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Pulmonary contusion is associated with TLR4 upregulation and decreased susceptibility to *Pseudomonas pneumonia* in a mouse model

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Abstract

Pulmonary contusion is a major cause of respiratory failure in trauma patients. This injury frequently leads to immune suppression and infectious complications such as pneumonia. The mechanism whereby trauma leads to an immune suppressed state is poorly understood. To further study this phenomenon, we developed an animal model of pulmonary contusion complicated by pneumonia and assessed the effect of pulmonary contusion and pneumonia on toll-like receptor expression in alveolar macrophages. Using a mouse model, pulmonary contusion (PC) was induced on the right lung and pneumonia was induced with *Pseudomonas aeruginosa* (Pa) injected intratracheally 48 hours after injury. Susceptibility to pneumonia was assessed by mortality at seven days. Uninjured animals were used as controls. Bronchoalveolar lavage (BAL) fluid and blood were assayed 48 hours after injury and 24 hours after Pa instillation to look at markers of systemic inflammation. Toll-like receptor (TLR) expression in the initial inflammatory response was analyzed by flow cytometry. Unexpectedly, injured animals subjected to intratracheal injection of Pa at 48h after pulmonary contusion demonstrated increased survival compared to uninjured animals. BAL cytokine expression was increased significantly after Pa administration but not after PC alone. TLR4 expression on alveolar macrophages was significantly elevated in the injured group compared to sham but not in neutrophils. Animals subjected to PC are more resistant to mortality from infection with Pa and display an enhanced cytokine response when subsequently subjected to Pa. Increased expression of TLR4 on alveolar macrophages and enhanced innate immunity is a possible mechanism of increased cytokine production and decreased susceptibility to pneumonia.

Introduction

Pulmonary contusion (PC) is a common injury in patients with blunt trauma. PC is present in 27% of patients with multiple injuries(1). In a recent meta-analysis, 10% of patients with blunt chest wall trauma developed pneumonia and the development of pneumonia was associated with a 5-fold increase in mortality(2) and a significant increase in hospital length-of-stay.(3) A separate study demonstrated that the presence of PC is an independent risk factor for mortality in trauma patients.(4)

Animal models have demonstrated that PC is associated with acute lung injury as evidenced by decreased oxygenation, pulmonary edema, increased pulmonary vascular resistance, poor

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pulmonary compliance and surfactant dysfunction.(5–7) PC has also been shown to increase susceptibility to an inflammatory stimulus. In a study of PC and gastric aspiration, lung injury was increased in the presence of both PC and aspiration compared to PC alone.(8) In another study, intratracheal injection of LPS enhanced the inflammatory response leading to increased production of cytokines and chemokines. (9) Alveolar macrophages from injured animals have recently been shown to produce an increased amount of cytokines compared to those from uninjured animals.(10) Toll-like receptors (TLR) are important in the response to PC(11) and pneumonia(12). TLR-4 is strongly implicated as a possible mechanism for the increased cytokine production in response to LPS after injury, but it is not clear why this response is seen.

These studies demonstrate that PC increases susceptibility to acute lung injury, presumably a pro-inflammatory state. However, it is not clear whether an increase in local inflammation enhances the ability of the animal to clear a bacterial challenge. The enhanced cytokine and inflammatory response might impart an increased resistance to infection, or it may simply represent a dysfunctional immune state that does not affect the organism's ability to control a pathogen. A previous study demonstrated that alveolar macrophage depletion improved 24-hour mortality in a model of *Pseudomonas pneumonia*, but worsened longer-term mortality.(13) This study suggests that alveolar macrophages contribute to early acute lung injury, but are necessary to ultimately clear a bacterial infection in the lungs. In order to better understand the relationship between local inflammation and resistance to infection, we conducted a study to evaluate the consequences of pulmonary contusion on the immune response in a mouse model of PC by assessing mortality after an infectious challenge. To determine whether TLR-4 is potentially involved in this response, we assessed the effect of the injury on TLR-4 expression in alveolar macrophages.

