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MONOPHOSPHORYL LIPID A ATTENUATES MULTIORGAN DYSFUNCTION DURING POST-BURN *PSEUDOMONAS AERUGINOSA* PNEUMONIA IN SHEEP

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ABSTRACT—**Background:** Monophosphoryl lipid A (MPLA) is a TLR4 agonist that has potent immunomodulatory properties and modulates innate immune function to improve host resistance to infection with common nosocomial pathogens in mice. The goal of this study was to assess the safety and efficacy of MPLA in a sheep model of burn injury and *Pseudomonas aeruginosa* pneumonia. The sheep provides a favorable model for preclinical testing as their response to TLR4 agonists closely mimics that of humans. **Methods:** Twelve chronically instrumented adult female Merino sheep received 20% total body surface area, third-degree cutaneous burn under anesthesia and analgesia. At 24 h after burn, sheep were randomly allocated to receive: MPLA (2.5 μg/kg i.v., n = 6), or vehicle (i.v., n = 6). At 24 h after MPLA or vehicle treatment, *Pseudomonas aeruginosa* pneumonia was induced. Sheep were mechanically ventilated, fluid resuscitated and cardiopulmonary variables were monitored for 24 h after induction of pneumonia. Cytokine production, vascular barrier function, and lung bacterial burden were also measured. **Results:** MPLA infusion induced small and transient alterations in core body temperature, heart rate, pulmonary artery pressure, and pulmonary vascular resistance. Pulmonary mechanics were not altered. Vehicle-treated sheep developed severe acute lung injury during *Pseudomonas aeruginosa* pneumonia, which was attenuated by MPLA as indicated by improved PaO_2/FiO_2 ratio, oxygenation index, and shunt fraction. Sheep treated with MPLA also exhibited less vascular leak, lower blood lactate levels, and lower modified organ injury score. MPLA treatment attenuated systemic cytokine production and decreased lung bacterial burden. **Conclusions:** MPLA was well tolerated in burned sheep and attenuated development of acute lung injury, lactatemia, cytokinemia, vascular leak, and hemodynamic changes caused by *Pseudomonas aeruginosa* pneumonia.

KEYWORDS—Burns, immunomodulation, inflammation, organ injury, pneumonia, sepsis, TLR4 agonists, vascular permeability

INTRODUCTION

Hospital-acquired infections are among the most pressing threats facing modern healthcare facilities (1–3). Critically ill, immunosuppressed, and high-risk surgical patients are most vulnerable, although anyone receiving hospital care is at risk. Patients with large total body surface area (TBSA) cutaneous burns are particularly susceptible to hospital-acquired infections, especially pneumonia and wound infections, due to loss of the skin barrier, invasive monitoring, mechanical ventilation, and immune dysfunction (4, 5). *Pseudomonas aeruginosa* (*P aeruginosa)* is the most common organism causing pneumonia in burn patients (6–9).

Other than infection control strategies such as hand washing, gowning, and aseptic technique, there are not any proven ways to decrease or prevent infection in burn patients. In general, the evidence that systemic antibiotic prophylaxis reduces the incidence of wound and invasive infections or infection-associated mortality is weak (10–12). Therefore, new strategies are needed to decrease the incidence and severity of infections in burn victims. Immunotherapy using toll-like receptor (TLR) agonists provides a means of achieving that goal. Monophosphoryl lipid A (MPLA) is a TLR4 agonist with negligible toxicity and pro-inflammatory effects but potent immunomodulatory properties (13, 14). MPLA is employed as an adjuvant in the human papilloma virus (Cervarix) and shingles (Shingrix) vaccines and has been administered safely to more than a million people worldwide in that application (15, 16). Our interest in MPLA is not as a vaccine adjuvant but a means of augmenting innate immunity against common opportunistic pathogens in vulnerable patients. Our published studies, in mice, show that treatment with MPLA confers resistance

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Reprints will not be ordered.

SF, AH, JKB, ERS, and PE designed and supervised the experiments; SF, KI, CS, RS, and PE performed the experiments; SF, AH, JKB, ERS, KI, CS, RS, and PE analyzed the data; SF, AH, JKB, KI, CS, RS, DNH, DSP and PE critically revised the article for important intellectual content; ERS wrote the manuscript. Drs PE and ERS serve as co-senior authors on the manuscript.

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against *P aeruginosa, S aureus*, and *C albicans* infection as well as polymicrobial sepsis caused by cecal ligation and puncture (17–21). MPLA-induced protection persists for at least 15 days and is independent of the adaptive immune system but highly dependent on innate immune function (19, 21, 22). Thus, treatment with MPLA induces a state of innate immune memory that confers resistance against common hospital-acquired pathogens. However, to develop MPLA for clinical use, further work is needed in models that closely replicate the clinical environment.

The sheep model provides an ideal model for testing of MPLA because both physiological and genomic responses of sheep to TLR4 agonists are essentially identical to that of humans (23–25). Consequently, sheep provide a model to test the systemic effects of MPLA and gain an understanding of how critically ill patients will respond to treatment. In this study, we evaluated the effect of MPLA infusion on hemodynamics and pulmonary function in sheep with skin burn. We then assessed the effect of MPLA on the response of those sheep to *P aeruginosa* pneumonia. Our study shows that burned sheep tolerate MPLA infusion well and that MPLA prophylaxis attenuates acute lung injury and sepsis severity during post-burn *P aeruginosa* pneumonia.

METHODS

Animals

Twelve adult female Merino sheep (body weight [BW] 35.4 ± 1.0 kg) were studied. The study was approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch and conducted in compliance with the guidelines of the National Institutes of Health (NIH) and the American Physiological Society for the care and use of laboratory animals.

Surgical preparation of sheep for study

Fasted animals were surgically prepared for chronic study under isoflurane anesthesia. Pre- and post-surgical analgesia was provided with long acting (72 h buprenorphine SR (0.05 mg/kg, SR Veterinary Technologies, Windsor, Colo). Catheters were placed into the right femoral artery and the left atrium for continuous measurement of blood pressure and left atrial pressure, respectively. A 7Fr Swan-Ganz thermal dilution catheter (Edwards Lifesciences LLC; Irvine, Calif) was introduced through the right external jugular vein and advanced into the pulmonary artery for measurement of cardiac output, and pulmonary artery and central venous pressures. Following the operative procedures, the sheep were given 5 to 7 days to recover. For admission into the protocol the animals must have: a PaO_2 >100 mm Hg on room air, a core body temperature greater than 38°C and less than 40°C, and a hematocrit >20%.

