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Systemic response induced by *Scorpaena plumieri* fish venom initiates acute lung injury in mice ☆

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Abstract

Scorpaena plumieri venomous fish inflicted severe injuries in humans characterized by systemic effects and cardiovascular abnormalities. Although cardiotoxic and hypotensive effects induced in rats by this venom have been studied, little is known about their effect on bronchial epithelial permeability and airway inflammation in mice. The primary goal of this study was to determine whether the intraplantar or intraperitoneal injection of *S. plumieri* venom results in systemic response, and whether this event initiates acute lung injury. We found that BALB/c mice developed neutrophilic infiltrates, areas of lung hemorrhage and alveolar macrophage activation within 24 h after injection with *S. plumieri* venom. These histopathological changes were associated with an early increase in BAL fluid protein and early induction of cytokines, chemokines and matrix metaloproteinases, followed by a later increase in BAL fluid neutrophils. These findings provide clear evidence that the injection of *S. plumieri* venom in footpad or peritoneal cavity of mice results in venom deposition in the airway and initiates a sustained inflammatory response in the lungs.

Keywords: *Scorpaena plumieri* venom; Lung injury; Inflammatory mediators; Alveolar macrophages

1. Introduction

Although venomous fish envenomations are less documented in Brazil than snake bites, some venomous species have been found. Here, special attention has been given to the venomous fish *Thallasophryne nattereri*, which provoke symptoms in fishermen and swimmers characterized by local pain and swelling, erythema, followed by intense necrosis that persists for several days (Fonseca and Lopes-Ferreirra, 2000). Murine models, which mimic the local manifestations of envenoming induced by *T. nattereri*, are used and show that low doses of the venom (0.3 μg/animal) induced local effects such as nociception and edema, similar to that described in humans (Lopes-Ferreira et al.,

☆ *Ethical statement*: All experiments are in accordance with the guidelines provided by the Brazilian College of Animal Experimentation, and were authorized by the Ethics Committee for Animal Research of the Butantan Institute (275/06).

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1998) and inflammatory response in footpad with cytokines such as TNF-α, IL-1β and IL-6 and a weak leukocyte influx (Lima et al., 2003). In addition, the venoms of the catfish *Cathorops spixii* or stingrays *Potamotrygon* cf. *scobina* and *Potamotrygon* gr. *orbignyi* induce local effects in mice mainly nociception and edema with moderate duration (Junqueira et al., 2007; Magalhaes et al., 2006).

The family Scorpaenidae represents a wide array of fish characterized by the ability to envenomate with various types of specialized spines (Kizer et al., 1985). This family includes the three genus: *Synanceja*, *Scorpaena* and *Pterois*. In a documented case Lehmann and Hardy (1993) described human envenomation by stonefish with pulmonary edema, developing within 35 min of envenomation that was confirmed radiographically. Wiener (1959) reported that injection of the venom from *Synanceja trachynis* into several animal species produced evidence of increased vascular permeability. In particular, acute pulmonary edema was observed in mice that were given the venom by intravenous injection.

Only members of the genus *Scorpaena* are found on the Brazilian coast, with *Scorpaena plumieri* being the most abundant specie found. The venomous apparatuses are composed of 13 dorsal, 3 anal and 2 pelvic short and thick spines with larger venom glands. The bizarrely shaped fish is often taken for a weed-covered stone, and accidents occur when swimmers, divers or fishermen step on the spines of the dorsal fin (Halstead, 1951; Roche and Halstead, 1972). After compression of the venom glands located at the base of the spines, venom travels from the glands through anterolateral depressions in the spines and into the wound. Symptoms are limited to severe local manifestations in the extremity involved (Haddad et al., 2003), and cardiotoxic and hypotensive effects induced in rats by this venom has been studied (Carrijo et al., 2005). The intensity of clinical features of *S. plumieri* envenomation is related to the concentration of venom injected in one or more puncture wounds. Patients can be stung by one or all spines present in the dorsal region of the fish, and each spine contains 5–10 mg of venom (Gwee et al., 1994).

The main objective of this study was to determine whether the injection of *S. plumieri* venom results in systemic response in mice, and whether this event initiates acute lung injury. Venom of *S. plumieri* was administered by an intraplantar or an intraperitoneal (i.p.) route in BALB/c mice, and remote lung injury was assessed by histopathology, increase of vascular permeability, neutrophil influx and production of the pro-inflammatory mediators such as IL-6, KC and MMPs, and the contribution of alveolar macrophages (AMs) was investigated.

2. Methods

2.1. Venom

Venom was obtained from specimens of *S. plumieri* (Fig. 1) collected in the coastal State of Espírito Santo, Brazil. The venom extraction was carried out according to Schaeffer et al. (1971). Protein content was determined by the colorimetric method of Bradford (1970) using bovine serum albumin (Sigma) as standard protein.

2.2. SDS–Polyacrylamide gel electrophoresis (SDS–PAGE)

The proteins (10 μg) of *S. plumieri* venom were analyzed by SDS–PAGE under non-reducing conditions by Laemmli (1970) using 12% gel and stained with silver. A pre-stained protein calibration mixture including β-galactosidase (116), bovine serum albumin (66), ovalbumin (45), lactase degydrogenase (35), Rease Bsp98I (25) and β-lactoglobulin (18.4) (Sigma Chemical Company, St. Louis, MO, USA) was used as molecular weight markers (MW, kDa).

2.3. Animals

Male BALB/c mice weighing 18–20 g were provided by the Instituto Butantan. The animals were maintained at the animal house facilities under specific pathogen-free conditions in temperature- and humidity-controlled rooms, and received food and water *ad libitum*. All the procedures involving mice were in accordance with the guidelines provided by the Brazilian College of Animal Experimentation (275/06).

