sterile disposable pipette tip into just one of the mouse nares. Mice were then immediately held upright to facilitate bacterial inhalation well past the return of normal breathing. A series of experiments were performed to determine the desired inoculum that would yield an LD$_{50}$ in healthy naive C57BL/6 mice. The determined LD$_{50}$ was 1 × 10$^6$ bacteria (data not shown). When indicated, pneumonia survival studies were carried out using the LD$_{50}$ inoculum. In all of the other studies, mice treated with bacteria received a sublethal inoculum (LD$_{10}$) of 1 × 10$^5$ bacteria.

**Bronchoalveolar lavage**

Bronchoalveolar lavage (BAL) was performed on mice as described previously (17) at 24 h after pneumonia induction. The BAL fluid (3 ml) was diluted in 50 ml aliquots at −80°C. Before flow cytometry, 20 μl of stock was dissolved in 650 μl of PBS to a final concentration of 30 g/ml. A total of 25 μl of working DHR123 solution was added to peritoneal cell isolates suspended in 200 μl PBS and incubated at 37°C for 5 min. Cells then were spun and washed twice with PBS. Subsequently, cells were stimulated with FITC-labeled PMA at 37°C and evaluated by flow cytometry analysis at various points over the subsequent 30 min period using an LSR flow cytometer (BD Biosciences). A minimum of 1 × 10$^6$ live, nondebris cells were collected for analysis.

**Flow cytometry**

Cells obtained from spleen, bone marrow, whole blood, and BAL fluid were analyzed by flow cytometry as described previously (6, 16) in sham and CLP mice. Abs included anti–GR-1 PerCp5.5 (Ly6G and Ly6C [RB6-8C5]), anti-Ly6C FITC, anti-Ly6G PE, anti-CD11b Pacific blue (Integri- mAb, chain Mac-1a chain [M170]), anti-F4/80 Ag allophycocyanin (pan-macrophage marker [BM8]), anti-CD31 PE (MEC 13.3), Fc-block (CD16/32 clone 93), and Sytox blue. F4/80–, CD11b–, and FcγR-specific Abs were purchased from eBioscience (San Diego, CA), and all of the other Abs were purchased from BioLegend (San Diego, CA). Spleen, whole blood, and bone marrow were harvested, and single-cell suspensions were created by passing the cells through 70-μm pore sized cell strainers (Falcon). Erythrocytes then were 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, San Jose, CA). A minimum of 5 × 10$^4$ live, nondebris cells (Sytox$^-$) were collected for analysis.

**Reactive oxygen species detection**

Spleen, bone marrow, and BAL fluid were harvested, and single-cell suspensions were created by passing the cells through 70-μm pore sized cell strainers (Falcon). Neutrophils, monocytes, and macrophages then were isolated using a Ficoll density gradient (1.104 specific gravity) and washed two times using PBS without calcium, phenol red, or magnesium. Samples then were labeled for surface Ags as described and washed twice with PBS. Reactive oxygen species (ROS) production was determined using dihydrorhodamine 123 (DHR123; Invitrogen, Carlsbad CA). The stock DHR123 was prepared by diluting the DHR123 1 mg/ml in DMSO, stored in 50 μl aliquots at −80°C. Before flow cytometry, 20 μl of stock was dissolved in 650 μl of PBS to a final concentration of 30 g/ml. A total of 25 μl of working DHR123 solution was added to peritoneal cell isolates suspended in 200 μl PBS and incubated at 37°C for 5 min. Cells then were spun and washed twice with PBS. Subsequently, cells were stimulated with FITC-labeled PMA at 37°C and evaluated by flow cytometry analysis at various points over the subsequent 30 min period using an LSR II flow cytometer (BD Biosciences). A minimum of 1 × 10$^6$ live, nondebris cells were collected for analysis.