Induction of burn injury

Instrumented sheep were anesthetized with intravenous ketamine (800 mg) and inhaled isoflurane via mask (to effect) and tracheostomy was performed. Pre- and postsurgical analgesia was provided with long acting (72 h) buprenorphine SR (0.05 mg/kg, SR Veterinary Technologies, Windsor, Colo) via the subcutaneous route as previously described (26). Anesthesia was maintained using inhaled isoflurane via tracheostomy. The 20% TBSA, third-degree flame burn was made on one flank by Bunsen burner as previously described (27,28). Afterward, anesthesia was discontinued and sheep were placed on mechanical ventilation and monitored for 24 h in a conscious state. Sheep were fluid resuscitated with lactated Ringer's solution (LR) per protocol (29). Sheep were studied in pairs to provide side-by-side assessment and were randomized to treatment with saline (control) or MPLA.

Monophosphoryl lipid A (MPLA) treatment

MPLA derived from *Salmonella enterica* serotype Minnesota Re 595 was purchased from Sigma-Aldrich Co (Catalog #: L6895, St. Louis, Mo), 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 saline solution (25 mL) and administered by intravenous infusion (2.5 μg/kg) over 50 min. Physiologic measurements were performed prior to and at 5, 10, 20, 30, 45, 60, 75, 90, 105, and 120 min after initiation of MPLA infusion in awake sheep. During this and previous (burn induction) phases, sheep were fluid resuscitated with LR using our standard formula (29).

Induction of pneumonia

At 24 h after MPLA or vehicle treatment, sheep were anesthetized (as described in Burn Induction Section) and *P aeruginosa* ($1.6 \sim 2.5 \times 10^{10}$ colony-forming units in 30-mL solution, strain; 27317, ATCC, Manassas, Va) was instilled into the airways through a bronchoscope as previously described (30–33). After instillation, sheep were maintained on mechanical ventilation with a pressure-regulated volume control, assist-control (PRVC A/C) mode, a tidal volume (TV) of 12 mL/kg, and a positive end-expiratory pressure of 5 cmH_2O and monitored in an awake condition throughout for 24 h. Physiologic measurements were performed at baseline and at every 3 h after infection out to 24 h. Sheep were fluid resuscitated with LR to maintain hematocrit at baseline levels (±3%) (15,16).

Multi-organ function assessment

To assess the severity of multi-organ dysfunctions during pneumonia, we modified the Sequential Organ Failure Assessment (SOFA) score (34). The modified sheep SOFA (mSOFA) scores included the values of mean arterial pressure (MAP) and PaO_2/FiO_2 ratio, total platelet count measured by HEMA-VET HV950FS (Drew Scientific Inc, Miami Lakes, Fla), and total bilirubin and creatinine concentrations in plasma as measured at the institutional clinical chemistry laboratory through spectrophotometric assay (Supplemental Table 1, http://links.lww.com/SHK/A872). To assess mental status, a simplified sheep neurological/alertness assessment scale was developed (Supplemental Table 2, http://links.lww.com/SHK/A873).

Bacterial clearance in lung

Lung tissue (100 mg from the dorsal edge of right middle lobe) was taken during the necropsy (72 h post-burn), homogenized with 1× PBS and plated (200 μL) onto soy agar plates. The plates were incubated for 24 h at 37°C and colony-forming units were counted.

Plasma interleukin-6 measurement

Arterial blood samples were collected into EDTA tubes (BD Vacutainer, Ref# 367861, Franklin Lakes, NJ) before instillation of the *P aeruginosa* into the lung, and at 6 and 24 h after the instillation. Blood was centrifuged at 1,800 g at 4°C for 10 min and plasma was aliquoted and frozen at −20°C until the day of assay. Quantification of interleukin 6 (IL-6) levels was performed using enzyme-linked immuno-sorbent assays (ELISA) kit (RPA079Ov01, Cloud-Clone Corp., Katy, Tex), according to the manufacturer's instructions. All samples and standards were assayed in duplicate.

Trans-endothelial electrical resistance assay

Pooled human dermal microvascular endothelial cells (Lonza, Basel, Switzerland) were primed with MPLA (10 μg/mL) or vehicle for 24 h, washed, and plated on 24-well ThinCert inserts (0.4 μm pore diameter, Greiner Bio-One, Kremsmünster, Austria) at 4×10^5 cells/mL. Prior to seeding, ThinCerts were coated with 0.01% Poly-L-Lysine (EMD-Millipore, Burlington, Mass), 50% glutaraldehyde (EMD-Millipore), and 0.25 mg/mL gelatin (Sigma, St. Louis, Mo). Inserts were placed inside pots containing 950 μL culture media and allowed to equilibrate. Trans-endothelial electrical resistance (TEER, $\Omega \bullet cm^2$) across the pots and inserts was measured using a cellZscope (nanoAnalytics, Münster, Germany), reading once an hour. After 24 h, LPS (1 μg/mL) or vehicle control was added as indicated and TEER was measured for another 16 h. TEER readings were normalized to a baseline value (the last reading prior to treatments) and percentage change from baseline for each group was plotted. The area under the curve (AUC) was calculated by taking the percent change in resistance (increase or positive and decrease or negative) relative to the baseline TEER measurement prior to LPS and adding them together to get the area of percent change over time.

Statistical analysis

All data were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, Calif). Variables are reported as mean ± standard error of mean (SEM).

Data were analyzed using Mann–Whitney *U* test or two-way ANOVA with repeated measures followed by Bonferroni or Tukey *post-hoc* tests. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

MPLA infusion caused transient physiologic alterations in burned sheep

Hemodynamics, pulmonary function, and core body temperature were not different at baseline or during the 24-h period following the cutaneous burn when comparing sheep ultimately randomized to vehicle or MPLA treatment (Supplemental Table 3, http://links.lww.com/SHK/A874).

The MPLA infusion caused changes in core body temperature and heart rate (HR) but mean arterial pressure and systemic vascular resistance index were not affected (Fig. 1). Mean pulmonary artery pressure (mPAP) and pulmonary vascular resistance index (PVRI) were significantly elevated at 30 to 60 min following MPLA infusion and left atrial pressure, left ventricular stroke work index (LVSWI), and stroke volume index (SVI) were significantly decreased compared to control beginning 30 min after MPLA infusion (Fig. 2). All parameters, with the exception of temperature and SVI, returned to baseline by 60 min after initiation of MPLA infusion.

