Published in final edited form as:

Ann Surg. 2012 January ; 255(1): 158–164. doi:10.1097/SLA.0b013e31823433ca.

Mechanisms of Indirect Acute Lung Injury: A Novel Role for the Co-Inhibitory Receptor, Programmed Death-1 (PD-1)

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Abstract

Objective—To determine the contribution of PD-1 in the morbidity and mortality associated with the development of indirect-acute lung injury

Summary Background Data—The immune cell interaction(s) leading to indirect-acute lung injury are not completely understood. In this respect, while we have recently shown that the murine cell surface co-inhibitory receptor, Programmed Cell death receptor (PD)-1, has a role in septic morbidity/mortality that is mediated in part through the effects on the innate immune arm. However, it is not known if PD-1 has a role in the development of indirect-acute lung injury and how this may be mediated at a cellular level.

Methods—PD-1 $-/-$ mice were used in a murine model of indirect-acute lung injury (hemorrhagic shock followed 24 h after with cecal ligation & puncture-septic challenge) and compared to wild type controls. Groups were initially compared for survival and subsequently for markers of pulmonary inflammation, influx of lymphocytes and neutrophils, and expression of PD-1 and its ligand, PD-L1. In addition, peripheral blood leukocytes of patients with indirect-acute lung injury were examined to assess changes in cellular PD-1 expression relative to mortality.

Results—PD-1 $-/-$ mice showed improved survival compared to wild type controls. In the mouse lung, CD4⁺, CD11c⁺ and Gr-1⁺ cells showed increased PD-1 expression in response to indirect-acute lung injury. However, while the rise in BAL fluid protein concentrations, lung IL-6, and lung MCP-1 were similar between PD-1 $-/-$ and wild type animals subjected to indirect acute lung injury, the PD-1 $-/-$ animals that were subjected to shock/septic challenge had reduced CD4:CD8 ratios, TNF- α levels, MPO activity, and caspase 3 levels in the lung. Comparatively, we observed that humans, who survived their acute lung injury, had significantly lower expression of PD-1 on T cells.

Conclusions—PD-1 expression contributes to mortality following the induction of indirect-acute lung injury and this appears to be associated with modifications in the cellular and cytokine profiles in the lung.

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Introduction

Acute lung injury (ALI) is characterized by bilateral pulmonary infiltrates and hypoxemia. It is either the result of a pulmonary insult (direct), such as pneumonia, or non-pulmonary inflammatory process (indirect, iALI) resulting from non-pulmonary sepsis, trauma, pancreatitis, or hypotensive shock.^{1,2} Estimates of incidence range as high as 75 cases per 100,000 people each year with mortality approaching 30%.² Currently, the only treatment shown to reduce mortality for patients with ALI, and its more severe form, acute respiratory distress syndrome (ARDS), is low tidal volume ventilation;³ thus, suggesting much remains to be understood about the pathobiology of ALI/ARDS if better therapeutic interventions are to be found.

There are currently two hypotheses that are proposed to explain pathologically how iALI develops; one dealing with neutrophils and, the other, focusing on native lung epithelial and/or endothelial cells and their interactions with the immune system. Neutrophils are thought to contribute to acute lung injury in response to an inciting event leading to a primed/partially-activated state. This model as described depends on a pre-dispositional insult like, trauma, shock/hemorrhage to prime neutrophils and recruit them to the lung so as to precipitate iALI, in the setting of a subsequent septic insult.⁴⁻⁶ The priming of neutrophils is likely due to their response to the pro-inflammatory milieu that results in potentiated respiratory burst capacity, a delay in apoptosis of the neutrophils, increased cytokines and postponement of the typical resolution of the inflammatory state.⁷ While not mutually exclusive, another theory that has been put forward to explain the development of iALI, proposes the epithelial and endothelial cells of the lung first undergo increased local activation, inducing pulmonary inflammation (which may enhance phagocyte recruitment), apoptosis, and/or epi/endothelial cell dysfunction leading to epithelial barrier dysfunction.⁸⁻¹⁰

Programmed death receptor 1 (PD-1) is a co-inhibitory surface protein of immune cells.^{11,12} PD-1 is classically described as controlling T cells in adaptive immune response to both self and non-self antigens. Thus, it has been documented to have roles in autoimmune diseases,^{13;11} tumor cell (cancer) survival and chronic viral infection (where over-expression appears to lead to the persistence of conditions)^{14;1516} suggesting the effect of PD-1 ligation may influence immune cells other than T cells.¹⁷