**Results**

**Susceptibility to P. aeruginosa pneumonia is increased due to impaired neutrophil clearance of bacteria early after CLP**

In the hospital setting, *Pseudomonas pneumonia* is a frequent cause of increased morbidity and organ failure in septic individuals at an increased risk of secondary infections (18). To determine whether mice are more susceptible to *Pseudomonas pneumonia* after sepsis, mice were challenged intranasally with sublethal *P. aeruginosa* 3 or 7 d after CLP or a sham procedure. In control animals, *Pseudomonas pneumonia* produced ~50% mortality. However, mice challenged with *P. aeruginosa* 3 d after exposure to polymicrobial sepsis experienced 100% mortality (p < 0.01) (Fig. 1A). In contrast, the CLP animals receiving *P. aeruginosa* 7 d after sepsis displayed no increase in mortality (Fig. 1B). To assess clearance of this organism 24 h after a pseudomonal challenge, BAL fluid was harvested and cultured overnight for viable bacterial colonies. Mice receiving intranasal *P. aeruginosa* 3 d after CLP were unable to clear the bacteria, whereas mice challenged at 7 d after CLP were able to clear the bacteria similar to sham-treated and naive animals (Fig. 1C, 1D).

To determine whether deficits in tissue-associated neutrophil numbers are associated with *Pseudomonas* persistence, we analyzed BAL fluid for relative neutrophil percentages and absolute numbers 24 h after *Pseudomonas* instillation (Fig. 2A, 2B). Animals challenged 3 d after CLP exhibited a significant reduction in the percentage and absolute number of neutrophils in the BAL fluid. On the contrary, mice administered *Pseudomonas* 7 d after CLP display neutrophil percentages and absolute numbers consistent with sham-treated mice (Fig. 2).

**Acute polymicrobial infection induces peripheral blood and bone marrow granulocytopenia**

Because a reduction in the number of BAL neutrophils was noted in response to a *P. aeruginosa* challenge 3 d after CLP, the effect of sepsis on blood, peritoneum, and bone marrow neutrophil populations was...
determined. Neutrophils were analyzed 1–10 d after CLP or a sham procedure to ascertain whether changes in circulating or bone marrow polymorphonuclear cells were responsible for the low number of neutrophils in the lungs. Twenty-four hours after CLP, there was a 50% reduction in the percentage of circulating neutrophils that persisted for 3 d in comparison with that in sham animals (Fig. 2). In contrast, from day 5 to day 10, CLP animals had a substantial recovery in the percentage of blood neutrophils as compared with earlier times and to sham-treated animals (Fig. 2).

The bone marrow myeloid compartment is the primary site of neutrophil production (19). During episodes of inflammatory stress and acute infection, the vast majority of bone marrow neutrophils, lymphocytes, and monocytes exit the bone marrow into the systemic circulation (20). To determine the kinetics of sepsis-induced changes in bone marrow cellularity, bone marrow, blood, and peritoneum neutrophils were monitored 1–10 d after CLP or sham procedures. As anticipated, there was a massive influx of neutrophils to the peritoneum in the immediate period after sepsis that remained elevated for several days (Fig. 2G). CLP animals experienced a 3-fold reduction in absolute bone marrow cellularity that persisted for 7 d. Although there was an increase in the relative percentage, the absolute number of neutrophils in septic mice did not achieve sham levels until 5 d after CLP (Fig. 2D, 2F).

The data indicate that although neutrophils represent the largest percentage of blood leukocytes after CLP, there is a relative deficiency in absolute bone marrow cellularity as well as neutrophil...
number in the bone marrow and lungs after sepsis. This extends over the first 5 d and is associated with increased mortality during pseudomonal pneumonia.

**Bone marrow and blood neutrophils are defective in respiratory burst early after sepsis**

Reduced BAL neutrophil numbers in response to pneumonia after sepsis do not provide insight into neutrophil function. One of the most important neutrophil functions is their ability to produce ROS through the NADPH oxidase complex in response to foreign pathogens (21). To assess the postsepsis ROS response, neutrophils from BAL fluid of septic and sham-treated mice with pneumonia were subjected to stimulation with phorbol esters. Three days after sepsis, there was a significant reduction in the respiratory burst of BAL neutrophils from CLP animals as compared with that of sham-treated animals. In contrast, BAL neutrophils from mice that were challenged with *P. aeruginosa* 7 d after CLP were 25% more capable of producing a respiratory burst than neutrophils from both sham-treated and control animals (Fig. 3A, 3B). Not only did the early post-CLP BAL neutrophils fail to produce ROS, but similar reductions in ROS production from bone marrow neutrophils were seen at 3 d after sepsis and 24 h postpneumonia. By 7 d after sepsis, the bone marrow neutrophils were able to mount an effective oxidative burst in response to phorbol stimulation, similar to sham-treated and control animals (Fig. 3C, 3D). The data indicate that in addition to the reduced number of tissue and systemic neutrophils after sepsis there is also a respiratory burst deficit that further impairs bacterial clearance.

