Efficacy of *Bothrops jararaca* venom on the changes in immune functions and TH1/TH2 cytokine balance in murine macrophages

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Abstract

Background: Hemorrhage, intravascular coagulation and cardiovascular shock are effects commonly observed in victims bitten by *Bothrops* snakes. The severity of the envenomation is dependent on the immunological condition of the victim. This study was designed to evaluate the effects of *Botrhops jararaca* venom on macrophage functions and TH1 / TH2 cytokine balance.

Methods: Various amounts of *B. jararaca* venom were used, and the activation of macrophages was determined by cytotoxicity assays, hydrogen peroxide production, cell expansion, and phagocytosis. The release of cytokines presents in macrophages treated with *B. jararaca* venom were measured by ELISA.

Results: The results showed that the *Bothrops jararaca* venom altered the function of peritoneal macrophage cultures, increasing cytotoxicity and production of H_2O_2. The venom was able to inhibit cell expansion and phagocytosis. The effects of the venom on immunological mediator production in peritoneal macrophage cultures resulted in an increase in TNF-α, IL-1β and IL-6 production at 24 hours of treatment. The maximum production of NO and IFN-γ was observed at 24–48 and 48-72 hours, respectively. The elevated production of IL-4 and -10 was observed from 24 up to 120 hours after venom exposition.

Conclusion: The combined data suggest that *Bothrops jararaca* venom has an immunomodulatory effect for the first 24 hours; of venom treatment was observed a balance between TH1 and TH2 cytokines. After this time period with a TH2-dominant response in the TH1/Th2 cytokines.

1. Background

The envenomation caused by a snake bite from *Bothrops jararaca* is a common medical problem in South America (1). The venom of these snakes has been described to have proteolytic action, with coagulant, cytotoxic and rhabdomyolytic effects (2). Recent studies have shown that *Bothrops jararaca* venom (VBj) is a complex mixture of compounds that induce various biological activities and has been previously noted to be involved in disordered homeostasis of the inflammatory response associated with envenomation (3-10). Immune system activation during envenomation attempts to maintain homeostasis of the victim's body; and septic shock can develop as a result of excessive immune responses, with the release of associated immune mediators (11). Various studies have shown that *Bothrops jararaca* venom is able to induce an increase in leukotriene B4, thromboxane A2, interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) and in various adhesion molecules (12,13). Another mediator observed after envenomation is nitric oxide, which has been associated with a number of conditions including severe poisoning (14,15).

As part of the inflammatory process, tissue damage after a snake bite occurs when the substances released in the affected area activate macrophages, which begin to devour damaged tissues (16-18). The more intense the inflammatory reaction is, the greater the tissue injury, because the first line of immune defense is macrophages, which get activated by the inflammatory process. After an injury or infection macrophages can produce many factors that influence their own physiology (19,20). They exhibit different phenotypes, which alter their cell morphology, antigen expression and their various surface proteins that trigger responses to environmental signals (21,22). There are two general phenotypic categories into which macrophages are classified: classical activation (M1) and alternative activation (M2) (17,23).

M1 macrophages can also be generated transiently, in response to stimuli by TH1 cytokines, such as interferon-gamma (IFN-γ), TNF-α and IL-6, as well as

by microbial triggers. Macrophages M1 are essential components in the host defense response, and their activation must be tightly controlled because excessive cytokines and other mediators can lead to damage to the host tissues (24). In contrast, M2 macrophages are involved in parasite containment and immunoregulatory functions by reducing secretion of proinflammatory cytokines (24). These cells are activated by IL-4, IL-13, glucocorticoids, immune complexes, and IL-10 – all factors representative of the TH2 type immune response. Early production of IL-4 leads resident macrophages to enter the M2 program, and is associated stimulation of characteristic arginase activity (25). Another characteristic observed in M2 macrophages is the secretion of high amounts of IL-10 and low levels of IL-12; they also have efficient phagocytic activity and high expression of scavenging molecules. Disease states are often associated with dynamic changes in the activation of macrophages, with M1 cells being involved in the initiation and maintenance of inflammation, and M2 cells being associated with processes of resolution or chronic inflammation (17).

Macrophages play key roles in the immune system by acting as antigen-presenting cells, producing cytokines and also having a very important role as phagocytic cells, with a high capacity that can be activated by soluble mediators or other substances as part of the host response to pathogens. The phagocytic process involves the participation of various cellular components and, is critical for nonspecific and specific host immune responses (26). Different factors are able to stimulate the phagocytic response, and the response can be induced to varying levels (27). Stimuli of phagocytosis include interleukins IL-1 and IL-6, while decreases in phagocytic function are often due to disorders of peroxidation (28,29). Three important cellular factors/processes are critical to phagocytosis: a) cytoskeletal elements, which mediate ingestion, b) maturation of the vacuole and c) the inflammatory response (30). The activation of appropriate effectors of the immune response determine the differentiation of precursor cells into TH1, TH2, TH17, T-regulatory T cells

(TH0/Treg), TH9, and follicular TH cells, and granulocyte/macrophage colony stimulating factor (GM-CSF) (31). The objective of the present study was to evaluate the efficacy of *Bothrops jararaca* venom on peritoneal macrophage activation.

