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Characterization and Modulation of the Immunosuppressive Phase of Sepsis[∇]

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Sepsis continues to cause significant morbidity and mortality in critically ill patients. Studies of patients and animal models have revealed that changes in the immune response during sepsis play a decisive role in the outcome. Using a clinically relevant two-hit model of sepsis, i.e., cecal ligation and puncture (CLP) followed by the induction of *Pseudomonas aeruginosa* pneumonia, we characterized the host immune response. Second, AS101 [ammonium trichloro(dioxoethylene-*o,o'*)tellurate], a compound that blocks interleukin 10 (IL-10), a key mediator of immunosuppression in sepsis, was tested for its ability to reverse immunoparalysis and improve survival. Mice subjected to pneumonia following CLP had different survival rates depending upon the timing of the secondary injury. Animals challenged with *P. aeruginosa* at 4 days post-CLP had ~40% survival, whereas animals challenged at 7 days had 85% survival. This improvement in survival was associated with decreased lymphocyte apoptosis, restoration of innate cell populations, increased proinflammatory cytokines, and restoration of gamma interferon (IFN- γ) production by stimulated splenocytes. These animals also showed significantly less *P. aeruginosa* growth from blood and bronchoalveolar lavage fluid. Importantly, AS101 improved survival after secondary injury 4 days following CLP. This increased survival was associated with many of the same findings observed in the 7-day group, i.e., restoration of IFN- γ production, increased proinflammatory cytokines, and decreased bacterial growth. Collectively, these studies demonstrate that immunosuppression following initial septic insult increases susceptibility to secondary infection. However, by 7 days post-CLP, the host's immune system has recovered sufficiently to mount an effective immune response. Modulation of the immunosuppressive phase of sepsis may aid in the development of new therapeutic strategies.

Severe sepsis with resultant organ dysfunction remains a life-threatening condition that is associated with significant morbidity and mortality. Understanding the changes in the host's immune response throughout the course of illness remains a major obstacle to the development of effective strategies in the treatment of sepsis. Sepsis results in a cascade of events, including the production of pro- and anti-inflammatory cytokines and a shift from a Th1 to a Th2 immune phenotype, as well as the induction of apoptosis of immune effector cells (5, 10, 17, 25). This immune response in sepsis has dramatic and deleterious effects on the host's ability to eradicate the infection.

The early phase of sepsis is dominated by a hyperinflammatory state mediated by systemic production of inflammatory cytokines, including interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNF- α), and gamma interferon (IFN- γ) (3, 7, 31). This hyperinflammatory "cytokine storm" may lead to significant end-organ damage and death in a subset of patients. Concomitant with the initial hyperinflammatory response is a nearly simultaneous production of anti-inflammatory cytokines, including IL-10, that serve to balance the inflammatory state. As a result, the majority of patients survive the initial hyperinflammatory state and progress to a protracted period of immune suppression (3, 7, 9, 28, 31). Animal studies, as well as recent human studies, have

highlighted the importance of delayed sepsis-induced immunosuppression and its contribution to the mortality of the syndrome (14). Several researchers have shown that the levels of both proinflammatory and anti-inflammatory cytokines can be correlated with the severity of illness (26, 27, 29). For example, IL-10, a potent anti-inflammatory cytokine, has been shown to predict poor prognosis in patients with sepsis, and modulation of IL-10 in selected animal models of sepsis improved survival (15, 16, 34; M. Ariefdjohan, K. Queensland, L. Weitzel, D. Heyland, and P. Wischmeyer, presented at the 32nd Annual Conference on Shock, San Antonio, TX, 2009). Conversely, there is also evidence that downregulation of IL-10 or absence of IL-10 (IL-10-null mice) is detrimental in some animal models of infection (21). These apparently contradictory findings point to the importance of understanding the overall balance between pro- and anti-inflammatory cytokines and their effects upon the hypo-/hyperinflammatory axis.

For decades, researchers have tried to develop new therapeutic strategies to treat sepsis and the resultant multiorgan failure. Unfortunately the majority, if not all, of these therapeutic modalities have failed, including those therapies that have shown significant promise in animal models (32). As a result of the protracted immunosuppression, patients with sepsis face a significant risk of developing secondary infections. Researchers have demonstrated this increased risk in both septic patients and animal models of sepsis (19, 22). However, there is little understanding of the duration of immune suppression and how the timing of secondary injury impacts overall mortality. Having previously developed a clinically relevant murine model of secondary infection, i.e., cecal ligation and puncture

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(CLP) followed by *Pseudomonas aeruginosa* pneumonia infection, our first aim was to further characterize the extent and duration of immune suppression following primary (CLP) injury in light of resistance to and recovery from secondary (*P. aeruginosa*) insult (22). In our previous work, we had established increased mortality in animals infected with either *P. aeruginosa* or *S. pneumoniae* 3 days following CLP (22). This more recent work set out to understand how the immune response evolves over time by evaluating the changes in the cellular composition of the spleen and the ability of the host to produce inflammatory cytokines and respond to a secondary insult over the first 7 days following CLP. Next, we examined the possibility of manipulating the cytokine milieu following primary injury. In particular, we used the immune modulator AS101 [ammonium trichloro(dioxoethylene-*o,o'*)tellurate], a compound known to block synthesis of IL-10 and to increase a number of proinflammatory cytokines. AS101 has been shown to be effective in multiple models, including both viral and bacterial infections, autoimmune diseases, and cancer (16, 34, 35). Due to its ability to modulate the inflammatory axis, we evaluated the potentially prosurvival effects of AS101 in a two-hit model of sepsis.

MATERIALS AND METHODS

Study design. (i) CLP model of sepsis. The CLP model of sepsis as developed by Chaudry et al. and as modified for use in mice by Baker et al. was employed as the initial septic insult (1, 2). Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), ~20 to 25 g body weight and 8 to 10 weeks of age, were housed for at least 1 week prior to use. The mice were anesthetized with isoflurane (5% induction, 2% maintenance), and the abdomen was prepped and draped. A midline laparotomy was performed, and the cecum was identified and exteriorized. The distal one-third of the cecum was ligated and punctured once with a 30-gauge needle. This level of injury was utilized in order to create a prolonged infection with relatively low mortality (<10%). The abdomen was closed in two layers, and 1 ml of 0.9% saline mixed with 0.05 mg/kg of body weight buprenorphine (Hospira Inc., Lake Forest, IL) was administered subcutaneously (s.c.) to replace third-space losses and provide pain control. A single dose of imipenem (25 mg/kg) was given s.c. 1 h post-CLP. Controls, i.e., sham-operated mice, were treated identically, except the cecum was neither ligated nor punctured. One day post-CLP, 1 ml of 0.9% saline was given s.c. to all animals.

(ii) The "second-hit" pneumonia model of sepsis. At 4 or 7 days post-CLP, the surviving mice were again anesthetized with isoflurane and held vertically in a "head-up" position. Using a pipette, 25 μ l of a bacterial suspension ($0.3 A_{600} = 5 \times 10^8$ CFU/ml; $0.5 A_{600} = 1 \times 10^9$ CFU/ml; $0.7 A_{600} = 3 \times 10^9$ CFU/ml) was slowly injected intranasally (divided equally between the nares) and observed to be aspirated on inhalation and held in position for 1 min. The control protocol for the pneumonia model consisted of mice that were treated identically except that normal saline was instilled intranasally instead of the bacterial suspension. Survival was recorded for 7 days following pneumonia. For all acute studies, 25 μ l of the $0.5 A_{600}$ *P. aeruginosa* suspension was used as the secondary injury.