Materials and Methods

Animals

Male ND4 mice, 6–8 weeks old and weighing ~30–35 g, obtained from Harlan Sprague Dawley (Indianapolis, IN), were housed in a room with an ambient temperature of $22\pm1^{\circ}\text{C}$ on a 12h light: dark cycle. Animals were allowed 7 days to acclimate and given *ad libitum* access to standard chow and tap water. The current study was conducted in accordance with the institutional guidelines for humane treatment of animals and was approved by the Institutional Animal care and Use Committee of Washington University School of Medicine, St. Louis. In all experiments, animals were sacrificed by cervical dislocation under anesthesia at the end of study.

Trauma model: Pulmonary Contusion

Mice were anesthetized under 5% isoflurane and then maintained at 2% isoflurane for induction of general anesthesia. Animals were laid in a prone position and injury was induced on the right lung by a 120mg projectile launched from a spring mechanism (velocity 55m/s) 2cm from the impact zone on the mouse. Immediately after the injury mice consistently experience a period of apnea that last 2–5 seconds. After the injury, mice received buprenorphine hydrochloride (0.1mg/kg) subcutaneously for pain relief and were allowed to recover. Sham animals were anesthetized only without being subjected to trauma.

Arterial blood was collected from the coeliac artery 48 hours post injury to estimate the circulation of the gases through the blood stream compared to shams. Whole blood was drawn in heparinized syringe and then collected in heparin tubes on ice to be analyzed by Stat Profile pHox Plus C Analyzer (Nova Biomedical, Waltham, MA) within half an hour. The gases were obtained while mice were breathing 98% oxygen and 2% isoflurane.

Secondary Infection model: Intra-tracheal instillation of Bacteria

For inducing secondary infection, *Pseudomonas aeruginosa* was administered intra-tracheally 48 hours after primary injury. *Pseudomonas aeruginosa* (ATCC#27853) was grown on blood agar (sheep blood plates) stored at 4°C and brought to room temp to sub. A sample of bacteria was taken 24 hours before use and put into a 50mL conical vial containing 10mL of Tryptic Soy Broth (TSB). The solution was placed in a rotating incubator overnight. The cells were spun at 4000 rpm for 5 minutes and the bacteria were resuspended in 10mL of 0.9% NaCl to remove the TSB from solution. The supernatant was discarded and the cells were resuspended in another 5mL solution of 0.9% NaCl. The diluted bacteria were then added slowly (drop by drop) to a clean 5mL tube of 0.9% NaCl until a reading of OD 0.3 was reached on a turbidity meter.

Under aseptic conditions, the trachea was exposed surgically on the ventral side of the neck. 30ul of *Pseudomonas* was administered via a needle inserted through the tracheal wall into the lumen just below the larynx. Animals were given pain reliever as stated previously and allowed to recover.

Delayed-Type Hypersensitivity model

For the sensitization phase, mice received 100uL of 10mM 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) subcutaneously. Two days later mice were subjected to PC using the model described previously. On the fourth day, animals were challenged with 40uL TNBS injected into the left and 40uL of PBS into the right footpad. On the fifth day, mice were sacrificed and footpads were measured for edema. Measurements were recorded in micrometers ($\pm$ SE) and reflect the difference between the left (TNBS challenge) and right footpad (PBS challenge). These values demonstrate the immune response to TNBS. (14)

Broncho-alveolar lavage

Following euthanasia, a tracheotomy was performed and a cannula inserted and tied. 1 ml of sterile saline was infused into the lung and the fluid was drawn by gentle suction for BAL cell counts and the rest were centrifuged and samples stored as aliquots at -80°C until assayed.

Cytokine analysis and WBC count

Animals were sacrificed at 24 hours post injury (Sham; PC) as well as 24 hours post infection (Sham + Pa; PC + Pa) for collecting blood and BAL fluid for cytokine analysis and WBC counts. Blood was drawn from the heart via cardiac puncture under anesthesia to measure WBC count (Hemavet in Siteman Cancer Center at Washington University in Saint Louis). The rest of the blood was centrifuged at $250 \times g$ for 10 min and plasma samples were frozen for later analysis of cytokines. Plasma and BAL IL-6, IL-10 and TNF α levels were determined using ELISA kits (Invitrogen Corp., San Diego, CA) according to the manufacturer's instruction.

