Role of G-CSF in monophosphoryl lipid A-mediated augmentation of neutrophil functions after burn injury

Julia K. Bohannon,*1 Liming Luan,* Antonio Hernandez,* Aqeela Afzal,† Yin Guo,‡ Naeem K. Patil,* Benjamin Fensterheim,‡ and Edward R. Sherwood*‡

Departments of *Anesthesiology, †Neurosurgery, and ‡Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee, USA

ABSTRACT

Infection is the leading cause of death in severely burned patients that survive the acute phase of injury. Neutrophils are the first line of defense against infections, but hospitalized burn patients frequently cannot mount an appropriate innate response to infection. Thus, immune therapeutic approaches aimed at improving neutrophil functions after burn injury may be beneficial. Prophylactic treatment with the TLR4 agonist monophosphoryl lipid A is known to augment resistance to infection by enhancing neutrophil recruitment and facilitating bacterial clearance. This study aimed to define mechanisms by which monophosphoryl lipid A treatment improves bacterial clearance and survival in a model of burn-wound sepsis. Burn-injured mice were treated with monophosphoryl lipid A or vehicle, and neutrophil mobilization was evaluated in the presence or absence of Pseudomonas aeruginosa infection. Monophosphoryl lipid A treatment induced significant mobilization of neutrophils from the bone marrow into the blood and sites of infection. Neutrophil mobilization was associated with decreased bone marrow neutrophil CXCR4 expression and increased plasma G-CSF concentrations. Neutralization of G-CSF before monophosphoryl lipid A administration blocked monophosphoryl lipid A-induced expansion of bone marrow myeloid progenitors and mobilization of neutrophils into the blood and their recruitment to the site of infection. G-CSF neutralization ablated the enhanced bacterial clearance and survival benefit endowed by monophosphoryl lipid A in burn-wound-infected mice. Our findings provide convincing evidence that monophosphoryl lipid A-induced G-CSF facilitates early expansion, mobilization, and recruitment of neutrophils to the site of infection after burn injury, allowing for a robust immune response to infection. J. Leukoc. Biol. 99: 629–640; 2016.

Introduction

Despite extensive use of topical and systemic antibiotics, infection remains the leading cause of prolonged hospitalization and death in burn patients [1]. Sepsis, from any cause, is a leading cause of multiorgan injury and death in burn patients [2–4]. Numerous reports have determined that severe burn injury induces immunosuppression and impaired antimicrobial immunity [5–7]. Loss of the skin barrier and impaired innate immunity increase the susceptibility of burn patients to serious infection and sepsis [8, 9]. The problem of infection is complicated further by the emergence of antibiotic resistant bacteria, such as Pseudomonas aeruginosa, as common pathogens in burn patients. Therefore, interventions aimed at improving antimicrobial immunity might provide significant benefit to infection-prone patients, such as those with major burns. Given the prevalence of immune dysfunction in burn patients, immunomodulatory regimens may provide an attractive approach to restore immune function as a means of controlling and preventing infections.

MPLA is a derivative of LPS that activates host innate and adaptive immune responses by binding and activating TLR4. MPLA is produced by successive acid and base hydrolysis of lipid A from Salmonella minnesota 595 or by de novo synthesis. The resulting removal of the C1 phosphate group renders a compound with low immunotoxicity that can be administered at doses of 1000–10,000 times greater than native diphosphoryl lipid A before inducing systemic proinflammatory activity in humans [10, 11]. Despite reduced toxicity, MPLA retains potent immunomodulatory activity, including augmentation of Th1 activity and antigen-induced T cell clonal expansion [12]. As such, MPLA is currently used as a component of the U.S. Food and Drug Administration-approved AS04 vaccine adjuvant.

Abbreviations: CBC = complete blood count, CD62L = cluster of differentiation 62 ligand, CFU-G = CFU-granulocyte progenitors, CFU-GM = CFU-granulocyte/macrophage progenitors, KC = keratinocyte-derived chemokine, LR = lactated Ringers, MFI = mean fluorescent intensity, MPLA = monophosphoryl lipid A, PVA = polyvinyl alcohol, SDF-1 = stromal cell-derived factor 1

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Anesthesiology Research Division, Vanderbilt University Medical Center, 1161 21st Ave., South, T-4202-MCN, Nashville, TN 37292-2520, USA. E-mail: julia.k.bohannon@vanderbilt.edu

0741-5400/16/0099-629 © Society for Leukocyte Biology


Volume 99, April 2016 Journal of Leukocyte Biology 629

Downloaded from www.jleukbio.org to IP 54.70.40.11. Journal of Leukocyte Biology Vol.99, No.4, pp.629-640, November, 2017
system, which is a component of commercially available human papilloma and hepatitis virus vaccines [13].

