Published in final edited form as:
J Immunol. 2011 January 1; 186(1): 195–202. doi:10.4049/jimmunol.1002104.

SEPSIS INDUCES EARLY ALTERATIONS IN INNATE IMMUNITY THAT IMPACT MORTALITY TO SECONDARY INFECTION

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Abstract

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, multi-organ 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/MCP1 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/

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Conflicts of Interest:
The authors state that they have no financial or equity conflicts of interest.

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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.

Keywords

myeloid cells; mouse; cecal ligation and puncture; innate immunity

Introduction

Sepsis is the leading cause of death in the critically ill population with more than 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 to 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 Th_1 cell function (6), increased suppressor cell activity (7) and reduced T cell receptor function (6) have been implicated in the 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 multi-organ 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 antigens, 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 following sepsis (10, 11). Furthermore, sepsis has also been demonstrated to reduce monocyte HLA-DR expression (12, 13) and antigen presentation (6), to reduce 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. Using a survivable, cecal ligation and puncture model (CLP) of polymicrobial sepsis, the role of neutrophils, monocytes and adaptive immunity to a secondary *Pseudomonas* pneumonia or *L. 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.

Methods

Mice

All 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 Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the University Of Florida College of Medicine and were studied between 8-12 weeks of age.

Cecal ligation and puncture (CLP)

For induction of polymicrobial sepsis, mice underwent sham laparotomy (laparotomy followed by extracorporeal cecum mobilization and intraperitoneal replacement) or CLP induced by ligation of the cecum and a double enterotomy created with a 27 gauge needle with a mortality of approximately 10-15% at ten days. Death occurred predominantly within the first three days; thereafter, surviving mice developed abscesses surrounding the devitalized cecum as previously described (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 mid-log phase. The cultures were centrifuged at 4000 × *g* for 15 min and the cell pellets washed twice with PBS. The bacterial pellet was diluted in its original volume and the optical density at 600λ (A600; BioRad SmartSpec 3000 spectrophotometer) 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 (BHI) medium (Sigma-Aldrich) diluted in PBS and injected *i.v.* into the retro-orbital space. Prior to experimentation, the LD_{50} and LD_{100} at 10 days after listeriosis induction in healthy naïve C57BL/6 mice were determined to be 1×10^5 (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). Serial dilutions were performed in BHI medium and 100 μL was plated onto BHI media plates incubated at 37 degrees Celsius overnight.

Pneumonia induction

Mice were anesthetized with inhaled isoflurane. The mice were then held supine and 50 μL of bacterial suspension were gently pushed 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 naïve C57BL/6 mice. The determined LD_{50} was 1×10^8 bacteria (*data not shown*). When indicated, pneumonia survival studies were carried out using the LD_{50} inoculum. In all other studies, mice treated with bacteria received sublethal inoculum (LD_0) of $1x10^6$ bacteria.

Bronchoalveolar lavage (BAL)

BAL was performed on mice as previously described (17) at 24 hours after pneumonia induction. The BAL fluid (3 ml) was diluted and plated on LB agar plates to obtain viable bacterial counts. Total cell counts were measured in the BAL fluid using a hemocytometer with manual counts in quadruplicate.

Listeriosis bacteremia

When indicated, the listeriosis bacteremia model was carried out by first conducting the CLP as previously described. Beginning at three days after the completion of the CLP, either a sublethal (1×10^4 bacteria), lethal inoculum of *L. monocytogenes* (1×10^6 bacteria) was

administered to the mice as described above. Survival analysis was carried out for 10 days from the time of listeriosis induction.

Flow cytometry

Cells obtained from spleen, bone marrow, whole blood and BAL fluid were analyzed by flow cytometry as previously described (6, 16) in sham and CLP mice. Antibodies included anti-GR-1-PerCp5.5(Ly6G and Ly6C (RB6-8C5)) anti-Ly6C FITC, anti-Ly6G PE, anti-CD11b-Pacific Blue (Integrin aM, chain Mac-1a chain (M1/70)), anti-F4/80 Antigen- APC (Pan Macrophage Marker (BM8)), anti-CD31-PE (MEC 13.3), Fc-Block (CD16/CD32 Fc g III/II Receptor (2.4G2)), c-Kit conjugated to either FITC or APC (2B8), FcγR-Pac Blue (CD16/32 clone 93), and Sytox Blue. F4/80, CD11b and FcγR specific antibodies were purchased from eBioscience (San Diego, CA), and all other antibodies were purchased from BD Pharmingen (San Jose, 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™, BD Biosciences). 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 (San Jose, CA)). A minimum of 5×10^4 live, non-debris cells ($Sytox^{negative}$) were collected for analysis.

Reactive oxygen species detection

Spleen, bone marrow, and BAL fluid were harvested and single cell suspensions created by passing the cells through 70 μm pore sized cell strainers (Falcon™). Neutrophils, monocytes and macrophages were then isolated using a ficoll density gradient (1.104 spec. grav.) and washed two times using PBS without calcium, phenol red, or magnesium. Samples were then labeled for surface antigens as described and washed twice with PBS. Reactive oxygen species (ROS) production was determined using dihydrorhodamine 123 (Invitrogen, Carlsbad CA). The stock DHR123 was prepared by diluting the DHR123 1 mg: mL in DMSO, stored in 50μL 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 DHR solution was added to peritoneal cell isolates suspended in 200 μL PBS and incubated at 37°C for 5 minutes. Cells were then spun and washed twice with PBS. Subsequently, cells were stimulated with FITC labeled phorbol-12-myristate-13-acetate (PMA) at 37°C and evaluated by flow cytometry analysis at various points over the subsequent 30 minute period using an LSRII flow cytometer (BD Biosciences). A minimum of 1×10^4 live, non-debris cells were collected for analysis.