2.4. Model of acute lung inflammation

S. plumieri venom dissolved in sterile PBS was administered by an intraplantar or an i.p. route. Venom (100 μg of protein) diluted in 500 or 40 μL sterile PBS were injected in two groups of mice i.p. or into the intraplantar region of the hind foot paw,

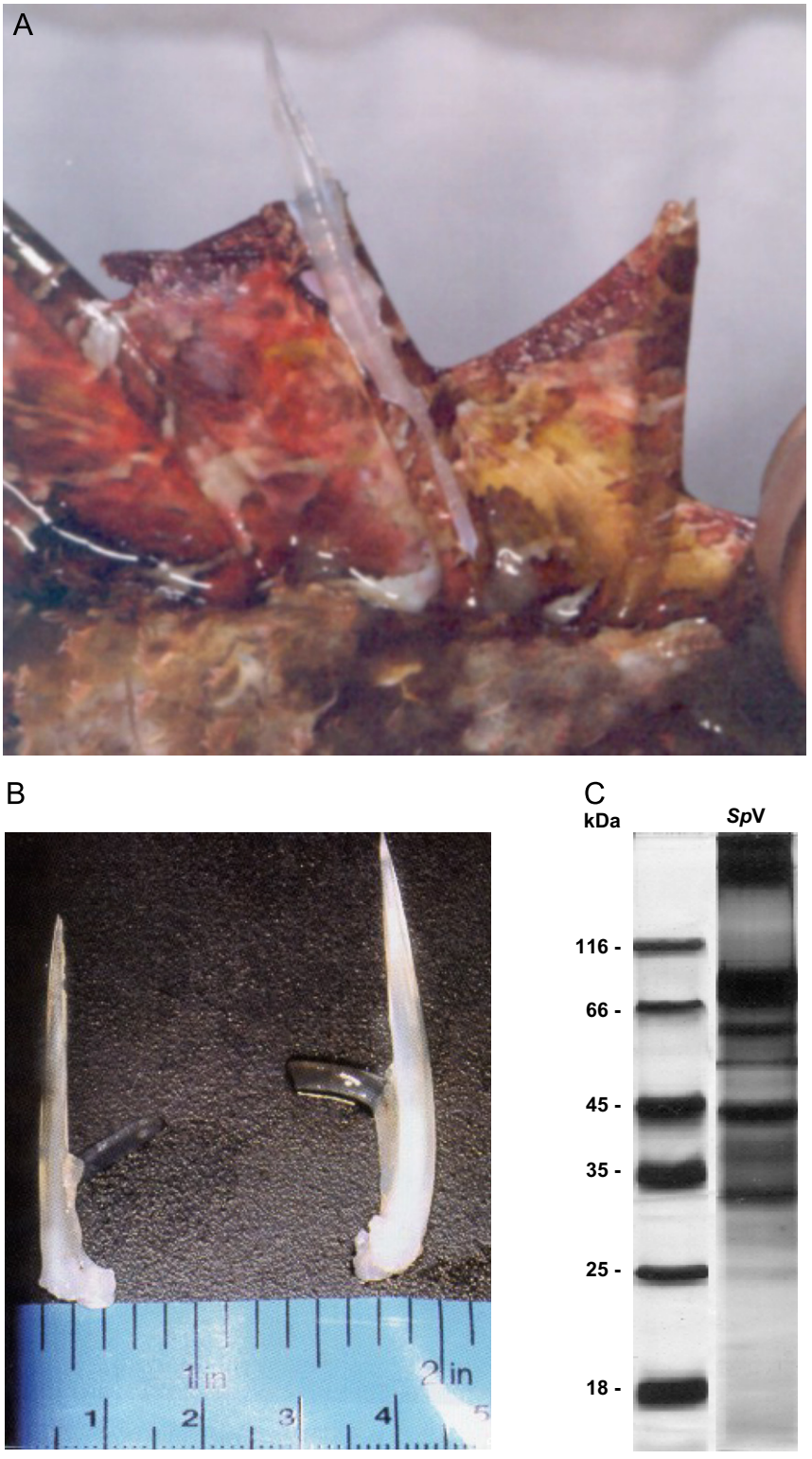

Fig. 1. Dorsal spines (A, B) of *Scorpaena plumieri*, the Brazilian venomous fish, and the eletrophoretical profile of the venom (C). Venom of *S. plumieri* (*Sp*V) was analyzed by SDS–PAGE gel 12% under no-reduction conditions, and revealed by Silver methods. Numbers on the left correspond to position of molecular weight markers (MW, kDa).

respectively. The control group was injected only with sterile PBS. All samples were collected 2, 6 and 24 h after injection.

2.5. Bronchoalveolar lavage (BAL) fluid

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in the BAL according to the described method (Lima et al., 2007). Mice were killed with i.p. pentobarbital injection (60 mg/kg; Sanofi, Libourne, France), the chest wall was opened and the tracheas were cannulated. The airway lumina were washed and the resulting BAL was immediately centrifuged at 500*g*, 4 °C, for 10 min. BAL cells were resuspended and supernatants were stored for further analysis. Cell counts were performed using a hemocytometer, and cytocentrifuge (Cytospin II; Shandon, Cheshire, UK) slides were stained (Hema 3, Scientific Products, Chicago, IL). For differential cell counts, 300 leukocytes were enumerated and identified as mononuclear cells or neutrophils, on the basis of staining and morphologic characteristics using a conventional light microscope (Axio Imager A1, Carl Zeiss, Germany). After BAL collection, the lungs were processed by homogenate collection and further analysis. The total protein concentration in BAL fluid was measured using the Bradford method (1970).

2.6. Quantification of IL-6, KC and MCP-21

Cytokines and chemokines were measured in lung tissue homogenates by a specific two-site sandwich ELISA, using the OpEIA Kit (BD-Pharmingen, San Diego, CA, USA). Detection limits were 31.3 pg/mL for IL-6, 7.8 pg/mL for MCP-1 and 15.6 pg/mL for KC.