The MPLA infusion transiently increased respiratory rate at 45 min after initiation of infusion but did not significantly affect peak/plateau airway pressures, and lung dynamic/static compliances (Fig. 3).

MPLA treatment improved oxygenation and pulmonary mechanics in sheep with P aeruginosa pneumonia/sepsis

The PaO_2/FiO_2 ratio was decreased in sheep at 3 h after *P aeruginosa* instillation and reached levels below 300 mm Hg (mild ARDS) at 9 to 24 h after bacterial instillation in vehicle-treated sheep (Fig. 4). Pulmonary oxygenation index and pulmonary shunt fraction were increased at 3 h after *P aeruginosa* instillation and remained increased throughout the study period in vehicle controls (Fig. 4). Preconditioning with MPLA significantly attenuated the worsening PaO_2/FiO_2 ratio, pulmonary oxygenation index, and shunt fraction after *P aeruginosa* instillation. Peak and plateau airway pressures were significantly increased and static compliance decreased in vehicle-treated sheep with pneumonia (Fig. 5). Those changes were attenuated in MPLA-treated sheep.

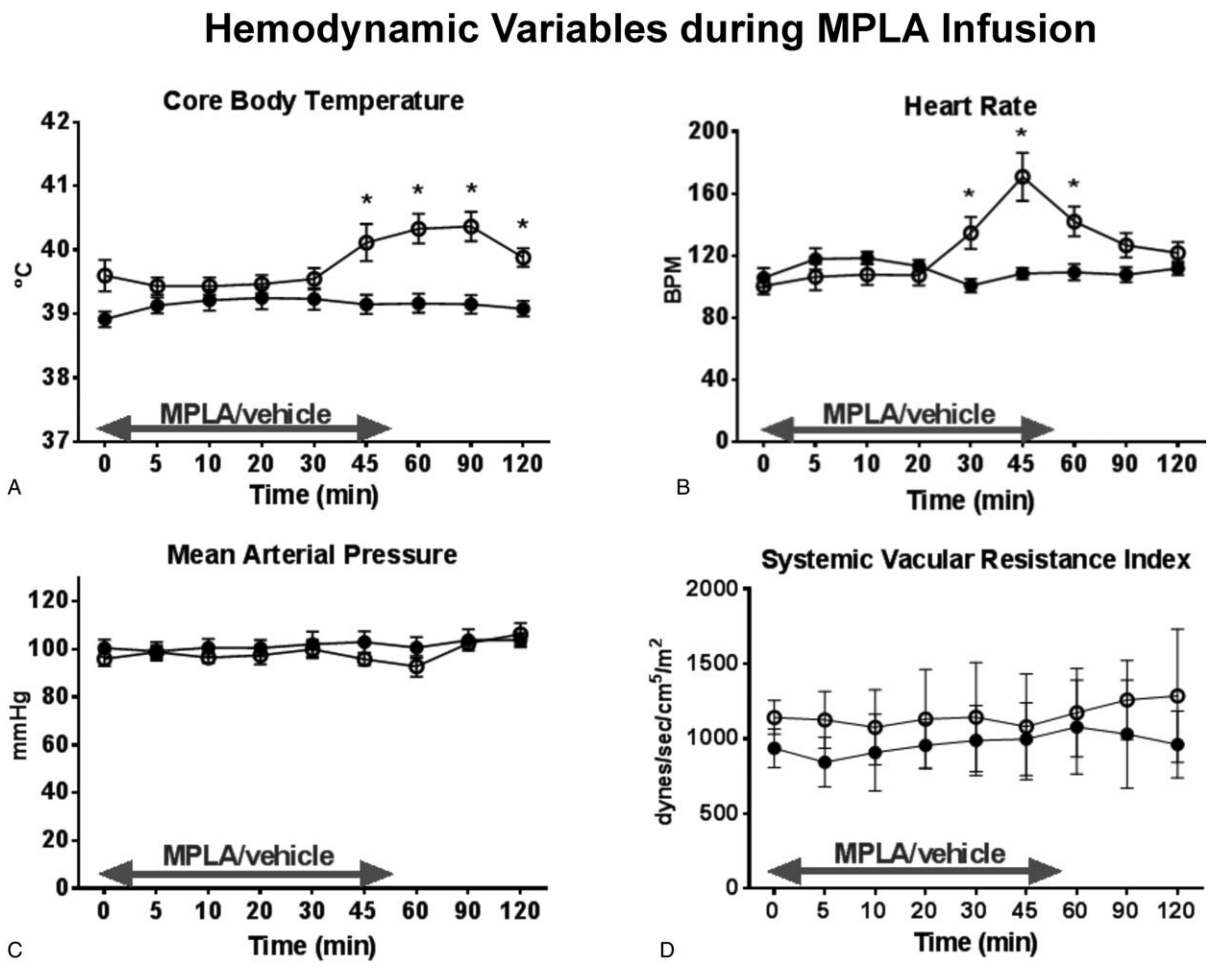

FIG. 1. **Hemodynamic variables during MPLA or vehicle infusion.** MPLA (2.5 μg/kg) or vehicle was infused over 50 min. Hemodynamics variables were measured for 2 h after initiation of infusion. (A) Core body temperature, (B) heart rate, (C) mean pulmonary artery pressure, (D) systemic vascular resistance index during the MPLA infusion. Open circles represent MPLA-preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM (*$P < 0.05$ vs. control). MPLA indicates monophosphoryl lipid A.