**NADPH oxidase-deficient mice fail to eradicate *P. aeruginosa* pneumonia similar to septic mice**

To demonstrate that a respiratory burst is essential for the clearance of *Pseudomonas* pneumonia, we examined outcome and response to CLP-induced sepsis and secondary *Pseudomonas* pneumonia in a mouse strain incapable of mounting a respiratory burst. NCF-1 null mice are devoid of a functional p47 subunit in the NADPH oxidase complex and display a predisposition to develop Gram-negative bacterial pneumonia (22). This is analogous to humans with chronic granulomatous disease whose neutrophils also have defects in their oxidative burst (23). To model the oxidative burst deficiency found in early postseptic mice, healthy naive NCF-1 null mice were given a sublethal intranasal instillation of *P. aeruginosa*. NCF-1 null mice failed to eradicate the bacteria and exhibited 100% mortality, similar to septic animals treated with pneumonia 3 d after CLP (Fig. 4A, 4D). NCF-1 null mice also died uniformly in response to the CLP. Mortality to *Pseudomonas* pneumonia occurred even though the percentage and absolute number of recruited BAL neutrophils were similar to those of wild-type mice (Fig. 4B). When NCF-1 null mice were reconstituted with healthy naive bone marrow neutrophils 1 h prior to the induction of pneumonia, the number of bacteria CFUs present in the BAL fluid was reduced to healthy control mouse levels (Fig. 4A). These findings support an overall model whereby both neutrophil recruitment and oxidative burst capacity are required for *Pseudomonas* eradication early after sepsis.

**Polymicrobial sepsis enhances *L. monocytogenes* eradication**

Our findings suggest that a sublethal septic challenge (CLP) results in increased mortality to a secondary *Pseudomonas* pneumonia during an early period of reduced neutrophil numbers and function. To determine if sepsis similarly impairs the clearance and killing of another opportunistic infection, we used the same modestly lethal CLP (LD10) model to evaluate the clearance of a secondary *L. monocytogenes* infection. In a sublethal *Listeria* infection, bacteria replicate until their numbers are controlled by activated macrophages. The development of *Listeria*-specific T cell responses is required to ultimately eliminate the bacteria and memory T cells that provide protection against reinfection (24, 25). Thus, sublethal listeriosis represents an excellent in vivo model of early innate and adaptive immune responses. Mice were challenged with *L. monocytogenes* (1 × 10³ bacteria, i.v.) 3 or 7 d after an LD10 CLP. Five days after listeriosis, the livers and spleens of surviving animals were harvested, and bacterial colonies were determined. Sham-treated mice that received *L. monocytogenes* 3 d after their procedures were unable to clear the organism from their spleens and liver, whereas mice receiving *L. monocytogenes* 3 d after polymicrobial sepsis were able to clear the bacteria (Fig. 5A, 5C).

Similar results were seen 7 d postsepsis where CLP animals exhibited little colonization with *L. monocytogenes*, whereas sham-

**FIGURE 3.** ROS production by BAL and bone marrow cells as well as splenic macrophages. BAL and bone marrow cells as well as splenic macrophages were harvested and enriched from naive, sham-treated, and CLP mice 3 (A, C, F) and 7 d (B, D, E) after sepsis and stimulated ex vivo with PMA in the presence of DHR123 at 37°C for up to 30 min. Rhodamine fluorescence was gated on neutrophil-enriched populations and presented as a percentage of control fluorescence. Data from CCR2γ-/- mice are included in E and F. Values represent the mean of 7–10 animals per group. Each experiment was confirmed three times. Significance between sham and CLP over time was determined by two-way ANOVA. *p < 0.01.
treated mice were again unable to clear the bacteria (Fig. 5B, 5D), carrying 5–10 times the bacterial load in their spleens and liver than septic animals. Similarly, when higher lethal doses of *L. monocytogenes* (10^6 CFU) were administered to mice either 3 or 7 d after the CLP, an otherwise 100% mortality was prevented (Fig. 5E, 5F).