2.7. Determination of MMP-9 levels by ELISA

MMP-9 levels in lung samples were analyzed by mouse ELISA kits following the manufacturer's instructions (R&D Systems, Minneapolis, MN). Detection limit was 0.63 ng/mL.

2.8. Determination of MMPs in lung after venom injection by gelatin zymography

Enzymatic activity in BAL or lung homogenates of mice injected or not with *S. plumieri* venom was analyzed by electrophoresis in polyacrylamide gels containing SDS and gelatin as described previously (Hibbs et al., 1985; Kleiner and Stetler-Stevenson, 1994). The SDS–PAGE was carried out using 10% polyacrylamide gels containing 1 mg/mL. After electrophoresis, the gels were stained with Coomassie Brilliant blue and destained by acetic acid in methanol and H_2O (1:3:6), both for 2 h, to visualize bands with gelatinolytic activity. Gels were then photographed by means of a digital camera (Nikon Coolpix S1; Nikon). The molecular mass (kDa) of the gelatinases was estimated against markers of known molecular mass (Sigma-Aldrich).

2.9. Pathological analysis of lungs

The left lung was ligated and removed, whereas the right lung lobes were washed once with ice-cold HBSS (BAL) then fixed (10% formaldehyde) and paraffin-embedded. Paraffin-embedded sections (5 μm) were stained with hematoxilin/eosin (H&E) to evaluate general morphology. All slides were examined with light microscopy at a magnification of ×40 (Axio Imager A1, Carl Zeiss, Germany) calibrated with a reference micrometer slide. For each group of six mice, four stained lung sections from each mouse were analyzed.

2.10. Immunohistochemistry for detection of venom in the lung

Venom was detected as described previously (D'Suze et al., 2002, with a slight modification). Sections were deparaffinized, rehydrated and incubated in 3% H_2O_2 in methanol for 10 min at room temperature to block endogenous peroxidase activity. The sections were then washed with PBS three times, incubated in 0.1% trypsin (Sigma Chemical) in 0.1% $CaCl_2$ at 37 °C for 10 min and rinsed with 4 M HCl for 15 min. After nonspecific binding blocking, the lung sections were incubated for 1 h with sera of mice immunized with *S. plumieri* with high titers of specific antibodies. After incubation, goat anti-mouse IgG HRP (R&D Systems) was detected by incubating the sections for 5 min with chromogenic substrate for the peroxidase 3,3′-diaminobenzidine (Sigma), providing a brown reaction product. Sections were counterstained with hematoxylin and mounted in Paramount. The control procedure was performed for venom detection using normal mice serum.

2.11. Stimulation of alveolar macrophages with S. plumieri venom

After BAL collection from normal BALB/c mice, cells were cultured for AM as described previously (Landsman and Jung, 2007; Vermaelen and Pauwels 2004; Gonzalez-Juarrero et al., 2003). Cells were incubated for 1 h at 37 °C, 5% CO_2 to allow attachment of macrophages. Isolated adherent AMs were identified by morphology and by specific antibodies and counted on a hemocytometer. All isolations were >95% macrophages. Murine AMs express the β-integrin CD11c, low levels of MHC II and are negative for CD11b and CX_3CR1 (data not shown). AMs (4×10^5) were stimulated with 100 μg protein of *S. plumieri* venom for 24 h for detection of IL-6.

2.12. Assessment of apoptosis

Apoptosis of AMs stimulated with 100 μg protein of *S. plumieri* venom for 24 h was assessed using annexin V staining and flow cytometry according to the manufacturer's directions (BD Pharmingen).

2.13. Statistical analysis

All values were expressed as mean ± SEM. Parametric data were evaluated using analysis of variance, followed by the Bonferroni test. Non-parametric data were assessed using the Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$. The SPSS statistical package (Release 13.0, Evaluation Version, 2004) was employed. Experiments were repeated at least three times.

3. Results

3.1. Protein profile of S. plumieri venom

To verify the eletrophoretical profile, the venom was submitted to 12% SDS–PAGE (10 μg of protein/well). *S. plumieri* venom presented nine important bands: one band located above 116 kDa, one band located above 66, two bands located between 45 to 66 kDa, one band located at 45 kDa, another located below 45 kDa, two located below 35 kDa and the last one located at 25 kDa (Fig. 1C).

3.2. Effect of S. plumieri venom on BAL protein, leukocyte recruitment and pro-inflammatory mediator production in lungs

As a functional measurement of alveolar epithelial disruption, we measured total protein in BAL fluids. Between 2 and 24 h after i.p. *S. plumieri* venom injection, the BAL total protein concentration was significantly increased in BALB/c mice compared with the control group of mice (Fig. 2A). However, 6 and 24 h after intraplantar injection of venom, the concentration of total protein in the BAL fluid was significantly increased (Fig. 2B).

After i.p. injection of venom, an accumulation of leukocytes, mainly macrophages, was observed in BAL at 2 h (Fig. 3A and B). In contrast, in intraplantar-injected mice the presence of AMs was evident at 6 h, remained elevated until 24 h (Fig. 4C). The macrophage influx was followed by the recruitment of neutrophils to the BAL 24 h after injection (Fig. 4B).

The potential release of monocyte or neutrophil chemoattractants into the lung after venom i.p. or intraplantar injection was investigated for several inflammatory mediators. High levels of IL-6 and MCP-1 were detected 2 h after i.p. injection (Fig. 5A and B). In order to determine MMPs protein in lung, an ELISA assay was used. The concentration of MMP-9 in lung homogenates recovered from venom-injected mice was 1.5 times greater than that detected in the lung from control mice, and remained elevated until 24 h (Fig. 5C). The injection of 100 μg of venom by intraplantar route only induced the production of significant levels of KC and MCP-1 at 2 h that returned to basal levels 6–24 h after venom administration (Fig. 6A and B). KC or IL-6 was not detected after i.p. or intraplantar injection of venom, respectively (data not shown).