2. METHODS

2.1 Chemicals, reagents and buffers: RPMI-1640 media, Actinomycin D, orthophenyldiamine (OPD), fetal calf serum (FCS), and recombinant TNF were purchased from Sigma (St. Louis, MO, USA). Cytokines (recombinant, antibodies for capture and detection) were purchased from BD Biosciences Pharmingen (USA).

2.2 Venom: Lyophilized venom from *Bothrops jararaca* (VBj) was obtained from Dr. R. Zucatelli Mendonça (Instituto Butantann – SP – Brasil), and stored at -20 °C. The lyophilized venom used in different assays always was reconstituted in RPMI-1640 media.

2.3 Animals: Females BALB/c mice (6 – 8 weeks old, weighing 15 to 20 g) were purchased from Bioterio – Instituto Nacional de Salud Publica (Cuernavaca, México). The animal studies were evaluated and approved by the Committee for Basic Research and the Clinical School of Medicine, with registration number 41MM2013-2. The animals were sustained and used under strict ethical conditions according to international recommendations.

2.4 Peritoneal cells: The peritoneal cells from groups of females BALB/c mice were obtained by the method previously described by Cohn & Benson, 1965 (32). Briefly, the peritoneal cavity of each animal was washed with RPMI-1640 medium and the cell suspension obtained was centrifuged at 400 rpm for 5 min. The cell pellet recovered was adjusted to a concentration of 1 X 10^6 cells/mL in RPMI-1640 with 10% FCS and was then distributed onto microplates. After 2 hours of incubation at 37 °C and 5% CO_2 the culture media was removed and cells were exposed to different amounts of VBj and cultivated under the same conditions for the indicated experimental times.

2.5 Activation assays

2.5.1 Cytotoxic assay: Macrophages cultures were treated with different amounts of VBj, and incubated for various times periods. The media was then removed and 100 µL of 2% crystal violet solution in 20% ethanol was added to each well. The optical density was determined with a microplate reader at a wavelength of 630 nm. The cytotoxic percentage was calculated using the formula: % cytotoxicity = (1 – Asample/Acontrol) X 100.

2.5.2 Determination of Hydrogen Peroxide levels: H_2O_2 levels produced by peritoneal macrophages exposed to VBj for different time intervals were determined using the method described by Pick & Mizel, 1981 (33). In brief, macrophages cultures treated with different amounts of VBj were incubated at 37ºC in an atmosphere of 5% CO_2. At each time point, 100 µL of phenol red solution [140 mM NaCl, 10 mM potassium phosphate buffer, 7.5 mM dextrose, 0.28 mM phenol red and 5.5 mM horseradish peroxidase] were added. The cells were incubated for 2 hours at 37 °C with 5% CO_2 and then 10 µL of 1M NaOH were added to each well and the optical density was measured with a 620 nm filter using a microplate reader. The absorbance obtained from the samples was compared with a standard curve of hydrogen peroxide to determine the concentration of H_2O_2 produced.

2.5.3 Cell Expansion: The cell expansion assay was performed with the method previously described by Arruda et al., 2004 (34). The peritoneal macrophages obtained as previously described were distributed at a concentration of 1 X 10^5 cells/well on microplates with submerged coverslips and were incubated at 37 °C with 5% CO_2. After incubation for 2 hours, the cells were washed and different concentrations of VBj were added and the cells were maintained in the same conditions for varying periods of time. Then, the media was removed and the coverslips were stained with crystal violet for 20 seconds. The presence of expanded cells was then analyzed under the microscope at a magnification of 40 X. The results were expressed in percent according

to the following formula: % Cell Expansion = (Number of expanded cell / total cell number) x 100. The cell expansion rate in cultures of macrophages exposed to VBj for the indicated time from 0 to 24 hours was determined by the following formula: Cell expansion rate = (CEfinal - CEinitial) / (Timefinal - Timeinitial), where CEinitial and CEfinal are the initial and final number of expanded cells, and Timeinitial and Timefinal are the initial and final times.

2.5.4 Measurement of phagocytic macrophage activity: The yeast phagocytosis assay was performed as described by Zebedee et al., 1994 (35). Briefly, microplates were prepared with submerged coverslips and 1 X 10^5 cells/well and incubated for 2 hours at 37 ºC in an atmosphere of 5% CO_2. Then, the media was removed and the cells were treated with different concentrations of VBj. At different time periods the cells were washed with PBS and opsonized yeast suspensions at a MOI of 5 were added and incubated for 90 minutes at 37 ºC and 5% CO_2. The coverslips were then washed with PBS and fixed with absolute methanol for 20 minutes at room temperature, and aqueous safranin was added for 35 seconds and the excess dye was removed with distilled water. The number of phagocytic cells was determined under the microscope at a magnification of 40 X. The phagocytic percentage was calculated with the following formula: % Phagocytosis (number phagocytic cells / total cell number counted) X 100.

The phagocytosis rate within the first 24 hours of exposure to VBj was calculated by the following formula: Phagocytosis rate = (Phagfinal - Phaginitial) / (Timefinal – Timeinitial), where Phaginitial and Phagfinal are the initial and final number of phagocytic cells, and Timeinitial and Timefinal are the initial and final times.