Recently, it has been shown that mice lacking the gene for PD-1 (PD-1 $-/-$) or those that were treated with a neutralizing antibody against PD-1 were better able to survive experimental sepsis.^{17,18} However, while animals and patients in septic shock clearly express substantial levels of PD-1 on both cells of the innate and adaptive immune system,¹⁷ controversy exists as to how the PD-1 mediates its actions. Is PD-1 anti-inflammatory/immune-suppressive or anti-apoptotic? What cells mediate its effects? And, is its role similar in other conditions of critical illness, such as iALI or ischemia/reperfusion injury? Here we test the hypothesis that PD-1 has a role in mediating the development of iALI and the associated mortality seen resulting from the combined insults of hemorrhagic shock followed by septic challenge. Further we begin to delineate what cells in the lungs appear to mediate PD-1's action in iALI as well as if such changes in PD-1 expression are evident in ICU patients that succumb to multiple organ failure with ALI.

Methods

Mice

C57BL/6 (wild type) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and PD-1 deficient mice (PD-1 $-/-$) were bred at Rhode Island Hospital after obtaining a

breeder colony (see acknowledgements). Male mice aged 10–12 weeks were used in all experiments and done according to guidelines from the National Institutes of Health (Bethesda, MD) and were approved by the Rhode Island Hospital animal use committee.

Indirect Acute Lung Injury

Hemorrhage (non-lethal shock) followed by cecal ligation and puncture (CLP) 24 hours later induced indirect acute lung injury as previously described.^{4,5,10,19–21} The control groups, including sham hemorrhage followed by sham CLP (Sham-Sham), hemorrhage^{5,10,19–21} followed by sham CLP (Hem-Sham) or sham hemorrhage followed by CLP (Sham-CLP) described previously in our laboratory, have been shown to be no different in parameters tested.⁴ Therefore, we chose the Sham-Sham group as control in this study.

Sample Collection

On the third experimental day (24 hours after CLP) mice were euthanized with a CO₂ overdose. The acquisition of bronchial alveolar lavage fluid was done as previously described.^{20,21} This fluid was centrifuged at 1500g at 4°C for 10 minutes and the supernatant was tested for protein concentrations. The lungs were then perfused as previously described^{20–23} and harvested for analysis.

Lung Histology

Mice for which histology was assessed on the lungs were euthanized with an isoflurane overdose. The lung was then fixed in 10% formalin and then paraffin embedded. Samples were then stained with hematoxylin and eosin and examined by light microscopy for lung morphology.^{4,19,21}

Flow Cytometry

The left lobe of the lung underwent an enzymatic digestion as previously described.^{20,21} After digestion, the cells were phenotyped and expression of PD-1 and PD-L1 assessed by flow cytometry (BD FACS Array). Antibodies purchased from eBioscience (San Diego, CA) include phycoerythrin (PE) labeled anti PD-1 (J43), PE labeled antiPD-L1 (MIH5), allophycocyanin (APC) labeled anti Gr1 (RB6-8C5), APC labeled anti B220 (RA3-6B2), APC labeled anti CD8 (53-6.7), and APC labeled anti CD115 (AFS95). Invitrogen (Carlsbad, CA) provided APC labeled anti CD11c (N418) and APC labeled anti CD4 (RM4-5). Antibodies and their isotype controls were used according to the manufactures recommendations.

Assessment of lung chemokines and cytokines

Lung tissue homogenate was analyzed for IL-6, IL-10, MCP-1, IFN- γ , TNF- α , and IL-12 levels using the commercially available BD Cytometric Bead Array mouse inflammation kit (BD Biosciences, Franklin Lakes, NJ).