3.3. Induction of active matrix metalloproteinases in BAL and lung after intraplantar venom injection

The profile of proteinases released into BAL fluid or lung homogenates from control- or venom-injected mice was analyzed using zymography. The intraplantar injection of venom increased the proteolytic activity of MMP-2 in BAL at 6 h and the activity of MMP-9 at 24 h (Fig. 6C, left) compared with control mice. Two and 24 h after intraplantar injection of venom the proteolytic activity of MMP-9 was observed in the lung homogenate (Fig. 6C, right). The proteolytic activity of MMP-2 in lung homogenates of

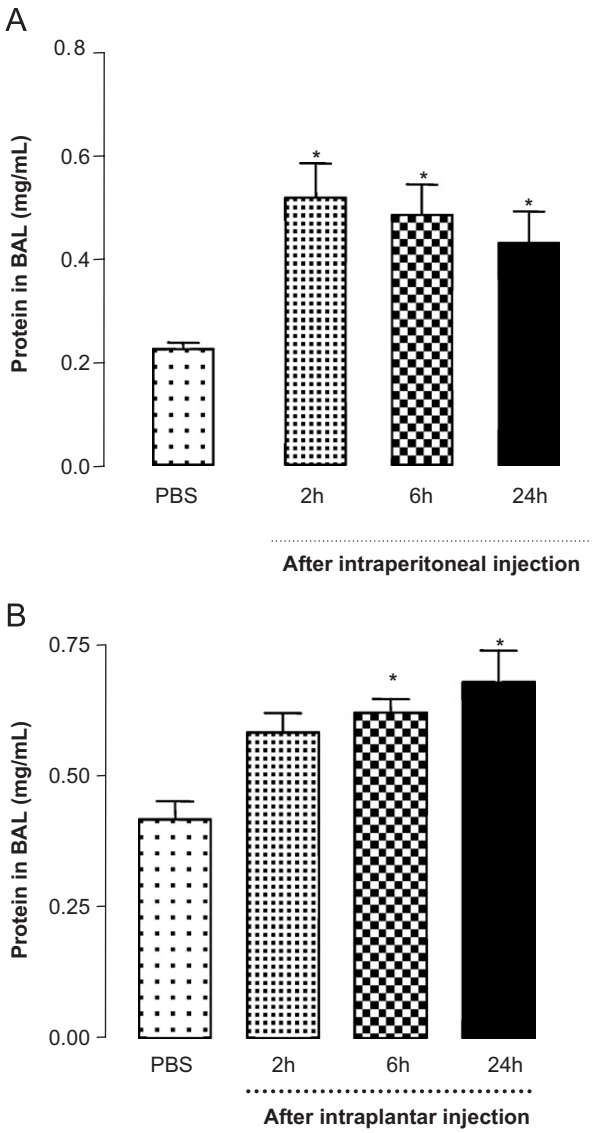

Fig. 2. Protein recovery in the bronchoalveolar lavage (BAL) in the mice injected with *S. plumieri* venom. Mice were i.p. (A) or intraplantar (B) injected with 100 μg of protein venom diluted in 500 or 40 μL sterile PBS, and a control group was injected only with PBS. After 2, 6 or 24 h, BAL was collected for total protein measurement by the Bradford method after 2, 6 or 24 h. $*p<0.05$ compared with control group.

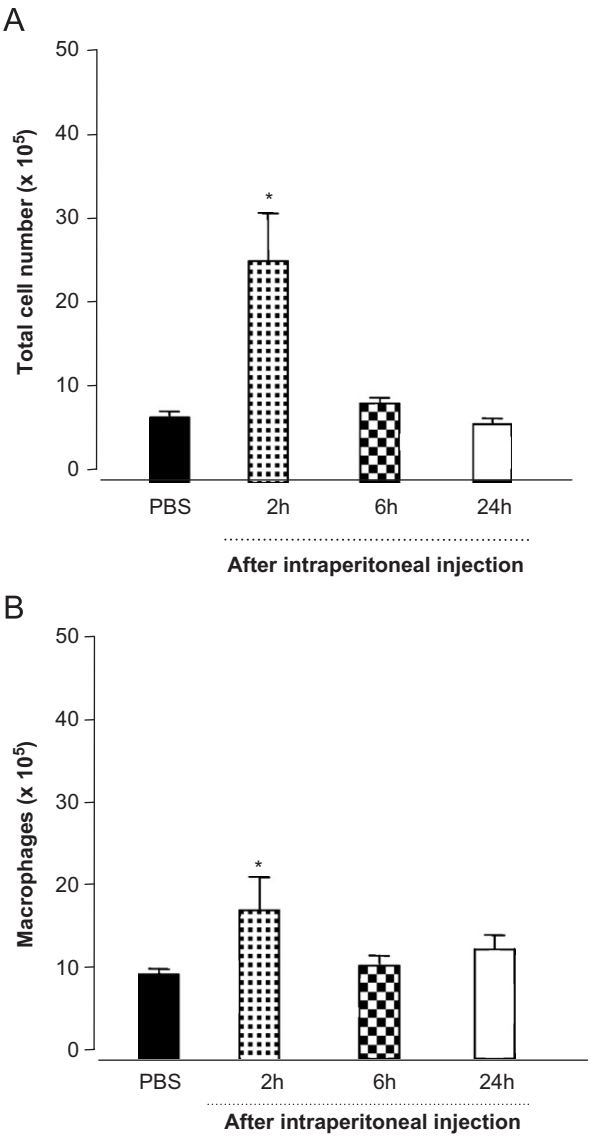

Fig. 3. Total BAL leukocytes (A) or macrophages counts (B) at 2, 6 or 24 h after the i.p. administration of 100 μg of *S. plumieri* protein venom diluted in 500 μL sterile PBS. $*p<0.05$ compared with the control group.

venom- or control-injected mice was similar. The data show that *S. plumieri* venom directly induces MMPs expression in AMs in the lung.