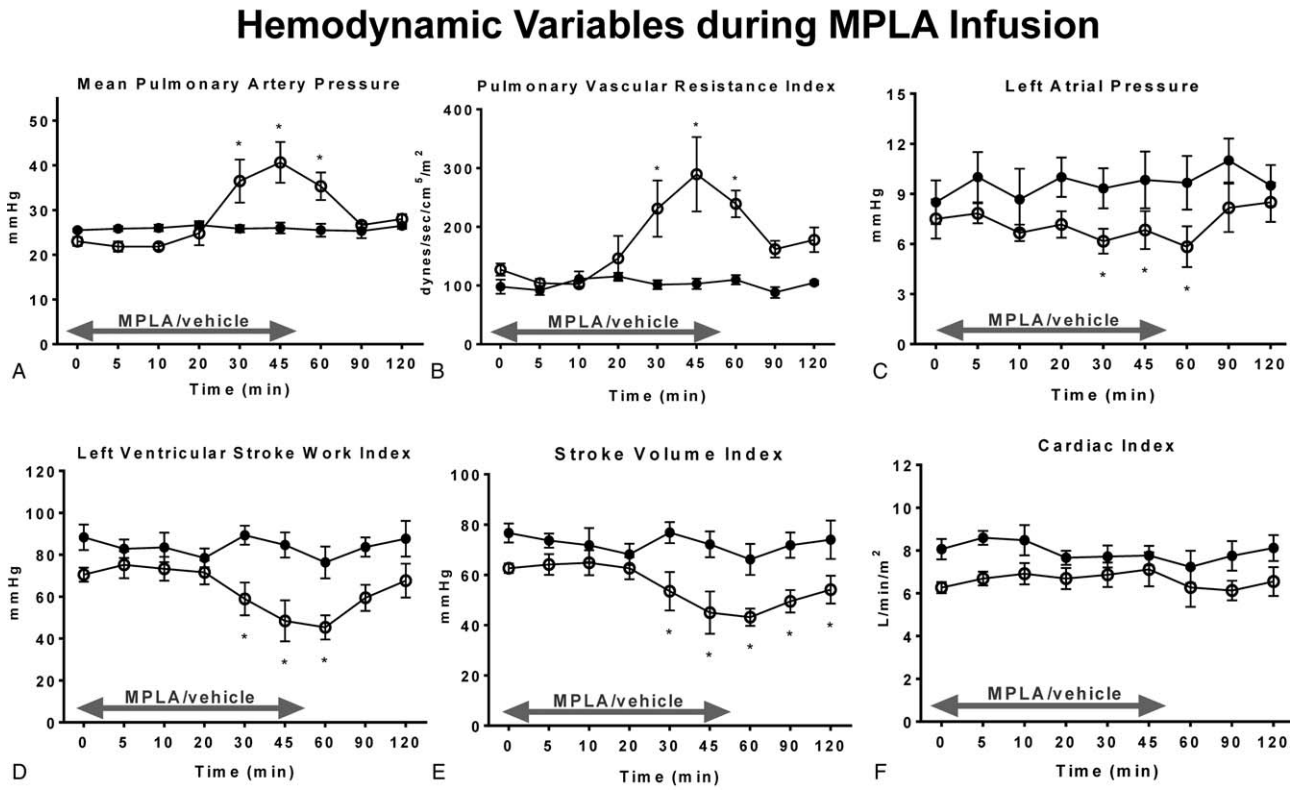

Fig. 2. **Hemodynamic variables during MPLA or vehicle infusion.** MPLA (2.5 μg/kg) or vehicle were infused over 50 min. Hemodynamics variables were measured for 2 h after initiation of infusion. (A) Mean pulmonary artery pressure, (B) pulmonary vascular resistance index, (C) left atrial pressure, (D) left ventricular stroke work index, (E) stroke volume index, (F) cardiac index. Open circles represent MPLA-preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM ($^*P < 0.05$ vs. control).

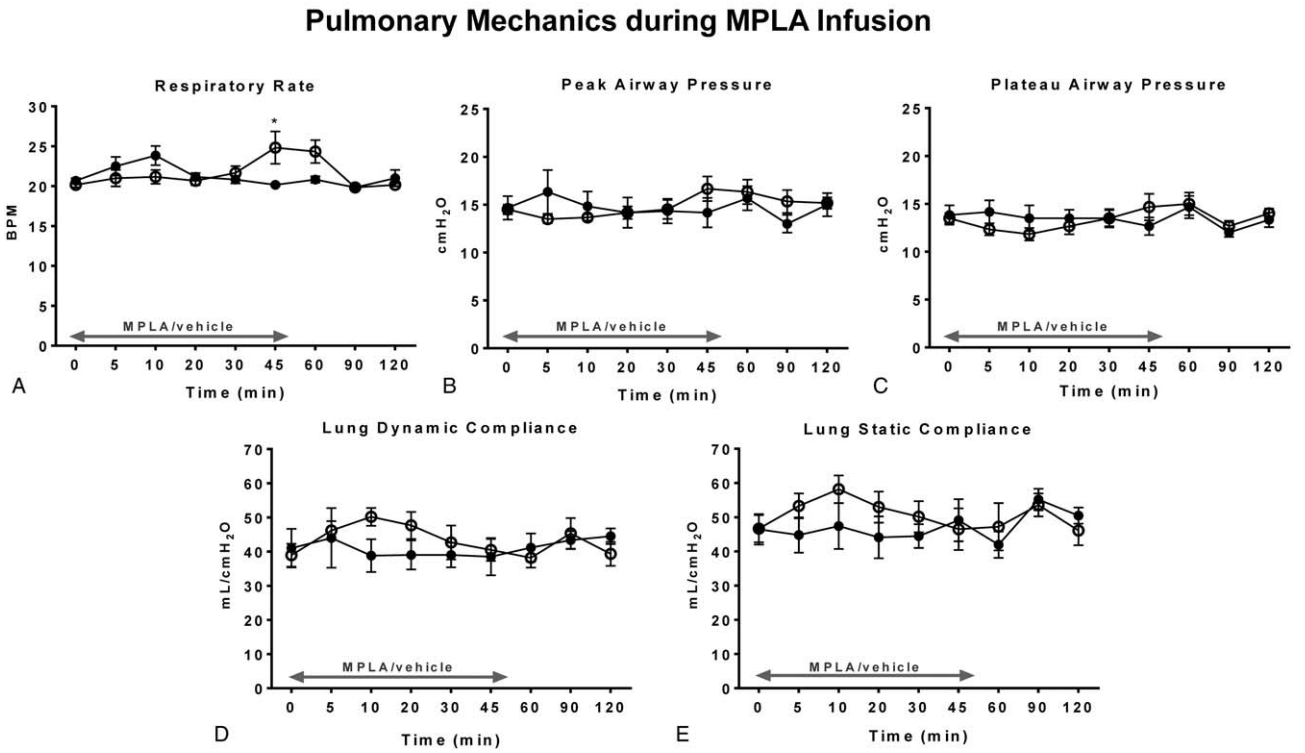

Fig. 3. **Respiratory variables during MPLA or vehicle infusion.** MPLA (2.5 μg/kg) or vehicle was infused over 50 min. Respiratory variables were measured for 2 h after initiation of infusion. (A) Respiratory rate, (B) peak airway pressure, (C) plateau airway pressure, (D) lung dynamic compliance, (E) lung static compliance during the MPLA infusion period. Open circles represent MPLA-preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM ($^*P < 0.05$ vs. control).