Myeloperoxidase Activity and Caspase 3 Activity

Lung tissue homogenate was also assessed for caspase 3 activity, as a marker of apoptosis, and myeloperoxidase activity, a correlate to neutrophil influx, as previously described.^{24,25}

Human samples

Patients admitted to the surgical or trauma intensive care unit between June of 2009 and May of 2010 were enrolled in the study. The diagnosis of acute lung injury was based upon criteria set by Bernard et al.²⁶ Plasma was obtained after whole blood was collected and centrifuged at 10,000 $\times$ g at 4°C for 10minutes. Red blood cells were lysed using a solution

of 8.26g NH₄Cl (Sigma, St. Louis, MO), 1g KHCO₃ (Sigma, St. Louis, MO), and 0.037g EDTA (Invitrogen, Carlsbad, CA) in 1L double distilled water. Once the red blood cells were lysed the lymphocytes were phenotyped using flow cytometry (BD FACSArray). Antibodies used to phenotype cells from the peripheral blood were APC anti CD3 (UCHT1, Beckman Coulter) and PE labeled anti PD-1 (Ms IgG1, Biolegend). The Rhode Island Hospital Institutional Review Board approved this portion of the study.

Statistical Analysis

Data was analyzed using SigmaPlot 10.0 (Systat Software, San Jose, CA). Paired t tests were done when two groups are compared. When multiple groups are compared one-way ANOVA or ANOVA on ranks tests were done with Student-Newman-Keuls correction. Kaplan-Meier Survival Analysis was done to assess a survival benefit. Graphs are displayed as mean with error bars representing the standard error. Alpha was set to 0.05

Results

Deficiency of PD-1 Improves Survival in Mice Subjected to the Hemorrhage followed by Septic Challenge

In response to iALI induced by hemorrhage followed by CLP, it was discovered that PD-1 ^{-/-} mice (n=17) had a significantly increased survival benefit as compared to wild type mice (n=16)(Figure 1). The survival benefit was seen as early as day 3 and continued through the rest of the 14-day observation period. After fourteen days the PD-1 ^{-/-} had a 70% survival compared to 31.25% survival in the wild type animals.

PD-1, but not PD-L1, Expression is Increased on a Number of Lung Injured Mouse Leukocyte Sub-populations

To determine the effect of the shock followed by septic challenge on the degree of PD-1 expression on cells in the lung, the number of cells expressing PD-1 was quantified in wild type animals with iALI or sham/sham animals using flow cytometry. There was an increased number of CD4⁺, CD11c⁺, and Gr1⁺ cells expressing PD-1 after hemorrhage followed by CLP when compared to the sham/sham wild type animals (Figure 2). Other cell types (CD8⁺, B220⁺, CD115⁺) did not exhibit a marked change in the expression of PD-1 in animals with iALI compared to animals that underwent sham procedures. In addition, the total number of cells expressing PD-L1 in the lung was not different among groups. Even when different sub-populations of immune cells (CD4⁺, CD8⁺, CD11c⁺, CD115⁺, Gr1⁺) were assessed, there was no significant difference in the number of each cell population expressing PD-L1 in the lung.

Deficiency of PD-1 Alters Indices of Indirect Acute Lung Injury

The protein concentration in the bronchial alveolar lavage (BAL) fluid is commonly used as a marker of lung injury in mice because of limitations of their size. In this study, the BAL fluid protein concentration was significantly elevated in the wild type animals that had iALI when compared to the sham/sham controls (Figure 3). The PD-1 knockout animal also shared a similar increase in BAL fluid protein when subjected to iALI as compared to sham treated animals (Figure 3).

Increases in inflammatory cytokines are also thought to be indices of local pulmonary inflammation/injury seen following hemorrhage and CLP. Inasmuch, we observed that the levels of cytokines IL-6, MCP-1, and TNF- α were significantly increased in the lung of the wild type animals that underwent hemorrhage and CLP as compared to the control animals (Figure 4). At 24 hours after the second insult, PD-1 knockout animals subjected to iALI had a similar increase in levels of IL-6 and MCP-1 when compared to the wild type animal

treated similarly (Figure 4A, 4B). However, the level of TNF- α was significantly lower in the PD-1 knockout mouse subjected to iALI when compared to wild type mice with iALI (Figure 4C). Other cytokines, such as IL-10, IFN- γ , and IL-12, showed no significant differences between experimental groups.

Previous work has also shown that the ratio of the number of CD4⁺ cells to CD8⁺ cells in the lung is increased in mice with iALI.²⁰ Here we observed this ratio was increased in the wild type mice that underwent hemorrhage/CLP compared to the sham/sham controls; however, the CD4⁺ to CD8⁺ ratio in PD-1 knockout mice subjected to hemorrhage/CLP was lower than wild type animals subjected to iALI and similar to the sham levels (Figure 5). Other cell types (B220⁺, CD115⁺ or CD11c⁺) showed no significant difference in the number of cells when all experimental groups were compared.

