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Low level laser therapy reduces acute lung inflammation in a model of pulmonary and extrapulmonary LPS-induced ARDS

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ABSTRACT

The present study aimed to investigate the effects low level laser therapy (LLLT) in a LPS-induced pulmonary and extrapulmonary acute respiratory distress syndrome (ARDS) in BALB/c mice. Laser (830 nm laser, 9 J/cm^2, 35 mW, 80 s per point, 3 points per application) was applied in direct contact with skin, 1 h after LPS administration. Mice were distributed in control ($n = 6$; PBS), ARDS IT ($n = 7$; LPS orotracheally 10 μg/mouse), ARDS IP ($n = 7$; LPS intra-peritoneally 100 μg/mouse), ARDS IT + Laser ($n = 9$; LPS intra-tracheally 10 μg/mouse), ARDS IP + Laser ($n = 9$; LPS intra-peritoneally 100 μg/mouse). Twenty-four hours after last LPS administration, mice were studied for pulmonary inflammation by total and differential cell count in bronchoalveolar lavage (BAL), cytokines (IL-1beta, IL-6, KC and TNF-alpha) levels in BAL fluid and also by quantitative analysis of neutrophils number in the lung parenchyma. LLLT significantly reduced pulmonary and extrapulmonary inflammation in LPS-induced ARDS, as demonstrated by reduced number of total cells ($p < 0.001$) and neutrophils ($p < 0.001$) in BAL, reduced levels of IL-1beta, IL-6, KC and TNF-alpha in BAL fluid and in serum ($p < 0.001$), as well as the number of neutrophils in lung parenchyma ($p < 0.001$). LLLT is effective to reduce pulmonary inflammation in both pulmonary and extrapulmonary model of LPS-induced ARDS.

1. Introduction

The acute respiratory distress syndrome (ARDS) is defined as respiratory failure from inflammatory response that leads to alteration of alveolar-capillary permeability, pulmonary edema and hypoxemia refractory to high oxygen flow [26,17]. Although several causes of ARDS result in a uniform pathology, in the last stage, evidence suggests that the pathophysiology may differ according to the type of primary insult. Thus, two forms of ARDS have been described: ARDS with direct effects on lung epithelial cells; ARDS reflecting lung involvement secondary to a systemic inflammatory response, being the center of the injury, the pulmonary endothelial cell [26,17].

Many studies show that the prevalence of intrapulmonary ARDS is higher when compared with extrapulmonary [23]. However [8] demonstrate an equal prevalence of both types, ant this issue remains controversial [8]. From pulmonary causes, pneumonia is the most direct cause of injury, followed by aspiration of gastric contents and pulmonary trauma [23]. The rate of death from pulmonary and extrapulmonary insults varies considerably, however, [25], shows an increase in mortality in the group of direct etiology, while [8] found a direct relationship between lung injury and increased mortality.

The scientific literature has reported anti-inflammatory effects of low-level laser therapy (LLLT) in models of acute lung injury [4–7]. Furthermore, a growing number of clinical studies are demonstrating the efficacy and safety of LLLT for different pulmonary diseases, as asthma and chronic obstructive pulmonary diseases (COPD) [15,9,10,14]. For instance, some studies also have demonstrated that application of LLLT for the treatment of patients with

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chronic obstructive bronchitis accelerates the elimination of clinical symptoms, increases its efficiency, promotes drainage function of the bronchi, facilitates standardization the immune status of the patient, and contributes to the optimization of lipid peroxidation [10,14].

Therefore, the present study was designed aiming to fill a lack of information regarding the effects of LLLT in a model of pulmonary and extrapulmonary LPS-induced ARDS in BALB/c mice.

2. Materials and methods

2.1. Animals and experimental groups

Thirty-eight male BALB/c mice weighing between 25 and 30 g were obtained from the Animal Facility of the Nove de Julho University. All experimental procedures with animals care followed the international recommendations for the use and care of animals and were approved by the local ethical committee. All mice were housed in bright rooms with controlled temperature (21–23 °C) and humidity (45–65%) and 12–12 h light/dark cycle, with access to food and water *ad libitum*.