**FIGURE 4.** Outcome and colonization of p47^-/- mice infected with *Pseudomonas* pneumonia. Naive C57BL/6 and p47^-/- mice were subjected to intranasal instillation of *Pseudomonas*, and 24 h later bacterial colonization of their BAL fluid was determined. *Pseudomonas* colonies in the naive p47^-/- mice were comparable to levels seen in mice 3 d after sepsis and markedly higher than levels seen in sham-treated or control wild-type mice despite normal numbers of BAL neutrophils (A–C). All p47^-/- mice succumbed to CLP prior to any intranasal instillation, whereas healthy mice uniformly died in response to *Pseudomonas* (D). Adoptive transfer of 5 x 10^6 healthy naive bone marrow neutrophils into p47^-/- mice reduced *Pseudomonas* colonization of the lungs (A). Survival studies were carried out with 20 mice per group, whereas seven mice per group were used for BAL fluid bacterial and neutrophil determination. Significance in survival was determined by Fisher’s exact test and in BAL CFUs and neutrophil counts by one-way ANOVA. *p < 0.01.

**FIGURE 5.** Liver and spleen colonization and outcome after listeriosis in septic, sham, and control mice. Three and seven days (A–D) after CLP-induced sepsis, mice were challenged with a sublethal *L. monocytogenes* infection (10^5 CFU). Five days later, *L. monocytogenes* colony counts were determined in the spleen and liver. When sham and septic mice were challenged with a lethal number of *L. monocytogenes* (10^6 CFU), only the septic animals survived (E, F). Bacterial colony counts represent the mean of seven animals per group, and the survival studies contained n = 20 mice per group. Significance between sham and CLP liver and spleen CFUs was determined by one-way ANOVA, whereas survival differences were determined by Fisher’s exact test. *p < 0.05.

*L. monocytogenes* clearance after CLP is not dependent on adaptive immunity but rather monocyte innate immune responses

To determine whether changes in adaptive immunity were responsible for the increased sepsis-induced resistance to *L. monocytogenes* infection, *Rag^-/-* animals, which lack functional lym-
L. monocytogenes eradication by innate immune effector cells, then results for Rag mice increased susceptibility to subsequent listeriosis. In contrast to the animals with defective monocyte migration should experience an sepsis-induced listeriosis protection is mediated through brisk its ligand MCP-1 are responsible for monocyte migration from the MCP-1 were evaluated in the CLP and listeriosis model. CCR2 and tion and mortality, mice with deficits in either CCR2 or its ligand monocytes in the blood 3–7 d after sepsis.

**FIGURE 6.** Survival to L. monocytogenes infection in RAG, CCR2−/−, and MCP-2−/− mice after polymicrobial sepsis. Wild-type and RAG, CCR2−/−, and MCP-2−/− mice underwent CLP to induce sepsis, and 3 d later, septic, sham-treated, and control mice were administered an otherwise lethal L. monocytogenes infection (10⁶ CFU) (A, B). Both wild-type and RAG−/− mice with sepsis survived the lethal L. monocytogenes challenge (A). Conversely, neither CCR2−/− nor MCP-2−/− mice were protected from the lethality of L. monocytogenes infection by prior sepsis (B). Values represent 20 animals per group. Each survival experiment was conducted four independent times. Significance between wild-type, RAG−/−, CCR2−/−, and MCP-2−/− mice was determined by Fisher’s exact test.

phocytes of adaptive immune cells, were used in our CLP and listeriosis model. After LD10 CLP, Rag−/− mice have a slightly higher mortality rate than immune competent animals. However, those Rag−/− mice that survived sepsis had no further increase in mortality to listeriosis than similar CLP wild-type animals in response to an LD100 inoculum of L. monocytogenes. In contrast, the sham-treated wild-type mice quickly succumbed to mortality early after L. monocytogenes infection (Fig. 6A). The results suggest that the adaptive immune system is not essential for the improved survival of L. monocytogenes infection seen after CLP.

We also examined the monocyte response to sepsis to determine whether it paralleled the early loss of neutrophils seen after a CLP. As shown in Fig. 7, although there was a total loss of bone marrow cellularity (Fig. 7B), monocyte numbers did not decline and actually increased with time. This also was reflected by a relative monocytosis in the blood 3–7 d after sepsis.