As stated previously, the influx of neutrophils to the lung has been suggested to play a role in acute lung injury.⁴⁻⁷ Interestingly, both wild type and PD-1 knockout mice demonstrated a marked influx of neutrophils into the lung as exhibited by an increase in the number of Gr1 positive cells in the lung after hemorrhage and CLP as compared to sham/sham animals (Figure 6A). However, myeloperoxidase activity, which has also been used as a surrogate index for neutrophil infiltration/activity, was increased in the lung tissue of wild type mice with iALI as compared to the sham control animals (Figure 6B); PD-1 knockout animals with acute lung injury showed levels of myeloperoxidase activity similar to those of the sham animals (Figure 6B).

Since studies have previously shown that pulmonary cell apoptosis is increased following iALI we attempted to assess the change in lung active caspase 3 levels as an index of this process.^{19,27} The active caspase 3 levels in the lung of the wild type animal after hemorrhage and CLP was increased as compared to animals who underwent sham procedures (Figure 7). The PD-1 knockout animals subjected to iALI had significantly lower caspase 3 activity than wild type iALI mice and were similar to control animals (Figure 7).

Morphologically, sections of lung stained with hematoxylin and eosin showed evidence of increased congestion and neutrophils influx in the wild type animal with iALI (Figure 8A) when compared to sham controls (Figure 8B, 8D). However, the PD-1 knockout animals subjected to iALI appeared to have lung histology (Figure 8C) more similar to sham controls.

Patients with Acute Lung Injury that Died, Exhibited Higher Number of PD-1⁺ T-cells in the Blood

In a sample of fifteen trauma/surgical intensive care unit patients with acute lung injury the level of expression of PD-1 on CD3⁺ cells in the peripheral blood was assessed and compared between survivors and non-survivors. In an attempt to control for factors that may influence mortality, variables such as severity of illness (PaO₂:FIO₂), underlying physiologic derangement and co-morbidities (APACHE II), age, and gender were compared. While there was no difference between the groups when comparing gender, PaO₂:FIO₂, and APACHE II scores (Table 1), we observed that those who died were more likely to be older (Table 1) and had significantly higher expression of PD-1 on their T-lymphocytes in peripheral blood than the patients who survived (Figure 9).

Discussion

Here, we have explored the role PD-1 (or deficiency) plays in a murine model of iALI resulting from non-lethal hemorrhage followed by septic challenge. The increased expression of PD-1 on the immune cell types that are of the CD4⁺, CD11c⁺, and Gr1⁺

lineage in the lungs of wild type animals with iALI supports the concept that PD-1 plays a role in the development of acute lung injury (Figure 2). However, in the PD-1 knockout animals, some indices of iALI were not changed when compared to the wild type animals with iALI. This indicates some aspects of pathology in the lung are either not affected by PD-1 gene loss or not different at the time of assessment. The lack of differences seen in IL-6, MCP-1 (Figure 4A, B) and protein leak (Figure 3) might relate to differences seen in the PD-1 system with regard to chronic versus acute inflammation, as well as the appreciation that only one time-point has been assessed here (since iALI is an ongoing process). In chronic inflammation models of tuberculosis and atherosclerosis using PD-1 deficient mice, MCP-1 and IL-6 are typically increased significantly.^{28–30} In addition, in the same models of tuberculosis and atherosclerosis, lack of functional PD-1 also led to increased TNF- α levels.^{29,31}

Nonetheless, it appears that PD-1 plays an important role in mediating the pathology contributing to acute lung injury. When PD-1 gene expression was absent, some markers (TNF- α , myeloperoxidase, caspase-3, CD4⁺:CD8⁺) return to levels seen in animals that underwent sham procedures. These variations in the markers of acute lung injury/inflammation seen in mice without PD-1 appear to support aspects of both the neutrophil mediated lung injury hypotheses and the loss of barrier function hypothesis of acute lung injury and are, as such, in concert with previous work.^{19,20,27,32,33}

As stated earlier, PD-1 knockout animals and animals treated with antibodies to PD-1 have been reported to exhibit improved survival in a murine model of sepsis.^{17,18} Importantly, while it has been shown previously that hemorrhagic shock potentiates the mortality seen in the CLP model,⁴ we report here that the lack of PD-1 gene expression attenuates the rise in mortality seen in the wild type animals in response to shock followed by sepsis (Figure 1).