The animals were divided into 5 groups: control ($n = 6$), LPS orotracheal ($n = 7$), intra-peritoneal LPS ($n = 7$), orotracheal LPS + laser ($n = 9$), intra-LPS Laser peritoneal + ($n = 9$).

2.2. Pulmonary and extrapulmonary model of LPS-induced ARDS

For the pulmonary model of LPS-induced ARDS, under anesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg), using a 100 μl micropipette, animals received LPS (10 μg/mouse) diluted in 50 μl of PBS through an orotracheal instillation as previously described [27]. For the extrapulmonary model of LPS-induced ARDS, animals received LPS (100 μg/mouse) diluted in 50 μl of PBS through an intra-peritoneal injection.

2.3. LLLT protocol

One hour after LPS administration, LLLT treated groups received infrared laser administration [continuous wave, 830 nm, 3 J/cm^2, 35 MW, 80 s per point (3 points per application)], where point 1 was in the end part of trachea, point 2 in the right lung and the point 3 in the left lung, in direct contact with skin. These 3 points application totalized 240 s and an energy of 9 J/cm^2. In total, LLLT groups received the LLLT as described above for 3 times, in a 1 h interval between each application.

2.4. Blood collection, processing and analysis

Under anesthesia, the abdomen was open the 1 ml of blood was collected via cava vein using a syringe without anti-coagulant and immediately centrifuged at 950 g, 4 °C, during 7 min. The serum was collected and stored at −70 °C for cytokines measurement.

2.5. Bronchoalveolar Lavage Fluid (BALF)

Aiming to access lung inflammation, the number of total and differential cells count in BALF was performed. Briefly, under anesthesia, mice were submitted to tracheotomy and cannulated. Then, using a 1 ml syringe, a 3× 0.5 ml PBS washing was applied and the recovery material was centrifuged at 800 g, at 4 °C during 7 min. The supernatant was stored at −70 °C for cytokines analysis and the cell pellet was resuspended in 1 ml PBS. The number of total cells was counted using a hematocytometer (Neubauer chamber) and the differential cells count were performed through a cytospin preparation, stained with Diff Quick and 300 cells were counted according to the hematological characteristic [13,20].

2.6. Inflammatory mediators in BALF and in serum

The levels of pro-inflammatory cytokines IL-1beta, IL-6, KC and TNF-alpha and of anti-inflammatory cytokine IL-10 was evaluated in the BALF according to the manufacturer's instructions.

2.7. Histomorphometric study

To evaluate the effects of LLLT on parenchymal inflammation, one the hallmarks of ARDS, the lungs were collected, fixed in 10% formalin and submitted to histological routine. Briefly, 5 μm ticks lung slices were stained with hematoxylin and eosin. Then, 15 aleatory fields of the lung parenchyma of each mouse were photographed. By using the software Image Pro Plus 4.0, the air and tissue area of all photomicrographs were determined. The number of polymorphonuclear (PMN) cells (notably neutrophils) was counted in each photo according the morphological criteria by an experienced research, blinded to the group's description. Then, the number of PMN cells per square millimeter of lung tissue was presented.

3. Results

3.1. Inflammation in Bronchoalveolar Lavage Fluid (BALF) and in lung tissue in the pulmonary model of ARDS

Fig. 1 shows the inflammatory profile in BALF (total cells – panel 1A; neutrophils – panel 1B) and the number of polymorphonuclear cells (notably neutrophils – panel 1C) and the representative photomicrographs of control (panel 1D), LPS IT (panel 1E) and LPS IT + laser (panel F) in the pulmonary (IT) model of ARDS. The results shows that intra-tracheal administration of LPS significantly increased the number of total cells ($p < 0.001$) and neutrophils ($p < 0.001$) in BALF when compared with control group. On the other hand, LLLT significantly reduced the number of total cells ($p < 0.001$) and neutrophils ($p < 0.01$) when compared with LPS group. LLLT also significantly reduced the number of polymorphonuclear cells in the lung parenchyma ($p < 0.001$; panels 1C until 1F).