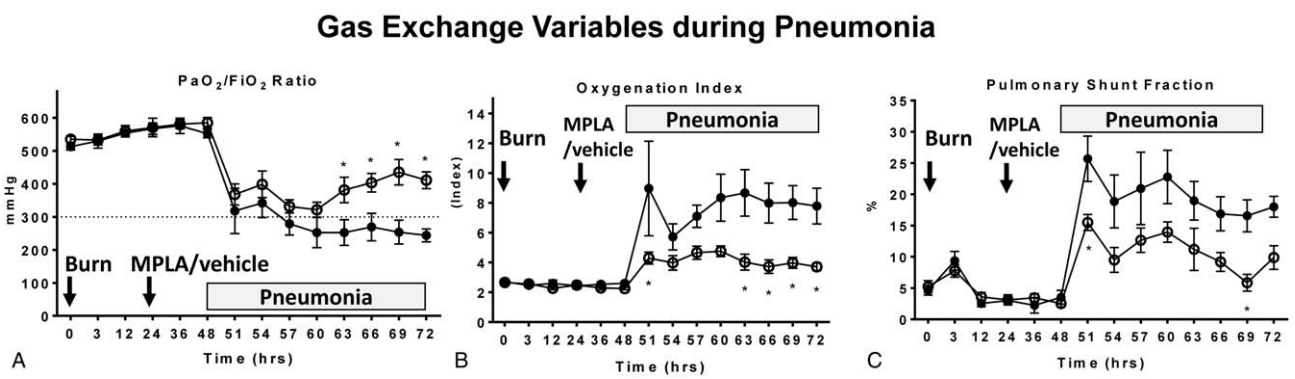

FIG. 4. **Lung injury and gas exchange following *P aeruginosa* instillation into lungs.** Sheep underwent 20% TBSA cutaneous burn at time 0 followed by vehicle or MPLA (2.5 μg/kg) infusion at 24 h after burn injury. *P aeruginosa* was instilled into the lungs at 48 h after burn injury. (A) PaO_2/FiO_2 ratio, (B) oxygenation index, (C) pulmonary shunt fraction. Open circles represent MPLA-preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM ($^*P < 0.05$ vs. control).

MPLA treatment attenuated pneumonia/sepsis-induced changes in hemodynamics, lactate production, and organ injury during post-burn pneumonia

Increases in cardiac index and decreases in systemic vascular resistance index caused by intrapulmonary *P aeruginosa* instillation were significantly attenuated by MPLA (Fig. 6). No significant differences were noted in heart rate or mean arterial pressure. Core body temperature, mPAP, pulmonary artery wedge pressure, PVRI, LVSWI, and right ventricular stroke work index were not different between groups (data not shown).

Instillation of bacteria significantly increased plasma lactate concentrations in control sheep, which were significantly lower in MPLA-preconditioned sheep (Fig. 7). mSOFA score was increased during pneumonia in control sheep and significantly improved by MPLA preconditioning (Fig. 7). The more favorable mSOFA score in MPLA-preconditioned sheep was related to improved PaO_2/FiO_2 ratio and less hypotension (Supplemental Table 4, http://links.lww.com/SHK/A875). Numbers of lung bacteria at 24 h after *P aeruginosa* instillation were significantly lower in MPLA-treated sheep compared to control (Fig. 7).

MPLA treatment attenuates systemic cytokine production and vascular permeability during post-burn pneumonia and sepsis

MPLA treatment significantly attenuated increases in IL-6 production at 6 and 24 h after *P aeruginosa* instillation (Fig. 8). Preconditioning with MPLA reduced *P aeruginosa*-induced plasma protein concentration changes and attenuated the increase in lung wet to dry weight ratio indicating less vascular leak (Fig. 9). To further assess the impact of MPLA on endothelial barrier function, human endothelial cell monolayers were primed with MPLA or vehicle prior to LPS challenge. Measurement of barrier integrity by Trans-endothelial electrical resistance (TEER, $\Omega \bullet cm^2$) showed significant improvement in human endothelial cell barrier function after MPLA-priming compared to control (Fig. 9).

DISCUSSION

The major findings of this study are that MPLA infusion is well tolerated in burned sheep and confers resistance to acute lung injury, lactatemia, and hemodynamic alterations during post-burn *P aeruginosa* pneumonia and sepsis. These findings

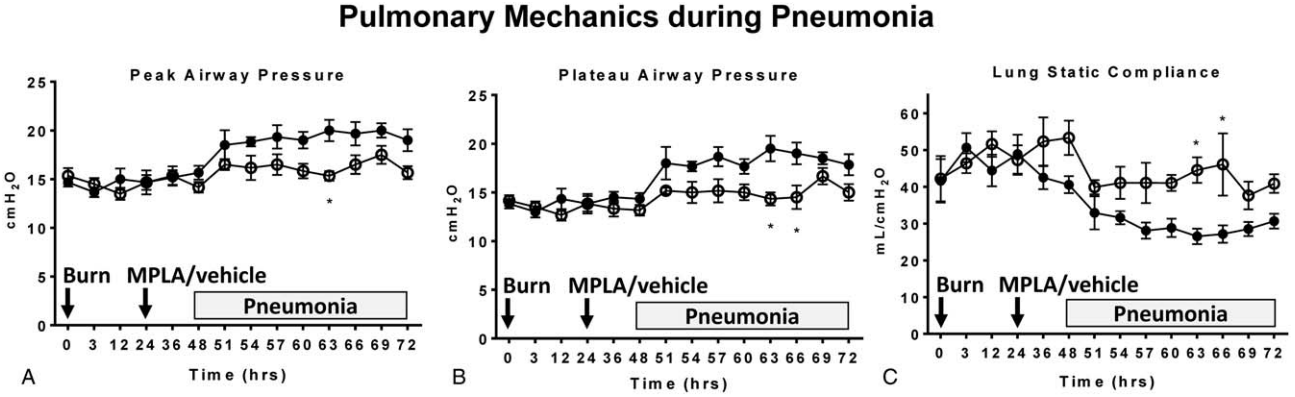

FIG. 5. **Pulmonary mechanics following *P aeruginosa* instillation into lungs.** Sheep underwent 20% TBSA cutaneous burn at time 0 followed by vehicle or MPLA (2.5 μg/kg) infusion at 24 h after burn injury. *P aeruginosa* was instilled into the lungs at 48 h after burn injury. (A) Peak airway pressure, (B) plateau airway pressure, and (C) lung static compliance during the whole study period. Open circles represent MPLA-preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM ($^*P < 0.05$ vs. control).