TNF- α is a cytokine that has received much attention with regard to its role in initiating numerous immune system derangements. TNF- α has been implicated as a priming agent of neutrophil function in numerous models of diseases.^{34–36} With respect to the lung, patients with ARDS (the most severe form of acute lung injury) have been reported to have higher levels of TNF- α in their BAL fluid,^{37–39} thus, suggesting a central role for TNF- α in ALI. The lower levels of TNF- α seen in the lungs of PD-1 knockout animals with iALI could explain the decrease in myeloperoxidase activity, since TNF- α has been shown to increase lysozyme release, degranulation, and respiratory burst of neutrophils in culture.^{40,41}

TNF- α has also been implicated in the induction of apoptosis. TNF- α has been shown to have a cytotoxic effect on cultured lung endothelial cells⁴² and TNF- α leads to increased neutrophil apoptosis in vitro.⁴³ Based on the concomitant decline in pulmonary cell apoptosis and the decrease in TNF- α levels, one could suggest that the loss of TNF- α directly and/or indirectly may have resulted in less apoptosis in the lung (Figure 6B) as seen in the PD-1 knockout mice with iALI.

Apoptosis of epithelial and endothelial cells leading to loss of barrier function in the lung has been proposed as another possible mechanism involved in the development of ALI. Although PD-1 was initially discovered in a search for proteins associated with apoptosis (as the name implies), it has been found, based on its structure and function, to be primarily a co-inhibitory receptor protein.¹² However, in these experiments the PD-1 knockout animals exhibited lower levels of active caspase 3, correlating to decreased apoptosis in the lung. Even though there was no significant difference in the levels of PD-L1 expression on the immune cell phenotypes assessed in the lung, there was an overall trend towards an increase in pulmonary cell expression of PD-L1 (data not shown). This suggests the possibility that expression on non-immune cell populations, like epi-/ endothelial cells, which might be lost

in the more overt expression of PD-L1 on immunocytes, may be effected. This however remains to be assessed. Nonetheless, PD-1 ligation with PD-L1 has been linked to induction of apoptosis^{44,45} and without PD-1 (as in the knockout animal) apoptosis may be decreased. PD-1 antibody administration after a septic challenge has also been shown to decrease apoptosis and increase Bcl-xL, an anti-apoptotic protein.¹⁸ This is in concert with the findings that ligation of PD-1:PD-L1 leads to a decrease in Bcl-xL.⁴⁶ If an antibody blocking PD-1 or a knockout animal is studied, one might predict an expected increase in Bcl-xL leading to less apoptosis, as seen in the lungs of PD-1 deficient mice with iALI (Figure 7); this, however, remains to be determined in our system.

Of note, the role of cells of the adaptive immune system, namely lymphocytes, in acute conditions like iALI has largely been ignored. Recently, our group and others have shown that there may be a role of lymphocytes in the murine model of iALI applied here. Specifically, it has been observed that there is an increase in the CD4⁺:CD8⁺ ratio in response to iALI indicating that CD4⁺ cells are preferentially recruited to the lung.^{20,47,48} Lymphocytes have also been shown to play a role in lung injury resulting from direct LPS instillation.⁴⁹ In our study, we show that the CD4⁺:CD8⁺ ratio in the PD-1 knockout animal with iALI is significantly reduced compared to the level in the wild type animal with iALI (Figure 5). This result was due to a combination of less CD4⁺ and more CD8⁺ expression since no significant change seen in each cell population on its own was seen (data not shown). Interestingly we have previously shown that CD8 knockout mice subjected to iALI exhibit increased lung apoptosis.²⁰ It is tempting to speculate that in PD-1 knockout animals with iALI, a relative increase in CD8⁺ cells coupled with the lack of PD-1 (less inhibition) may have contributed to the decrease in apoptosis seen in these animals.