Statistics

Continuous variables were first tested for normality and equality of variances. Differences among groups in flow cytometric analyses were evaluated by analysis of variance for multiple groups and Student's t-test for two groups. Post-hoc comparisons were performed using Student-Newman Keuls multiple range tests. In all cases, significance was designated at the 95% confidence level using a two-tailed test. Statistical analysis for survival was performed using a Fisher's Exact test of significance.

Results

Susceptibility to Pseudomonas 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 increased risk of secondary infections (18). To

determine whether mice are more susceptible to *Pseudomonas* pneumonia following experimental sepsis, mice were challenged intranasally with sublethal *P. aeruginosa* three or seven days after CLP or a sham procedure. In control animals, *Pseudomonas* pneumonia produced approximately 50% mortality. However, mice challenged with *P. aeruginosa*- three days after exposure to polymicrobial sepsis experienced 100% mortality ($p<0.01$) (Figure 1, Panel A). In contrast, the CLP animals receiving *P. aeruginosa* seven days after sepsis displayed no increase in mortality (Figure 1, Panel B). To assess clearance of this organism 24 hours after a *pseudomonal* challenge, BAL fluid was harvested and cultured overnight for viable bacterial colonies. Mice receiving intranasal *P. aeruginosa* three days after CLP were unable to clear the bacteria, whereas mice challenged at seven days after CLP were able to clear the bacteria similar to sham-treated and naïve animals (Figure 1, Panels C and D).

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 hours after *Pseudomonas* instillation (Figure 2, Panels A and B). Animals challenged three days after CLP exhibited a significant reduction in the percentage and absolute number of neutrophils in the BAL fluid. On the contrary, mice administered *Pseudomonas* seven days after CLP display neutrophil percentages and absolute numbers consistent with sham-treated mice (Figure 2).

Acute polymicrobial infection induces peripheral blood and bone marrow granulocytopenia

Since a reduction in the number of BAL neutrophils was noted in response to a *P. aeruginosa* challenge three days after CLP, the effect of sepsis on blood, peritoneum and bone marrow neutrophil populations was determined. Neutrophils were analyzed one to ten days following CLP or sham procedure in order 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 which persisted for three days in comparison to sham animals (Figure 2). In contrast, from day 5 to day 10, CLP animals had a substantial recovery in the percentage of blood neutrophils as compared to earlier times and to sham treated animals (Figure 2, Panel C).

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 peritneum neutrophils were monitored one to ten days following CLP or sham procedures. Septic animals experienced a three-fold reduction in absolute bone marrow cellularity that persisted for seven days. Although there was an increase in the relative percentage, the absolute number of neutrophils in septic mice did not achieve sham levels until five days following CLP (Figure 2, Panels D-F). As anticipated, there was a massive influx of neutrophils to the peritoneum in the immediate 6-12 hour period after sepsis that remained elevated for several days (Figure 2, panel G).

The data indicate that although neutrophils represent the largest percentage of blood leukocytes following 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 five days 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 reactive oxygen species (ROS) through the NADPH-oxidase complex in response to foreign pathogens (21). To assess the post-sepsis ROS response, neutrophils from BAL fluid of septic and sham-treated animals 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 to sham-treated animals. In contrast, BAL neutrophils from mice that were challenged with *P. aeruginosa* seven days after CLP were 25% more capable at producing a respiratory burst than neutrophils from both sham-treated and control animals (Figure 3, Panels A and B). 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 three days after sepsis and twenty four hours post-pneumonia. By seven days following 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 (Figure 3, Panels C and D). The data indicates that in addition the reduced number of tissue and systemic neutrophils following 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 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 post-septic mice, healthy naïve NCF-1 null mice were given a sublethal intranasal instillation of *Pseudomonas aeruginosa.* NCF-1 null mice failed to eradicate the bacteria and exhibited 100% mortality, similar to septic animals treated with pneumonia three days after CLP (Figure 4 Panels A and D). 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 wild-type mice (Figure 4; Panel B). When NCF-1 null mice were reconstituted with healthy naïve bone marrow neutrophils one hour prior to the induction of pneumonia, the number of bacteria colony forming units present in the BAL fluid was reduced to healthy control mouse levels (Figure 4 panel A). These findings support an overall model whereby both neutrophil recruitment and oxidative burst capacity are required for *Pseudomonas* eradiation early after sepsis.

Polymicrobial sepsis enhances Listeria 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 utilized the same modestly lethal CLP (LD_{10}) model to evaluate clearance of a secondary *L. monocytogenes* infection. In a sub-lethal *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 provide protection against re-infection (24, 25). Thus, sublethal listeriosis represents an excellent in vivo model of early innate and adaptive immune responses. Mice were challenged with *L. monocytogenes* (1×10^4 bacteria, *i.v.*) three or

seven days after an LD_{10} 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* three days after their procedure were unable to clear the organism from their spleens and liver, whereas mice receiving *L. monocytogenes* three days after polymicrobial sepsis were able to clear the bacteria (Figure 5, Panels A, and C).