2.6 Production of Mediators

2.6.1 Cytokine ELISA assays: The levels of TH1 and TH2 cytokines present in the media from macrophages with or without venom exposure was determined using a two-site sandwich enzyme-linked immunosorbent assay (ELISA) (36). The assays were performed according to the manufacturer's instructions. The minimum

levels of each cytokine that was detectable in assay conditions were: 0.1 pg/10^6 cells for IL-1β and IL-4, and 1 pg/10^6 cells for IFN-γ, L-10 and IL-6.

2.6.2 TNF assay: The presence of TNF in the media of macrophages with or without VBj exposure was determined with a standard assay using L929 cells, as described by Ruff & Gifford,1980 (37). The cytotoxicity percentage was calculated by the formula: (1 – Asample/Acontrol) X 100. TNF activity was expressed as pg/mL as determined by a comparison to a standard made with mouse recombinant TNF.

2.7 Statistical analyzes: The statistical significance of differences between the groups was obtained by the analysis of variance (ANOVA) test complemented by Dennett´s test; $p < 0.05$ and $p < 0.01$ were considered to be significant, respectively.

3. RESULTS

Effect of venom on activation status

To evaluate the ability of VBj to activate macrophages, different assays including cytotoxicity, cell expansion, hydrogen peroxide concentration and phagocytosis were performed. Groups of mice were sacrificed for peritoneal cell collection, and the cells were exposed to different amounts of VBj for various time periods.

Figure 1 shows the cytotoxicity percentage from macrophage cultures treated with varying amounts of VBj over the course of 120 hours. In macrophages treated with VBj the cytotoxicity percentages were significantly higher than what was seen in control cultures ($p <0.001$). It was also observed that the cytotoxic effect of the venom was dose-dependent.

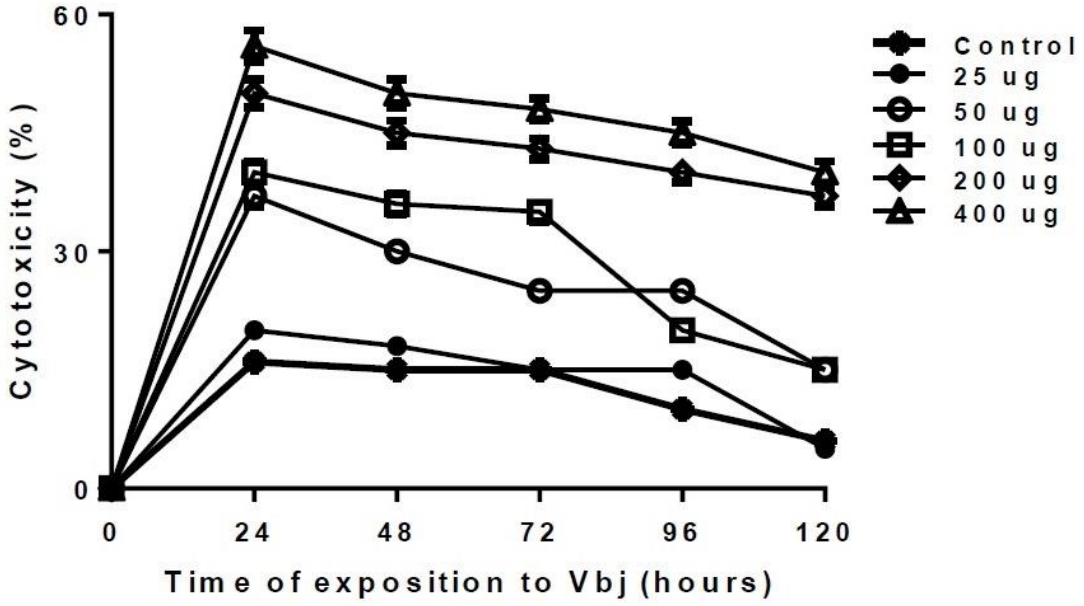

Figure 1: Cytotoxicity Percentage. Peritoneal macrophages were obtained, maintained and treated with different concentrations of VBj over the course of 120 hours. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

Figure 2 shows the results of the hydrogen peroxide production of groups of cells exposed to venom, versus control cells. The hydrogen peroxide production present in cultures treated with venom was significantly higher as compared to control ($p < 0.001$). It was also observed that the hydrogen peroxide production in cultures treated with venom was dose-dependent.