To confirm that innate immune effectors were responsible for enhanced postsepsis protection from L. monocytogenes colonization and mortality, mice with deficits in either CCR2 or its ligand MCP-1 were evaluated in the CLP and listeriosis model. CCR2 and its ligand MCP-1 are responsible for monocyte migration from the bone marrow to the periphery during bacterial infection (26). If sepsis-induced listeriosis protection is mediated through brisk L. monocytogenes eradication by innate immune effector cells, then animals with defective monocyte migration should experience an increased susceptibility to subsequent listeriosis. In contrast to the results for Rag−/− animals, both CCR2−/− and MCP-1−/− septic mice experienced increased mortality to listeriosis when compared with that of septic wild-type mice (Fig. 6B). These data indicated that the recruitment of monocytes, key effectors of innate immunity, is necessary for efficient postsepsis L. monocytogenes clearance and survival.

Sepsis-induced enhancement of L. monocytogenes eradication requires splenic monocyte recruitment and monocyte ROS production

To characterize the postsepsis cellular changes occurring in the spleens of mice lacking an intact CCR2/MCP-1 signaling axis, spleens of CCR2−/− animals 5 d after CLP were evaluated for monocyte maturation phenotypes. Although CLP-treated CCR2−/− mice experienced the same increase in GR-1highCD11bhigh splenocytes as CLP-treated wild-type mice, the CCR2−/− animals failed to expand the GR-1intermediateCD11bintermediate (monocyte) splenocyte population (Supplemental Fig. 1A). In addition, CLP-treated CCR2−/− mice presented with a 7-fold reduction in the frequency of CD11bLy6GLy6C splenocytes when compared with that of CLP-treated wild-type mice (Supplemental Fig. 1B). These findings demonstrate that after initiation of sepsis splenic monocyte expansion occurs simultaneously and protects against listeriosis in wild-type mice. In contrast, CCR2−/− animals, which lack a robust splenic monocyte expansion, are unable to survive a subsequent listeriosis infection.

A key monocyte function is their ability to produce ROS through the NADPH oxidase complex in response to foreign pathogens (21). To assess the role of ROS in the post-CLP monocyte response, splenic and bone marrow monocytes from wild-type and CCR2−/− CLP-treated animals were subjected to stimulation with phorbol esters followed by DHR123 staining. After CLP, there

**FIGURE 7.** Blood monocyte and tissue macrophage response to polymicrobial sepsis. At intervals after polymicrobial sepsis, mice were sacrificed, and blood and bone marrow CD11bLy6C cells were determined by flow cytometry. A, Total CD11bLy6C% numbers in blood. B and C, Bone marrow CD11bLy6C%F4/80% percentages and absolute numbers. Values represent the mean of 5–7 animals per group. Each experiment was confirmed at least three times. Significance between naive, sham, and CLP at days 3 and 7 was determined by one-way ANOVA. Significance between sham and CLP over time was determined by two-way ANOVA. *p < 0.01.
was a significant increase in the respiratory burst in both wild-type and CCR2−/− monocytes (Fig. 3E, 3F). In contrast, splenic and bone marrow monocytes from sham and unmanipulated mice generated 2-fold less ROS. These findings support the suggestion that CLP augments splenic monocyte expansion and oxidative burst capabilities, both of which are required for survival to secondary infection with *L. monocytogenes* after sepsis.

**Discussion**

Survival from polymicrobial sepsis has improved only modestly over the past 30 years, despite significant progress in our understanding of its underlying derangements in innate and adaptive immunity (1, 2). Early research focused on the exaggerated inflammatory and innate immune responses, after which anti-inflammatory therapies entered the clinic with only limited success (4). In general, resuscitation and improvements in emergent care have improved so greatly that the majority of individuals with severe sepsis now survive the initial inflammatory event (2), only to succumb days or weeks later to a seemingly inconsequential bacterial infection and organ failure.

In contrast to earlier reports that focused on the early immune response to severe sepsis using models that induce near 100% lethality, the present report focuses on immune aberrations that follow a more survivable (LD50) septic event. It is thought that this model more closely recapitulates the clinical setting where mortality from severe sepsis is only 20–40% (16). With this model, the goal of the present studies was not to examine the factors affecting outcome to the early inciting event but rather to examine how sepsis subsequently alters the host response to secondary and opportunistic infections. By varying the length of the cecal ligation and the number of enterotomies, polymicrobial sepsis can be induced that is only 10% lethal (16), and the requirements for a successful response to a secondary infectious challenge in septic animals can be explored.