3.4. Histopathological evidence of lung injury induced by S. plumieri venom

The histological examination of paraffin-embedded lung sections of mice receiving i.p. or intraplantar injection of PBS revealed a normal cellular architecture (Fig. 7A and D). However, 2 h after i.p. injection of venom, mice presented a significant perivascular hemorrhage (Fig. 7B) and venom deposition throughout the lung interstitium (Fig. 7E). Interestingly, in intraplantar-injected mice, areas of neutrophilic infiltrate characterized the lung injury (Fig. 7C) with venom adhering to the endothelium of venules in the alveolar walls (Fig. 7F). Intraplantar injection of the venom also induced airway constriction (Fig. 7C).

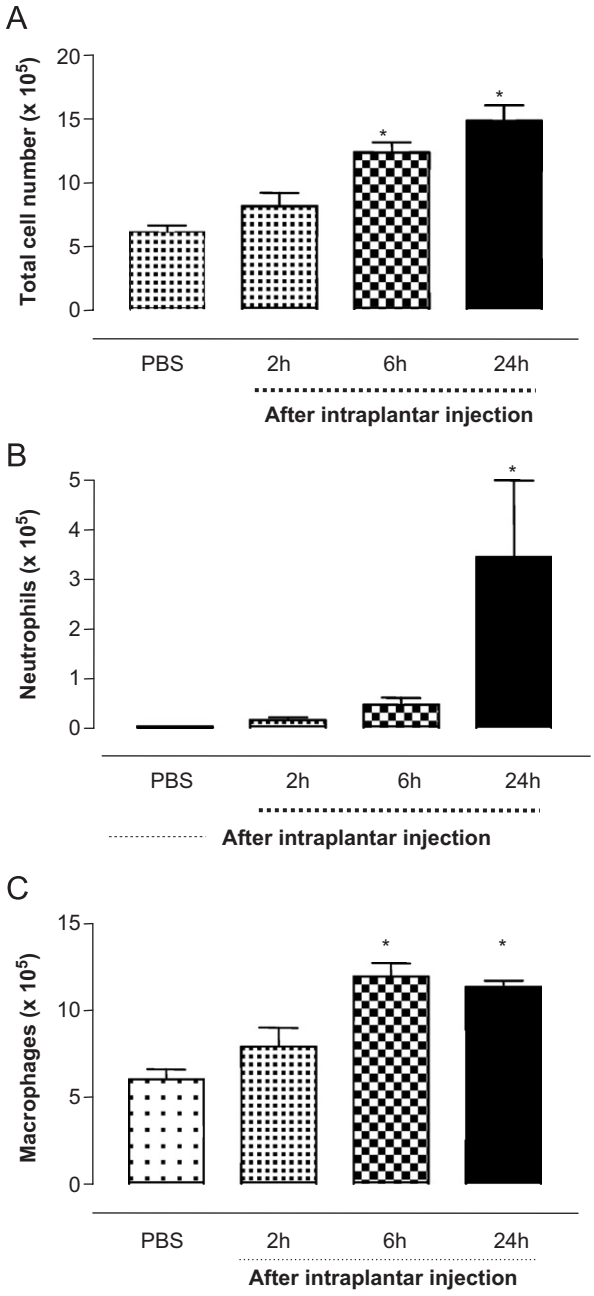

Fig. 4. Neutrophilic inflammation induced by intraplantar administration of 100 μg of *S. plumieri* protein venom after 2, 6 or 24 h. Total BAL leukocytes (A), neutrophils (B) and macrophages were count (C). *$p < 0.05$ compared with control group.