Similar results were seen seven days post sepsis where CLP animals exhibited little colonization with *L. monocytogenes* while sham-treated mice were again unable to clear the bacteria (Figure 5, Panels B, and D), carrying 5 to 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 three of seven days after the CLP, an otherwise 100% mortality was prevented (Figure 5, Panels E and F).

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 lymphocytic adaptive immune cells, were used in our CLP and listeriosis model. Following LD_{10} 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 the listeriosis than similar CLP wild-type animals in response to an LD_{100} inoculum of *L. monocytogenes*. In contrast, the sham-treated wild-type mice quickly succumbed to mortality early after *L. monocytogenes* infection (Figure 6 panel A). 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 $CD11b^+Ly6C^+$ (monocyte) response to sepsis to determine whether it paralleled the early loss of neutrophils seen after a CLP. As shown in Figures 2 and 7, although there was a total loss of bone marrow cellularity (Figure 2, panel E), monocyte numbers did not decline and actually increased with time (Figure 7). This was also reflected by a relative monocytosis in the blood three to seven days after sepsis.

To confirm that innate immune effectors were responsible for enhanced post-sepsis protection from *L. monocytogenes* colonization and mortality, mice with deficits in either chemokine receptor 2 (CCR2) or its ligand, monocyte chemoattractant protein-1 (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 a 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 $MCP\text{-}1^{-/-}$ septic mice experienced increased mortality to listeriosis when compared to septic wild-type mice (Figure 6, Panel B). These data indicated that recruitment of monocytes, key effectors of innate immunity, are necessary for efficient post-sepsis *L. monocytogenes* clearance and survival.

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

To characterize the post-sepsis cellular changes occurring in the spleens of mice lacking an intact CCR2/MCP-1 signaling axis, spleens of $CCR2^{-/-}$ animals five days after CLP were evaluated for monocyte maturation phenotypes. Although CLP treated $CCR2^{-/-}$ mice experienced the same increase in $GR\text{-}1^{high}CD11b^{high}$ splenocytes as CLP treated wild type

mice, the $CCR2^{-/-}$ animals failed to expand the GR-$1^{intermediate}CD11b^{intermediate}$ (monocyte) splenocyte population (Supplemental Figure 1, Panel A). In addition, CLP treated $CCR2^{-/-}$ mice presented with a seven-fold reduction in the frequency of $CD11b^{+}Ly6G^{-}Ly6C^{+}$ splenocytes, when compared to CLP-treated wild-type mice (Supplemental Figure 1, Panel B). These findings demonstrate that following 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. Following CLP, there was a significant increase in the respiratory burst both in the wild-type and $CCR2^{-/-}$ monocytes (Figure 3, Panels E and F). In contrast, splenic and bone marrow monocytes from sham and un-manipulated mice generated two-fold less reactive oxygen species. 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 thirty 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 (LD_{10}) septic event. It is thought that this model more closely recapitulates the clinical setting where mortality from severe sepsis is only 20-40% (16). Using 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, seven days 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 significantly increased three days after sepsis, Muenzer *et al.* also reported that mice had increased susceptibility to *Streptococcus* and *Pseudomonas* pneumonia three days after a survivable CLP (27, 28). Steinhauser and colleagues also reported that one day after CLP mice were more susceptible to *Pseudomonas* pneumonia (29). We have provided an in depth analysis of the mechanism(s) responsible for the selective increased *Pseudomonas* pneumonia mortality following 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 seven days after CLP, and mortality from *Pseudomonas* pneumonia was no longer increased as compared to from sham-treated animals, a finding also reported by Muenzer and Hotchkiss (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 antigen-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 Th_1 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 (Figure 6 and (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/MCP1 signaling pathway.

Clinical implications

Due to improvements in supportive care and resuscitation, initial survival to severe sepsis has significantly improved. 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 here do not support the conclusion that the adaptive immune response is critical to resistance to secondary infections after polymicrobial sepsis. Rather, this data implicate the importance of sepsis-induced alterations in myeloid cell populations as crucial to host resistance to infection in the post-septic 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/MCP1 recruitment of myeloid cells to the spleen and liver, and, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants R01 GM-40586 and R01 GM-81923 (LLM), awarded by the National Institute of General Medical Sciences. U.S.P.H.S. MJD and AGC were supported by a training grant in burn and trauma research (T32 GM-08431); RDW was supported by a training grant in surgical oncology (T32 CA-106493) awarded by the National Cancer Institute. SMW was supported by NRSA grant DE-016509. MAW was supported by a training grant in rheumatology (T32 AR-007603) and the Laura McClamma Fellowship for Immune Deficiency.