3.2. Cytokines levels in BALF in the pulmonary model of ARDS

Fig. 2 shows the levels of IL-1beta, IL-6, KC, TNF-alpha and IL-10 in BALF in a pulmonary model of ARDS (panels 2A–E, respectively). Panel 2A–D shows that LLLT significantly reduced intra-tracheal LPS increased IL-1beta, IL-6, KC and TNF-alpha ($p < 0.05$). Panel 2E shows that no differences in the levels of IL-10 were found when all groups were compared ($p > 0.05$).

3.3. Cytokines levels in serum in the pulmonary model of ARDS

Fig. 3 shows the serum levels of IL-6 and TNF-alpha in a pulmonary model of ARDS (panels 3A and 3B, respectively). In the panel 3A, the results show that LLLT significantly reduced intra-tracheal LPS increased IL-6 levels ($p < 0.01$). In panel 3B, the results show that LLLT significantly reduced intra-tracheal LPS increased TNF-alpha levels ($p < 0.001$).

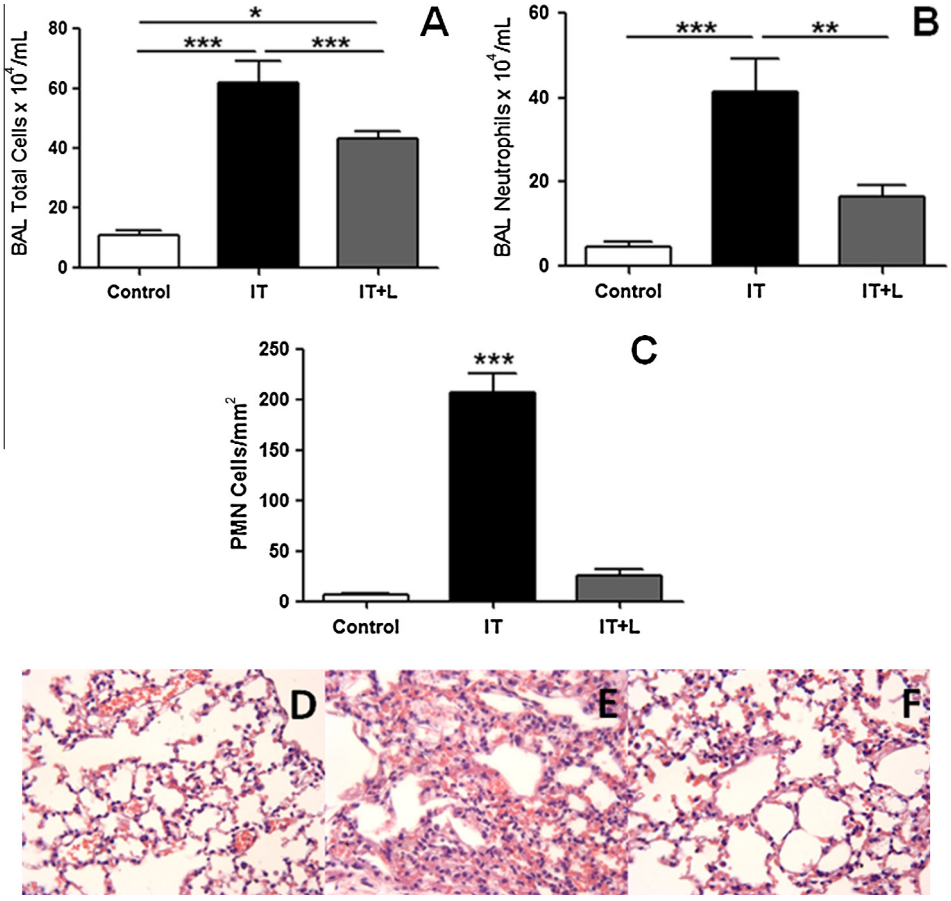

Fig. 1. Inflammatory profile in BALF (total cells – panel A; neutrophils – panel B) and the number of polymorphonuclear cells in the lung parenchyma (notably neutrophils – panel C) and the representative photomicrographs of control (panel D), LPS i.t. (panel E) and LPS i.t + laser (panel F) in the pulmonary (IT) model of ARDS. In panel A, B and C, $^{***}p < 0.001$; $^{**}p < 0.01$ and $^{*}p < 0.05$.