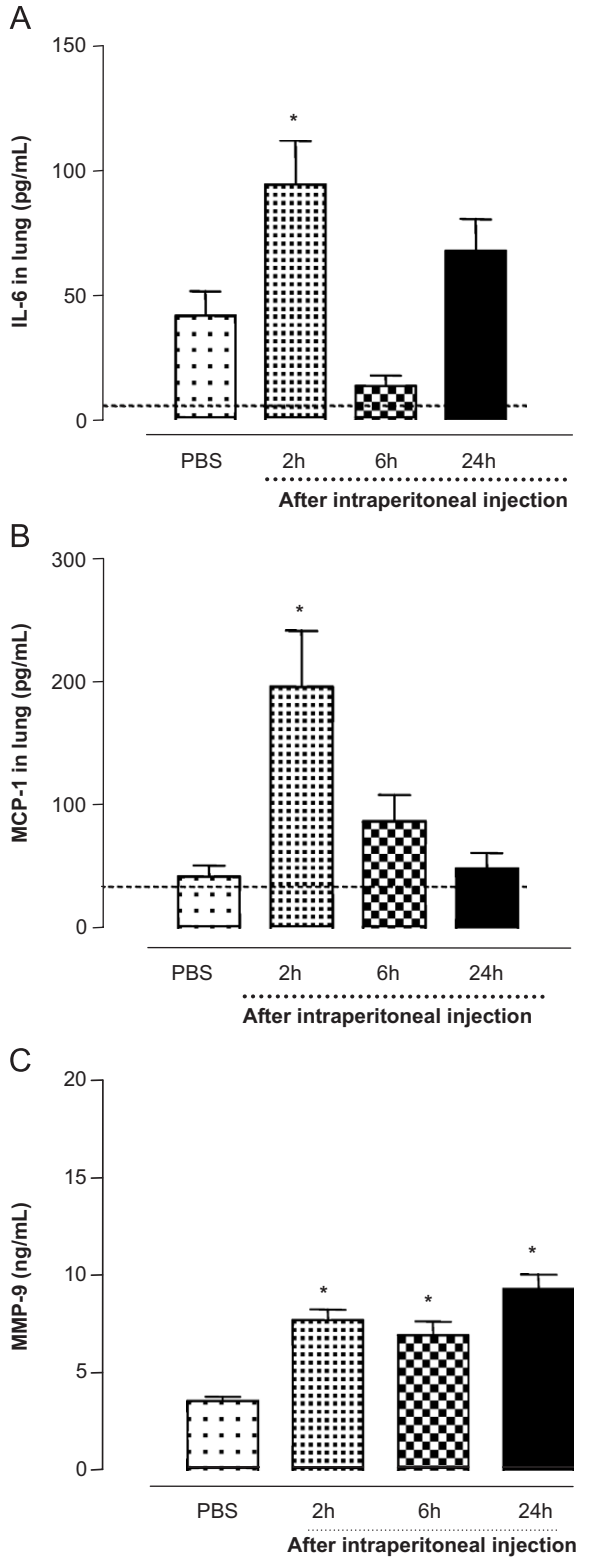

Fig. 5. Analysis of IL-6, MCP-1 and MMP-9 levels by ELISA in lung of mice injected by intraperitoneal route. After 2, 6 or 24 h of i.p. injection of 100 μg venom, lung homogenates were collected for IL-6 (A), MCP-1 (B), and MMP-9 (C) determination by ELISA. *$p < 0.05$ compared with control group.

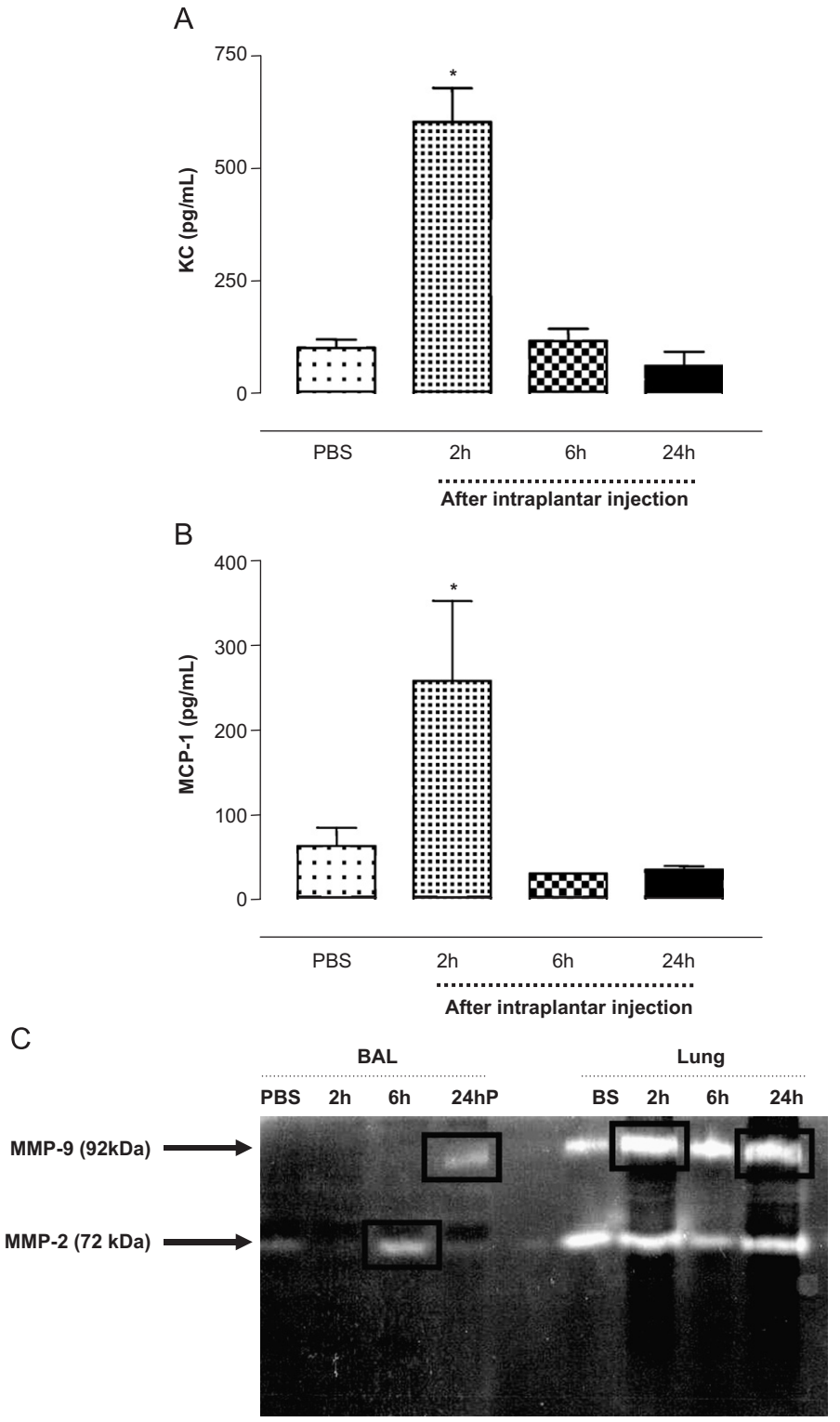

Fig. 6. KC and MCP-1 levels and activity of MMP-2 and MMP-9 in lung after intraplantar injection of *S. plumieri* venom. After 2, 6 or 24 of intraplantar injection of 100 μg venom, lung homogenates were collected for KC (A) and MCP-1 (B) determination by ELISA, and MMPs (C) determination by gelatin zymography. $*p<0.05$ compared with control group.