Microbiologic preparation. The bacteria were prepared as previously described (8). *P. aeruginosa* was selected because it is a Gram-negative bacterium that is one of the most common causes of nosocomial pneumonia (6). Briefly, *P. aeruginosa* (ATCC 27853) was grown overnight at 35°C with constant shaking to stationary phase in Trypticase soy broth. Bacteria were harvested by centrifugation, and the pellet was washed and resuspended in sterile phosphate-buffered saline (PBS) to absorbencies of 0.3, 0.5, and $0.7 A_{600}$. Serial dilution and colony counts of inocula corresponded to densities of 5×10^8 CFU/ml, 1×10^9 CFU/ml, and 3×10^9 CFU/ml, respectively.

Cytokine analysis. Whole blood was drawn from all mice at the time of harvest. Plasma was collected for cytokine analysis and analyzed in duplicate using a cytometric bead array for 23 cytokines. The cytokines measured were IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-18, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , KC, monocyte chemoattractant protein 1 (MCP-1), MIP-1 α , RANTES, and TNF- α (BD Pharmingen, San Diego, CA). An IFN- γ enzyme-linked immunosorbent assay (ELISA) was performed on supernatants from cultured and stimulated

splenocytes (R&D Systems, Minneapolis, MN). The splenocytes were cultured in the presence of CD3 and CD28 for 6 h prior to measurement.

Cell surface markers. Spleens were harvested at the time of sacrifice from all groups of mice. Total cell counts were obtained using a Beckman-Coulter (Fullerton, CA) Vi-Cell cell counter. For specific cell population counts, antibodies were used to identify lymphocyte subpopulations (CD3 and b220), NK cells, dendritic cells, and neutrophils (BD Pharmingen, San Diego, CA). Flow cytometric analysis was performed on a FACScan (BD Biosciences, San Jose, CA).

Apoptosis. Apoptosis was quantified using a commercially available antibody against active caspase 3 (Cell Signaling Technology, Inc., Beverly, MA) and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) as previously described (21). Mouse T- and B-cell populations were identified using fluorescein-labeled anti-mouse CD3 and fluorescein-labeled anti-mouse b220 antibodies (BD Pharmingen, San Diego, CA). Flow cytometric analysis (50,000 events/sample) was performed on a FACScan (BD Biosciences, San Jose, CA) as previously described (12).

BAL fluid and blood bacterial counts. Mice were deeply anesthetized with isoflurane. Following sterile preparation, blood was obtained by direct cardiac puncture, placed in sterile vials, and diluted from equal volumes for culture. For bronchoalveolar lavage (BAL), animals were placed supine and the trachea was exposed by dissection. A 25-gauge angiocatheter was inserted into the trachea, and 1 ml of sterile saline was injected and immediately withdrawn. The fluid was placed in sterile vials and diluted from equal volumes for culture.

IFN- γ blockade. In survival studies using IFN- γ -blocking antibody, 250 μ g of antibody or control IgG (clone R4-6A2; BioXcell, West Lebanon, NH) was given intraperitoneally (i.p.) just prior to secondary injury at 7 days.

Anti-IL-10. The immune modulator AS101 (Toctris, Ellisville, MO), pH. 7.4, was diluted to an appropriate concentration in PBS (10 μ g/100 μ l). For survival studies, the animals ($n = 17$ per group) received either 10 μ g AS101 (100 μ l) or PBS s.c. starting 24 h after CLP and continuing every 24 h throughout the survival study. In survival studies using anti-IL-10 antibody, the animals were given 200 μ g of anti-IL-10 or control IgG (clone JES5-2A5; BioXcell, West Lebanon, NH) antibody i.p. at days 1, 3, and 5 post-CLP. For acute studies, AS101 or PBS was given s.c. in the same manner following CLP until the time of harvest at 16 h after the induction of pneumonia ($n = 6$ per group). Methods for measurement of plasma and stimulated cytokines, histology, apoptosis, and BAL fluid and blood cultures were the same as described above.

Histologic evaluation. Histologic analysis of hematoxylin and eosin (H&E)-stained lung specimens was performed to confirm the presence of pneumonia. The degree of infiltration and consolidation was scored by a pathologist (R.E.S.) blinded to the group assignment. The groups evaluated included animals that received secondary injury (pneumonia) at 4 and 7 days post-CLP and animals treated with AS101 (including controls). The scores ranged from 0, representing no consolidation or infiltration, up to 5, which represented severe infiltration with multiple areas of consolidation.

Statistical analysis. Student's *t* test or the Mann-Whitney U test, whichever was appropriate, was used for studies in which two groups were compared. For studies comparing three or more groups, one-way analysis of variance or the Kruskal-Wallis test was used, whichever was appropriate. The chi-square test was used for survival studies. Significance was accepted at $P < 0.05$.

Animal studies. Animal studies were approved by the Washington University School of Medicine Animal Studies Committee.

RESULTS

Analysis of immune cells revealed significant changes in innate and adaptive cell populations. In order to determine the changes in immune cell numbers following single injury, cellular components of the spleen were quantitated at 1, 4, and 7 days post-CLP (including sham-operated controls). Evaluation by flow cytometry revealed significant changes in the cellular composition of whole spleens at these time points. Staining for cell surface markers revealed that NK cells returned to sham-operated levels by 4 days following CLP and had significantly increased by 7 days ($P < 0.05$) (Fig. 1A). Dendritic cells remained depleted at 4 days but had significantly increased by 7 days ($P < 0.001$) (Fig. 1A). Neutrophils had significantly increased at both 4 and 7 days following CLP ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 1A). It should be noted that all

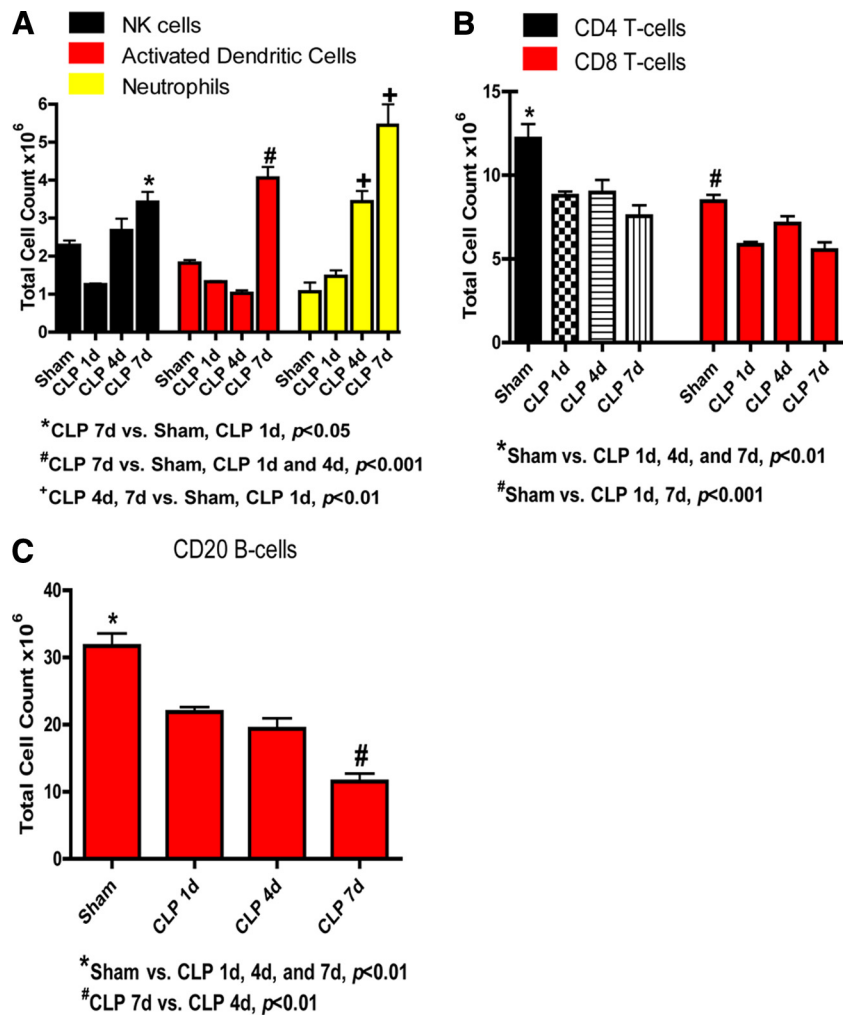


FIG. 1. Evaluation of immune effector cells from spleens harvested from sham-operated mice 1 day after CLP (CLP 1d), 4 days after CLP (CLP 4d), and 7 days after CLP (CLP 7d). (A) NK cells returned to sham-operated levels by 4 days following CLP and had significantly increased by 7 days; dendritic cells remained depleted at 4 days but had significantly increased by 7 days. Neutrophils had significantly increased at both 4 and 7 days following CLP. (B) CD4⁺ lymphocytes had significantly decreased by 1 day and remained depleted through 7 days compared to sham-operated animals, while CD8⁺ lymphocyte counts were significantly depleted at 1 and 7 days post-CLP. (C) CD20⁺ B lymphocytes showed continuous loss over the 7-day period. $n = 10$ per group (except $n = 6$ for neutrophils). The error bars indicate standard deviations.

three innate cell types (NK cells, dendritic cells, and neutrophils) showed significant losses following secondary injury at both 4 and 7 days (data not shown).

