Original Paper

Acute Kidney Injury Reduces Phagocytic and Microbicidal Capacities of Alveolar Macrophages

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Key Words

Alveolar macrophages • Pulmonary inflammation • Killing • *Klebsiella pneumonia* • Ischaemia and reperfusion injury

Abstract

Background/Aims: Renal ischaemia-reperfusion injury (IRI) is a systemic inflammatory process in which Th1 responses predominate affecting other organs including the lungs. The present study explored the phagocytic and microbicidal capacity of macrophages in rats with lung inflammation that underwent IRI. **Methods:** The alveolar macrophages of rats sensitised to OVA were evaluated for phagocytosis and bacterial killing 24h after antigen challenge in animals with or without prior submission to 60 min of renal ischaemia. **Results:** Bronchoalveolar lavage had a high level of cellular infiltrate in immunised animals (420%) compared with control animals; IRI significantly reduced this infiltration (52%). Macrophages from animals immunised and challenged with OVA presented a 10x increase in phagocytic capacity compared to the control group, whereas immunised animals subjected to IRI showed a reduction in the phagocytic index of 68%. The killing of *Klebsiella pneumoniae* by macrophages from immunised animals was higher (56%) compared with the control group but reduced in animals submitted to IRI (45%). Immunised and challenged group showed an increase in gene expression levels of IL-10(450%), HO-1 (259%), INF- γ (460%) and MCP-1 (370%) compared to the immunised group subjected to IRI. **Conclusions:** Renal ischaemia and reperfusion injury apparently alters the phagocytic and microbicidal capacity of macrophages, reducing lung inflammation to OVA.

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Introduction

Several types of cells can contribute to the lung defence system, including structural cells, such as epithelial cells and fibroblasts, resident macrophages (alveolar macrophages) and haematopoietic cells that migrate into the lung. Among these cells, itinerant leukocytes, such as neutrophils, dendritic cells, lymphocytes and macrophages, can play an important role in the local immune response [1].

Macrophages are classically associated with a variety of activities, including the induction of inflammation, engulfment of microorganisms and dead cells, antigen presentation and the regulation of extracellular components. Alveolar macrophages also produce and secrete inflammatory cytokines, chemokines and lipid mediators, playing an important role in pulmonary innate immunity [2, 3]. Alveolar macrophages also play an important role in the reduction of microorganisms in pulmonary inflammation [4]. The phagocytosis of opsonised particles is triggered by the interaction of opsonins with specific receptors on the surface of phagocytes. One of the most important and well-studied phagocytic receptors is the Fc γ receptor (Fc γ R), which binds to the Fc portion of immunoglobulins (Ig) that coat the target [5]. In this sense, efficient phagocytosis is achieved by opsonisation of the targets by Ig and complement proteins [6, 7].

Acute kidney injury (AKI) is characterised by an abrupt decline in renal function, resulting in the inability of the kidneys to perform their basic functions of excretion and maintenance of fluid and electrolyte homeostasis in the organism [8, 9]. Ischaemia-reperfusion injury (IRI) is one of the major causes of AKI. The pathogenesis of renal IRI is complex and not yet completely understood, but the inflammatory response is now accepted as an important pathogenic component, such as endothelial injury and reactive oxygen species (ROS) [10, 11]. Patients with AKI have a higher incidence of complications, such as respiratory failure [12]. In mice and rats, after ischaemic AKI, lung injury characterised by neutrophil infiltration, pulmonary oedema and increased chemokine and cytokine expression can be observed [13-15]. AKI can cause increased pulmonary permeability, pulmonary oedema and increased pulmonary neutrophil recruitment, all of which are consistent with acute lung injury.

Although there are several mechanisms through which AKI could lead to lung injury, little is known about the effects of AKI on the mechanism of immune response in the lungs. In this paper, we analyse the effects of systemic AKI in the function of alveolar macrophages during antigen-driven lung inflammation.

Materials and Methods

Animals

Eight- to twelve-week-old male Wistar rats (250-300 g) from our colony were housed in a $22 \pm 1^\circ\text{C}$ environment at 60% humidity and maintained on a 12-h light-dark cycle with food and water provided *ad libitum*. All procedures used in this study were approved and performed in accordance with guidelines established by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biomedical Sciences Institute/USP-Ethical Committee for Animal Research (CEEAA).