Much to our surprise, we observed that mice surviving polymicrobial sepsis (CLP) were not globally more susceptible to a secondary infection. Three days after polymicrobial sepsis, mice demonstrated a greater susceptibility to *P. aeruginosa* pneumonia, while they were simultaneously more resistant to *L. monocytogenes* infection. In addition, 7 d after CLP, mice were no longer sensitive to the *P. aeruginosa* challenge as well as maintaining their enhanced clearance of *L. monocytogenes* infection. These findings suggest that sublethal polymicrobial sepsis may induce changes in the innate and adaptive immune responses, but such changes are complex and do not translate to an overall increased susceptibility to secondary infections.

Similar to our finding that susceptibility to *Pseudomonas* pneumonia was increased significantly 3 d after sepsis, Muenzer et al. (27, 28) also reported that mice had increased susceptibility to *Streptococcus* and *Pseudomonas* pneumonia 3 d after a survivable CLP. Steinhauser et al. (29) also reported that 1 d after CLP mice were more susceptible to *Pseudomonas* pneumonia. We have provided an in-depth analysis of the mechanism(s) responsible for the selective increased *Pseudomonas* pneumonia mortality after sepsis and found that BAL fluid from animals with *Pseudomonas* infection had a 10-fold increase in bacteria along with a simultaneous reduction in neutrophil numbers and respiratory burst capacity. These alterations correlated temporally with deficits in bone marrow neutrophil production. All of these deficits resolved within 7 d after CLP, and mortality from *Pseudomonas* pneumonia was no longer increased as compared with that from sham-treated animals, a finding also reported by Muenzer et al. (28).

In models of severe sepsis with lethality near 100%, there is little doubt that impairment of adaptive immunity contributes to adverse outcomes (6). Such animals have deficits in both Ag-specific and nonspecific T cell proliferative responses (6). However, there is less convincing data to suggest whether intact adaptive immunity is required for a sufficient host response to secondary infections in a septic host. The response to listeriosis, which is presumed to be dependent upon a functioning Th1 response as well as upon intact innate immune recognition (24, 25), is enhanced in animals after polymicrobial sepsis. Surprisingly, Rag−/− mice, which are more susceptible to lethality from polymicrobial sepsis (Fig. 6) (30), display the same protection from *L. monocytogenes* after sepsis that is demonstrated by wild-type mice. This increased listericidal response is not dependent upon T or B cells but rather upon recruitment and expansion of myeloid populations in the spleen and liver, requiring the CCR2/MCP-1 signaling pathway.

**Clinical implications**

Due to improvements in supportive care and resuscitation, initial survival to severe sepsis has improved significantly. Unfortunately, patients often succumb to subsequent secondary infections and organ failure. Although there is little doubt that an exaggerated inflammatory response and impairment of adaptive immunity contribute to the adverse early outcomes to severe sepsis, the mechanisms leading to increased susceptibility to secondary nosocomial infections and late mortality in sepsis appear to be more dependent upon innate immunity. The findings reported in this study do not support the conclusion that the adaptive immune response is critical to resistance to secondary infections after polymicrobial sepsis. Rather, these data implicate the importance of sepsis-induced alterations in myeloid cell populations as crucial to host resistance to infection in the postseptic period. Relative neutropenia and loss of neutrophil oxidative function early after sepsis contribute to the decreased bacterial clearance and increased mortality secondary to *Pseudomonas* pneumonia. Similarly, the increased bactericidal response to listeriosis after sepsis is dependent upon CCR2/MCP-1 recruitment of myeloid cells to the spleen and liver rather than the presence of T or B cells. Treating the septic patient who has survived the initial event may require more precise monitoring and manipulation of innate immune processes in general and neutrophil number and function in particular.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure 1 - Enhanced monocyte recruitment to the spleen in polymicrobial sepsis. Seven days after sepsis or sham treatment, splenic monocyte numbers from wild-type and CCR2−/− mice were determined by flow cytometry analysis. Panel A is a representative scatter plot depicting the reduced number of splenic monocytes in the CCR2−/− mice. Panel B demonstrates the relative difference in splenic monocyte percentages following sepsis in CCR2−/− and wild type mice. Values represent 7 animals per group. Significance between groups was determined using a Student’s t test, * = p<0.01.