Evaluation of T and B lymphocytes revealed significant changes over the course of 7 days post-CLP compared to their sham-operated controls. CD4⁺ lymphocytes had decreased by day 1 and remained depleted through 7 days ($P < 0.01$) (Fig. 1B), while CD8⁺ lymphocyte counts were significantly depleted at both 1 and 7 days post-CLP ($P < 0.001$) (Fig. 1B). Finally, CD20⁺ B lymphocytes showed a continuous loss over the 7-day period ($P < 0.001$) (Fig. 1C).

Restoration of stimulated splenocyte IFN- γ production 7 days post-CLP. Next, we evaluated the ability of splenocytes to produce IFN- γ at 4 and 7 days following CLP to determine the host's ability to mount an effective inflammatory response prior to a secondary insult. Following stimulation by CD3 and CD28, supernatants from harvested and plated splenocytes were measured for IFN- γ . The IFN- γ measurements revealed an 8-fold

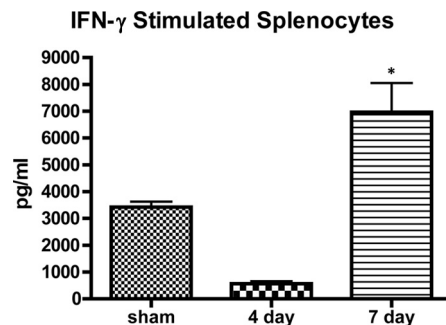


FIG. 2. Splenocytes from animals harvested and stimulated just prior to secondary pneumonia infection at 4 days (96 h after CLP) revealed significant decreases in IFN- γ secretion compared to splenocytes from sham-operated animals or animals 7 days post-CLP. *, $P < 0.01$; $n = 6$ per group. The error bars indicate standard deviations.

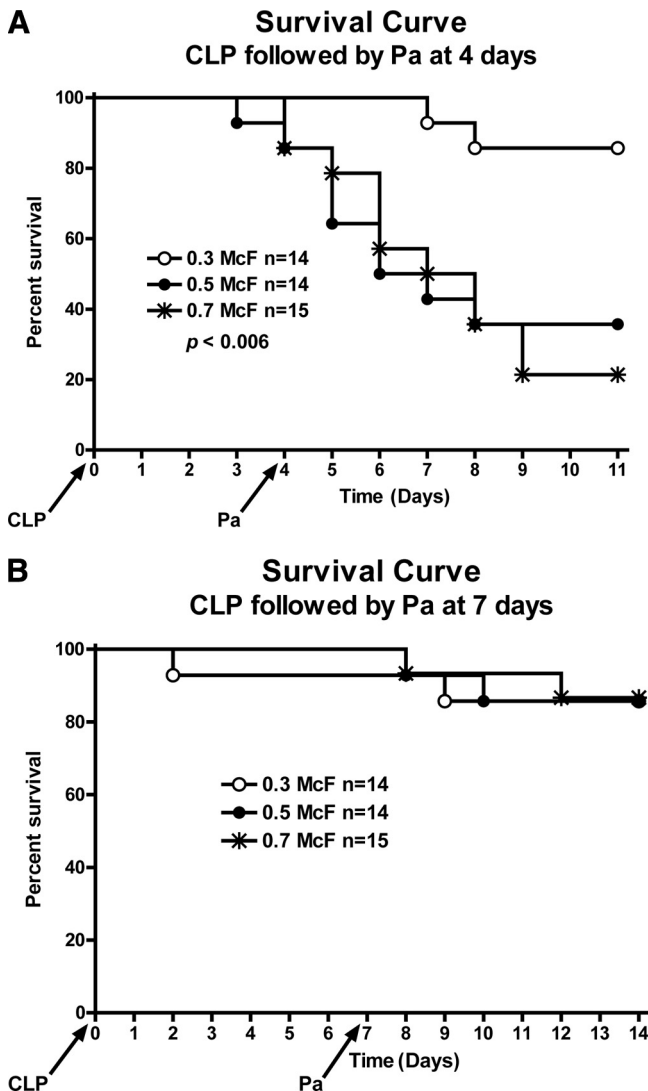


FIG. 3. (A) Animals that underwent secondary pneumonia infection 4 days after CLP showed increasing mortality with increasing doses of *P. aeruginosa* (Pa) ($0.3 A_{600} = 5 \times 10^8$ CFU/ml, $0.5 A_{600} = 1 \times 10^9$ CFU/ml, and $0.7 A_{600} = 3 \times 10^9$ CFU/ml; $P < 0.006$). (B) Animals that underwent secondary pneumonia at 7 days post-CLP showed no significant difference in survival with increasing doses of bacteria. McF, McFarland standard.

increase in the supernatant from animals 7 days post-CLP compared to 4-day animals and a 2-fold increase compared to sham-operated animals (Fig. 2).

Enhanced survival of mice infected with pneumonia 7 days following induction of CLP. Given the significant changes in the immune cell composition of the spleen at selected time points and the exaggerated return of IFN- γ production post-CLP (7 days), the survival differences in animals undergoing secondary infection at 4 and 7 days postinjury were evaluated. Peritonitis induced by CLP with a 30-gauge needle alone or pneumonia induced by *P. aeruginosa* (0.3, 0.5, or 0.7 A_{600}) alone resulted in 85 to 95% survival (data not shown) at 7 days. We then evaluated the survival of mice given *P. aeruginosa* pneumonia at the three different densities administered 4 and

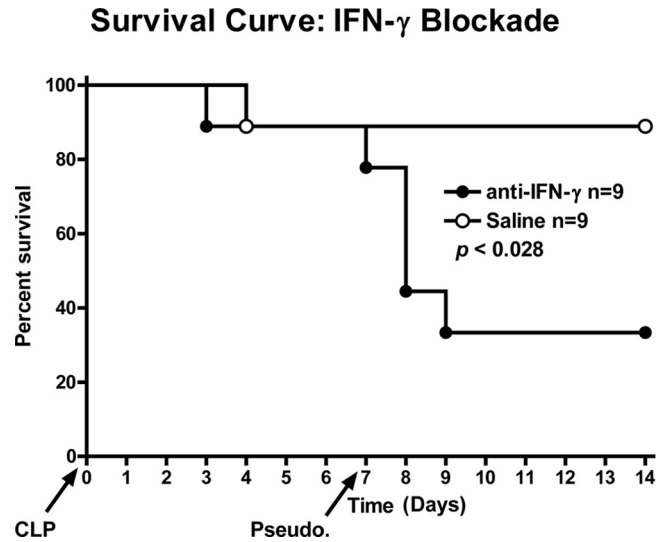


FIG. 4. Animals 7 days post-CLP and under IFN- γ blockade at the time of secondary injury showed increased mortality compared to control animals. $P < 0.028$; $n = 9$ per group. Pseudo, *P. aeruginosa*.