Immunisation protocol

Rats were sensitised on days 0 and 7 by an intraperitoneal injection of a mixture containing 50 mg of ovalbumin and 1 mg of Al(OH) $_3$ in saline (a total volume of 0.7 ml). At 14 days after the first immunisation, the animals were challenged by exposure to an aerosol of ovalbumin (grade III, Sigma) generated by an ultrasonic nebuliser (ICEL US-800, SP, Brazil) delivering particles of 0.5–10 μm diameter at approximately 0.75 ml/min for 20 min. The concentration of ovalbumin in the nebuliser was 2.5% (w/v). The control group consisted of animals immunised as described and challenged with saline solution or non-immunised animals challenged with OVA aerosol as above.

Experimental model of IRI

Surgery was performed 24 hours after the OVA challenge. Rats were anesthetised with ketamine-xylazine (Agribands do Brazil, São Paulo, Brazil). A midline incision was made, and both renal pedicles were cross-clamped. In the ischaemia period, animals were kept well hydrated with saline and at a constant temperature (37°C) through a heating pad device. Sixty minutes later, the microsurgery clamps were removed, the abdomen was closed in two layers, and the animals were placed in single cages and warmed by indirect light until complete recovery from anaesthesia. Control rats were subjected to the surgical procedure without clamping of renal pedicles (sham animals). The animals were kept under adjustable conditions until sacrifice 24 hours after reperfusion.

Analysis of renal function

Serum creatinine was used for the evaluation of renal function after IRI. Blood samples were collected 24 hours after ischaemic surgery from the abdominal inferior cava vein immediately before induced death. Serum creatinine levels were determined through absorbance readings at 520 nm with a spectrophotometer using a modified Jaffé technique.

Bronchoalveolar lavage

The animals were anesthetized with an injection of ketamine/xylazine 24h after ischaemia or sham surgery. A tracheal cannula was inserted via a midcervical incision, and the airways were flushed five times with 10 ml of phosphate-buffered saline (PBS, pH 7.4 at 4°C).

Total and differential cell counts

Bronchoalveolar lavage (BAL) fluid was centrifuged at 170 *g* for 10 min at 4°C, the supernatant was removed, and the cell pellet was resuspended in 0.5 mL of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hemacytometer. Differential cell counts were performed after cytocentrifugation and staining with haematoxylin-eosin (Hema 3).

Cell isolation and culture

Resident macrophages were obtained from rats via *ex vivo* lung lavage, as previously described [3], and resuspended in RPMI-1640 (Gibco-Invitrogen, Carlsbad, CA) to a final concentration of 2×10^5 cells/mL. The cells were allowed to adhere to culture plates for 1 hour (37°C, 5% CO₂) followed by one wash with warm RPMI-1640. More than 99% of the adherent cells were identified as alveolar macrophages according to a modified Wright-Giemsa stain. Cells were cultured overnight in RPMI-1640 supplemented with 2% fetal bovine serum (FBS) and washed twice with warm medium to remove non-adherent cells.

Phagocytosis of red blood cells opsonised with IgG and evaluation of the phagocytic index.

Red blood cells (Bioboavista Laboratories, Brazil) were opsonised with a subagglutinating concentration of IgG rabbit anti-sheep erythrocyte antibody (IgG-RBCs) (Cappel Organon Teknika, Durham, NC) as previously described [16]. The phagocytosis of lamb red blood cells by rat alveolar macrophages was evaluated according to a method previously described by Aronoff et al. [17]. Briefly, 2×10^5 cells were added to each well of a 24-well culture plate and allowed to adhere for 1 hour (37°C, 5% CO₂). The cell monolayer was washed to remove non-adherent cells and then cultured for 18 hours in RPMI containing 2% fetal bovine serum (HyClone, Logan, UT-USA), according to Serezani et al. [3].

The alveolar macrophages were washed with PBS at 37°C and incubated with or without LTC₄ (100 nM) (Biomol Inc., Palo Alto, CA-USA) for 10 minutes. Following the incubation, the supernatant was removed, and blood cells that were opsonised or not opsonised (internalisation control) were added to the alveolar macrophages at a concentration ratio of 50:1. The samples were incubated for 90 min at 37°C and 5% CO₂ in PBS. After phagocytosis, the cells were washed three times with PBS and stained with haematoxylin and eosin. The phagocytic index (PI), which was derived by multiplying the percentage of positive macrophages (alveolar macrophages containing at least one ingested target) by the mean number of phagocytised targets per positive macrophage, was determined by optical microscopy (x1000).