An emerging body of literature reveals that priming with TLR4 agonists can augment innate antimicrobial responses in a variety of infection models [14–19]. Romero et al. [20] showed that treatment with MPLA improved survival and bacterial clearance in several models of sepsis, including cecal ligation and puncture-induced polymicrobial peritonitis and systemic \textit{P. aeruginosa} challenge and in a lethal \textit{P. aeruginosa} burn-wound infection model. This protection was partially mediated by increased infiltration of myeloid phagocytes into sites of infection. More specifically, depletion of neutrophils with anti-Ly6G antibody after MPLA treatment ablated the protective effects of MPLA during systemic \textit{P. aeruginosa} challenge [20]. This evidence suggests a critical role of neutrophils in mediating protection against infection after MPLA treatment. However, the molecular mechanisms by which MPLA augments neutrophil responses in the burn-injured host are unclear.

In the present study, we evaluated the effect of MPLA on the expansion, mobilization, and recruitment of neutrophils necessary for local and systemic clearance of bacteria in response to infection after severe burn injury by use of a murine model of \textit{P. aeruginosa} burn-wound infection. \textit{P. aeruginosa} is the most common organism causing infection in immune-compromised patients, including those with severe burn injuries [21, 22]. Furthermore, we evaluated the role of G-CSF as a mediator of MPLA-induced neutrophil responses. G-CSF is known to play a critical role in mediating protection during systemic infection [20]. This evidence suggests a critical role of neutrophils in mediating protection against infection after MPLA treatment. However, the molecular mechanisms by which MPLA augments neutrophil responses in the burn-injured host are unclear.

In the present study, we evaluated the effect of MPLA on the expansion, mobilization, and recruitment of neutrophils necessary for local and systemic clearance of bacteria in response to infection after severe burn injury by use of a murine model of \textit{P. aeruginosa} burn-wound infection. \textit{P. aeruginosa} is the most common organism causing infection in immune-compromised patients, including those with severe burn injuries [21, 22]. Furthermore, we evaluated the role of G-CSF as a mediator of MPLA-induced neutrophil responses. G-CSF is known to play a pivotal role in the expansion and differentiation of neutrophils in the bone marrow and their subsequent release into the blood circulation in response to inflammatory stimuli. Prior studies have linked induction of G-CSF by LPS stimulation with emergency granulopoiesis or accelerated expansion and release of neutrophils out of the bone marrow and into the periphery in response to stress [23, 24].

**MATERIALS AND METHODS**

**Mice and burn injury**

All animal procedures were consistent with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Male 8- to 10-wk-old BALB/c mice, purchased from Harlan Laboratories (Indianapolis, IN, USA), were housed in an institutional care facility and allowed to acclimate for 1 wk after arrival. A well-developed technique for induction of full-thickness burn wounds was used for these studies [25–28]. In brief, mice were anesthetized during all procedures with 2.5% isoflurane delivered in oxygen. Buprenorphine (0.1 mg/kg) was given 30 min before burn injury for analgesia. The dorsal surface of mice was shaved, and 1 ml saline was injected subcutaneously along the spinal column to avoid unnecessary injury to the spinal cord during the burn procedure. Each mouse was placed supine and secured in a protective template with an opening unnecessary injury to the spinal cord during the burn procedure. Each mouse was placed supine and secured in a protective template with an opening corresponding to 35% of the total body surface area. The exposed skin was immersed in 97–98°C for 10 s. Following the injury, 2 ml LR solution was administered into the peritoneal cavity for fluid resuscitation.

**Bacterial infection**

\textit{P. aeruginosa} was obtained from American Type Culture Collection (Manassas, VA, USA; ATCC 19660). The culture was grown in tryptic soy broth and resuspended in sterile saline solution before inoculation. Mice were inoculated with $1 \times 10^6$ CFU of \textit{P. aeruginosa} on d 4 postburn. For wound infection, \textit{P. aeruginosa} was applied topically to burn wounds. This model represents a slowly progressive wound infection that develops as the bacteria gradually colonize the burn wound and then spread systemically into the surrounding tissues, blood, and eventually, distant organs, primarily the lungs [25, 29]. In some experiments, mice received intraperitoneal challenge with \textit{P. aeruginosa} to allow for assessment of neutrophil recruitment and local bacterial clearance. Bacterial burden in the blood, peritoneal lavage fluid, and lungs was quantified by performing, serial dilutions of blood, peritoneal lavage fluid, or lung-tissue homogenates, followed by culture (37°C) on tryptic soy agar overnight. For survival studies, mice were monitored daily for 8 d following wound inoculation.