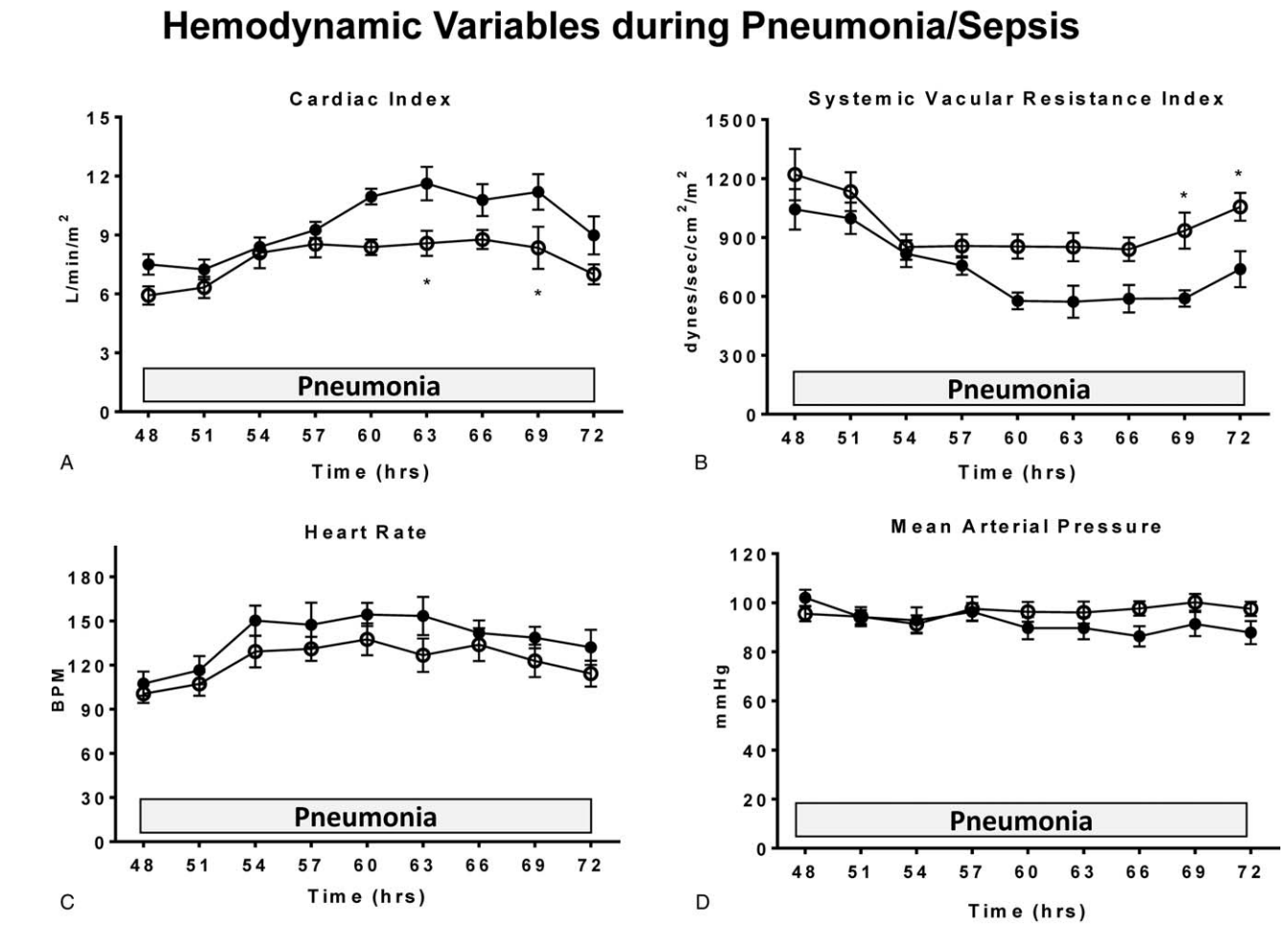

Fig. 6. **Hemodynamic variables following *P aeruginosa* instillation into lungs.** Sheep underwent 20% TBSA cutaneous burn at time 0 followed by vehicle or MPLA (2.5 μg/kg) infusion at 24 h after burn injury. *P aeruginosa* was instilled into the lungs at 48 h after burn injury and hemodynamics were measured for 24 h. (A) Cardiac index, (B) systemic vascular resistance index, (C) heart rate, (D) mean arterial pressure. Open circles represent MPLA-preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM (*$P < 0.05$ vs. control).

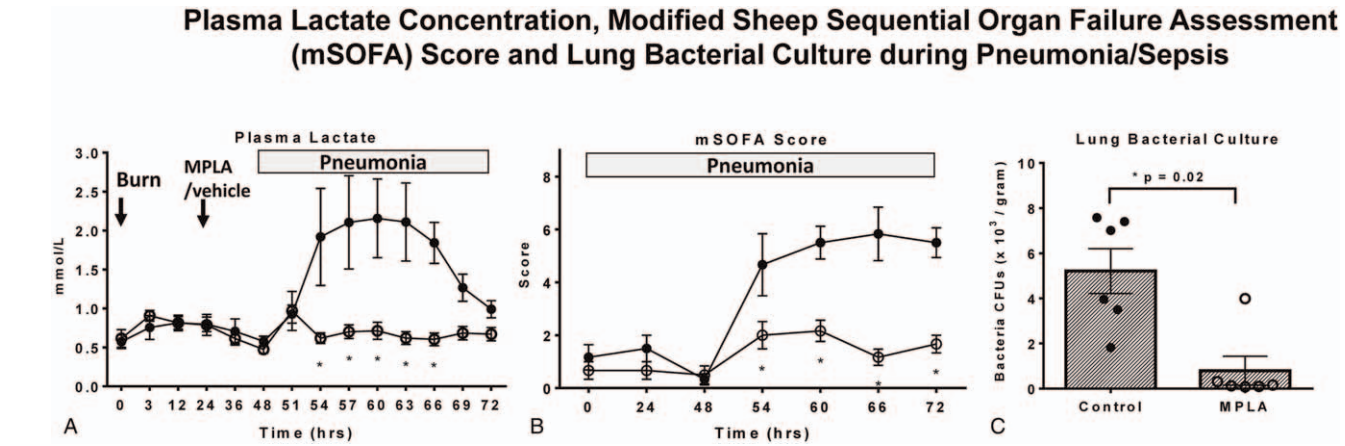

Fig. 7. **Plasma lactate, mSOFA score, and lung *P aeruginosa* CFU following *P aeruginosa* instillation into lungs.** Sheep underwent 20% TBSA cutaneous burn at time 0 followed by vehicle or MPLA (2.5 μg/kg) infusion at 24 h after burn injury. *P aeruginosa* was instilled into the lungs at 48 h after burn injury The figure shows (A) plasma lactate concentration and (B) modified sheep Sequential Organ Failure Assessment (mSOFA) score during the whole study period and (C) numbers of the bacteria in lung culture (at 24 h after *P aeruginosa* infection). Open circles represent MPLA preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6. Data are expressed as mean ± SEM (*$P < 0.05$ vs. control).