Bacterial killing assay

Klebsiella pneumoniae 43816 serotype 2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Bacterial stocks were thawed, inoculated into broth, and grown to log phase. The internalisation and intracellular killing of *K. pneumoniae* was monitored using a modification of the assay described by Bidani et al. [18]. Briefly, 2×10^5 macrophages were suspended in RPMI and infected with *K. pneumoniae* at a concentration ratio of 30:1 bacteria per cell at 37°C for 1 h. The cell suspensions were centrifuged (300 xg, 10 min, 4°C), the supernatants were collected, and the cells were washed twice in PBS and lysed. Quantitative bacterial cultures were performed for the supernatants and cell lysates to determine the colony-forming units (CFUs) in the extracellular and intracellular environments. CFUs were determined by serial dilution on agar plates. *K. pneumoniae* was grown in BBL brain heart infusion broth and nutrient agar (DIFCO; Becton Dickinson) and incubated at 37°C for 18–24 h.

Measurement of nitric oxide

To evaluate nitric oxide (NO) production, the nitrite concentrations in the supernatants of the alveolar macrophage cultures were measured using the standard Griess reaction. Briefly, 50 µl of supernatant culture medium was added to 50 µl of Griess reagent [equal volumes of 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 0.1% (w/v) naphthylendiamine-HCl] and incubated at room temperature for 10 min. The absorbance at 540 nm was then measured in a microplate reader (MultiskanEX), and the nitrite concentration was calculated based on a standard curve of sodium nitrite. All assays were performed in triplicate.

Cytokine gene profiles

Lung samples were quickly frozen in liquid nitrogen. Total RNA was isolated from lung tissue using TRIzol Reagent (Invitrogen, USA), and the RNA concentration was determined by absorbance readings in a spectrophotometer at 260 nm and 280 nm. First-strand cDNA was synthesised using MML-V reverse transcriptase (Promega, USA). All experimental protocols for real-time PCR were based on the manufacturer's recommendation using the TaqMan gold RT-PCR Core Reagents Kit (PerkinElmer/Applied Biosystems). Primers and probes were purchased from Applied Biosystems. Cycling conditions were as follows: 10 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The amount of the target gene was first normalised to an endogenous reference (HPRT) and then normalised relative to a calibrator (sample with the lowest expression, namely, sham-operated animals) using the $2^{-\Delta\Delta C_t}$ method. Therefore, steady-state mRNA levels were expressed as an n-fold difference relative to the calibrator. Analyses were performed with Sequence Detection Software 1.9 (SDS).

Statistical analysis

The data are expressed as the means $\pm$ S.E.M. Statistical evaluation of the data was carried out by an analysis of variance (ANOVA), and the sequential analysis of differences among means was performed by Tukey's contrast analysis. A P value less than 0.05 was considered to be significant. $P < 0.05$, $P < 0.01$ and $P < 0.001$ are indicated with one, two and three asterisks, respectively. All of the experiments were independently performed at least three times.

Results

Renal dysfunction

Rats were subjected to 60 minutes of renal ischaemia. After 24 h of reperfusion, blood was collected to evaluate renal function by measuring serum creatinine and urea levels. The ischaemic group showed increased serum creatinine levels compared to the controls (sham animals) (2.503 ± 0.39 vs. 0.776 ± 0.07 mg/dl). Serum creatinine levels were also increased in the ischaemic immunised group; however, these levels were not different from the IRI group. Similar results were observed in the urea levels (Fig. 1A and B).

Fig. 1. Creatinine and urea levels in animals subjected to renal ischaemia and reperfusion (I/R). Animals subjected to I/R were assessed after 24 h of reperfusion. The results represent the mean $\pm$ S.E.M of 6 animals per group, * $p < 0.001$ in comparison with the control group.

Fig. 2. Effect of I/R on bronchoalveolar lavage cells. Bronchoalveolar lavage was performed 24 h after I/R. Eosinophils and neutrophils were counted in a cytocentrifuged preparation of bronchoalveolar lavage cells stained with haematoxylin/eosin. The results are the mean $\pm$ S.E.M of 5 animals per group. * $P < 0.01$ in comparison with the control group, and # $P < 0.05$ in comparison with the immune group.

Cells in BAL after IRI

Immunised rats were challenged with ovalbumin and submitted to IRI 24 h later. Bronchoalveolar lavage was performed 24 h after surgery. The control group consisted of immunised and saline-challenged rats.

Although the number of eosinophils and neutrophils in the BALF of the control group was low (less than 1 cell/ml), significant increases in total cells (260.7 ± 21.6 to 994.3 ± 172.4 cells $\times 10^4$ /ml), neutrophils (5.9 ± 0.5 to 349.7 ± 74.8 cells $\times 10^4$ /ml) and eosinophils (0.5 ± 0.2 to 114.9 ± 25 cells $\times 10^4$ /ml) were observed in the immunised and antigen-challenged rats (Fig. 2A, B and C). Figures 2A, B and C show the significantly decreased number of total cells (47%), neutrophils (30%) and eosinophils (21%) in the BALF of immunised rats subjected to IRI.