**MPLA treatment**

MPLA derived from \textit{Salmonella enterica} serotype Minnesota Re 595 was purchased from Sigma-Aldrich (St. Louis, MO, USA). MPLA was solubilized in sterile water containing 0.2% triethylamine solution (1 mg/ml) and sonicated for 1 h at 40°C. For administration, MPLA was diluted in LR solution (100 µg/ml) and was administered by intraperitoneal injection (20 µg in 0.2 ml). In G-CSF neutralization experiments, mice received intravenous injection with 100 µg anti-mouse G-CSF-blocking mAb (clone 67604; R&D Systems, Minneapolis, MN, USA) or purified rat monoclonal IgG1 isotype control (R&D Systems), 1 h before MPLA treatment.

**Evaluation of neutrophils in bone marrow, blood wound, and peritoneal cavity**

To examine the effect of MPLA treatment on neutrophil mobilization and recruitment, neutrophils were quantified in bone marrow, blood, peritoneal cavity, and the burn wound. All cell counts were performed by use of a TC20 automated cell counter (Biorad Laboratories, Hercules, CA, USA). Bone marrow was harvested by flushing excised femurs with 5 ml cold PBS. Cells were counted, centrifuged (300 g for 10 min at 4°C), and resuspended in PBS at desired cell concentrations. Whole blood was harvested by carotid artery laceration under isoflurane anesthesia and collected in heparinized syringes. Blood was collected in K3 EDTA tubes (Greiner Biosciences, San Diego, CA, USA) and chilled on ice for CBC. CBC measurements were performed by use of a Forecyte veterinary hematology analyzer (Oxford Science, Oxford, CT, USA). Remaining blood was centrifuged (4750 rpm for 15 min at 4°C) to collect plasma for cytokine analyses. Peritoneal lavage fluid was obtained by lavage of the peritoneal cavity with 2 ml cold PBS. Cells were counted, centrifuged (300 g for 10 min at 4°C), and resuspended in PBS at desired cell concentrations. Burn-wound cells were collected by use of PVA sponges, implanted subcutaneously at the margin of the burn wound. For sponge implantation, mice underwent general anesthesia by use of 2.5% isoflurane, and a 1 cm incision was made at the border of the burn wound. A bloodless subcutaneous pocket was formed at the margin of the burn-wound site. Four PBS-soaked PVA sponges (Medtronic Xomed, Mystic, CT, USA; 5 mm thick by 5 mm diameter) were inserted aseptically into the subcutaneous pockets along the burn-wound margin, and the incision was closed with sutures (Becton Dickinson, Sparks, MD, USA). Three hours after sponge implantation, mice were euthanized, and sponges were removed. Cells were washed from the sponges with cold PBS. Cells were counted, centrifuged (300 g for 10 min at 4°C), and resuspended in PBS at desired concentrations.

**Flow cytometry**

Bone marrow, intraperitoneal, and wound leukocytes were suspended in cold PBS ($1 \times 10^7$ cells/ml) and incubated with anti-mouse CD 16/32 (eBioscience, San Diego, CA, USA; 1 µl/ml) for 5 min to block nonspecific FcR-mediated antibody binding. One million cells were then transferred to polystyrene tubes, and fluorochrome-conjugated antibodies or isotype control antibodies (0.5 µg/10^6 cells/0.1 ml) were added. Cells were incubated at 4°C for 30 min, washed with 2 ml cold PBS, and resuspended in 250 µl cold PBS. Samples were run immediately on an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA, USA). Data were analyzed by use of Accuri C6 software. Antibodies used for these studies included anti-F4/80-PE/Cy7 (clone 53.1.5)
BM8; eBioscience), anti-Ly6G-PE (clone 1A8; BD Biosciences), anti-CXCR4- allopheycocyanin (clone 2B11; eBioscience), and appropriate isotype controls (eBioscience and BD Biosciences).

Hematopoietic progenitor cell measurement
Hematopoietic progenitor cell proliferation was quantified by use of MethoCult colony-forming cell assays (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer’s specifications. In brief, 1 femur/mouse was flushed with 5 ml cold PBS + 2% FBS. RBCs were lysed by use of ammonium chloride solution. Cells were counted and resuspended to 10^5 cells/ml in MethoCult medium and were plated into pretested culture dishes. Colony types were evaluated and quantified by use of an inverted microscope after incubating at 37°C and 5% CO2 for 9 d.

Assessment of neutrophil adhesion molecule expression
The adhesion markers CD11b and CD62L (p-selectin) were measured on bone marrow neutrophils (F480 Ly6G+) by flow cytometry after ex vivo stimulation with varying concentrations of MPLA. Bone marrow cells were collected by flushing of femurs with 5 ml cold PBS. Cells were plated in 24-well plates at a concentration of 10^6/ml in FBS-supplemented RPMI-1640. MPLA was then added at concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, or 1 µg/ml, and cells were incubated at 37°C with 5% CO2 for 2 h. Cells were washed, and then bone marrow neutrophils (F480 Ly6G+) were stained for adhesion markers CD11b and CD62L (p-selectin; eBioscience) and analyzed by the Accuri C6 flow cytometer (BD Biosciences).