7 days following induction of CLP. Animals receiving CLP followed by the induction of pneumonia at 4 days at $0.3 A_{600}$ density showed an 80% survival at 7 days after induction of pneumonia, $0.5 A_{600}$ resulted in 35% survival, and $0.7 A_{600}$ resulted in 20% survival ($P < 0.006$) (Fig. 3A). Animals infected with pneumonia (all three densities) 7 days after CLP had survival rates similar to those of animals receiving CLP or pneumonia alone (~85%) (Fig. 3B).

IFN- γ blockade prior to secondary injury abrogates survival at 7 days post-CLP. Having found significant differences in IFN- γ production and survival between animals receiving secondary injury at 4 versus 7 days post-CLP, we performed survival experiments looking at the blockade of IFN- γ prior to secondary injury at 7 days. When an IFN- γ -blocking antibody was given just prior to secondary injury, we saw a significant increase in mortality compared to animals receiving an IgG control antibody (33.3% versus 88.9%; $P < 0.028$) (Fig. 4).

Increases in lymphocyte apoptosis following primary and secondary injury. Following septic injury, there is a significant loss of immune cells by apoptosis. Evaluation of apoptosis by flow cytometry demonstrated significant changes in levels of immune cell death following septic injury by both caspase 3 and TUNEL staining. In animals undergoing CLP alone, analysis of T-cell apoptosis at 4 days revealed significant increases in both caspase 3 and TUNEL (18.6 versus 1.9% and 12.0 versus 1.2%, respectively; $P < 0.001$) (Fig. 4). CLP followed by the induction of pneumonia at 4 days (0.5 McFarland standard) had an additive effect, as these animals, when evaluated on day 5 (16 h postpneumonia), had even greater levels of apoptosis (24.8 versus 18.6% [$P < 0.05$] and 19.2 versus 12% [$P < 0.01$]) (Fig. 4). By 7 days post-CLP, there was a reduction of T-cell apoptosis in both single- and double-injury animals, i.e., CLP at 7 days and CLP followed by the induction of pneumonia on day 7. Similar to 4-day animals, double-injury animals at 7 days had an increase in T-cell apoptosis compared to their single-injury counterparts. However, double-injury animals at 7 days compared to 4 days had significantly decreased

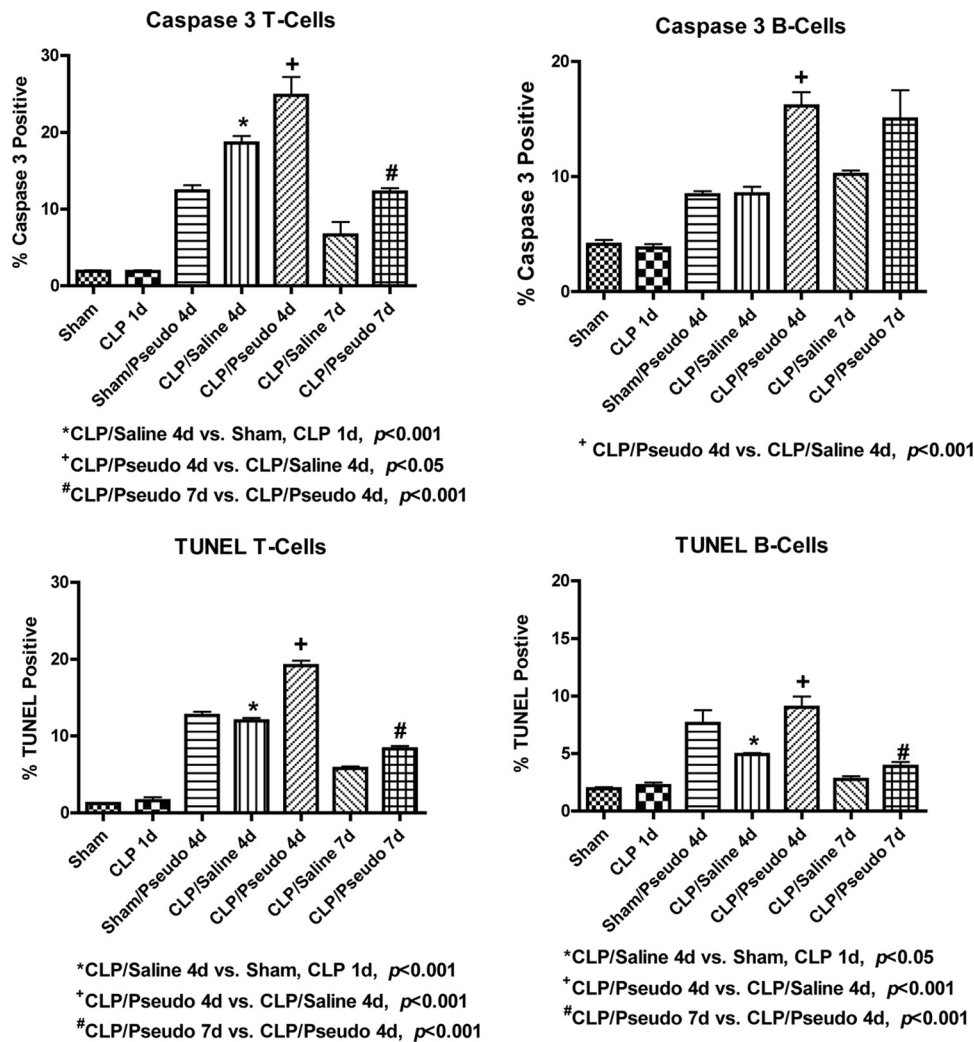


FIG. 5. Following septic injury, there is significant loss of immune cells by apoptosis. In animals that underwent CLP alone, analysis of splenic T-cell apoptosis at 4 days revealed significant increases in both caspase 3 and TUNEL. CLP followed by the induction of pneumonia at 4 days (0.5 McFarland standard) had an additive effect, as these animals, when evaluated on day 5 (16 h after the induction of pneumonia), had even greater levels of apoptosis. By 7 days post-CLP, there was a reduction of T-cell apoptosis. Similar to 4-day animals, double-injury animals at 7 days had an increase in T-cell apoptosis compared to their single-injury counterparts. Double-injury animals at 7 days compared to 4 days had significantly decreased levels of T-cell apoptosis. Analysis of B-cell apoptosis from spleens at 4 days postinjury revealed significant increases in apoptosis in animals that underwent CLP alone. As seen in the T cells, CLP followed by the induction of pneumonia at both 4 and 7 days had an additive effect upon injury-related cell death. $n = 6$ per group. The error bars indicate standard deviations.

levels of T-cell apoptosis (18.6 versus 12.3% and 12.0 versus 8.3%; $P < 0.001$) (Fig. 5).

Analysis of B-cell apoptosis in spleens at 4 days postinjury revealed increases in apoptosis in animals undergoing CLP alone. As seen in the T cells, CLP followed by the induction of pneumonia at both 4 and 7 days had an additive effect upon injury-related cell death. The overall pattern of decreased apoptosis by 7 days in both single- and double-injury animals was also seen in B cells by TUNEL; however, caspase 3 staining revealed similar levels of apoptosis.

Cytokine profiles are dependent on the timing of the secondary injury. Primary sepsis induces a profound production of circulating cytokines, both pro- and anti-inflammatory. Following a second septic insult, evaluation of plasma cytokines revealed differences in cytokine levels that were dependent on

the timing of the secondary injury. Animals undergoing *P. aeruginosa* pneumonia at 4 days post-CLP had very low levels of inflammatory cytokines, including IL-1 α , IL-6, IFN- γ , and G-CSF, and higher levels of the anti-inflammatory cytokine IL-10 16 h after secondary injury (Fig. 6). In contrast, animals infected with pneumonia 7 days post-CLP had significant increases in IL-1 α , IL-6, and G-CSF compared to animals infected with pneumonia at 4 days (880 versus 223 pg/ml, 903 versus 188 pg/ml, and 5,139 versus 1,321 pg/ml, respectively; $P < 0.01$). Seven-day animals also had no increase in IL-10 16 h after the induction of pneumonia (Fig. 6).