3.4. Inflammation in Bronchoalveolar Lavage Fluid (BALF) and in lung tissue in the extrapulmonary model of ARDS

Fig. 4 shows the inflammatory profile in BALF (total cells – panel 4A; neutrophils – panel 4B) and the number of polymorphonuclear cells (notably neutrophils – panel 4C) and the representative photomicrographs of control (panel 4D), LPS IP (panel 4E) and LPS IP + laser (panel 4F) in the extrapulmonary (IP) model of ARDS. The results shows that intra-peritoneal (IP) administration of LPS significantly increased the number of total cells ($p < 0.001$) and neutrophils ($p < 0.001$) in BALF when compared with control group. On the other hand, LLLT significantly reduced the number of total cells ($p < 0.001$) and neutrophils ($p < 0.001$) when compared with LPS group. LLLT also significantly reduced the number of polymorphonuclear cells in the lung parenchyma ($p < 0.001$; panels 4C until 4F).

3.5. Cytokines levels in BALF in the extrapulmonary model of ARDS

Fig. 5 shows the levels of IL-1beta, IL-6, KC, TNF-alpha and IL-10 in BALF in a pulmonary model of ARDS (panels 5A–E, respectively). Panel 5A shows that intra-peritoneal LPS administration significantly increased the levels of IL-1beta ($p < 0.001$), while LLLT significantly its levels, compared with LPS group ($p < 0.01$). Panel 5B and 5C shows that intra-peritoneal LPS administration significantly increased the levels of IL-6 ($p < 0.001$) and KC ($p < 0.001$), respectively, while LLLT significantly its levels, compared with LPS group ($p < 0.001$). Panel 5D shows that while intra-peritoneal LPS administration significantly increased the levels of TNF-alpha ($p < 0.01$), LLLT significantly reduced its levels ($p < 0.01$). Similarly to intra-tracheal model of intra-pulmonary ARDS, in the extrapulmonary model of ARDS (intra-peritoneal LPS administration), no differences were observed in the levels of IL-10 ($p > 0.05$).

3.6. Cytokines levels in serum in the pulmonary model of ARDS

Fig. 6 shows the serum levels of IL-6 and TNF-alpha in an extrapulmonary model of ARDS (panels 6A and 6B, respectively). In the panel 6A, the results show that LLLT significantly reduced intra-peritoneal LPS increased IL-6 levels ($p < 0.001$). In panel 6B, the results show that LLLT significantly reduced intra-peritoneal LPS increased TNF-alpha levels ($p < 0.05$).

4. Discussion

The present study showed for the first time the effects of LLLT (830 nm) reducing the acute pulmonary inflammation in a pulmonary and extrapulmonary model of LPS-induced ARDS in BALB/c mice, revealing that LLLT (830 nm) may inhibit acute pulmonary inflammation independent of etiology of primary insult.

Acute respiratory distress syndrome (ARDS) presents high rates of morbidity and mortality and the amount and the state (activation and apoptosis rate) of the neutrophils may be correlated with the diseases severity and prognosis [11]. In the present study, we found that both models (pulmonary and extrapulmonary) of LPS-induced ARDS significantly increased the migration