Flow Cytometric analysis for TLR2 and TLR4 expression

At 24 hours post-infection, contused and uninjured lungs were harvested, minced and homogenized with a sterile 70- μ m nylon mesh (Becton Dickinson) in a media containing Collagenase and Hyaluronidase (Worthington) and DNase for 1hr at 37°C. Cells were then washed, counted and stained with GR-1, F4/80, and either TLR-2 or TLR-4 antibodies (Imgenex, San Diego, CA; Monoclonal antibody against TLR2 (Toll-like receptor 2)/CD282 FITC conjugate; Monoclonal Antibody to human TLR4 (Toll-like receptor 4)/CD284 FITC Conjugate), and acquired on a FACScan flow cytometer (BD).

Statistical Analysis

Data was analyzed using Graph Pad prism (v 4.00 San Diego, CA) and presented as the mean $\pm$ SEM. One-way Anova was used to compare three or more groups followed by Tukey's multiple comparison mean values. T-test was used for comparison between the means of two groups. A value of $P < 0.05$ was considered significant. Flow files were analyzed using Winlist 6.0 (verity), and statistical analysis was performed in graph Pad Prism 4.

Results

Pulmonary Contusion

Gross inspection of the lung after injury revealed considerable hemorrhage within the right lung and chest cavity [Fig. 1]. After induction of pulmonary contusion, the severity of the injury was assessed by measurement of blood gases. In whole blood (Sham $n=10$; PC $n=13$) collected 48 hours after injury, PO₂ was significantly decreased in the PC group compared to the sham ($p < 0.05$.) However, there was no difference in the pCO₂ between the 2 groups [Table 1].

Survival

Survival after *Pseudomonas aeruginosa* instillation was measured over a seven day time course and is shown in figure 2. There was a significant increase in survival in animals with the injury ($n=15$) compared to sham ($n=15$) at 48 hours after contusion (73% v 20%, $p=0.001$.) No mice died in the control group of injured animals injected with saline instead of Pa ($n=7$.)

Delayed-type hypersensitivity response

In order to assess the effects of PC on the systemic immune system, the delayed-type hypersensitivity response was assayed ($n=25$.) Animals that were not sensitized but were challenged with TNBS ($n=5$) and animals that received a sham injury ($n=15$) were used as controls and compared to the PC group. Footpads in the DTH mice were measured to evaluate the difference in immune response between the sham and the PC group. Footpad measurements showed that the pulmonary contusion and sham animals varied significantly in their response to TNBS. The difference between the TNBS-injected footpad and the saline-injected footpad demonstrated more swelling in the TNBS-injected footpad of PC animals as compared to sham groups ($p < 0.001$) (Fig. 3).

Cytokines and Cytology

In order to assess the response to injury, blood and BAL fluid were collected from animals at 48 hours after injury (PC) using uninjured animals as a control (sham.) To assess the subsequent response to infection blood and BAL fluid were collected from injured (PC+Pa) and uninjured animals (sham+Pa) at 24 hours after infection. Blood and BAL fluid were assayed for TNF-alpha and IL-6 as well as cytology. These experiments consisted of four groups: sham ($n=5$), PC ($n=5$), sham + Pa ($n=6$) and PC + Pa ($n=10$).

Blood lymphocyte counts were not significantly different in PC vs. sham alone after the primary injury but lymphocyte counts dropped dramatically in the sham + Pa group 24 hours after infection, compared to sham alone. No significant change was observed between PC + Pa and PC alone; however, the decrease in blood lymphocytes in the sham + Pa group was significant when compared to PC + Pa ($p < 0.05$) [Fig. 4A]. Neutrophil circulation in the blood was also measured 48 hours after contusion and then again at 24 hours after Pa administration. PC group was found to have higher counts of neutrophils 48 hours after

contusion, but the difference was not significant compared to sham. Neutrophil counts were significantly elevated in the PC + Pa group vs. sham + Pa ($p < 0.05$) at 24 hours after infection, although there was minimal change in neutrophil count from the pre-infection group [Fig. 4B]. Neutrophils were also found at significantly greater numbers 24 hours after pneumonia in the BAL fluid of contused mice than in their sham counterparts ($p = 0.004$) [Fig. 4C].

Local levels of TNF- α and IL-6 recovered in the BAL fluid were not increased 48 hours after contusion as compared to the shams. Interestingly, 24 hours after Pa administration, a considerable and significant increase was noted in the PC + Pa mice of both TNF α and IL-6 ($p < 0.001$) when compared to the sham animals that received Pa [fig. 5A and B]. IL-6 in blood was elevated in the PC group only after Pa infection but not before [Fig. 5C].