Effect of IRI on alveolar macrophage phagocytosis

Eight hours after the incubation of red blood cells with AMs from sensitised rats, a significant increase in phagocytosis via Fc γ R was observed (247 ± 35.4 to 826.5 ± 91.6 versus the control group), which persisted at high levels until 48 h (247 ± 35.4 to 1649 ± 107.3 versus the control group) (Fig. 3A). The immunised and sensitised rats showed an increase in phagocytosis compared to the control group (247.0 ± 35.4 to 2875 ± 217). Sensitised rats subjected to IRI showed decreased AM phagocytosis (2875 ± 217 to 924.9 ± 66.1), as shown in Fig. 3B.

Fig. 3. Phagocytosis assay of red blood cells opsonised with IgG and evaluation of the phagocytic index. Resident macrophages from male Wistar rats were obtained by *ex vivo* bronchoalveolar lavage 24 h after I/R injury. (A) AMs from sensitised rats demonstrated a significant increase in phagocytosis via Fc γ R, which persisted up to 24 h. (B) I/R reduced the rate of AM phagocytosis in the immunised group. The results represent the mean $\pm$ S.E.M. of 5 animals per group. * $P < 0.01$ compared with the control group, and # $P < 0.05$ compared with the immunised group.

Fig. 4. Killing of *Klebsiella pneumoniae* by alveolar macrophages after AKI. AMs were infected with *K. pneumoniae* *in vitro*. After 3 hours, the macrophages were removed by scraping, and aliquots were cultured to determine the number of viable organisms by quantitative culture. The results represent the mean $\pm$ S.E.M. of 5 animals per group. * $P < 0.01$ compared with the control group, and # $P < 0.05$ compared with the immunised group.

Fig. 5. Production of nitric oxide in cell culture supernatants. Nitric oxide production was measured using the Griess colorimetric reaction following the addition of sheep red blood cells (SRBCs) to AMs. The results represent the mean $\pm$ S.E.M. of 6 animals per group. * $P < 0.001$ compared with the control group, and # $P < 0.01$ compared with the immunised group.

Effect of IRI on macrophage killing and nitric oxide generation

The rate of killing internalised bacteria was increased in AMs from sensitised rats (187 ± 16.2 to 87.6 ± 7.2 CFU $\times 10^5$); however, AMs from sensitised rats subjected to IRI showed decreased killing (87.6 ± 7.2 to 127.3 ± 6.1 CFU $\times 10^5$) (Fig. 4).

The culture supernatant of macrophages from sensitised rats showed increased nitric oxide generation compared to the control group (4.2 ± 0.7 to 18.3 ± 2.9 $\mu\text{mol NO}_2^-$). However, the macrophages of rats with AKI showed significantly reduced nitric oxide production in the culture supernatant (18.3 ± 2.9 to 11.5 ± 1.9 $\mu\text{mol NO}_2^-$) (Fig. 5).

Cytokine expression

Analysing the immune response and the concentrations of cytokines, we evaluated the mRNA expression of cytokines in the lung tissue of rats. We observed a significant increase in MCP-1 (9.3 ± 3.2 to 2.2 ± 1.6) and IFN- γ (2.6 ± 1.2 to 0.3 ± 0.04) in animals with pulmonary

Fig. 6. Gene expression of cytokines present in lung tissue. Gene expression of MCP-1 (A), IFN γ (B), IL-6 (C) and TNF (D) in lung tissues of animals subjected to I/R. The data are expressed as the mean of $2^{-\Delta\Delta Ct} \pm SD$ of 6 rats per group. * $p < 0.01$ compared with the IM+I/R group.

inflammation, but when these animals were subjected to IRI, we observed a significant reduction of MCP-1 and IFN- γ (Fig. 6), without changes in IL-6 and TNF expression.

Discussion

Due to the constant presence of germs in the respiratory tract, an effective antimicrobial control system is extremely important to the host. In the periphery of the lungs, AMs attempt to control infections, maintain the sterility of the mucous and eliminate microorganisms on the surface of the alveoli through phagocytosis, microbicidal activity and the release of cytokines and bioactive lipids [19].

In this study, we evaluated the mechanisms through which alveolar macrophages participate in phagocytosis and killing through the Fc γ receptor present in their membranes and the changes after secondary stimuli. We also evaluated the changes in rat lungs after the induction of allergic inflammation to ovalbumin and examined the role of alveolar macrophages in this process.

Wistar rats are usually used in allergic airway inflammation studies [4, 20, 21]. Furthermore, it has already been demonstrated the relationship between lung inflammation and ischemia-reperfusion injury in Wistar rats [22, 23].