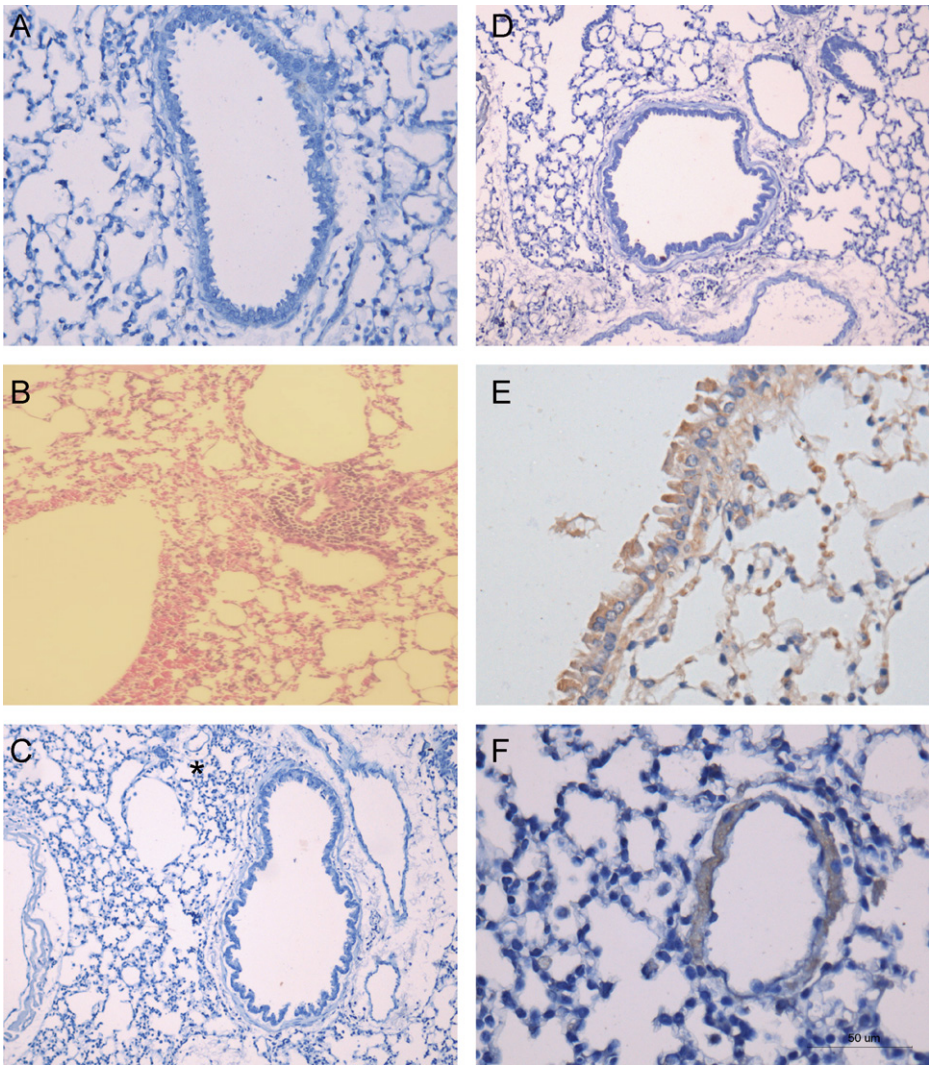

Fig. 7. Pathological analysis and detection of venom in the lungs of mice 2 h after i.p. or intraplantar administration of venom. H&E staining of lung tissue of venom-injected mice shows the presence of erythrocytes around the bronchial tissue (B, asterisk) and leukocytes in all interstitium with airway constriction (C). Imunohistochemistry shows the presence of venom dispersed in the lung compartment and epithelial cells (E) or in the arterial (F) after i.p. or intraplantar injection of venom (respectively). Control animals showed normal tissue architecture with no leukocyte infiltration or venom depots (A and D).

3.5. S. plumieri venom induced IL-6 production by alveolar macrophages and apoptosis

As seen in Fig. 8A, S, *plumieri* venom (100 μg) induced IL-6 production by AMs after 24 h of *in vitro* stimulation. AMs after 24 h of venom stimulation become apoptotic cells, as compared with macrophages without treatment (Fig. 8B).

4. Discussion

In humans, acute lung injury is characterized by epithelial and endothelial injury, neutrophilic alveolitis, and hyaline membrane formation. Neutrophil activation lead to the accumulation of oxidants and proteases in the lungs, which damage the cells of the alveolar environment. *Scorpaena plumieri* venomous fish inflicted severe injuries in humans characterized by systemic effects and cardiovascular abnormalities. Although cardiotoxic and hypotensive effects induced in rats by this venom have been studied, little is known about their effect on bronchial epithelial permeability and airway inflammation in mice. The primary goal of this study was to determine whether the intraplantar or i.p. injection of *S. plumieri* venom results in systemic response,

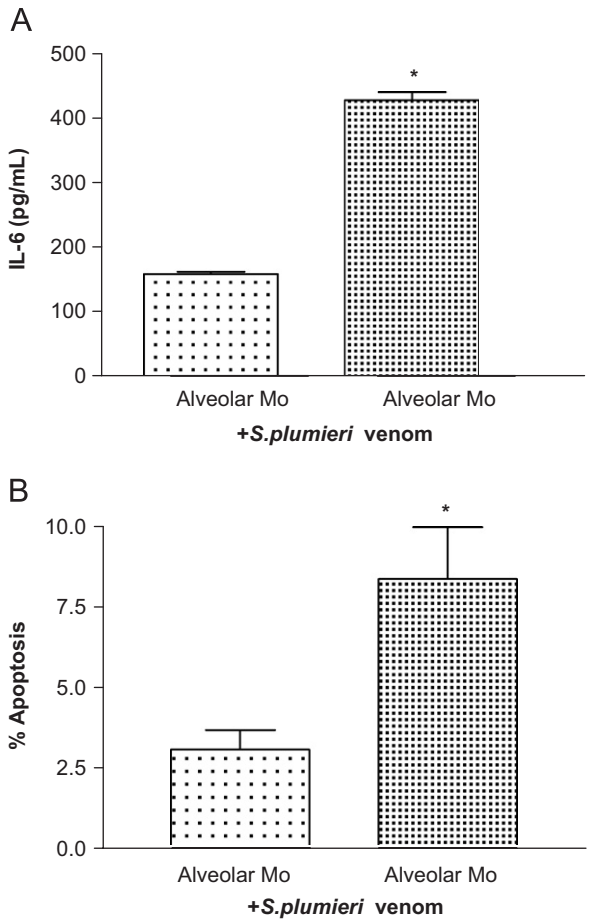

Fig. 8. *S. plumieri* venom induced IL-6 production (A) by alveolar macrophages and apoptosis (B). Increased percentage of apoptotic alveolar macrophages induced by *S. plumieri* venom was assessed via flow cytometry for their ability to bind annexin V.