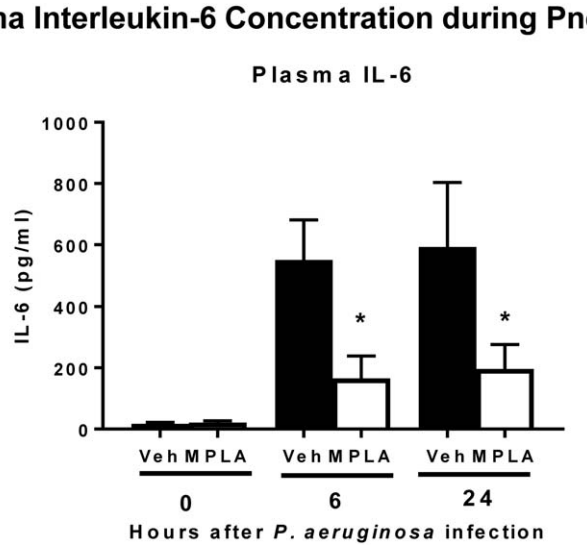

FIG. 8. **Plasma interleukin-6 during post-burn pneumonia.** Sheep underwent 20% TBSA cutaneous burn at time 0 followed by vehicle or MPLA (2.5 μg/kg) infusion at 24 h after burn injury. *P aeruginosa* was instilled into the lungs at 48 h after burn injury Plasma IL-6 concentrations were measured at 0, 6, and 24 h after *P aeruginosa* challenge. Data are expressed as mean ± SEM (*$P < 0.05$ vs. vehicle (veh)).

support the clinical relevance of applying MPLA, and other TLR4 agonists, to prevent and decrease the severity of serious infections and organ injury in high-risk populations such as those suffering major burns. In the present study, MPLA treatment was initiated after burn injury but before microbial challenge. Our model is clinically relevant since one could envision treating burn victims with MPLA early during burn shock resuscitation, or shortly thereafter, to improve downstream resistance to organ injury and infection. Burn patients are particularly susceptible to infections and lung injury and represent a population that could benefit significantly from MPLA prophylaxis (6, 9, 35). Sepsis and respiratory failure are the most common causes of morbidity and mortality in burn victims that survive the acute phase of injury (9). Furthermore, the lungs are the most common site of serious infections in severely burned patients and *P aeruginosa* is the most common pathogen (9, 36). The Centers for Disease Control (CDC) defines *P aeruginosa* as the most common cause of pneumonia in ICUs and the second most common gram-negative pathogen causing hospital-acquired infections (36–38). *P aeruginosa* is of particular clinical concern because of its extraordinary resistance to antibiotics resulting in the CDC classifying it as a ''SERIOUS'' threat to public health (39). Thus, the ability of MPLA to confer resistance to *P aeruginosa* pneumonia has clinical implications beyond application in burn patients alone. Any patient group that is at risk of developing *P aeruginosa*

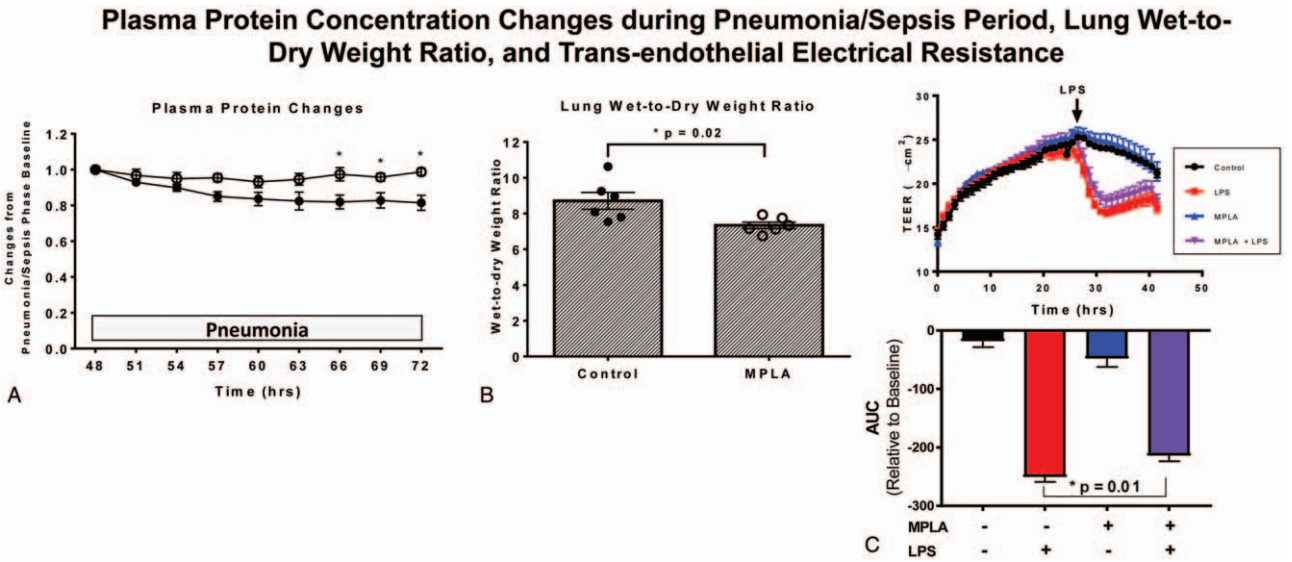

FIG. 9. **The graph shows that (A) plasma protein concentration changes during pneumonia/sepsis period (48–72 h), (B) lung wet-to-dry weight ratio (at 72 h), and (C) trans-endothelial electrical resistance in human dermal microvascular endothelial cells.** Open circles represent MPLA preconditioned treatment group animals. Closed circles represent control group. The animal numbers in both groups are n = 6 in graph (A) and (B). Data are expressed as mean ± SEM (*$P < 0.05$ vs. control).

pneumonia could benefit including intubated critically ill patients and persons suffering major trauma, high-risk surgery, or prolonged hospitalization.