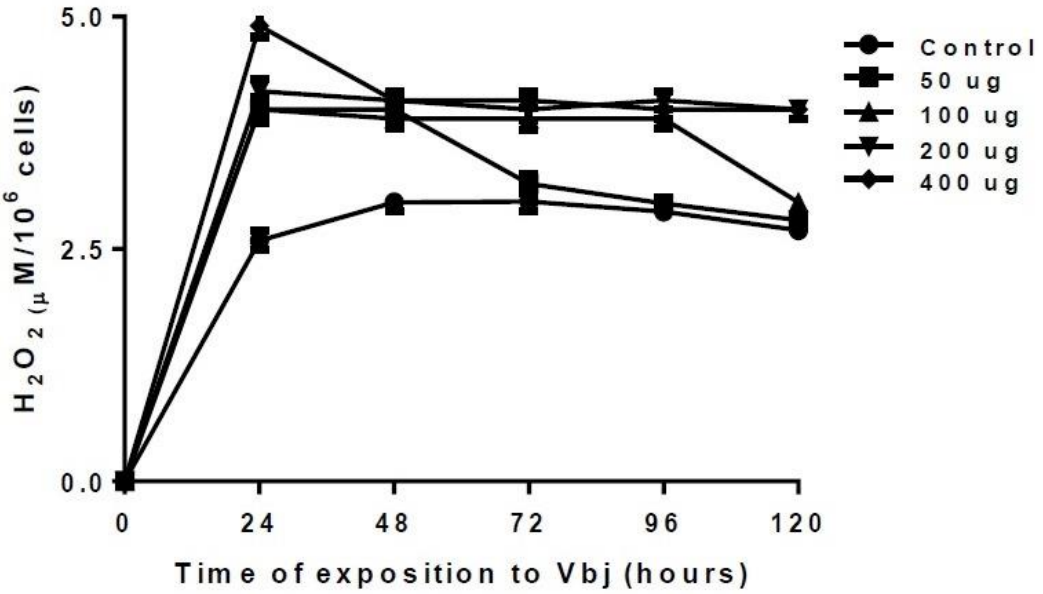

Figure 2: Hydrogen peroxide production. Peritoneal macrophages were exposed to different concentrations of VBj for 120 hours and hydrogen peroxide levels were determined. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

The venom was able to activate peritoneal macrophages in a dose dependent manner. The $DICT_{50}$ was found to be 200 µg of venom. To assess the optimal interaction between venom and cells we used a dose below the lethal dose of VBj. All kinetic experiments subsequently used 50 µg of VBj for the treatment of macrophages cultures. VBj reduced the viability of the macrophages and the largest increase in cytotoxicity percentage of macrophages exposed to venom was observed at 24 hours (Fig. 3A). In culture of macrophages treated with 50 µg of venom the highest production of H_2O_2 was observed at 24 and 48 hours (Fig. 3B). The cytotoxic percentage and the production of hydrogen peroxide in macrophages exposed to venom were significantly higher as compared to control ($p < 0.01$).

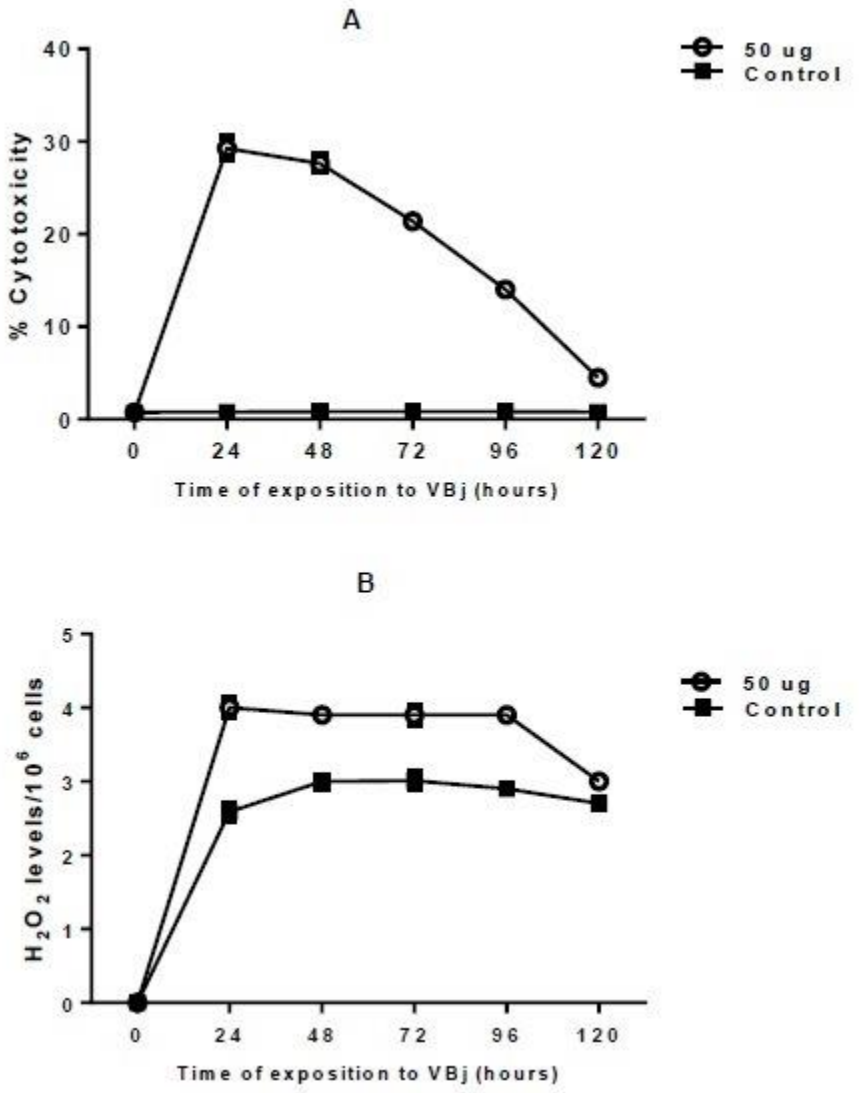

Figure 3: Percentage of cytotoxicity and hydrogen peroxide levels. Peritoneal macrophages were treated with 50 µg of VBj for different time periods. 3A) cytotoxic percentage and 3B) hydrogen peroxide levels. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