Flow Cytometry

Flow cytometry was used to assess TLR2 and TLR4 expression on cells from BAL fluid in animals subjected to PC ($n = 9$) using uninjured animals as control ($n = 6$). Cells were analyzed further based on GR-1 and F4/80 expression to differentiate neutrophils from macrophages. In the assays of differential expression on GR-1⁺ F4/80⁻ cells, there were significantly more cells from PC mice that stained positive for TLR-2 than control. [Fig. 6] There was no difference in TLR-4 presentation (data not shown). In F4/80⁺ cells, significantly more cells were positive for TLR-4 in PC mice than control (Fig. 4), while there was no difference in TLR-2 presentation (data not shown). Interestingly, among F4/80⁺ cells there appeared a GR-1^{hi} subgroup in PC mice that was nearly absent in control animals [Fig. 7]. Further analysis with and without this subgroup showed similar significant difference in TLR-4 expression.

Discussion

In severely-injured patients, the inflammatory state leads to acute lung injury as well as an apparent increase in susceptibility to infection.(15) This study demonstrates that in a model of pulmonary contusion, injured animals are more resistant to mortality from infection when subjected to trauma. Previous studies in rodent models of pulmonary contusion have shown that animals, like patients, develop evidence of acute lung injury. However, the effect of the pro-inflammatory state on susceptibility to infection has not been studied extensively. In a mouse model of burn injury, mice were less susceptible to intraperitoneal infection.(16) Other models of trauma followed by secondary injury with either intratracheal LPS or simulated aspiration have demonstrated increased mortality due to the inflammatory effects, without producing obvious pneumonia or sepsis.(8, 9)

In the present study, the heightened systemic immune response to an infectious challenge was confirmed by the increased DTH response in these animals. This finding suggests that pulmonary contusion induces a systemic immune response, and that the animals' resistance to pneumonia is not related simply to local mechanical factors. The DTH response is a crude measurement of systemic immune function and relies on both the innate immune system for antigen presentation and the acquired immune system for T-cell proliferation, cytokine production, and other effector function. While the DTH response provides no information about the mechanisms by which the immune response is enhanced, it does confirm that pulmonary contusion has an effect to enhance overall global immunity.

Although levels of TNF- α and IL-6 were not significantly different after PC when assessed at the time of infectious challenge, the subsequent response to administration of Pa led to marked increase in cytokine production. This enhanced response is a possible cause of the decreased mortality seen in the injured animals and coincides with increased expression of

TLR-4 on alveolar macrophages. While previous studies found that both cytokine response(9) and neutrophil recruitment(17) correlate with acute lung injury, our study demonstrates that this response may enhance the ability of the organism to fight an actual infectious challenge. Both acute lung injury and susceptibility to infection seem to be related to the robustness of the cytokine response and understanding the balance between these two effects will be crucial to determining the physiologic response to trauma.

The presence of the GR-1⁺ sub-population of the F4/80⁺ cells was unexpected. This sub-population of cells was only present in the injured animals. It is possible that this group of cells represents “inflammatory monocytes” described elsewhere.(18–20) As this population of cells was not present in the uninjured animals, a direct comparison of TLR expression on this sub-population between injured and uninjured animals was not possible. These cells may play a role in the protective effect of injury, but the limitations of the current study do not allow us to make such an assumption. However, the effect of these cells may provide a useful area of study in the future.

While an enhanced immune response likely occurs in humans in response to particular injuries, an overall immune suppressed state predominates in the most severely injured patients.(15) Because patients receive aggressive resuscitation and intensive care, they may survive more severe injuries than what can feasibly be modeled in a rodent. The severity of the injury in this model may not be sufficient to suppress the immune response.

Furthermore, this study demonstrates an enhanced innate immune response represented by enhanced cytokine production and changes in alveolar macrophages. Though the enhanced DTH response suggests that the adaptive immune response was not blunted, the enhanced innate response may mask a suppression of the adaptive response. Also, the local immune response to pulmonary contusion is not well described in humans and there is no evidence that human alveolar macrophages are affected in the same manner as those in the mouse. Despite these limitations, this model can potentially replicate one of the most common infectious complications of pulmonary contusion and severe trauma.