Neutrophil respiratory burst
Neutrophil respiratory burst was measured by use of the Neutrophil/Monocyte Respiratory Burst Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer’s specifications. In brief, bone marrow cells were collected by flushing of femurs with 5 ml cold assay buffer, RBCs were lysed by use of RBC lysis buffer, and cells were resuspended to a concentration of 10^6 cells/ml in assay buffer. Cells were incubated at 37°C with dihydroorhodamine 123 assay reagent for 15 min. Cells were stimulated with PMA assay reagent for 45 min. Cells were then analyzed by flow cytometry by use of the Accuri C6 flow cytometer to measure rhodamine 123 fluorescence on gated neutrophils.

Cytokine measurements
Concentrations of IL-6, G-CSF, MIP-2, and KC in plasma were measured by use of a Bio-Plex Multiplex Assay and MagPix Multiplex Reader (Bio-Rad Laboratories). Results were analyzed with Bio-Plex Manager Software 6.1, and graphs were made with GraphPad Prism Software 6 (GraphPad Software, San Diego, CA, USA).

Statistics
All data were analyzed with GraphPad Prism Software 6. Data from multiple group experiments were analyzed by use of 1-way ANOVA, followed by Tukey multicomparison post hoc test. Comparisons between 2 groups were analyzed by use of Student’s t test. All data values are presented as means or means ± SEM, except for bacterial counts, for which median values are designated. P ≤ 0.05 was considered statistically significant.

RESULTS

MPLA treatment promotes rapid neutrophil mobilization from the bone marrow into the blood
Neutrophil numbers were measured in the bone marrow and blood of burn-injured mice following MPLA treatment. Bone marrow cells were harvested from femurs at 0, 3, and 6 h after vehicle or MPLA treatment (Fig. 1A). Neutrophils were identified by flow cytometry as F4/80 Ly6G+ cells (Fig. 1B). Bone marrow neutrophils were decreased significantly at both 3 and 6 h after MPLA injection when compared with vehicle treatment. In contrast, peripheral blood neutrophil numbers increased significantly by 3 h and remained significantly elevated at 6 h after MPLA treatment (Fig. 1C). The proportion of bone marrow neutrophils expressing CXCR4 decreased significantly at 3 and 6 h after MPLA administration and was decreased significantly compared with vehicle-treated controls (Fig. 1D). CXCL12 levels in the bone marrow did not change after MPLA treatment (Supplemental Fig. 1).

G-CSF is essential for MPLA-induced mobilization of neutrophils from the bone marrow into the blood
G-CSF and IL-6 concentrations were measured in the plasma at 0, 2, 4, 6, and 24 h after MPLA treatment (Fig. 2). MPLA treatment potently increased G-CSF concentrations in the plasma by 2 h and maintained elevated plasma concentrations for up to 24 h (Fig. 2). Among the time points measured, the highest G-CSF concentrations were observed at 4–6 h after MPLA treatment. In contrast, MPLA marginally induced IL-6 release into plasma at 2 h, with levels returning to baseline by 4 h after MPLA treatment.

To determine the role of G-CSF in MPLA-induced neutrophil mobilization, G-CSF was neutralized by intravenous administration of a neutralizing mouse anti-G-CSF mAb (100 µg, i.v.) at 1 h before MPLA treatment (Fig. 3A). Nonspecific IgG1 served as an isotype control. Anti-G-CSF antibody effectively neutralized G-CSF levels in the plasma of vehicle- and MPLA-treated mice at 3, 6, and 24 h after MPLA, as determined by the absence of detectable G-CSF in plasma (Supplemental Fig. 2A and B). Anti-G-CSF antibody prevented the MPLA-induced decrease in bone marrow neutrophil numbers (Fig. 3B) and CXCR4 expression (Fig. 3C), as well as the increase in blood neutrophil numbers (Fig. 3D) at 2 and 6 h after MPLA treatment, whereas IgG1 isotype control had no impact on these parameters.

G-CSF is essential for MPLA-induced expansion of bone marrow progenitors and mobilization of neutrophils after MPLA treatment
Neutrophils in the bone marrow, blood, and burn-wound site were analyzed 24 h after 2 MPLA doses (Fig. 4A). G-CSF was neutralized by intravenous injection of anti-G-CSF antibody, 1 h before the first vehicle or MPLA injection. MPLA induced a significant decrease in bone marrow neutrophil numbers in control IgG1-treated mice, an effect that was ablated by treatment with anti-G-CSF (Fig. 4B). The proportion of bone marrow neutrophils expressing CXCR4 was decreased significantly in IgG1-treated, but not anti-G-CSF-treated mice at 24 h after MPLA treatment (Fig. 4C). An analysis of myeloid progenitor proliferation in the bone marrow after MPLA treatment revealed an expansion of myeloid progenitors, primarily CFU-G and CFU-GM. This expansion was blocked when mice were treated with anti-G-CSF antibody before MPLA treatment (Fig. 4D). At the same time, neutrophil numbers in the blood were elevated significantly in IgG1-treated mice following MPLA treatment, a response that was eliminated by treatment with anti-G-CSF (Fig. 4E). The blocking of G-CSF
before MPLA treatment also prevented the accumulation of neutrophils at the burn-wound site compared with isotype-treated mice (Fig. 4F).