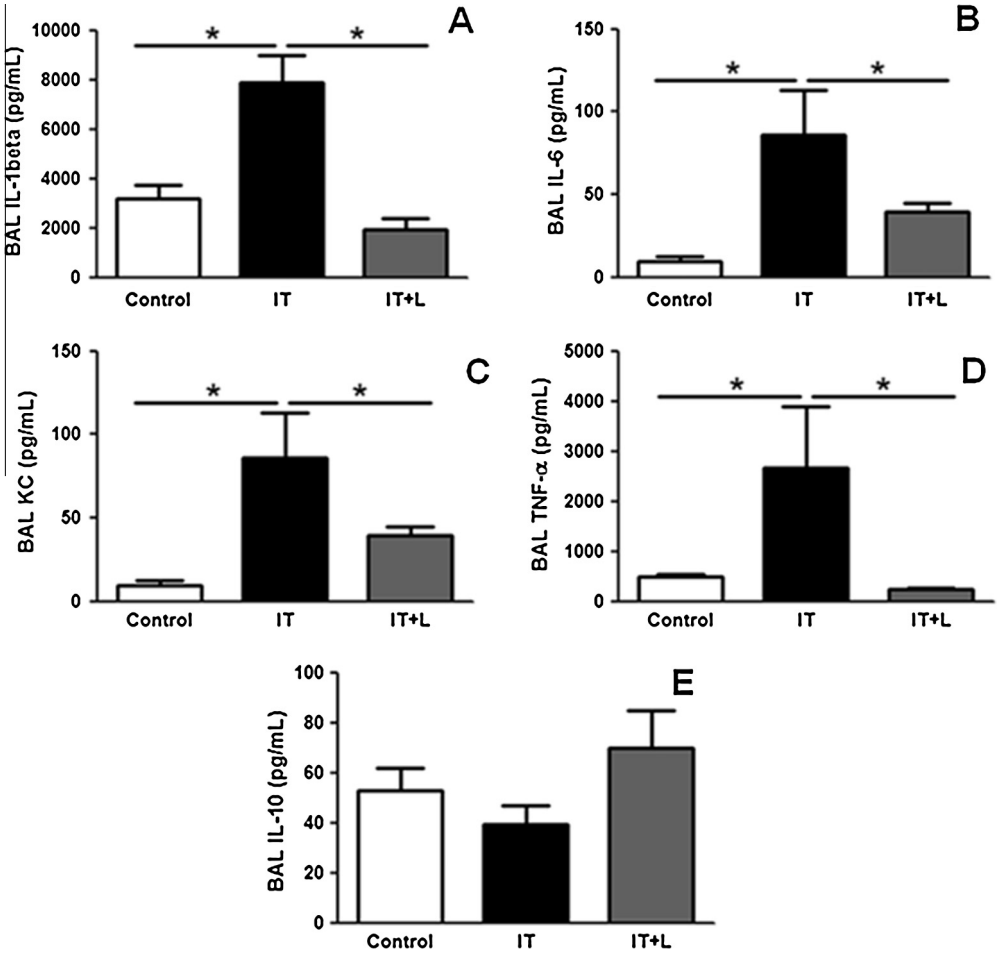

Fig. 2. Cytokines levels (IL-1beta, IL-6, KC, TNF-alpha and IL-10) in BALF in a pulmonary (IT) model of ARDS. In panel A, B, C and D, $^{*}p < 0.05$.

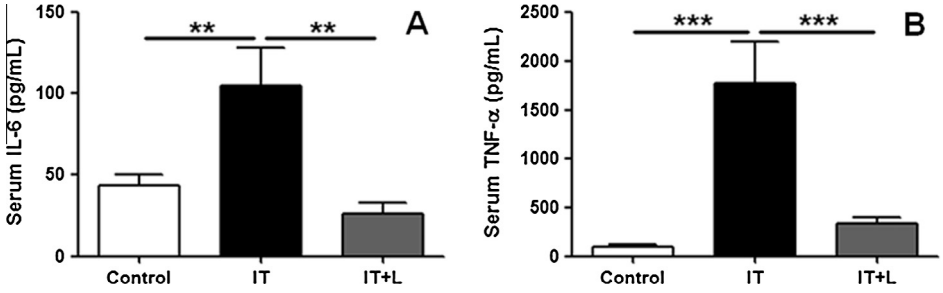

Fig. 3. Cytokines levels (IL-6 and TNF-alpha) in serum in a pulmonary (IT) model of ARDS (panels A and B, respectively). In panel A, $^{**}p < 0.01$ and in panel B, $^{***}p < 0.001$.

of neutrophils to the lungs, accordingly to the previous studies [17,23,13,20]. In the physiopathology of ARDS, neutrophils contribute to the lung injury releasing several mediators, i.e. free radicals, proteases, cytokines and chemokines [17]. Furthermore, the activation of neutrophils has been directly linked with ARDS' severity and mortality [11]. In this way, our results showed that LLLT was effective to reduce the migration of neutrophils to the lungs, as demonstrated through neutrophils counting in bronchoalveolar lavage and also by the quantitative analysis of the neutrophils number in the lung parenchyma. These anti-inflammatory effects of LLLT on neutrophils recruitment is particularly important, since that such effect was observed in pulmonary and extrapulmonary model of LPS-induced ARDS, reinforcing the beneficial effects of LLLT independent of the diseases etiology. This results are also in agreement with previous studies that have demonstrated that LLLT was able to reduce neutrophils migration in model of intestinal ischemia-reperfusion induce ARDS [4–7].