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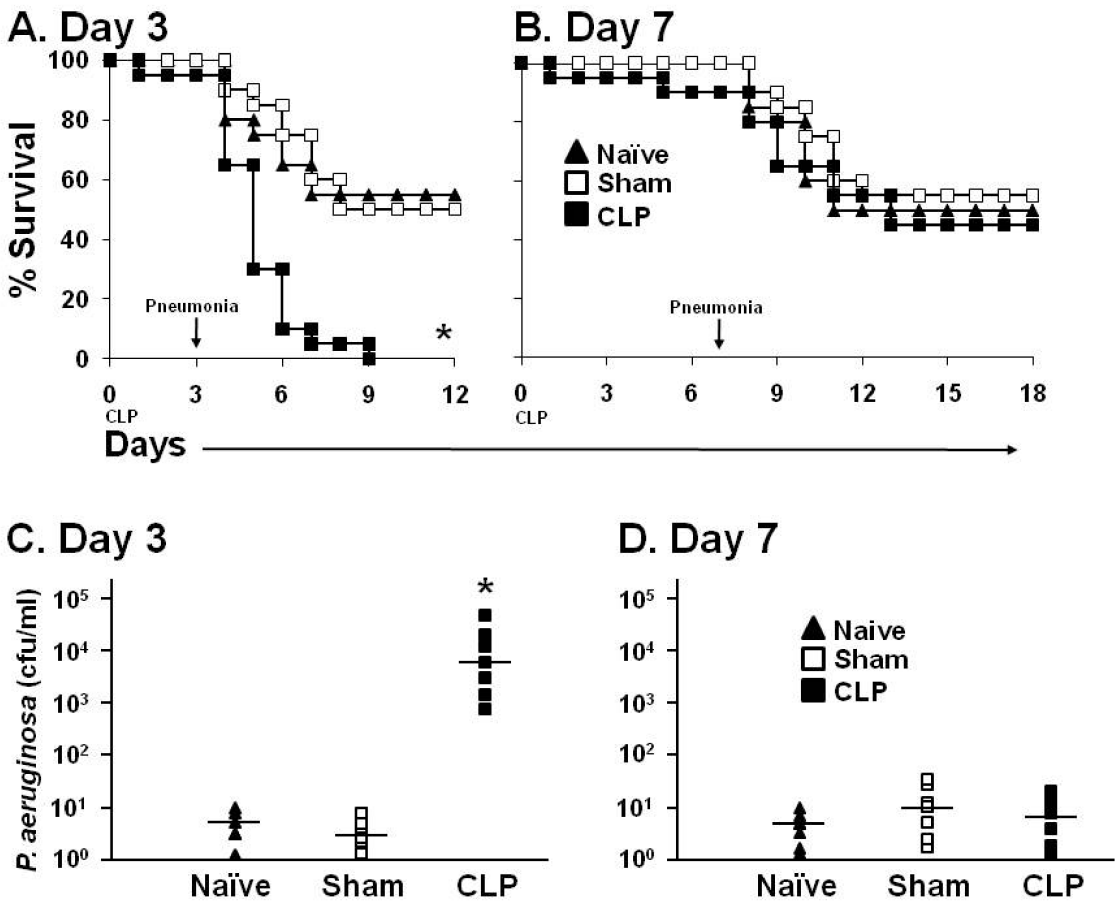

Figure 1. Survival and bacterial colonization to intranasal Pseudomonas aeruginosa infection in post-septic mice

On days three (Panel A) and seven (Panel B) post-CLP, mice were challenged with an intranasal administration of 10^8 cfu of *Pseudomonas aeruginosa* (□ healthy control animals, ▲ sham-treated animals, ■ CLP-treated animals). Survival was evaluated over the next nine days. Additional animals were sacrificed 24 hours after *Pseudomonas* pneumonia and bacterial colony counts in the bronchoalveolar lavage (BAL) fluid were determined (Panels C and D). Survival studies represent the outcome of n=20 animals per group, while BAL fluids represent n=7 per group. Each experiment was repeated at least three times. Significant differences in survival were determined by Fisher's exact test, and in cfu, by ANOVA and multiple range test * = $p<0.01$.