In a separate set of experiments, to confirm the impact of MPLA treatment on neutrophil expansion, mobilization, and recruitment, neutrophil numbers were measured in the bone marrow, blood, and wound after 2 doses of vehicle or MPLA treatment (Supplemental Fig. 3A). The results showed a significant decrease in bone marrow neutrophil numbers, an effect also evident in sections of femurs stained for neutrophils (brown stain) at 24 h after MPLA treatment compared with vehicle treatment (Supplemental Fig. 3B). CXCR4 expression was decreased significantly on bone marrow neutrophils, and blood neutrophil numbers were elevated significantly (Supplemental Fig. 3C and D). Burn-wound neutrophils were also increased significantly in MPLA-treated mice (Supplemental Fig. 3E).

**G-CSF is essential for protection against a burn-wound infection in MPLA-treated mice**

To determine the role of G-CSF in MPLA-mediated resistance to *P. aeruginosa* wound infection, survival, bacterial clearance, and cytokine responses were measured after induction of a burn-wound infection (Fig. 5A). In IgG1-treated mice, MPLA treatment significantly improved survival to 70% compared with 8% in vehicle-treated mice. Treatment with anti-G-CSF ablated the survival benefit provided by MPLA treatment (Fig. 5B).

Additionally, blood and lungs were harvested from the same mice, either at the point of morbidity or on d 8 for surviving mice. Mice treated with IgG1 and MPLA had lower bacterial burden in the blood and lungs compared with mice treated with vehicle and IgG1 (Fig. 5C). In MPLA-treated mice, only 3 of 13
and 4 of 13 mice had measurable numbers of bacteria in the blood and lungs, respectively. In contrast, 7 of 8 and 12 of 13 vehicle-treated mice had bacteria in the blood and lungs, respectively. All mice in vehicle- and MPLA-treated groups exhibited high bacterial burdens in the blood and lungs when anti-G-CSF was administered before MPLA or vehicle treatment (Fig. 5C).

Plasma cytokines and chemokines were also measured on d 3 postwound inoculation, as shown in Fig. 5D. Plasma IL-6, MIP-2, and KC concentrations were attenuated in MPLA and IgG1-treated mice compared with mice treated with vehicle and IgG1. This effect was ablated in mice treated with anti-G-CSF before MPLA treatment for all 3 mediators (Fig. 5D).

G-CSF is essential for MPLA-mediated neutrophil mobilization into the blood during infection and for MPLA-mediated neutrophil recruitment to the site of infection

In a second model of infection, burn-injured mice received an intraperitoneal challenge with *P. aeruginosa* to assess the effect of MPLA treatment on recruitment of neutrophils into the site of infection (Fig. 6A). MPLA and IgG1-treated mice showed a significant increase in blood neutrophils and an accumulation of neutrophils in the peritoneal cavity, 6 h after intraperitoneal inoculation, compared with mice treated with vehicle and IgG1 (Fig. 6B and C). Blockade of G-CSF ablated infection-induced mobilization of neutrophils into blood and peritoneal cavity, especially in MPLA-treated mice (Fig. 6B and C). However, the blocking of G-CSF did not reverse MPLA-mediated reduction in bacterial burden, as MPLA-treated mice in both IgG1 and anti-G-CSF groups had comparably lower bacterial counts at 6 h after infection compared with vehicle-treated mice (Fig. 6D). Furthermore, neutralization of G-CSF did not affect MPLA-mediated attenuation of plasma IL-6 levels at 6 h after intraperitoneal *P. aeruginosa* inoculation (Fig. 6E).

In a separate set of experiments, to confirm the impact of MPLA treatment on neutrophil responses, neutrophil numbers were measured in the blood and peritoneal lavage fluid of mice that had received 2 doses of vehicle or MPLA treatment but did not receive either IgG1 or anti-G-CSF treatment (Supplemental Fig. 4A). The results showed a significant increase in blood and peritoneal neutrophil numbers, both before and after intraperitoneal infection, in mice treated with MPLA (Supplemental Fig. 4B and C). MPLA-treated mice also showed significantly improved bacterial clearance and attenuated plasma IL-6 levels (Supplemental Fig. 4D and E).