The kinetics of cellular expansion and phagocytosis percentages in cultures of macrophages exposed to 50 µg of venom was significantly lower at all time intervals as compared to control macrophage cultures (data not shown). To determine the rate of cellular expansion and phagocytosis murine peritoneal macrophages were treated with

venom for 0 to 24 hours. Figure 4 shows that maximum rates were observed at 2 hours of venom exposure. The rates of cellular expansion and phagocytosis were significantly lower as compared to control ($p < 0.001$).

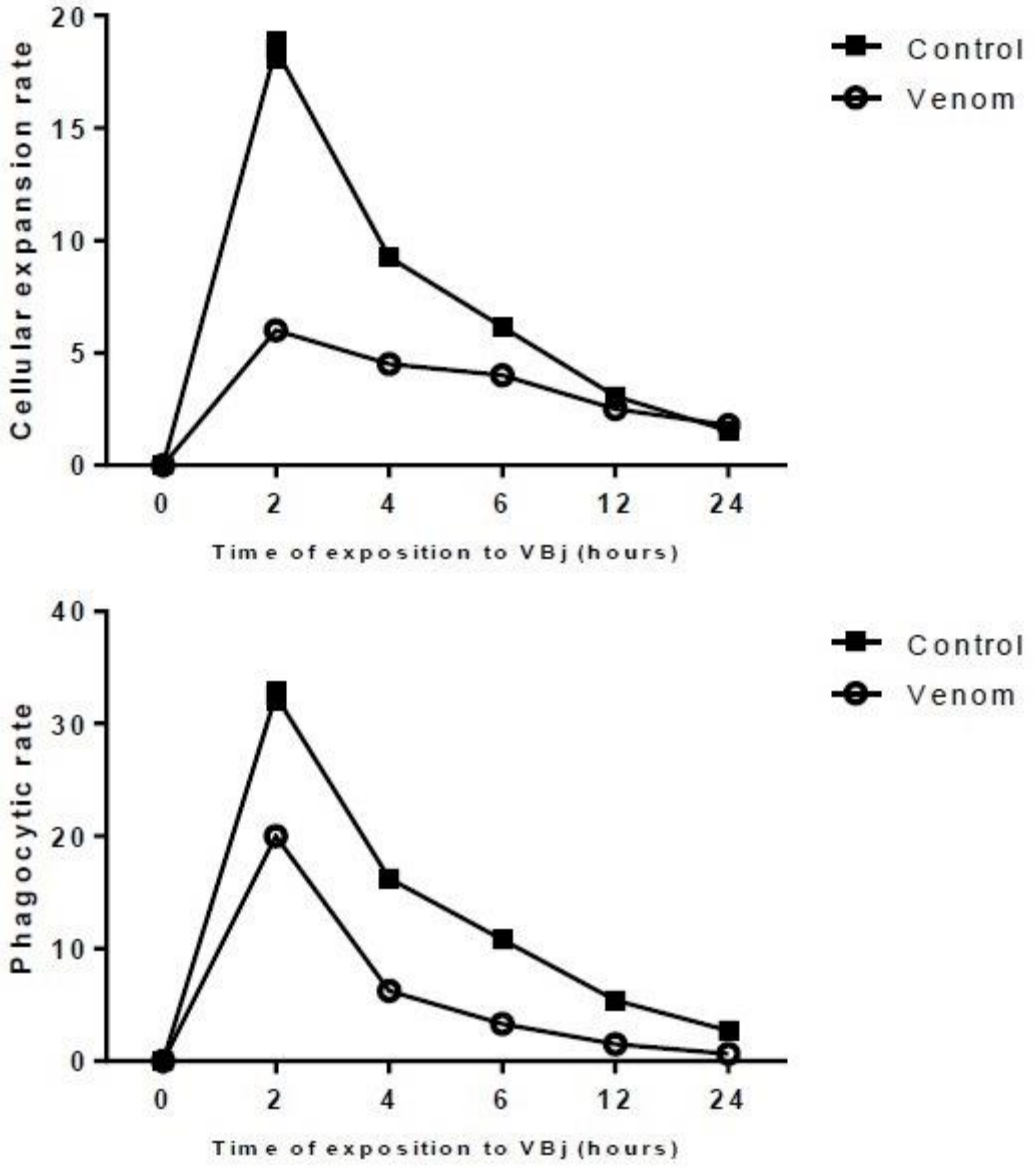

Figure 4: Rates of cellular expansion and phagocytosis. Peritoneal macrophages were treated with 50 µg of VBj for different time periods to determine the various rates. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

Effect of venom on the TH1 cytokines

To determine the production of TH1 cytokines, macrophages were incubated with 50 µg of VBj for different time periods; the results obtained are presented in Figure 5. The results observed were: a) the highest production of TNF-α, IL-1β and IL-6 were obtained at 24 hours and b) the maximum production of IFN-γ was observed from 24 to 72 hours (Fig. 5). The production of all of these mediators was significantly higher than what was seen in control macrophages at the same time point ($p < 0.001$).