Systemic and localized bacterial growth. The immunoparalysis caused by sepsis is associated with a decreased ability of the host to clear bacteria. Given this, we evaluated the ability of the host to clear *P. aeruginosa* from the lungs and blood following

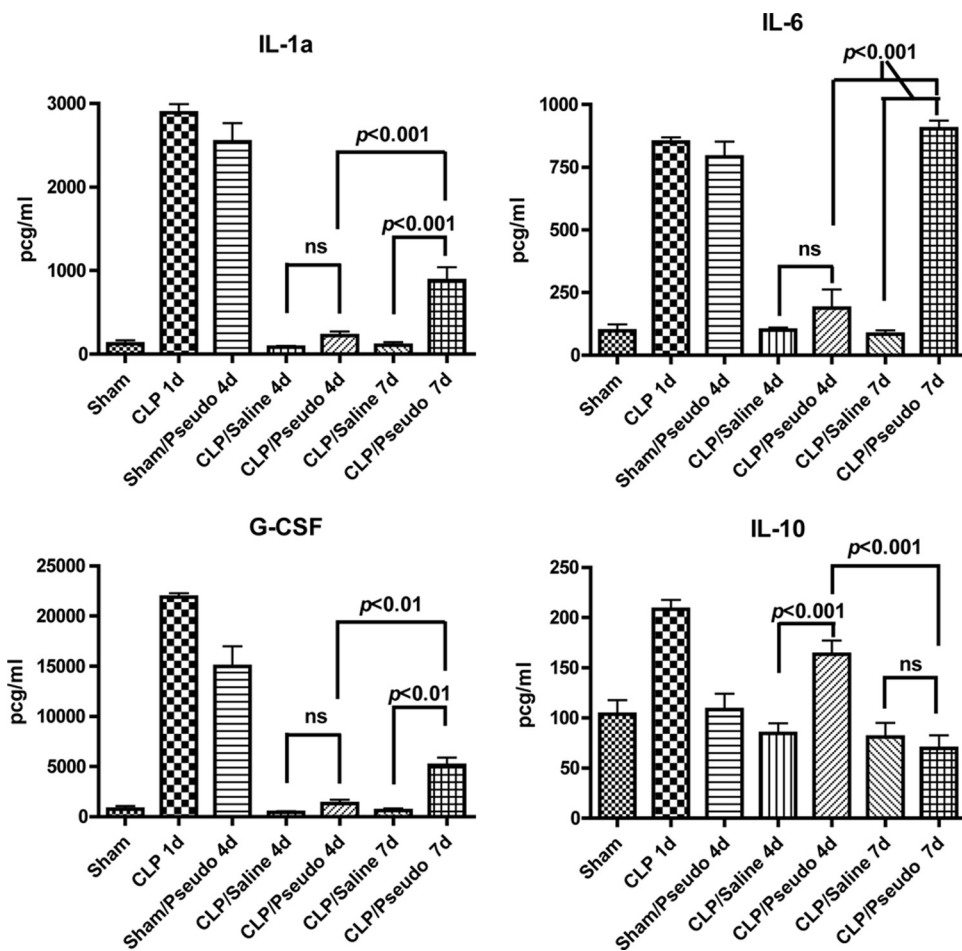


FIG. 6. Following a second septic insult, evaluation of plasma cytokines revealed differences in cytokine levels that were dependent on the timing of the secondary injury. Animals infected with *P. aeruginosa* pneumonia at 4 days post-CLP had very low levels of IL-1α, IL-6, IFN-γ, and G-CSF and higher levels of the anti-inflammatory cytokine IL-10. Animals that were infected with pneumonia 7 days post-CLP had significant increases in IL-1α, IL-6, and G-CSF compared to animals that underwent pneumonia infection at 4 days. Seven-day animals also had no increase in IL-10 16 h after the induction of pneumonia. *n* = 6 per group. The error bars indicate standard deviations. ns, not significant.

single and double injury. Animals undergoing the induction of pneumonia alone had no bacteria recovered from their blood and 2 log₁₀ CFU (100 CFU) recovered by BAL (Fig. 7). Animals undergoing CLP followed by *P. aeruginosa* infection at 4 days were found to have significant increases of bacterial growth in both blood and BAL fluid compared to single-injury animals and animals infected with pneumonia 7 days following CLP (Fig. 7) (*P* < 0.001). Consistent with improvement in survival, animals infected with pneumonia 7 days following CLP had a significant reduction in the bacterial load in both the blood and BAL fluid (*P* < 0.001) (Fig. 7). As expected, no *P. aeruginosa* was recovered from the blood or lungs of animals receiving CLP or sham surgery alone (Fig. 7).

Decreased lung infiltration and consolidation in 7-day animals. Light microscopy examination of lungs from double-injury animals at both 4 and 7 days were scored by a pathologist blinded to the group assignment. Analysis of lung sections revealed significant decreases in lung infiltration and consolidation in animals receiving secondary injury 7 days post-CLP (3.3 ± 0.75 versus 1.9 ± 0.31; *P* = 0.04) (Fig. 8).

Inflammatory cytokine modulation increases survival after secondary injury. Researchers have previously shown the importance of IL-10 as a classic anti-inflammatory cytokine (34). More importantly, it has been reported that the immune modulator AS101, by its direct inhibition of IL-10 production, can increase survival in multiple models of infection, including single-injury CLP (16). Given that immunosuppression, as evidenced by elevated levels of IL-10 coupled with decreased inflammatory cytokine levels, appeared to be a key factor in survival at 4 days post-CLP, we explored the effect of IL-10 inhibition by AS101 or an anti-IL-10 antibody in our two-hit model of sepsis. Mice were given daily injections of AS101 starting 24 h after CLP and continuing through the induction of *P. aeruginosa* pneumonia at 4 days post-CLP. Mice receiving anti-IL-10 antibody were treated on days 1, 3, and 5 following CLP.

Survival. Treatment with AS101 following CLP significantly improved survival following secondary injury (89% versus 54% survival; *P* < 0.017) (Fig. 9A). Treatment with anti-IL-10 antibody following CLP improved survival after

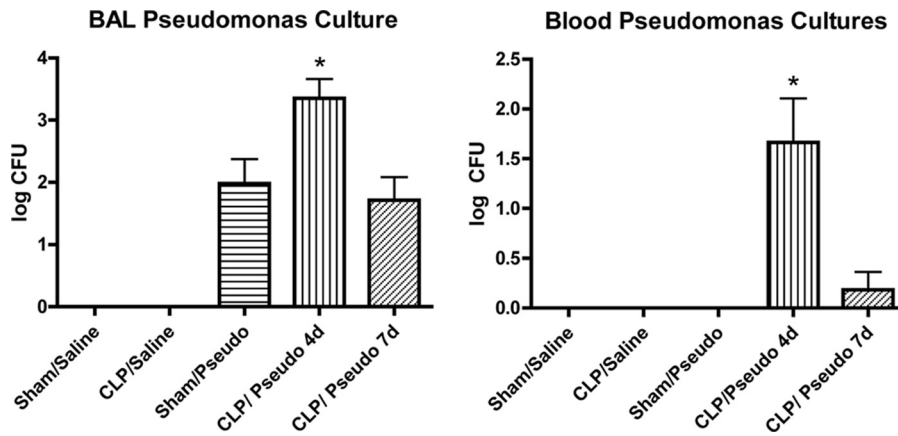


FIG. 7. BAL fluid and blood cultures showed increased growth in animals that underwent secondary pneumonia infection 4 days post-CLP compared to 7-day double-injury or single-injury animals. *, $P < 0.001$. $n = 10$ per group. The error bars indicate standard deviations.

secondary injury similar to that seen in animals treated with AS101 (87% versus 40% survival; $P < 0.011$) (Fig. 9B).