The modulation of neutrophilic inflammation in ARDS have been attributed to release of several pro-inflammatory cytokines, for instance, IL-1beta, IL-6, IL-8 and TNF-alpha [17]. Interleukin 1 beta (IL-1beta) is a potent pro-inflammatory cytokine and its increased levels in patients developing ARDS are related with poor prognosis of disease [19]. IL-1beta is thought to play a central role in the beginning of inflammatory process and the neutrophils to be the main source of IL-1beta release in during diverse inflammatory response [3]. IL-1beta also increases neutrophils survival, contributing for non-resolution of the inflammation [3]. In the present study we found increased levels of IL-1beta in both, pulmonary

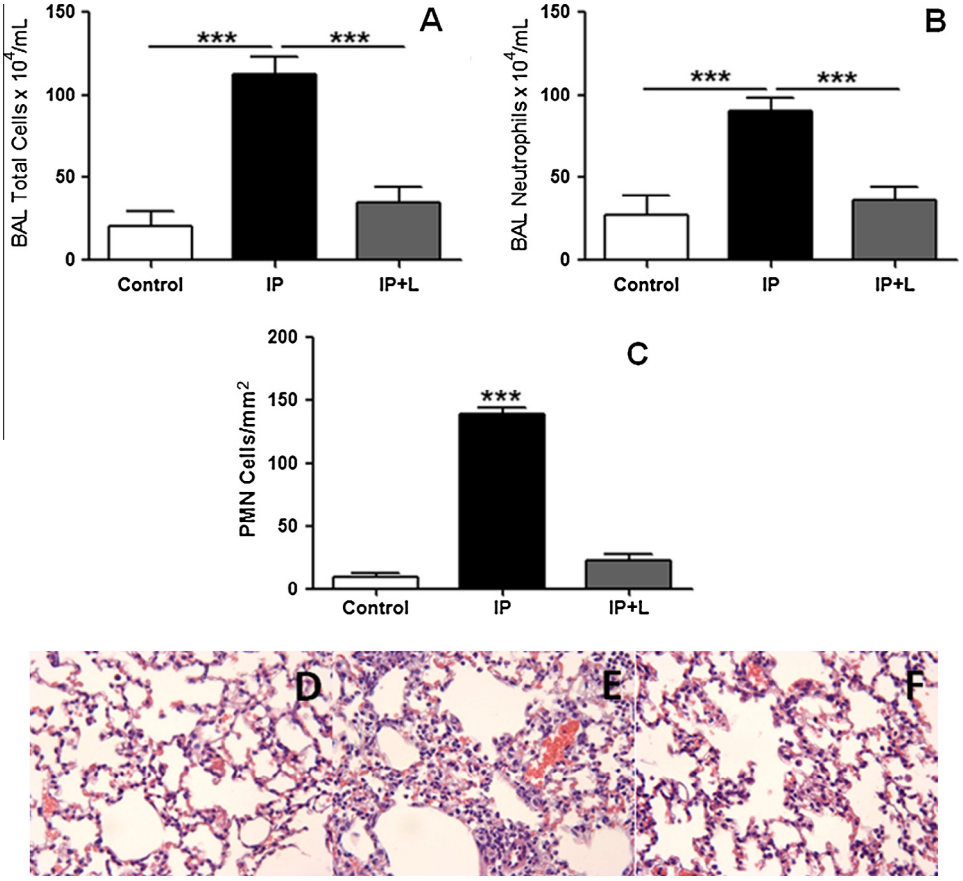

Fig. 4. Inflammatory profile in BALF (total cells – panel A; neutrophils – panel B) and the number of polymorphonuclear cells in the lung parenchyma (notably neutrophils – panel C) and the representative photomicrographs of control (panel D), LPS i.t. (panel E) and LPS i.t + laser (panel F) in the extra-pulmonary (IP) model of ARDS. In panel A, B and C, $^{***}p < 0.001$.