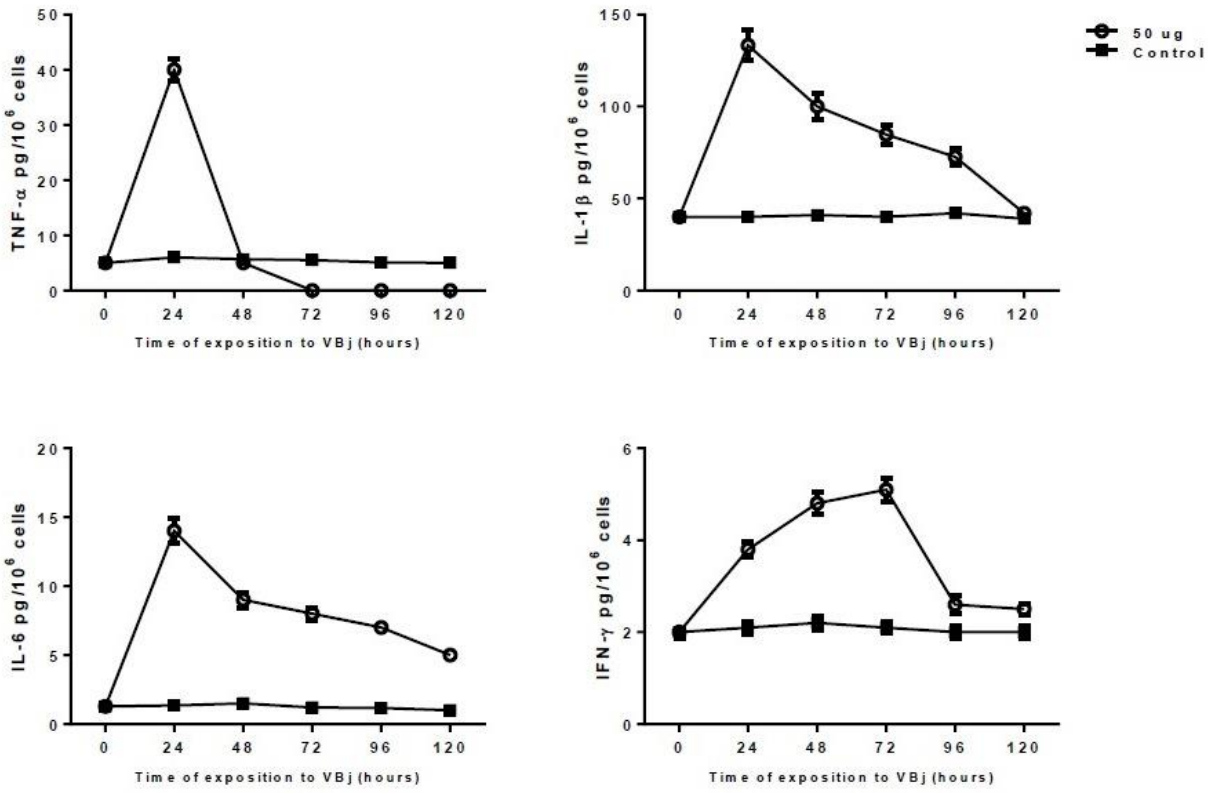

Figure 5: TH1 cytokines. Peritoneal macrophages were treated with 50 µg of VBj for different time periods and TNF-α, IL-1β, IL-6 and IFN-γ levels were determined. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

Effect of venom on TH2 cytokines

To determine the production of TH2 cytokines that were induced by the venom, the production of IL-4 and IL-10 were evaluated in cultures of macrophages exposed to the VBj for different time periods (Fig. 6). The venom induced both IL-4 and IL-10 production. The maximum production of IL-4 was observed at 24 hours of treatment with venom (Fig. 6). IL-10 production increased during the first 24 hours and remained elevated for up to 120 hours of exposure to the venom. The levels of IL-4 and IL-10 in macrophages exposed to VBj were significantly higher as compared to control macrophages ($p < 0.001$) (Fig. 6).

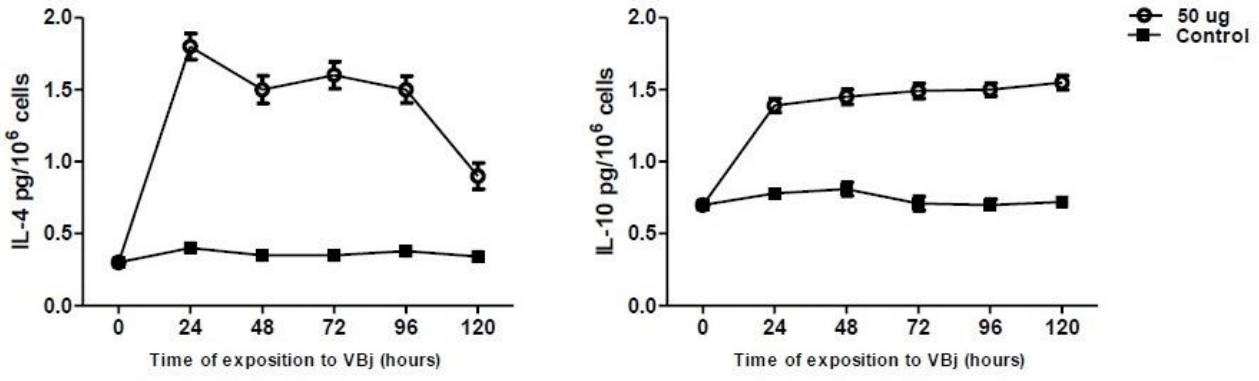

Figure 6: TH2 cytokine production. Peritoneal macrophages were treated with 50 μg of VBj for different time periods for and levels of IL-4 and IL-10 were determined. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