Our data concerning the cellular infiltrate in the BAL of animals challenged with OVA confirm previous studies in the same model, demonstrating an increase in inflammatory cells, which is characteristic of the induction of allergic pulmonary inflammation, with a predominantly Th2 response pattern [24].

Acute respiratory distress syndrome and renal IRI frequently co-exist in patients in intensive treatment units, and this combination is associated with higher mortality [25, 26].

Previous evidence from the literature has shown that distinct Th1-related pathologies could modulate the inflammatory response observed in asthma [27, 28]. Recently, our group demonstrated that renal IRI could also modulate lung function in a model of allergic inflammation in mice [13].

Our results showed that acute kidney damage can significantly alter the cellular infiltrate in immunised and challenged mice because the induction of renal ischaemia causes a reduction in the migration of cells to the lung tissue, primarily polymorphonuclear cells.

These results corroborate our recent studies that show that IRI could down-regulate allergic lung inflammation in mice [13].

Studies on phagocytosis after stimulation with LPS or ovalbumin showed a significant increase in degraded lipid uptake by macrophages [29], apoptotic neutrophils [30], and increased phagocytosis of *K. pneumoniae* [4, 31]. We demonstrated that the macrophages of rats sensitised and challenged with ovalbumin aerosol had an increased capacity of phagocytosis of IgG- and complement-opsonised targets to kill *K. pneumoniae*, and the induction of IRI in these animals significantly reduced the capacity of phagocytosis. A possible explanation for this result could be that after IRI induction, the animal showed reduced polymorphonuclear cell migration to the lung tissue and the consequent reduction of inflammatory mediators released by these cells, most likely by altering the Th1/Th2 balance. These findings corroborate our previous findings that the activation of alveolar macrophages by LTC₄ increases phagocytosis and microbicidal activity in macrophages from immunised and challenged rats [4].

The initial improvement in the inflammatory process is mediated by neutrophils and macrophages recruited to and activated at the site of injury [32]. Increased activity or a decrease in the population of these cells leads to an increased susceptibility to lung infection [33, 34]. Our data indicate the increased survival of bacteria in alveolar macrophages from animals with an established pulmonary inflammation process and reduced cellular infiltration, primarily polymorphonuclear cells, after acute renal failure. These data suggest a greater resistance of the pathogen in the host, making these animals more susceptible to lung infection.

Circumstantial evidence suggests that the production of NO is part of an effective host response to infection and is stimulated by proinflammatory cytokines, such as IFN- γ , TNF- α , and IL-2, and microbicidal products. The ability of AMs to produce NO with bactericidal effect has been shown in several clinical and experimental data [35, 36]. Our data indicate that the greater "killing" ability observed in previously immunised and challenged rats could be related to the increased generation of NO by AMs from this group.

Initially, it was thought that the increase in IFN- γ was associated with a reduction in allergic disease. However, subsequent studies showed that increased levels of IFN- γ can be detected in the BAL of asthmatics [37]. In addition, T cells generating IFN- γ can exacerbate airway inflammation in animal models [38]. The host response to pathogens is characterised by the infiltration of specific populations of leukocytes, including neutrophils and monocytes, into the injured tissue. This process is predominantly mediated by chemokines [39]. Among these cytokines, the CC chemokine family, including MCP-1 and MIP-1 α , have been implicated in the pathogenesis of chronic bronchitis [40, 41] and interstitial lung disease [42].

In a previous study, we demonstrated that IL-1 β , IL-2 and IL-12 expression was increased only in ischaemic mice, whereas IL-4, IL-5 and IL-13 were increased in immunised and challenged mice; however, in asthmatic mice subjected to renal IRI, the expression of Th2 cytokines was reduced to levels similar to that of ischaemic mice [13]. Some studies have demonstrated that the Th1 pattern in IRI is deleterious, while the Th2 pattern is protective [43, 44]. Although we observed only a trend toward increased gene expression of TNF- α and IL-6 in animals with AKI after immunisation with OVA, these data suggest that the presence of these cytokines could modulate the inflammatory response, contributing to the less established activity of alveolar macrophages to eliminate invading microorganisms. This is because the pro-inflammatory molecules contribute to tissue damage, and anti-inflammatory mediators interfere with the host's ability to eliminate invading microorganisms.

In summary, our results show an influence of AKI in the inflammatory lung, modulating the cellular activity of alveolar macrophages and reducing their capacity of phagocytosis and pathogen killing. These results offer a new opportunity for understanding the mechanisms involved in the systemic effects of AKI, and its participation in the lung inflammatory response has been established.

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