Apoptosis. Analysis of T and B splenocytes at 16 h post-secondary infection revealed a decrease in levels of apoptosis in animals treated with AS101 compared to controls. Staining of splenic T-cell and B-cell subpopulations for

caspase 3 showed decreased levels of apoptosis 16 h post-secondary injury (5.4% versus 9.2% and 5.0% versus 7.5%, respectively; $P > 0.03$) (data not shown).

Cytokine analysis. Analysis of plasma cytokines revealed a significant decrease in IL-10 and a significant increase in IL-1 α and IL-12p40 in animals treated with AS101 (31 pg versus 229

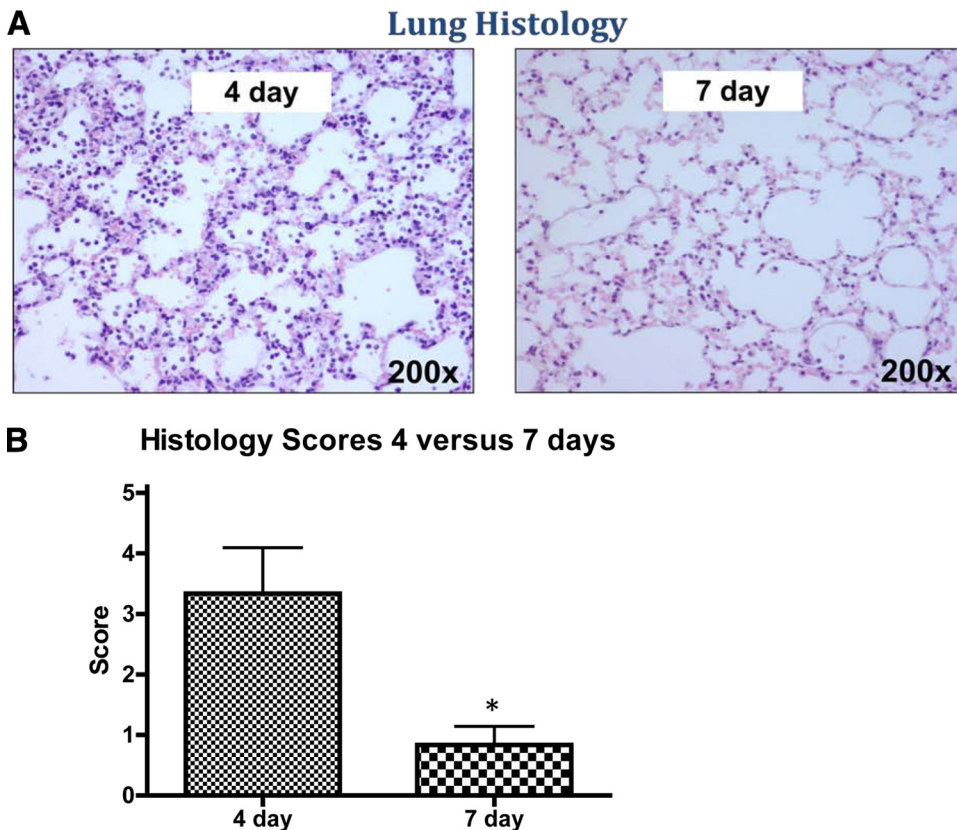


FIG. 8. (A) Light microscopic examination was performed on lung specimens harvested 18 h following the induction of pneumonia. Animals infected with pneumonia 4 days following CLP showed increased consolidation of tissue with extensive polymorphonuclear and mononuclear cell infiltration compared to animals infected with pneumonia 7 days post-CLP. (B) Evaluation of light microscopy by a pathologist blinded to sample identity revealed significantly decreased scores for infiltration and consolidation in animals infected with pneumonia 7 days after CLP. $P < 0.04$; $n = 6$ per group. The error bars indicate standard deviations.

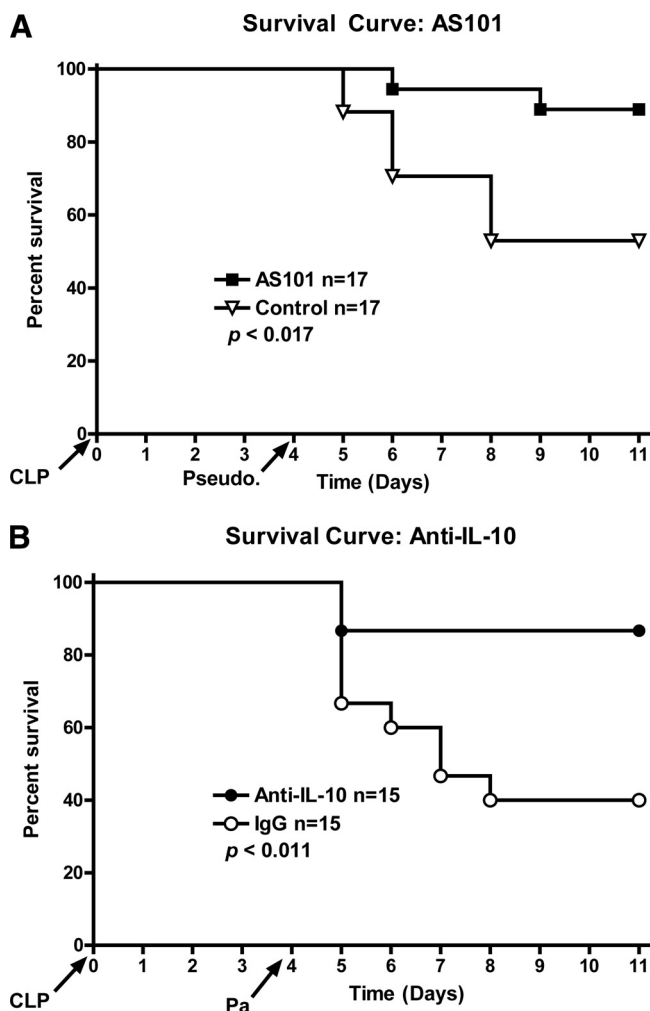


FIG. 9. (A) Treatment with AS101 following CLP significantly improved survival after secondary injury; $P < 0.017$; $n = 17$ per group. (B) Treatment with anti-IL-10 antibody following CLP improved survival after secondary injury similar to that seen in animals treated with AS101. $P < 0.011$; $n = 15$ per group.

pg/ml, 408 pg versus 108 pg/ml, and 291 pg versus 17 pg/ml, respectively; $P < 0.003$) (Fig. 10A). In addition, the ability to restore splenocyte IFN- γ production following IL-10 blockade was examined. Splenocytes from AS101-treated animals harvested 4 days after CLP (just prior to secondary injury) showed a 2.5-fold increase in IFN- γ production compared to untreated CLP animals ($P < 0.001$) (Fig. 10B).

BAL and blood culture. Previous literature had shown that high levels of IL-10 were associated with decreased bacterial clearance and increased spread of infection and that modulation of IL-10 could improve bacterial clearance (15, 16, 34). Given these data, we evaluated BAL fluid and blood cultures from animals that received AS101 and compared them to control animals at 16 h post-secondary injury. Treatment with AS101 resulted in decreased bacterial growth from both BAL fluid and blood compared to animals receiving PBS alone (1.1 versus 2.9 \log_{10} CFU and 0.03 versus 1.6 \log_{10} CFU, respectively; $P < 0.03$) (Fig. 11).

Histology. Light microscopy examination of lungs from double-injury animals treated with AS101 was compared to that of animals treated with vehicle alone. Lungs from control animals revealed increased consolidation, with extensive polymorphonuclear and mononuclear cell infiltration (Fig. 12A). Scoring by a pathologist who was blinded to sample identity on a 0- to 5-point scale revealed significantly increased infiltration and consolidation in the control group (3.3 ± 1.3 versus 1.8 ± 1.03 ; $P < 0.05$) (Fig. 12B).