To facilitate the comparison of production levels of cytokines the percentages of the various mediators and the resulting cytokine balance were calculated; results are shown in Figure 7. Increased levels of the cytokines TNF-α, IL-1β and IL-6 were observed for 24 hours, while IFN-γ was increased for 72 hours. In contrast, the levels of IL-4 were elevated for up to 96 hours and IL-10 levels remained elevated for up to 120 hours. In the first 24 hours of venom treatment was observed a balance between TH1 and TH2 cytokines. After this time period was elevated the TH2 cytokines.

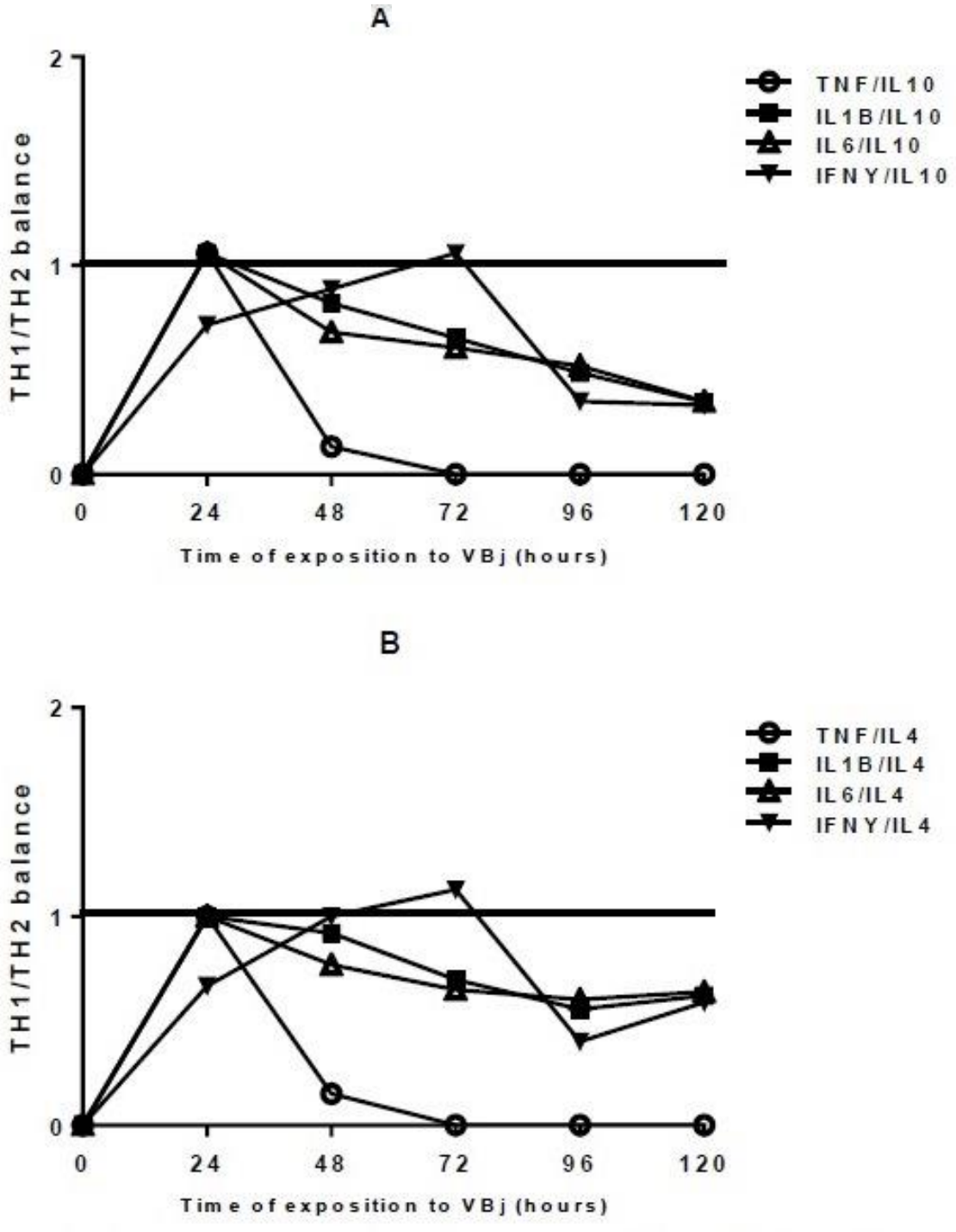

Figure 7: Cytokine balance. The comparison of the profiles of TH1 and TH2 cytokines and the percentages of each mediator were calculated and plotted. Each data point represents the mean ± the standard deviation of 4 different experiments ($p < 0.001$).