Sepsis is the leading cause of death in non-cardiac intensive care units (ICU) and accounts for 40% of ICU expenditures (40, 41). Patients who survive sepsis suffer long-term physical and cognitive disabilities and have a high 1-year mortality rate (42, 43). Over the past two decades the incidence of sepsis has increased and that trend is likely to continue due to our aging population, increased use of immunosuppressive drugs and invasive procedures and the emergence of antibiotic-resistant pathogens (41, 44). Attempts at effectively treating sepsis have proven exceedingly difficult. No drugs are currently approved by the FDA for the treatment of sepsis due to the repeated failure of clinical trials (45, 46). Therefore, new strategies are needed to decrease the burden of hospital-acquired infections and sepsis. The addition of immunoadjuvant therapy, such as that provided by treatment with TLR4 agonists, has significant potential to augment existing prophylactic approaches.

MPLA treatment attenuated acute lung injury during *P* aeruginosa pneumonia in sheep preconditioned with MPLA compared with vehicle-treated controls as indicated by more favorable PaO_2/FiO_2 ratio, oxygenation index, shunt fraction, and airway pressures. Those findings were paralleled by lower systemic inflammation reflected by lower plasma IL-6 concentrations in MPLA-treated sheep. The results indicate that MPLA treatment lessened pulmonary and systemic inflammation, both of which can precipitate or contribute to acute lung injury. This is clinically important since acute lung injury is among the most common causes of mortality in severely burned patients (9). Three factors are likely to contribute to the decreased inflammation and lung injury observed in MPLA-preconditioned sheep. First, MPLA augmented clearance of bacteria from the lungs as indicated by lower *P aeruginosa* CFU in lung cultures from MPLA-treated sheep. This is consistent with previous studies showing that MPLA facilitates clearance of bacteria at sites of infection and decreases the dissemination of bacteria to distant sites (19, 21, 22). MPLA-induced protection persists for at least 15 days and is independent of the adaptive immune system but highly dependent on innate immune function (19, 21, 22). The mechanisms of improved microbial clearance are multifactorial and include expansion of myeloid cell numbers in bone marrow and blood in association with increased myeloid cell recruitment to sites of infection and augmented microbial phagocytosis and killing (18, 20, 21, 47). Second, MPLA is known to induce endotoxin tolerance, a state of attenuated cytokine production during periods of inflammation and infection. Our cytokine measurements showed significantly decreased plasma IL-6 concentrations in MPLA-primed sheep compared to controls (18, 48, 49). That finding connotes decreased local and systemic inflammation in sheep receiving MPLA treatment, which is likely to translate into attenuated lung injury (50, 51). Third, it appears that MPLA treatment helps to maintain endothelial barrier integrity since plasma protein leak was attenuated in MPLA-treated mice. Loss of endothelial barrier function is a contributing factor to the development of pulmonary edema during acute lung injury and the acute respiratory distress syndrome. This is likely due to the ability of MPLA to modulate the endothelial cell response to microbial products and cytokines. Our previous studies, using human vascular endothelial cells, show that MPLA priming will attenuate TLR agonist-induced cytokine production by endothelial cells and improve barrier function through mechanisms activated via the MyD88-dependent signaling pathway (49, 52, 53).

The sheep model is advantageous for preclinical testing of TLR4 agonists because the physiologic and genomic response of sheep to TLR4 agonists is nearly identical to that of humans and sheep provide a model in which common clinical variables can be measured in real time (23, 54, 55). Findings from the present study support the validity of sheep as a robust model for preclinical testing of TLR4 agonists. The response to MPLA observed in this study is highly similar to that observed in humans. The dose of MPLA chosen for our study was based on results published by Astiz et al. (48) for normal human volunteers. They reported that human subjects did not experience subjective side effects until MPLA was administered at doses of 10 μg/kg or greater. At 20 μg/kg, humans experienced mild to moderate symptomatology in association with increases in HR, temperature and elevated plasma TNFα, IL-6 and IL-8 concentrations (48). Subjects in the 20 μg/kg group did not require therapy or intervention. We chose a dose of MPLA below the 10 μg/kg threshold because we employed burned, rather than normal, uninjured subjects in our study. Due to the inflammatory state induced by burns, we predicted that the physiologic and inflammatory responses to MPLA would be heightened. We observed that hemodynamics and respiratory function were minimally impacted by MPLA infusion at a dose of 2.5 μg/kg and that the response of sheep closely mimicked that seen in humans at similar doses. The most notable physiologic changes induced by MPLA infusion were increases in mean pulmonary artery pressure and pulmonary artery resistance. Those alterations persisted for about 30 min after MPLA perfusion and then returned to baseline. Although the burned sheep tolerated those alterations well, it is possible that burn victims suffering inhalation injury and a significant respiratory insult could respond unfavorably. Future studies using a burn and smoke inhalation model would be useful in assessing that possibility.

Our results provide important information about the host response to systemic MPLA infusion and the ability of MPLA to improve resistance pulmonary infection. However, further work is needed. We employed a single dose of MPLA that was guided by previous studies from humans. However, dose finding studies are needed to identify the dose of MPLA that is best tolerated and provides optimal immunomodulatory effect. The 2.5 μg/kg dose is a good starting point since MPLA was well tolerated and effective at that dose. Questions also remain about optimal timing of MPLA treatment. In this study, MPLA was administered 24 h after burn injury and 24 h prior to bacterial challenge. In previous studies in mice, we have initiated MPLA treatment 2 to 3 days after burn injury and 24 h prior to *P aeruginosa* burn wound infection (47). Results of the present study indicate that it is generally safe to administer TLR4 agonists within 24 h of a major burn injury. We do not know if administration of MPLA during early fluid resuscitation will have significant physiologic impact. We also do not

know if administration of MPLA at the time of infection initiation will be beneficial. Our previous studies suggest that prophylactic administration of MPLA 24 h prior to infection provides optimal protection and that the beneficial effects persist for at least 15 days (18, 21). Results of the present study provide proof of concept that MPLA, and other monophosphorylated TLR4 agonists, can be administered safely in a model that closely mimics human critical illness and induces protection from organ injury caused by pneumonia and sepsis.

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