DISCUSSION

In the current study, we first demonstrated that survival in a clinically relevant two-hit model of sepsis was dependent upon the timing of the secondary injury. This time-dependent survival was associated with a dynamic, evolving immune system that exhibited significantly different phenotypes at 1, 4, and 7 days postinjury. Several important characteristics should be noted. These changes include differences in splenocyte cell composition, cytokine production (plasma and stimulated IFN- γ), and levels of apoptosis. Consistent with previous studies, sepsis (in this case CLP) induced an early inflammatory profile that was dominated by a hyperinflammatory phenotype that, over time, shifted to a profound hypoinflammatory phenotype (10, 27). As evidenced by the current study, this state of immunoparalysis lasts at least 4 days, followed by a partial reconstitution of the immune response by day 7 post-CLP. As a result of this reconstitution at the 7-day time point, mice challenged with pneumonia at 7 days had improved survival compared to that for mice challenged at 4 days. This increased survival at 7 days post-CLP was associated with significantly decreased pulmonary and circulating bacterial counts. Furthermore, we demonstrated the ability to alter the immune response within the 4-day hypoinflammatory window. Through the administration of the immune modulator AS101, we were able to alter the immune response by decreasing IL-10, increasing IL-1 α and IL-12p40, and restoring the ability of splenocytes to make IFN- γ . These changes in the immune profile were associated with decreased growth of *P. aeruginosa* from the lungs and blood of treated animals. Finally, AS101 therapy following CLP led to significantly increased survival when the host was subjected to a secondary injury. This improved survival benefit was also seen in animals treated with an IL-10-blocking antibody.

Evaluation and characterization of the complex immune response in sepsis has led to a better understanding of both the hyper- and hypoinflammatory states. Many researchers now postulate that the signals for both the hyper- and hypoinflammatory responses begin concurrently in the early stages of sepsis; however, the timing and mechanisms that determine whether the host's phenotype is hyperinflammatory or immunosuppressed are less well understood (10, 30). The current study again establishes that risk of infection is increased when the host is in a state of relative anergy or immunosuppression. More importantly, the current findings provide evidence that the severity of and survival after a secondary infection depend upon the time of onset and the immunologic state of the host. The relative abundances of pro- and anti-inflammatory cytokines are thought to have a critical effect on how immune effector cells respond to infection. During the hyperinflamma-

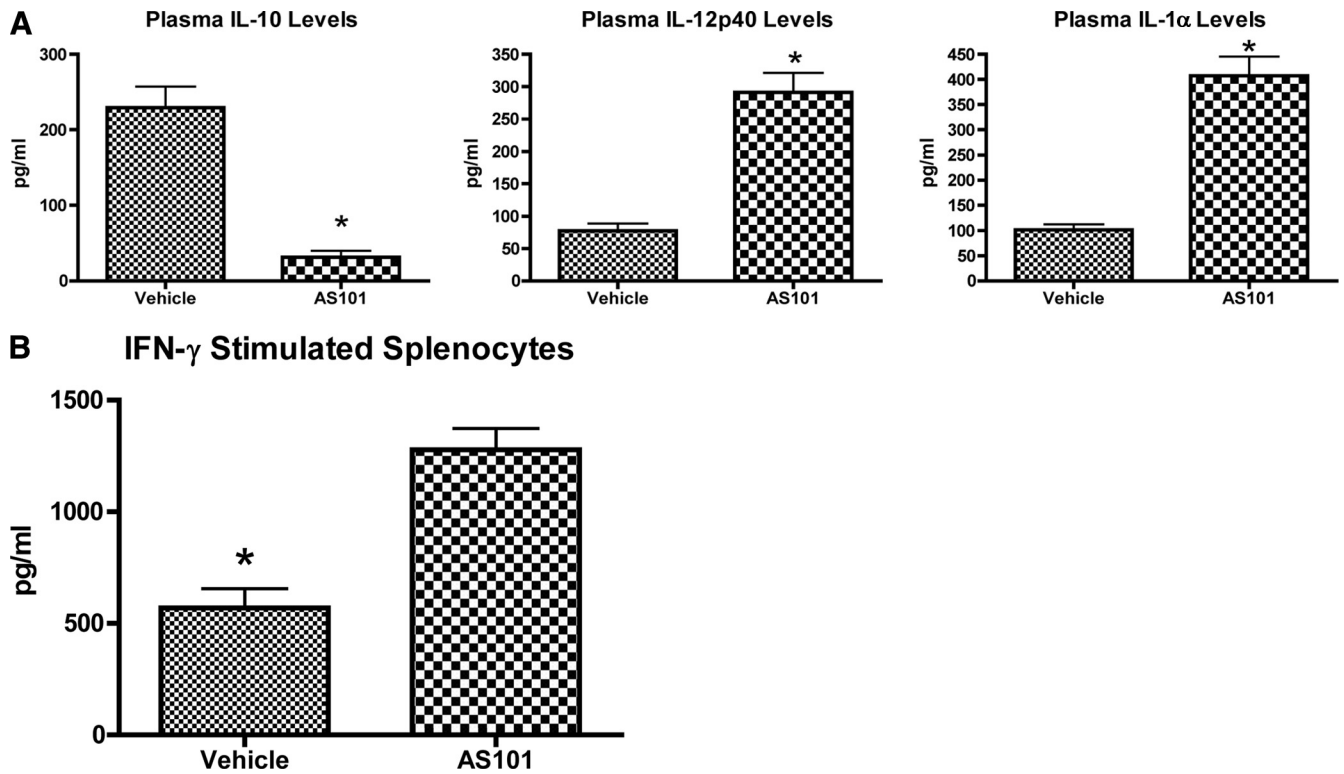


FIG. 10. (A) Blood collected 18 h following the induction of pneumonia showed significantly decreased plasma IL-10 and significantly increased IL-1 α and IL-12p40 levels in animals treated with AS101. *, $P < 0.003$. (B) Splenocytes from animals treated with AS101 showed significant increases in IFN- γ production compared to controls 4 days post-CLP; *, $P < 0.001$. $n = 6$ per group. The error bars indicate standard deviations.

tory-dominated stage of the septic response, cytokines such as IL-6, IFN- γ , TNF, and IL-12, have been shown to mediate bacterial clearance and upregulate immune effector cell function (18, 23). During the immunosuppressive phase of sepsis, cytokines such as IL-4 and IL-10 lead to suppression of macrophage cytokine secretion and decreased activity of macrophage and neutrophil functions (4, 20, 34). The current study has demonstrated that survival after a secondary injury is dependent on the ability of the host to mount an effective immune response. Four days following the initial injury, animals are unable to produce inflammatory cytokines in response to a secondary insult, including failure of stimulated splenocytes to

produce IFN- γ . However, when that insult is given at 7 days after the initial injury, the host is able to produce high enough levels of inflammatory cytokines to overcome the secondary infection. This need for an inflammatory response to secondary injury to improve survival is also supported by our survival study showing that blocking of IFN- γ just prior to the secondary injury removes the survival benefit seen at 7 days post-CLP. The importance of IFN- γ production in response to infection has been demonstrated by multiple authors. Hotchkiss et al. showed in adoptive transfer experiments that increased survival of animals receiving necrotic cells following CLP was due to the upregulation of IFN- γ , as this survival benefit was lost in

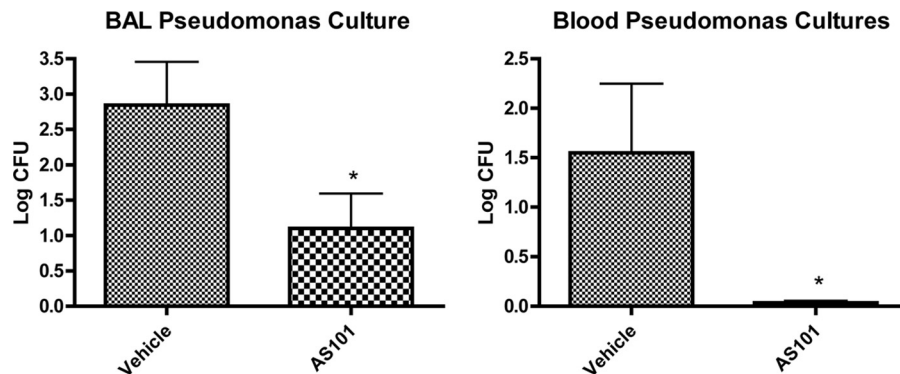


FIG. 11. BAL fluid and blood cultures showed decreased growth with AS101 treatment versus vehicle. *, $P < 0.03$. $n = 6$ per group. The error bars indicate standard deviations.