**MPLA induces increased CD11b expression and shedding of l-selectin by bone marrow neutrophils and increases neutrophil respiratory burst**

Bone marrow neutrophils were incubated with increasing concentrations of MPLA ex vivo, and the expression of CD11b and l-selectin was measured (Fig. 7A). Because all bone marrow neutrophils express CD11b, 2 h of stimulation with increasing concentrations of MPLA did not have an effect on the proportion of F480−Ly6G− cells expressing CD11b. However, the MFI, an indicator of number of molecules per cell, of CD11b expressed on neutrophils increased in a dose-dependent manner after
MPLA stimulation, rising significantly above untreated controls at doses of 100 and 1000 ng/ml (Fig. 7B). Additionally, MPLA stimulation induced a dose-dependent decrease in the proportion of bone marrow neutrophils expressing L-selectin and the MFI of L-selectin on bone marrow neutrophils (Fig. 7C).

Neutrophil respiratory burst potential was measured at 24 h after a 2-dose regimen of MPLA or vehicle treatment (Fig. 8A). Bone marrow neutrophils from MPLA-treated mice showed significantly increased respiratory burst activity after PMA stimulation compared with neutrophils from vehicle-treated mice (Fig. 8B).

**DISCUSSION**

Neutrophils are among the first responders to infection, where they function to remove bacteria via phagocytosis and killing. Neutrophils also produce soluble factors that initiate activation and recruitment of additional neutrophils and other leukocytes to the site of inflammation and infection [30–32]. The antimicrobial function of neutrophils has been ascribed to their robust oxidative burst, production of antimicrobial peptides, and phagocytosis [33]. Burn injury has been shown to induce functional alterations in neutrophils, most notably, defects in the migratory capacity [34, 35]. Interventions that reverse burn injury-induced neutrophil dysfunction may significantly reduce infection rates in burn patients.

Our results support the hypothesis that MPLA augments antimicrobial protection, in part, by inducing G-CSF production, which enhances neutrophil expansion in bone marrow and mobilization into blood and sites of infection. Earlier data from our laboratory showed that treatment with MPLA improves survival and bacterial clearance in multiple models of sepsis, including cecal ligation and puncture-induced polymicrobial peritonitis and *P. aeruginosa* burn-wound infection, but the mechanisms by which MPLA mediates protection were unclear [20]. In this study, we show that treatment of burn-injured mice with MPLA significantly enhanced neutrophil expansion and mobilization from the bone marrow and recruitment to the site of infection via early induction of G-CSF. The enhanced neutrophil recruitment led to improved bacterial clearance and survival during *P. aeruginosa* burn-wound infection. Additionally, MPLA significantly increased neutrophil antimicrobial respiratory burst capacity and expression of surface markers involved in neutrophil adhesion and migration.

G-CSF, produced by macrophages, endothelial cells, fibroblasts, bone marrow stroma, and tissue mesenchymal cells, is...
known to be essential for the differentiation, proliferation, and survival of neutrophils from the hematopoietic stem cell level through the full maturation process [36–39]. Furthermore, the release of neutrophils from the bone marrow is regulated, in part, by G-CSF. Prior studies have indicated that agents capable of inducing transient increases in G-CSF, including alum and IL-17, facilitate neutrophil mobilization and granulopoiesis within the bone marrow compartment [40, 41]. Induction of G-CSF by LPS has been linked directly to the initiation of emergency granulopoiesis from the bone marrow [23, 24]. We found that MPLA treatment potently induced G-CSF production. Furthermore, we found that MPLA treatment increased both neutrophil progenitor CFUs in the bone marrow and the percentage of hematopoietic stem cells (Lin<sup>-</sup>Sca1<sup>+</sup>ckit<sup>+</sup>) in the bone marrow (data not shown), an effect that was absent in anti-G-CSF-treated mice. These studies suggest that the MPLA-induced elevation in blood neutrophil numbers is linked to the proliferation of neutrophil precursors in the bone marrow and that this effect is mediated through the early induction of G-CSF. Additionally, our study provides convincing evidence that MPLA-induced G-CSF-expanded neutrophils are subsequently released into the circulation and can be recruited to local sites of infection.