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Figure 1.
Lungs after pulmonary contusion showing intraparenchymal hemorrhage of the right lung

Figure 2.

Survival curve of ND4 mice infected with *Pseudomonas aeruginosa* by the intratracheal route, 48 hours after primary injury. Mortality rate in Sham + Pa group was significantly higher than the PC + pa group ($p=0.0012$); PC- Pulmonary contusion, Pa - *Pseudomonas aeruginosa*, NS- Normal saline

Figure 3.

Delayed Type hypersensitivity response. The difference between footpad size (left and right) was recorded for all three groups. Edema in the pulmonary contused group was significantly elevated compared to both control groups (* $P < 0.001$).

Figure 4.

Blood lymphocytes and neutrophils were measured at two time points: 24h after injury and 24h post infection. **A.** Comparison of Blood lymphocytes between sham + Pa group vs. sham alone (* $P < 0.05$); comparison between PC + Pa group compared to Sham + pa group (# $P < 0.05$); **B.** comparison of Blood Neutrophils between PC + Pa group compared to Sham + pa group (* $P < 0.05$) **C.** BAL neutrophils were measured only 24h after infection (* $P = 0.004$); PC- Pulmonary contusion, Pa- Pseudomonas Aeruginosa

Figure 5.

Cytokine analysis performed at two time points in blood and bronchoalveolar fluid, 24h after injury and 24h post infection. **A.** TNF- α and **B.** IL-6 were measured in BAL with comparison between sham + Pa and PC + pa group; PC and PC + Pa group. * $P < 0.001$ for both **A** and **B** (One-way ANOVA + Tukey). **C.** Comparison of blood IL-6 between PC vs. PC + Pa (* $P < 0.05$ using One-way ANOVA + Tukey)

Figure 6.

Percent of surface TLR 4⁺ and TLR 2⁺ expression on macrophages and granulocytes in shams and pulmonary contused mice. Lung tissues were harvested, homogenized and stained using methods described. **A.** % of GR-1⁺ expressing TLR2 was significantly higher in the PC group vs. Sham (*p=0.0038 using unpaired t-test.) **B.** There was no difference in % of F4/80⁺ expressing TLR2 in the PC group vs. Sham. **C.** There was no difference in % of GR-1⁺ expressing TLR4 in the PC group vs. Sham. **D.** % of F4/80⁺ expressing TLR4 was significantly elevated in the PC group vs. Sham (*p<0.0001 using unpaired t-test).

Figure 7.

Flow cytometric analysis of lung cells. A. Dot-plot histogram of GR-1 x F4/80 for a control animal. B. Dot-plot histogram of GR-1 x F4/80 for a PC animal. The F4/80⁺ GR-1^{hi} population in the circle appears in PC mice but not control mice. Whether this population is included in the analysis f4/80⁺ cells are still significantly more positive for TLR-4 (PC: CTL, 40.97 ± 2.215 ; 10.20 ± 0.8688 $p < 0.0001$ including GR-1^{hi} vs 40.28 ± 2.271 ; 8.183 ± 0.7246 $p < 0.0001$ without GR-1^{hi}). C and D. The filled grey region represents Isotype control, the thin line represents a sample control animal, the thick line represents a sample PC animal. C. F4/80⁺ cells measured for TLR-4 (left) and TLR-2 (right). D. GR-1⁺ F4/80⁻ cells measured for TLR-4 (left) and TLR-2 (right).

Table 1
Arterial blood gas data obtained at 48 hours after injury compared to sham

PO₂ content was significantly lower in the PC group compared to sham(*P<0.0272), Hemoglobin and hematocrit values were significantly elevated in the PC group compared to sham; *P<0.0105 for Hb, *p<0.0067 for hematocrit

Groups/n	Sham (n=10)	Pulmonary contusion (n=13)
Analyte	Mean ± SEM	Mean ± SEM
pH	7.37±0.02	7.33±0.01
PCO ₂ (mmHg)	33.24±1.85	36.95±0.99
PO ₂ (mmHg)	487.54±20.17	414.58±21.99*
Hb (g/dL)	12.87±0.22	13.59±0.15*
Hct (%)	38.6±0.62	40.85±0.45*