and extrapulmonary models of ARDS, in agreement with the current literature [17,3]. The present study also revealed that LLLT was capable to decrease the levels of IL-1beta in both models of ARDS, pointing out the inhibitory effects of LLLT on the pro-inflammatory mediators involved in the physiopathology of ARDS. Of note, a study has been found similar results concerning the suppressive effects of LLLT on the levels of IL-1beta, however, in a model of extra-pulmonary LPS-induced ARDS in rats [2].

Interleukin 6 (IL-6) is considered a pleiotropic cytokine, presenting a central role in the physiopathology of ARDS, beyond to be correlated with poor prognostic for disease [19,3,22,21]. The levels of IL-6 are increased in the lungs and also in the blood of humans and also in animal' models of ARDS [19,3,22,21]. In the present study we found increased levels of IL-6 in Bronchoalveolar Lavage Fluid and in serum of mice in both pulmonary and extrapulmonary model of LPS-induced ARDS. Of note, in both models, LLLT was able to significantly reduce IL-6 levels in Bronchoalveolar Lavage Fluid and also in serum, to values very close to values of control group. These findings are extremely relevant, since that increased levels of IL-6 are involved in the perpetuation of the inflammatory state and also in pro-coagulant response in ARDS [19,12].

Interleukin 8 (IL-8) and its functional homologue in mice (CXCL1/KC) present a central role in the physiopathology of ARDS, primarily mediating the chemotaxis for neutrophils [19,3]. However, IL-8 and CXCL1/KC also presents other important effects in the inflammatory process in ARDS, for instance, increasing of neutrophils survival [19,3,18], and also are related with ARDS severity and mortality. In the present study we found that in both models (pulmonary and extrapulmonary) of LPS-induced ARDS the pulmonary levels of CXCL1/KC are significantly elevated. On the other hand, in the present study, we also found that LLLT significantly reduced the pulmonary levels of CXCL1/KC, event that may be involved in the anti-inflammatory effects of LLLT.

Tumor necrosis factor alpha (TNF-alpha) is a cytokine involved in neutrophils adhesion and activation, and coagulation and edema formation, especially during events of acute lung inflammation [24,1]. This cytokine is accredited to be involved in IL-6 stimulation and release, playing a central role in the inflammatory process in ARDS [24,1]. Also, increased levels of TNF-alpha are found in the lungs and also in the systemic circulation of patients developing ARDS, reinforcing its role in the pathophysiology of the disease [19,22,21]. In the present study we found that the pulmonary and the extra-pulmonary model of LPS-induced ARDS coherently induced increases in the BALF and serum levels of TNF-alpha. On the contrary, LLLT significantly reduced the TNF-alpha levels in both models and also in both sites, in the lungs (in BALF) and also in the systemic circulation (in serum). These inhibitory effects of LLLT are particularly important, considering the potent pro-inflammatory effects and the central role of TNF-alpha in the pathophysiology of ARDS. Also, these results are in agreement with previous studies that have demonstrated that LLLT significantly reduced the mRNA expression of TNF-alpha in a model of immune-complex induce lung injury [1] and also in an *ex-vivo* study using rat bronchi, where LLLT reduced bronchi hyper reactivity to cholinergic agonist through a TNF-alpha dependent mechanism [16].