Under steady-state conditions, interactions between CXCR4 and SDF-1/CXCL12 play a key role in maintaining neutrophil

Figure 5. G-CSF is required for MPLA-mediated protection against a burn-wound infection and attenuation of cytokine production. (A) At 48 h after burn injury, mice received intravenous injection of 100 μg anti-G-CSF or IgG1 control, followed 1 h later by 2 doses of LR (vehicle) or 20 μg MPLA by intraperitoneal injection, 24 h apart. At 24 h after the second dose of MPLA, a × 10<sup>6</sup> CFU of <i>P. aeruginosa</i> was applied to burn wounds. (B) Survival was monitored over 8 d (n = 13 mice/group). (C) Blood and lung tissue homogenates were collected and cultured between 3 and 8 d after wound inoculation to determine bacterial burden (n = 7–15 mice/group). (D) IL-6, MIP-2, and KC concentrations in plasma were measured by Bio-Plex Multiplex Assay. Data show median (C) or means ± SEM, n = 7–13 mice/group. ***P ≤ 0.0001, significantly different from all other groups; *P ≤ 0.05, significantly different from vehicle control; #P ≤ 0.05, significantly different from IgG1-MPLA.
homeostasis through retention of neutrophils in the bone marrow [42]. Decreased expression of CXCR4 in the bone marrow is associated with release of bone marrow neutrophils into the blood [43, 44]. Exogenous treatment with G-CSF has been shown to lower CXCR4 expression by bone marrow neutrophils and facilitate the mobilization of neutrophils out of the bone marrow and into the peripheral blood [43, 45]. Upon MPLA treatment, we observed a significant decrease in the percentage of bone marrow neutrophils expressing CXCR4 in parallel with increased G-CSF production. There was no change in SDF-1/CXCL12 levels in the bone marrow or plasma after MPLA treatment. Therefore, MPLA-induced mobilization of neutrophils appears to be mediated, in part, by down-regulation of CXCR4 on bone marrow neutrophils.

Additionally, we found that G-CSF is essential for MPLA-mediated augmentation of neutrophil recruitment to the site of infection. Neutrophil recruitment to the peritoneal cavity after P. aeruginosa inoculation was markedly impaired by G-CSF neutralization in MPLA-treated mice. However, we did not see a reversal of MPLA-induced augmentation of bacterial clearance from the peritoneum in mice treated with anti-G-CSF. This is likely a result of concomitant recruitment of monocytes by MPLA. Our studies show that intraperitoneal treatment with MPLA potently induces the recruitment of monocytes into the peritoneal cavity (data not shown), and monocyte recruitment can occur independently of G-CSF [46, 47]. In the context of a more progressive burn-wound infection, G-CSF was required for MPLA-mediated control of bacterial clearance and survival. This suggests that in the burn-wound infection model, neutrophils are critical for bacterial clearance and improved survival by MPLA.

In previous studies, it has been proposed that MPLA induces endotoxin tolerance, a phenomenon characterized by attenuated production of proinflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF-α, in response to infection [20]. We observed that MPLA-treated mice showed attenuated production of the inflammatory marker IL-6, as well as the neutrophil chemoattractants MIP-2 and KC following a burn-wound infection with P. aeruginosa. One possible explanation for this observation is that in line with previous reports, pretreatment with MPLA induced an endotoxin-tolerant phenotype in these mice. A second possibility is that these inflammatory mediators were attenuated in MPLA-treated mice as a result of enhanced bacterial clearance and a decrease in the severity of the infection. The latter explanation is supported by our observations that burn-wound-infected mice that received anti-G-CSF treatment did not show the same attenuation of cytokine production after MPLA treatment as mice treated with control IgG and did not have improved bacterial clearance; mice that received anti-G-CSF treatment showed partial attenuation of...
IL-6 and MIP-2 and had improved bacterial clearance. It has often been thought that induction of endotoxin tolerance and subsequent suppression of inflammatory cytokines would interfere with normal antimicrobial immune responses and render the host unable to properly clear infection [48–50]. The results of this study challenge this perception. Here, we show that priming animals with MPLA, which is known to induce endotoxin tolerance, allows for more effective antimicrobial responses and enhanced bacterial clearance.

Lastly, we showed that MPLA treatment facilitates neutrophil oxidative burst and augments neutrophil adhesion molecule expression. CD11b and L-selectin are key adhesion molecules on neutrophils and other myeloid cells that facilitate adhesion to the endothelium during margination and migration [51, 52]. Ex vivo stimulation of neutrophils with MPLA induced a dose-dependent increase in the MFI of CD11b and a decrease in the MFI of L-selectin on neutrophils. This is consistent with the observed enhancement of neutrophil recruitment induced by MPLA. We also found that respiratory burst or the rapid production of reactive oxygen species by neutrophils, which is required to mediate bacterial killing, was increased in neutrophils from MPLA-treated mice for up to 24 h after treatment compared with neutrophils from vehicle-treated mice. This is consistent with a prior study showing increased oxidative burst potential following