CD4⁺ knockout animals with iALI have also been shown to have an increased myeloperoxidase²⁰ activity, suggesting a role for CD4⁺ in the recruitment/activation of neutrophils. In this experiment, PD-1 knockout animals with iALI had lower myeloperoxidase activity, possibly as a result of the loss of CD4⁺ cells, exhibiting reduced inhibitory function.²⁰ This in turn might have had a greater effect on neutrophil recruitment/activation in the lung. Typically myeloperoxidase activity has been used as a correlate for influx of neutrophils in the lung.^{10,19–21} However, myeloperoxidase can also be produced by cells of the monocyte lineage.⁵⁰ In addition, Gr1 has been used as a marker of peripheral neutrophils,⁵¹ but it can be expressed on other cells of the myeloid lineage. These two observations may explain the differing results relative to the lack of change in the number of Gr1⁺ cells, coupled with decreased myeloperoxidase seen in the PD-1 knockout animal with iALI. As stated earlier TNF- α is reported to stimulate neutrophils.^{34–36} Lower levels of TNF- α seen in the lungs of PD-1 knockout animals with iALI may also have led to neutrophils that were less activated and thus, released less myeloperoxidase; thereby accounting for the increase in the neutrophil number along with a decrease in myeloperoxidase activity.

This is not to say that the increase in PD-1 on cells of the innate immune response may not also be one of the targets of PD-1 gene deficiencies actions. Since in this study we also noted that the number of pulmonary cells expressing Gr1 and CD11c (cell differentiations markers that have been associated with resident alveolar macrophages and/or tissue dendritic cells, in the case of CD11c⁵² or neutrophils [primarily] and to a lesser extent select macrophage and/or plasmacytoid dendritic cells, with respect to Gr1⁵³) also expressed high levels of PD-1 in response to iALI. Thus, we do not rule out the possibility that, as we observed in our acute model of polymicrobial septic shock that PD-1 deficiency appeared to be mediating its effects through its expression on cells of the monocyte/macrophage lineage,¹⁷ that it might also be acting through innate immune phagocytic cell subpopulations in the lung in this model of iALI.

The survival study of PD-1 knockout animals with iALI compared to wild type controls illustrates a deleterious effect of PD-1 expression on the onset of multiple organ failure when complicated with ALI. In humans with ALI, PD-1 expression on circulating lymphocytes was decreased in the patients who survived compared to patients who died (Figure 9). While this is only a small, single centered observational study, these results are comparable to the mouse results, in so much as humans with lower levels of PD-1 expression (akin to a PD-1 knockout animal) were more likely to survive an episode of ALI than patients with high levels of PD-1 (comparable to the wild type animal).

Finally, we feel the survival benefit seen in PD-1 knockout animals with iALI illustrates a here-to-for unappreciated potential role of immune co-inhibitory molecules in this disease process. Although it is typically thought that lack of inhibition of immune cells should result in severe inflammation with drastic effects on subsequent organ injury; here we show the lack of inhibition (PD-1 knockout) actually leads to decrease in pulmonary pro-inflammatory cytokine burden (TNF- α), decreased neutrophil activity (less myeloperoxidase), and less pulmonary cell apoptosis (decreased caspase 3). We also show that the lack of PD-1 gene expression in response to indirect acute lung injury also alters pulmonary immune cell make up as shown in the difference in the CD4⁺:CD8⁺ ratio. Taken together with the observational data from patients with ALI that documents an association between increased PD-1 expression and decreased survival; this suggests that the PD-1:PD-L1 may play a novel role in the developing morbidity seen in the critically ill patient with acute lung injury.

Acknowledgments

This work was funded in part by NIH HL 73525 (A.A.) as well as the Armand D. Versaci Research Scholar in Surgical Sciences Award (S.F.M., R.K.T.). The PD-1 $-/-$ mice were generously provided by T. Honjo (Kyoto University Graduate School of Medicine, Kyoto, Japan) via M. Sykes (Massachusetts General Hospital, Transplantation Biology Research Center, Boston, MA, USA).

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Figure 1.

Fourteen-day survival in PD-1 knockout mice (solid line, n=17) compared to wild type (C57BL/6) mice (dashed line, n=16) after hemorrhage followed by CLP. *p<0.05 versus wild type (Kaplan Meier Survival Analysis done each day).

Figure 2.