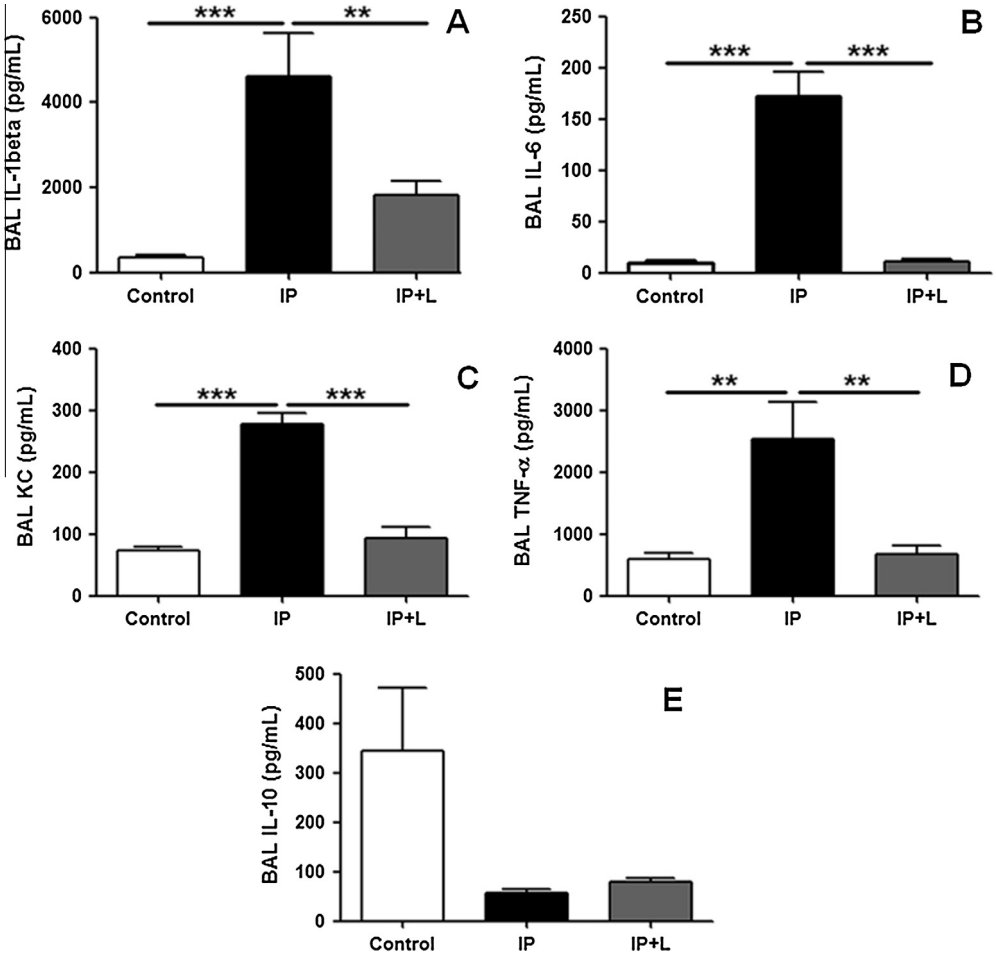

Fig. 5. Cytokines levels (IL-1beta, IL-6, KC, TNF-alpha and IL-10) in BALF in a extrapulmonary (IP) model of ARDS. In panel A, B, C and D, $^{***}p < 0.001$ and $^{**}p < 0.01$.

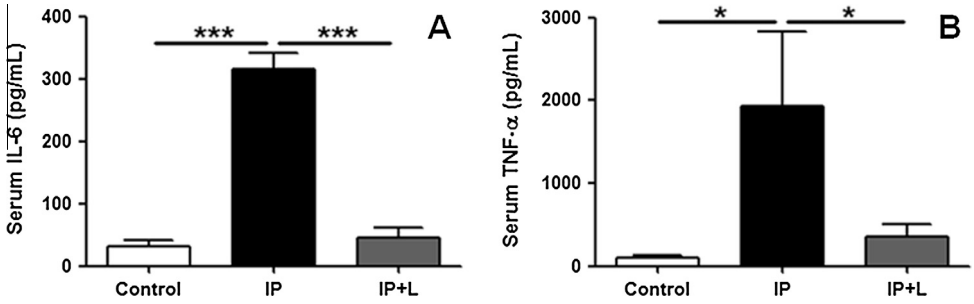

Fig. 6. Cytokines levels (IL-6 and TNF-alpha) in serum in an extrapulmonary (IP) model of ARDS (panels A and B, respectively). In panel A, $^{***}p < 0.001$ and in panel B, $^{*}p < 0.05$.

Therefore, we conclude that LLLT present important anti-inflammatory effects against the LPS-induced acute respiratory distress syndrome, independent of etiology of disease.

Conflict of interest

None.

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