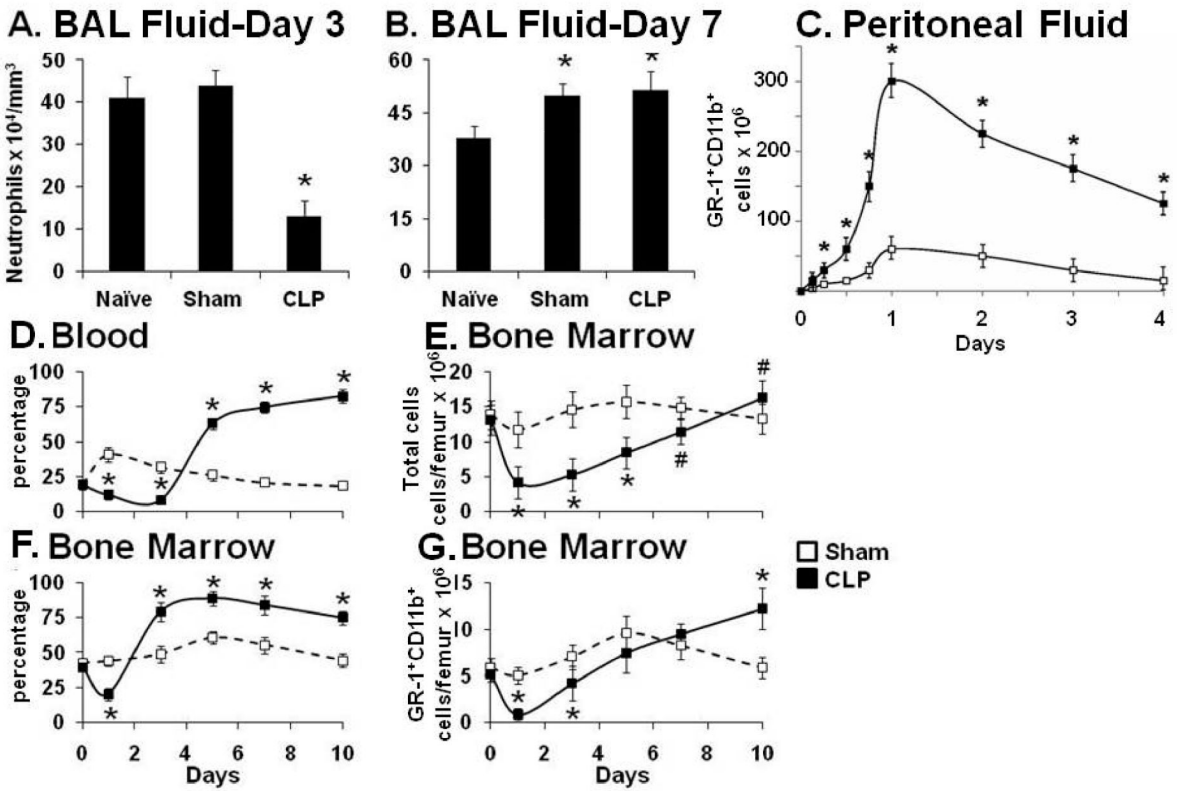

Figure 2. Blood, BAL, peritoneal lavage and bone marrow neutrophil responses to polymicrobial sepsis

At intervals after polymicrobial sepsis, mice were sacrificed and blood, BAL and bone marrow neutrophils ($CD11b^+$,$GR\text{-}1^+$) were determined by flow cytometry Panels A and B present total BAL neutrophil numbers while Panels C-G represent peritoneal lavage, blood and bone marrow neutrophil percentages and absolute numbers. Within three to five days, there was a relative rapid influx of neutrophils into the peritoneal cavity associated with deficiency in blood, BAL and bone marrow neutrophils that returned to baseline or above by seven days after sepsis. Values represent the mean of five to seven animals per group. Each experiment was confirmed at least three times. Significance between naïve, sham and CLP at day 3 and 7 was determined by one way ANOVA. Significance between sham and CLP over time was determined by two way ANOVA. * = $p<0.01$ and # = $p<0.05$.

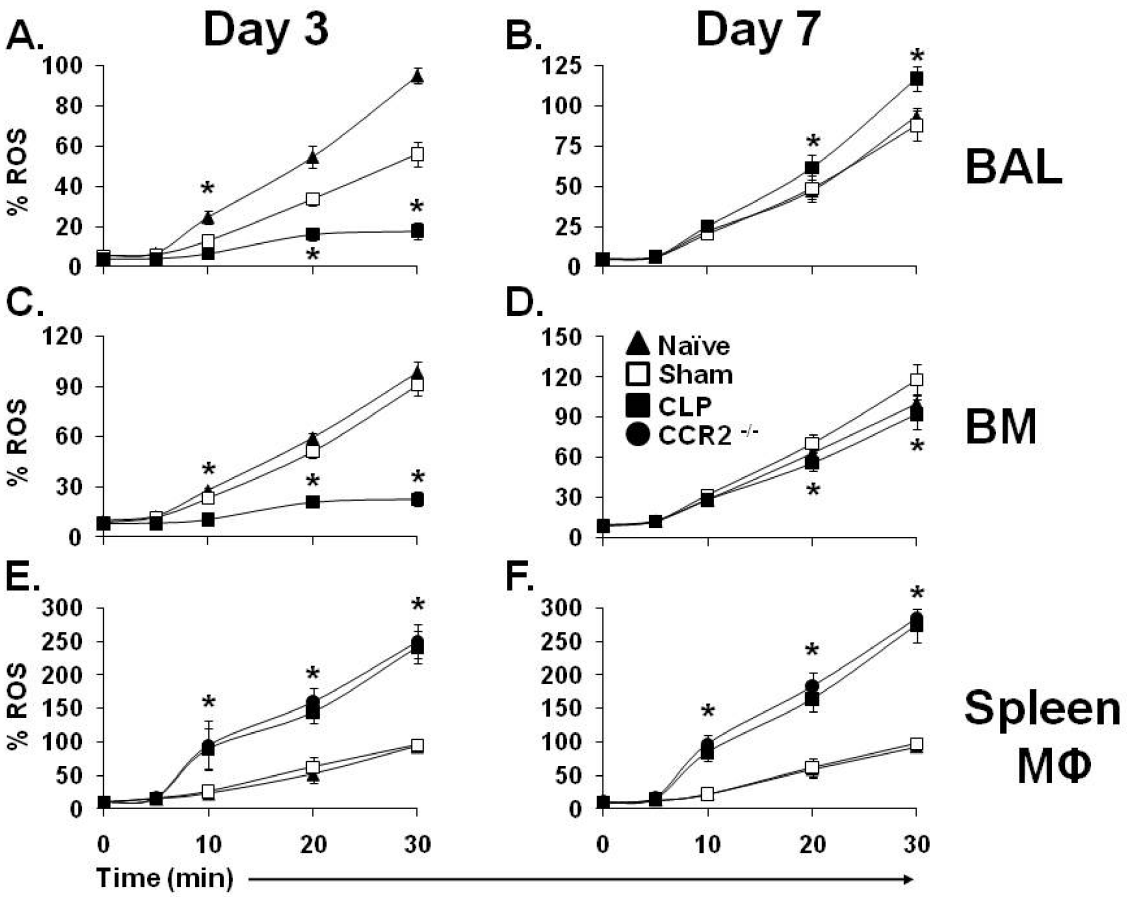

Figure 3. Reactive oxygen species (ROS) production by BAL and bone marrow cells as well as splenic macrophages

BAL and bone marrow cells as well as splenic macrophages were harvested and enriched from naïve, sham-treated and CLP mice three (Panels A, C, F) and seven days (Panels B, D, E) after sepsis, and stimulated ex vivo with PMA in the presence of dihydrorhodamine at 37° C for up to 30 mins. Rhodamine fluorescence was gated on neutrophil enriched populations and presented as a percentage of control fluorescence. Data from $CCR2^{-/-}$ mice are included in Panels 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* = $p<0.01$.