Number of cells expressing PD-1 on the surface of CD4+, CD11c+, and Gr1+ cells isolated from the lungs in wild type mice after indirect acute lung injury (Hem+/CLP+) as compared to mice that underwent sham procedures. N=7 per group. Analysis was done using t test.

*P<0.05 when compared to sham/sham animal.

Figure 3.

Quantification of protein in the bronchial alveolar lavage fluid of wild type and PD-1 knockout mice who had undergone hemorrhage followed by CLP or sham procedures. N=7 per group. Statistical analysis involved one-way ANOVA followed by the Student-Newman-Keuls test. *p<0.05 compared to wild type sham/sham animal. †p<0.05 compared to PD-1 knockout sham/sham animal

Figure 4.

(A) Lung tissue levels of IL-6 in wild type and PD-1 knockout mice that had undergone hemorrhage followed by CLP or sham procedures. (B) Lung tissue levels of MCP-1 in the same conditions. (C) Lung tissue levels of TNF-α under similar conditions. N=7 per group. Statistical analysis involved one-way ANOVA followed by the Student-Newman-Keuls test.

*p<0.05 compared to wild type sham/sham animal. †p<0.05 compared to PD-1 knockout sham/sham animal ‡p<0.05 compared to PD-1 knockout hem/CLP animal

Figure 5.

Ratio of the number of CD4+ cells to the number of CD8+ cells identified in the lung by flow cytometry in wild type and PD-1 knockout mice that had undergone hemorrhage followed by CLP or sham procedures. N=7 per group. Statistical analysis involved one-way ANOVA followed by the Student-Newman-Keuls test. *p<0.05 compared to wild type sham/sham animal. †p<0.05 compared to PD-1 knockout sham/sham animal ‡p<0.05 compared to PD-1 knockout hem/CLP animal

Figure 6.

(A) Number of Gr1+ cells (neutrophils) in the lungs of wild type and PD-1 knockout mice that had undergone hemorrhage followed by CLP or sham procedures. (B) Lung tissue myeloperoxidase activity in the same conditions. N=7 per group. Statistical analysis involved one-way ANOVA followed by the Student-Newman-Keuls test. *p<0.05 compared to wild type sham/sham animal. †p<0.05 compared to PD-1 knockout sham/sham animal. ‡p<0.05 compared to PD-1 knockout hem/CLP animal

Figure 7.

Caspase 3 activity in the lungs of wild type and PD-1 knockout mice that had undergone hemorrhage followed by CLP or sham procedures is displayed. N=7 per group. Statistical analysis involved one-way ANOVA followed by the Student-Newman-Keuls test. *p<0.05 compared to wild type sham/sham animal. †p<0.05 compared to PD-1 knockout sham/sham animal ‡p<0.05 compared to PD-1 knockout hem/CLP animal

Figure 8.
(A–D). Representative sections of lung from wild type and PD-1 knockout animals that underwent hemorrhage followed by cecal ligation and puncture (CLP), or sham control procedures and stained with hematoxylin and eosin. (A) Wild type animals with indirect acute lung injury show disrupted alveolar structure, increased congestions, and more neutrophils. (B) Wild type animals after sham procedures show more normal lung histology but some neutrophils. (C) PD-1 knockout animals with indirect acute lung injury exhibited architecture more similar to controls but more neutrophils are seen. (D) The PD-1 knockout animal after sham procedures ore normal architecture. Magnification is 400X.

Figure 9.

Number of CD3+ cells in the peripheral blood that also expressed PD-1 among survivors (n=10) and those who died (n=5) and had acute lung injury. Analysis was done using t test.

*P<0.05 when compared acute lung injury patients who died.

Table 1

Demographic and clinical data of the 15 surgical or trauma intensive care unit patients with acute lung injury. Gender, age, severity of acute lung injury ($\text{PaO}_2\text{:FIO}_2$), and underlying physiologic derangement and co-morbidities (APACHE II), were compared as they were likely to contribute to mortality.

Table 1	Dead (n=5)	Alive (n=10)	p value
Female	60%	20%	0.251
Age (mean, standard deviation)	73.0, 7.842	54.1, 13.86	0.015
$\text{PaO}_2\text{:FIO}_2$ (mean, standard deviation)	209.1, 78.65	207.5, 56.95	0.965
APACHE II (mean, standard deviation)	24, 7.071	19.9, 3.143	0.136