and whether this event initiates remote lung injury. We found that BALB/c mice developed neutrophilic infiltrates, areas of lung hemorrhage and alveolar macrophage (AM) activation within 24 h after injection with *S. plumieri* venom. These histopathological changes were associated with an early increase in BAL fluid protein and early induction of cytokines, chemokines and matrix metalloproteinases, followed by a later increase in BAL fluid neutrophils. These findings provide clear evidence that the injection of *S. plumieri* venom in the footpad or peritoneal cavity of mice results in venom deposition in the airway and initiates a sustained inflammatory response in the lungs.

The increase in protein permeability across the endothelial and epithelial barriers of the lung is an early characteristic of lung injury. The present study shows that the injection of *S. plumieri* venom in the footpad or peritoneal cavity of mice lead to the deposition of venom in the lung and to endothelial barrier dysfunction and microvascular hyperpermeability, causing an early increase in the BAL fluid total protein concentration and alveolar edema.

Inflammatory response is characterized by initial activation of pro-inflammatory cytokine/chemokine genes and release of soluble proteins, followed by leukocyte accumulation. Leukocyte recruitment was seen after 2, 6 and 24 h of the venom injection (intraplantar or i.p.) and was accompanied by increased release of pro-inflammatory mediators as IL-6, MCP-1 and KC. This contrasts with IL-1β, which was not detected following venom administration. All mediators can be produced by the activated macrophages in the lung (Liu, 2001; Simon and Paine, 1995) and are all potent inducers of MMPs (Gan et al., 2001; Miyajima et al., 2001). AMs located at the air–tissue interface of the lung are strategically positioned to respond to the local appearance of microbial or inflammatory agents, thus contributing to the initiation of lung inflammation and removing injured cells. The late recruitment of neutrophils after intraplantar injection of *S. plumieri* venom is consistent with the murine model of acute lung injury induced by intranasal instillation of anti-FAS antibody (Matute-Bello et al., 2001).

These results suggest that *S. plumieri* venom elicited a remarkable adhesion molecule engagement among leukocytes and the endothelium, and significant levels of chemokines thought to integrate inflammatory signals for transmigration. The infiltration we observed was broadly consistent with the induction of KC and MCP-1, a potent murine neutrophil or monocyte chemoattractant protein (Henderson et al., 2003), and increased levels of IL-6 protein, which plays a critical role in mediating the transition from neutrophilic to monocytic inflammation (McLoughlin et al., 2004). Thus, adherent neutrophils produce and release oxygen free radicals and enzymes, such as proteases (e.g., elastase and metalloproteinases) and myeloperoxidase, exacerbating damage to endothelial cells and to subendothelial matrix proteins and inducing tissue injury (Weiss, 1989).

After neutrophils are sequestered in the pulmonary vasculature, activated neutrophils adhere to the pulmonary endothelial cell layer and migrate through the interendothelial cell junctions down to the underlying basement membrane. The gelatinases

MMP-2 and MMP-9 cleave type IV collagen and allow neutrophils to pass freely into lung tissues and contribute to airway inflammation (Owen et al., 2003). Macrophages are the most abundant defense cells present in both normal or injured lung tissue, with the capacity to degrade and remodel the extracellular matrix and the basement membrane through synthesis and secretion of proteinases, including MMPs (Hibbs et al., 1987). Although macrophages are known to produce MMP-2 and MMP-9 (Mautino et al., 1997; Vu and Werb, 1998), MMP-9 expressed on the cell surface of murine neutrophils may be up-regulated after stimulation with KC chemokine (Masure et al., 1991). These data correlated well with the significant production and activity of MMP-2 and MMP-9 in BAL and lungs after intraplantar injection of venom, suggesting that AMs are the initial source of MMPs followed by neutrophils.

AMs are activated at an early stage (within 30 min) of acute lung injury (Eppinger et al., 1997) when they secrete the chemoattractant for neutrophils (KC) and the MCP-1, which is specific for monocyte chemotaxis and activation. These findings support the hypothesis that *S. plumieri* venom may mediate the migration of neutrophils into the lung by acting directly on AMs.

In our model, the presence of venom proteins in the lung interstitium after intraplantar injection of *S. plumieri* venom leads to neutrophilic inflammation with AM activation (IL-6 production) and apoptosis. Lung cellular apoptosis is a dominant mechanism of lung injury (He et al., 2005). In addition to cytokine production and antigenic particle uptake (Tao and Kobzik, 2002), induction of AM apoptosis may represent a mechanism by which pro-inflammatory signals, generated by the interaction of venom with lung cells, are down-regulated. AM apoptosis could, therefore, also play a critical role in limiting lung injury and resolution of the inflammatory response *in vivo* (Haslett, 1999).

In conclusion, our data show that envenomation by *S. plumieri* venom can induce systemic response in a BALB/c mice and lung injury, triggering alveolar edema and neutrophilic inflammation. In addition, AM and neutrophils act as a source of MMPs that together play a key role in the cascade of events leading to lung injury. Our findings also confirm a central role for macrophage and neutrophils in the pathogenesis of venom-induced lung injury and also the importance of AMs in the resolution of this process triggered by *S. plumieri* fish venom.

Acknowledgments

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