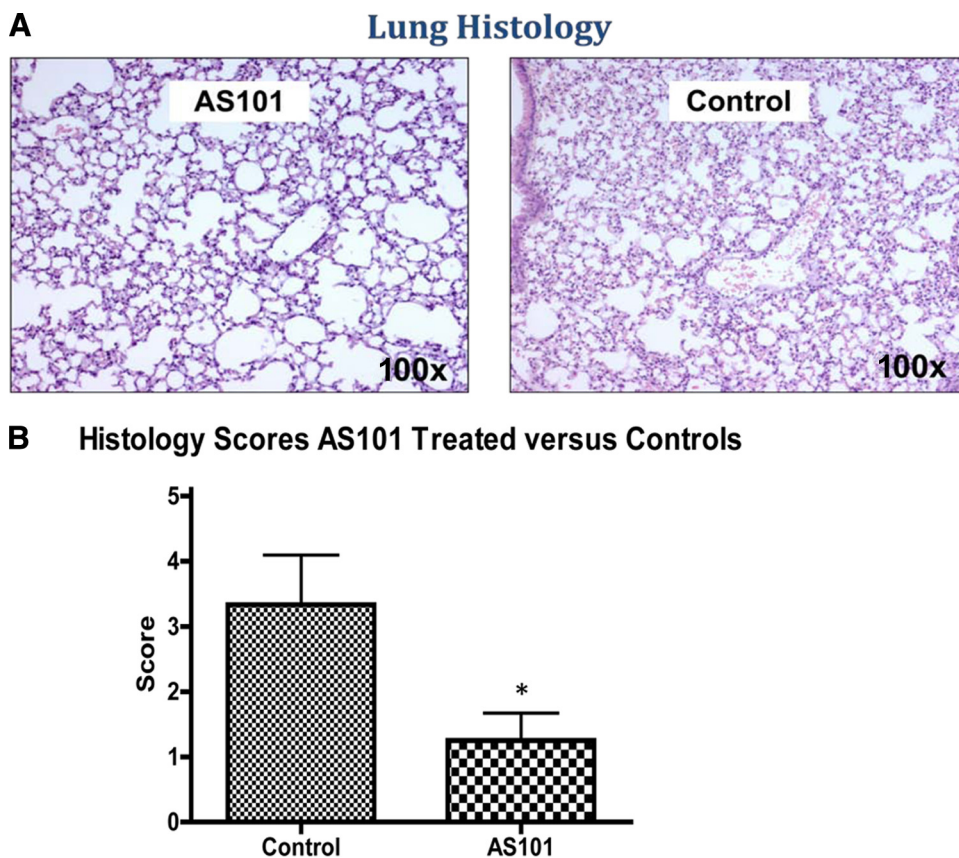


FIG. 12. (A) Light microscopic examination was performed on lung specimens harvested 18 h following the induction of pneumonia. Control animals showed increased consolidation of tissue with extensive polymorphonuclear and mononuclear cell infiltration. (B) Evaluation of light microscopy by a pathologist blinded to sample identity revealed significantly decreased scores for infiltration and consolidation in animals treated with AS101. *, $P < 0.05$; $n = 6$ per group. The error bars indicate standard deviations.

IFN- γ knockout animals or in animals treated with an anti-IFN- γ antibody (11). The clinical importance of IFN- γ was described by Docke et al., who showed that treatment with IFN- γ in septic patients restored monocyte activation and improved the clinical course (3).

In addition to changes in cytokine production, multiple research studies of animal models and patients dying from sepsis have demonstrated profound loss of immune effector cells by apoptosis (9, 10, 13, 36). This loss of lymphocytes and dendritic cells is thought to be partially responsible for the profound immunosuppression seen in sepsis (6, 9, 10). Recovery of specific immune cell populations in sepsis is likely correlated with improved outcome. However, the mechanisms by which these cellular compartments recover in sepsis are poorly understood. In this study, we demonstrated that there is a continued loss of lymphocytes following the initial injury; however, the innate cell populations in the spleen revealed that neutrophils and NK cells were increased over baseline by 4 days. Furthermore, dendritic cells, although still depleted at 4 days, were 2-fold above baseline levels by 7 days. We speculate that this return of innate cells, particularly dendritic cells, may be a global marker of immune status and correlated with the hyperinflammatory, pro-survival response seen in animals undergoing secondary injury at 7 days. Future studies, including depleting and/or adoptive transfer of

these cell types, may further characterize the role these innate cells play in determining the immune profile of the host.

Our hypothesis that immunosuppression at 4 days is a significant contributor to decreased survival when faced with a secondary bacterial challenge is further supported by results showing that blocking of IL-10 by AS101 improves the outcome. IL-10 has been shown by others to be an important mediator of immune function and is known to downregulate monocyte and neutrophil functions, as well as to decrease inflammatory cytokine production by T cells (33, 34). Consistent with work by Kalechman et al., who showed that administration of AS101 at least 6 h post-CLP improved survival and immune cell function, the present work showed that blockage of IL-10 by AS101 is equally efficacious in a two-hit model (16). This finding is particularly important because in many intensive-care units the majority of sepsis-related deaths are due to secondary nosocomial infections. However, these results are different than those suggested by Murphey and Sherwood, who showed that mice that underwent anti-IL-10 treatment or IL-10 knockout mice did not have improved bacterial clearance or survival in a two-hit model (24). Given these disparate findings, we evaluated survival in our model using an anti-IL-10 antibody in order to show that the primary survival benefit of AS101 treatment was its ability to downregulate

IL-10. As a result, our model showed improved survival in animals treated with anti-IL-10 antibody, similar to what was observed in AS101-treated animals. In addition, there are several differences between the model described here and the model used by Murphey and Sherwood (24). First, Murphey and Sherwood used a primary CLP injury with a mortality of around 50% compared to our study, in which CLP alone produced 90% survival. Next, in their study, intravenous *Pseudomonas* alone led to 75% mortality, whereas our secondary injury, when given alone intranasally, produced 90% survival. Finally, Murphey and Sherwood gave the second injury 5 days post-CLP (24). These differences in study design, particularly the large disparities in survival after a single injury and the route of administration, would likely lead to changes in the hyper/hypoinflammatory axis. It is also important to note that AS101 has other effects that may account for some of the findings described here. Strassman et al., who evaluated the effect of AS101 on mouse peritoneal macrophages, found that it inhibits the production of IL-10 at the mRNA level and also augments levels of IL-1 α and TNF- α (35). Our work found significant alterations in IL-1 α but no differences in TNF- α levels.

In conclusion, our findings provide further understanding of the development and recovery from sepsis-induced immunosuppression. Given the continued impact of sepsis syndromes and risks of secondary infection on morbidity and mortality, it is important to continue to develop therapeutic strategies that modulate the hyperinflammatory/hypoinflammatory axis. Additionally, a better understanding of the host's current immune status may be useful when determining specific goal-oriented therapies. Modulation of IL-10 may be one specific target for drug development to favorably alter the host immune status.

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