Figure 7. MPLA stimulation induces neutrophil activation, alters adhesion marker expression, and enhances antimicrobial functions. (A) Bone marrow (BM) cells were harvested 2 d after burn injury. Bone marrow cells were stimulated ex vivo with increasing concentrations of MPLA for 2 h. Cells were then washed and stained with fluorochrome-conjugated antibodies against F4/80, Ly6G, and either CD11b or L-selectin (CD62L) and were analyzed by flow cytometry. (B) CD11b expression was evaluated on gated neutrophils (F4/80^+Ly6G^+). The percentage of neutrophils expressing CD11b, as well as the MFI of CD11b expression on gated neutrophils, is presented. Histograms show MFI of CD11b on neutrophils. (C) L-selectin expression was evaluated on gated neutrophils (F4/80^+Ly6G^+). The percentage of neutrophils expressing L-selectin, as well as the MFI of L-selectin expression on gated neutrophils, is presented. Histograms show MFI of L-selectin on neutrophils. Data show means ± SEM; bone marrow cells were plated at 7 wells/group from the femurs of 4 pooled mice. *P < 0.05, significantly different from indicated groups.
stimulation with LPS, a phenomenon that was dependent on TLR4 activation [53]. These results show that MPLA directly augments neutrophil function, leading to enhanced recruitment and augmented antimicrobial functions.

In conclusion, this study demonstrates an important role for G-CSF in MPLA-induced neutrophil mobilization and activation, leading to protection against infection after severe burn injury. Prophylactic treatment of burn-injured mice with MPLA induced significant expansion, mobilization, and activation of neutrophils from the bone marrow to the site of infection. Neutralization of G-CSF with an antibody administered in vivo reversed MPLA-mediated augmentation of neutrophil recruitment and resistance to infection. A summary of our findings is illustrated in the schematic presented in Fig. 9. It is likely that much of the impact of MPLA on neutrophil functions is mediated through its effects on cells other than neutrophils, as blockade of G-CSF largely ablated MPLA-induced neutrophil expansion, mobilization, and recruitment. G-CSF is produced by a multitude of cells, including macrophages, endothelial cells, bone marrow stroma, and tissue mesenchymal cells. Neutrophils have not been described as a significant source of G-CSF. Thus, it is unlikely that autocrine mechanisms function to mediate neutrophil expansion and mobilization. Nevertheless, neutrophils have been shown to express TLR4, and it is possible that some MPLA-induced effects, such as increased adhesion molecule expression and respiratory burst activity, are mediated through direct activation of neutrophils by MPLA [54, 55]. The significant antimicrobial potential of MPLA makes it an attractive therapeutic candidate for clinical use in burn patients for the prevention of deadly postburn infections. This study significantly expands our knowledge of the immunomodulatory properties of MPLA and further identifies it as a novel, therapeutic agent that could protect burn patients from serious antibiotic-resistant infections. The results of this study also provide new insights into the molecular mechanisms by which MPLA mediates antimicrobial protection against infections after severe burn injury.

Figure 8. MPLA treatment increases the oxidative burst capacity of neutrophils. (A) Starting at 48 h after burn injury, mice received 2 doses of LR (vehicle) or 20 μg MPLA by intraperitoneal injection, 24 h apart. Mice were euthanized, and bone marrow was harvested, 24 h after the second MPLA injection. (B) Respiratory burst activity was measured by production of rhodamine 123 from dihydrorhodamine 123 upon stimulation with PMA. Rhodamine 123 was measured in the fluorescent 1 channel on neutrophils gated by forward-scatter (FSC-A) versus side-scatter (SSC-A). Data show mean from bone marrow cells collected from 4 mice/group, plated in triplicate. *P ≤ 0.05, significantly different from indicated groups.

Figure 9. Proposed cellular mechanisms of MPLA-mediated protection against a burn-wound infection. MPLA treatment in burn-injured mice increases plasma levels of G-CSF. Our data indicate that G-CSF facilitates expansion and increased mobilization of bone marrow neutrophils into the peripheral blood. Decreased expression of CXCR4 on bone marrow neutrophils assists with neutrophil egress. Increased neutrophil numbers in the peripheral blood and subsequent recruitment to sites of infection, along with improved activation and antimicrobial functions, aid in bacterial clearance and subsequently promote improved protection against infection.
REFERENCES


Role of G-CSF in monophosphoryl lipid A-mediated augmentation of neutrophil functions after burn injury


Access the most recent version at doi:10.1189/jlb.4A0815-362R

Supplemental Material

http://www.jleukbio.org/content/suppl/2015/11/03/jlb.4A0815-362R.DC1

References

This article cites 54 articles, 23 of which can be accessed free at:
http://www.jleukbio.org/content/99/4/629.full.html#ref-list-1

Subscriptions

Information about subscribing to Journal of Leukocyte Biology is online at http://www.jleukbio.org/site/misc/Librarians_Resource.xhtml

Permissions

Submit copyright permission requests at:
http://www.jleukbio.org/site/misc/Librarians_Resource.xhtml

Email Alerts

Receive free email alerts when new an article cites this article - sign up at http://www.jleukbio.org/cgi/alerts

© Society for Leukocyte Biology