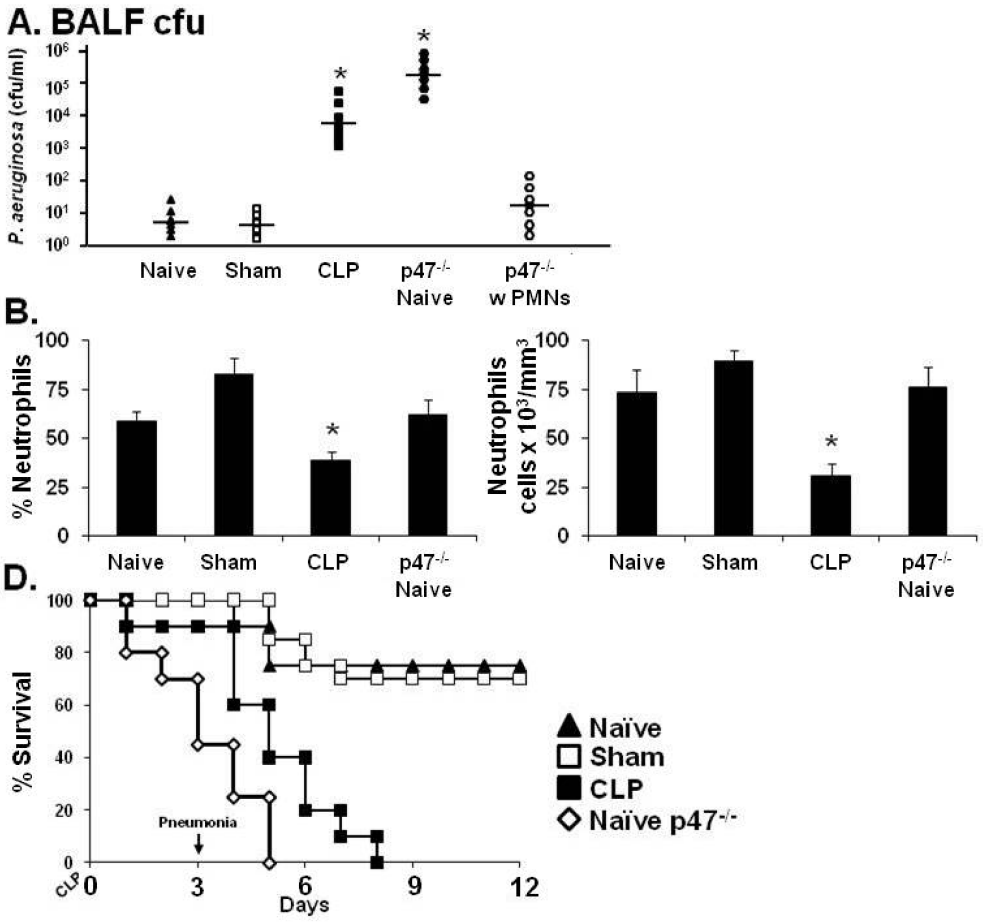

Figure 4. Outcome and colonization of p47$^{-/-}$ mice infected with Pseudomonas pneumonia

(Panel A) Naïve C57BL/6 and p47$^{-/-}$ mice were subjected to intranasal instillation of *Pseudomonas* and 24 hours later bacterial colonization of their BAL fluid was determined. *Pseudomonas* colonies in the naïve p47$^{-/-}$ mice were comparable to levels seen in mice three days after sepsis and markedly higher than levels seen in sham-treated or control wild-type mice despite normal numbers of BAL neutrophils (Panels A, B and C). All p47$^{-/-}$ mice succumbed to CLP prior to any intranasal instillation while healthy mice uniformly died in response to *Pseudomonas* (Panel D). Adoptive transfer of 5×10^6 healthy naïve bone marrow neutrophils into p47$^{-/-}$ mice reduced *Pseudomonas* colonization of the lungs (Panel A). Survival studies were carried out with 20 mice per group while 7 mice group were used for BALF 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. * = $p<0.01$