4. DISCUSSION

Bothrops jararaca venom is involved in immune and inflammatory responses and is believed to regulate cellular and vascular events to stimulate the activation and trafficking of cells to damaged tissue, resulting in leukocyte accumulation and infiltration of inflammatory cells (38,39). Its effect on the viability of immune cells is the result of lesions in the plasma membrane caused by calcium influx with a resulting increase in cytosolic calcium concentration that favors the activation of calcium dependent proteases and phospholipases, culminating in cell death (40,41).

In the present study, the $DICT_{50}$ was found to be 200 ug/mL of VBj. The highest percentage of cytotoxicity was observed in the first 24 hours of exposure to the venom. These data corroborate the data obtained by others studies that showed that the venom has a cytolytic capacity (42). These results agree with those studies that demonstrated that the snake venom is able to induce cytotoxicity in mouse cells (43,44). Others studies have shown changes in the morphology of human red blood cells or fibroblasts caused by crude venom or specific cytolytic toxins. These changes lead to an inhibition of cell membrane function, which can produce intracellular side effects, leading to vacuolization and cell death (39,45-47).

The results of this study showed that the cell expansion of macrophages was also affected by the exposure to the venom. These results are in accord with other investigations that have described that *Bothrops* venom contains a variety of toxins capable of affecting the integrity of the plasma membrane and thus cell expandability (41,48).

Macrophages have a remarkable ability to generate free radicals, specifically H_2O_2 and reactive oxygen species (42). These species are formed extracellularly and can act directly on cell membranes by altering the permeability of the target area and are also involved in apoptosis and necrosis (43). In the present study it was also observed that in macrophages exposed to venom an increased production of H_2O_2 in the first 24 hours was observed. The

mechanism of diffusion of hydrogen peroxide is an important interaction between phagocytes and the particles to be swallowed (24).

The phagocytic process involves several sequential steps that are common to cells, such as chemotaxis, adhesion, endocytosis and intracellular biochemical and physical changes (26,28,29,). These changes are generally accurate and occur in cases of conflict between phagocytes and particle size. In these cases, the phagocytes make contact with the target particles, which stimulates cytokine production. In the case of ingestion of toxic substances, there is destruction of the antigen particle, which can also damage the surrounding tissue (45). Different studies have shown that *B. jararaca* venom is able to stimulate phagocytosis in murine macrophages (4,10,46).

The envenomating process may result in an unbalanced immune response in the body of the victim and various studies have shown that cytokines are a group of immunoregulatory proteins involved in modulating the immune response. During the inflammatory process, some cytokines initiate and amplify the response, whiles other attenuate the response, and some help in the resolution of the inflammatory process. The pattern of secretion of these molecules determines the type of immune response that will be generated against a particular immune insult and, in this case in particular, against the components of *B. jararaca* venom (46,47). In the present study, it was shown that that cytokine production in macrophages was induced by exposure to VBj. Under the conditions used in this study, it was observed that 50 µg/mL of VBj could alter the production of mediators such as TNF-α, and IFN-γ , with peaks at 24 and 72 hours of exposure, respectively. Our results also demonstrated that IL-1β and IL-6 levels peaked at 24 hours of exposure. The results obtained in this study showed that immune response mediators as cytokines such as IL-6, IFN-γ and TNF-α are strongly implicated in the pathogenic processes of *Bothrops jararaca* venom, as well as in the severity of the envenomation reaction and its ability to trigger the immune response. However, the incubation of macrophages with VBj had

ability to induce high levels of IL-4 and IL-10 at all times points. These data corroborate the data obtained by others studies (11,15,20). As mentioned earlier, the inflammatory reaction induced by cytokines is due to the components present in the venom of *Bothrops*, and the cytokine balance determines the inflammatory state of the cells. The ratio of the TH1/TH2 cytokines in the first 24 hours suggests that the balance equilibrated. In contrast, with the increasing exposure time to venom the ratio favored the TH2 type, which would result in an activation of alternative pathway macrophages. The exact mechanisms by which different concentrations of Vbj modulates the immune response is complex, and they involve a disruption of macrophage homeostasis, altering their function and their response to toxic substances such as the protein components of venom.

Conclusion:

The results obtained in this study demonstrate that macrophages seem to be major players in the immune system by preventing excessive inflammation while down-regulating host protection against different pathogens. Combined data suggest that *Bothrops jararaca* venom is responsible for modifications in the phagocytic pathway of macrophages and has an immunomodulatory effect for the first 24 hours of venom treatment was observed a balance between TH1 and TH2 cytokines. After this time period with a TH2-dominant response in the TH1/Th2 cytokines.

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Author contributions: All of the authors contributed to this work. Vera L. Petricevich initiated and designed the study and performed the statistical analyses. Rosario del Pilar Nuñez Valderrama Nuñez Valderrama conducted the study. Lluvia Arteaga Figueroa provided technical support. Ronaldo Zucatelli Mendonça donated the venom. All of the authors prepared the manuscript and approved this version of the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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