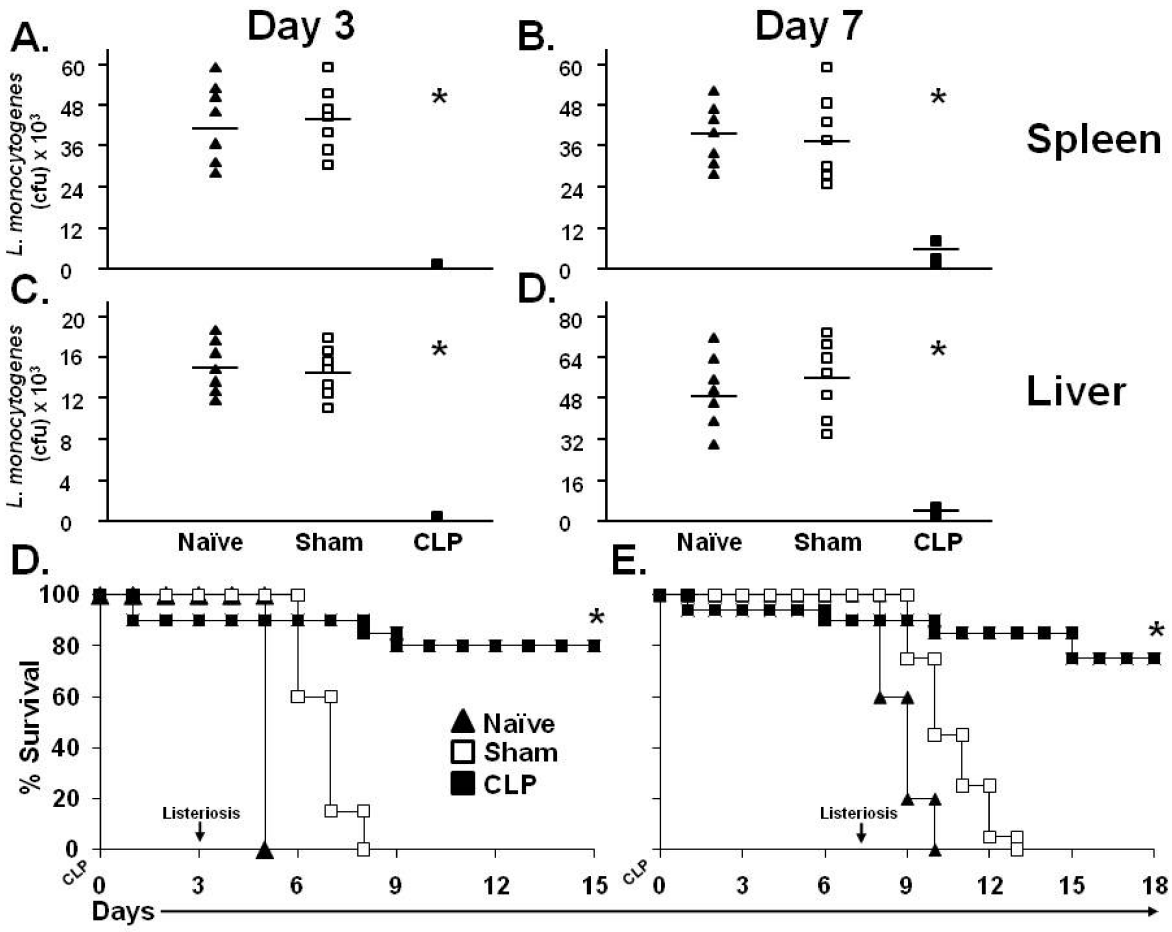

Figure 5. Liver and spleen colonization and outcome following listeriosis in septic, sham and control mice

Three and seven days (Panels A-D) after CLP-induced sepsis, mice were challenged with a sublethal *L. monocytogenes* infection (10^4 cfu). Five days later, *L. monocytogenes* colony counts were determined in the spleen and liver. Animals that had sepsis induced three and seven days earlier had markedly reduced *L. monocytogenes* infection 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 (Panels 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 cfu was determined by one way ANOVA, while survival differences were determined by Fisher's exact test, * = $p<0.05$

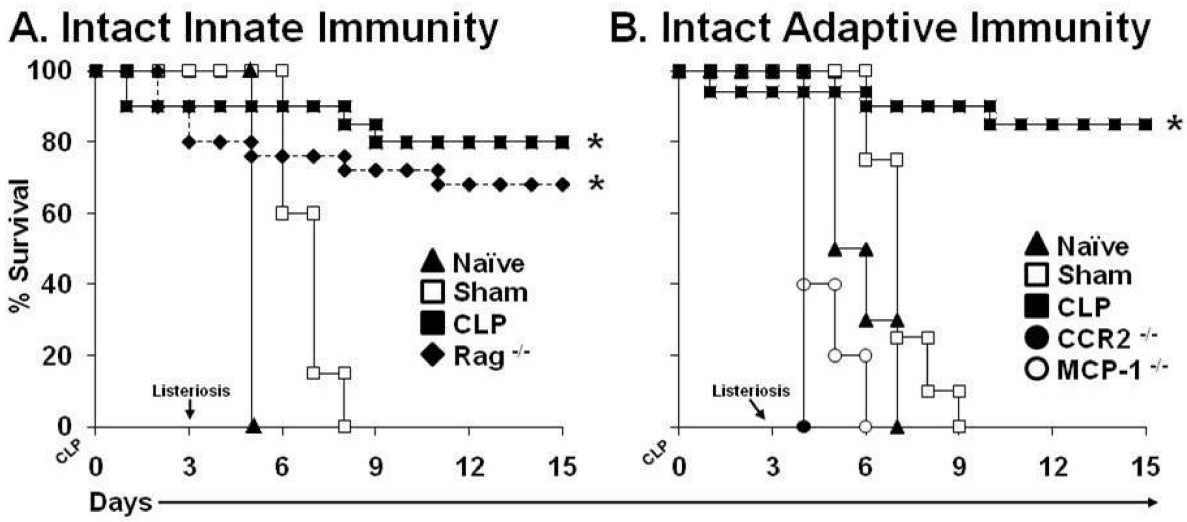

Figure 6. Survival to *L. monocytogenes* infection in RAG$^{-/-}$, CCR2$^{-/-}$ and MCP-2$^{-/-}$ mice after polymicrobial sepsis

Wild-type and RAG$^{-/-}$, CCR2$^{-/-}$ and MCP-2$^{-/-}$ mice underwent CLP to induce sepsis and three days later, septic, sham-treated and control mice were administered an otherwise lethal *L. monocytogenes* infection (10^6 cfu) (Panels A and B). Both wild-type and RAG$^{-/-}$ mice with sepsis survived the lethal *L. monocytogenes* challenge (Panel A). Conversely, neither CCR2$^{-/-}$ and MCP-2$^{-/-}$ mice were protected from the lethality of *L. monocytogenes* infection by prior sepsis (Panel B). Values represent 20 animals per group. Each survival experiment was conducted 4 independent times. Significance between wild-type, RAG$^{-/-}$, CCR2$^{-/-}$ and MCP-2$^{-/-}$ mice was determined by Fisher's exact test.

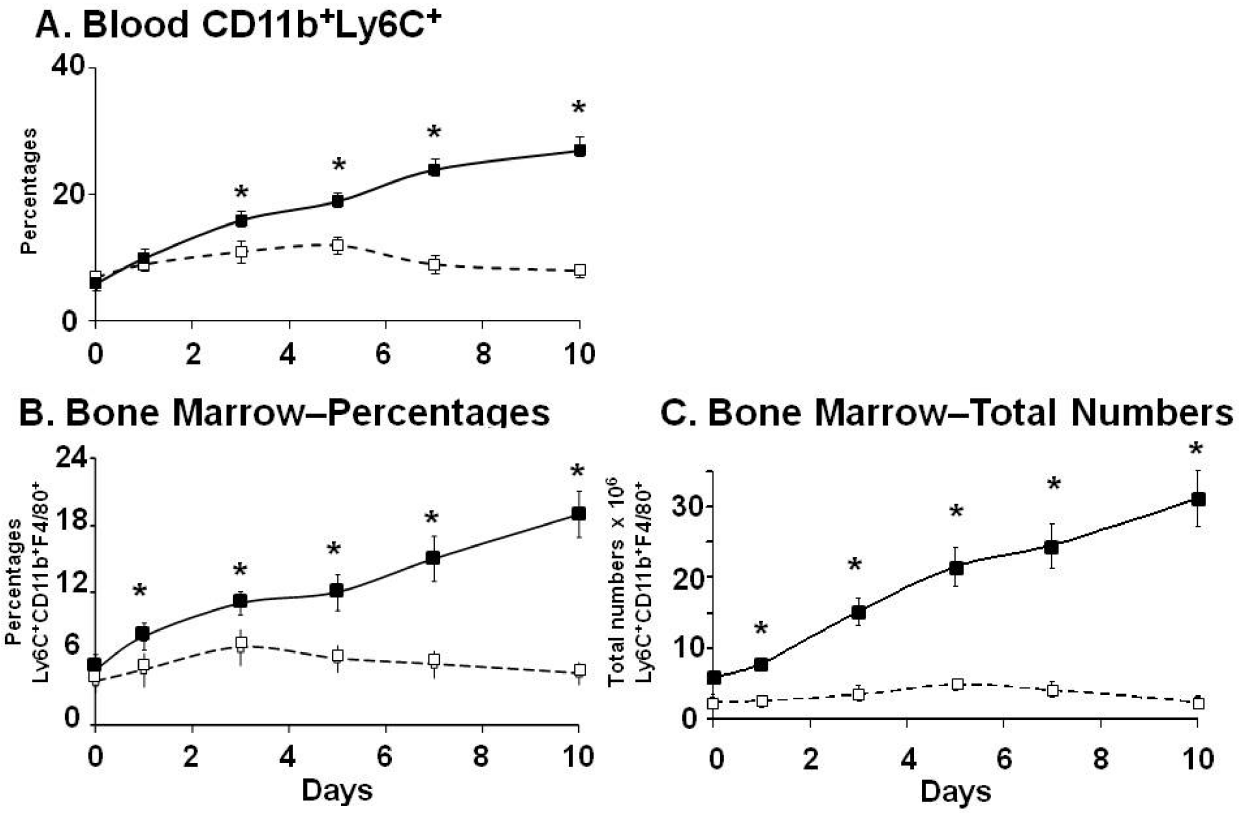

Figure 7. Blood monocyte and tissue macrophage response to polymicrobial sepsis

At intervals after polymicrobial sepsis, mice were sacrificed and blood and bone marrow $CD11b^+Ly6C^+$ cells were determined by flow cytometry Panels A presents total $CD11b^+Ly6C^+$ numbers in blood while Panels B-C represent bone marrow $CD11b^+Ly6C^+F4/80^+$ percentages and absolute numbers. Values represent the mean of five to seven animals per group. Each experiment was confirmed at least three times. Significance between naïve, sham and CLP at day 3 and 7 was determined by one way ANOVA. Significance between sham and CLP over time was determined by two way ANOVA. * = $p<0.01$ and # = $p<0.05$.