

Components from spider venom activate macrophages against glioblastoma cells: new potential adjuvants for anticancer immunotherapy

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ABSTRACT

Immunomodulation has been considered an important approach in the treatment of various types of malignant tumors. However, adaptive immune cells have received more attention, while the modulation of innate immune cells is still an underexplored tool. A recent study by our group demonstrated that the *Phoneutria nigriventer* spider venom (PnV) administration increased the circulating NK cells and monocytes and the infiltration of macrophage in glioblastoma, in addition to decreasing the tumor size in a preclinical model. The hypothesis that PnV would be modulating the innate immune system led us to the main objective of the present study: to elucidate the effects of PnV and its purified fractions on cultured macrophages. After differentiation from bone marrow precursors, cells were pre-activated with IFN- γ and treated as follow: Control (untreated); LPS (1 $\mu\text{g/mL}$); PnV (14 $\mu\text{g/mL}$); PnV-fractions F1, F2 and F3 (1 $\mu\text{g/mL}$) or PnV-subfractions (1 $\mu\text{g/mL}$). Results showed that PnV and the three fractions activated macrophages. Further purification generated twenty-three subfractions named Low Weight (LW-1 to LW-12) and High Weight (HW-1 to HW-11). LW-9 presented the best immunomodulatory effect. Treated cells were more phagocytic, migrated more, showed an activated morphological profile and induced an increased cytotoxic effect of macrophages on tumor cells. However, while M1-controls (LPS) increased IL-10, TNF-alpha and IL-6 release, PnV, fractions and subfractions did not alter any cytokine, with the exception of LW-9 that stimulated IL-10 production. These findings suggest that molecules present in LW-9 have potential to be used as immunoadjuvants in the treatment of cancer.

Keywords: cancer, glioma, immunomodulation, innate immune system, phagocytosis, *Phoneutria nigriventer*.

1 INTRODUCTION

Immunotherapy against cancer has been considered a fundamental and important pillar to improve the prognosis of malignancies. This therapy uses the abilities of the immune system components, including cytokines, antibodies and cells, to recognize and eliminate malignant cells (Alatrash et al., 2013). The first antibody therapy was approved by the FDA in the late 1990s for cancer treatment, to be used alone or in combination with surgery, chemotherapy, and radiation (Thorsson et al., 2019; Burugu et al., 2017; Gotwals et al., 2017; Helmy et al., 2013). Strategies targeting the adaptive immune system, such as specific antibodies against T lymphocyte-associated antigen 4 (CTLA-4) or against programmed cell death 1 (PD-1) and its ligand PD-L1, were the first approved therapies. Adoptive cell therapies, such as with TCR-T (T-cell receptor-engineered T cell) and CAR-T (chimeric antigen receptor T-cell), are also an approved approach (Maus and June, 2016; Zhang and Wang, 2019). These are now as the first line of therapy for some types of cancer, such as melanoma, non-small cell lung cancer and Hodgkin's lymphoma (Looi et al., 2019; Burugu et al., 2017; Mulder et al., 2019). However, such strategies can induce resistance and there is evidence of severe adverse effects (Burugu et al., 2017; Harris et al., 2018; Xu et al., 2018; Mulder et al., 2019).

Drugs that act on myeloid and lymphoid cells of the innate immune system, for example, natural killer (NK) cells and macrophages, or that can act on both innate and adaptive systems, have recently emerged as relevant therapies in many types of solid tumors and hematopoietic cancers (Burugu et al., 2017; Mulder et al., 2019). It has already been described that modulating macrophages may be a more promising strategy compared to try killing tumor cells, because macrophages kill malignant cells, reorganize the extracellular matrix, recycle debris originated from cells and molecules, start signaling to cell growth, and stimulate the migration of cells to repair the tissue (Weigert et al., 2016). However, modulating macrophages as a strategy to treat cancer is an underexplored field.

Macrophages are sentinel cells of the innate immune system and multifunctional antigen-presenting cells (Komohara et al., 2016; Kumar et al., 2018). They have the ability of engulf and brake down pathogens, toxins, cellular debris among other targets, providing the first line of immune defense. These properties make macrophages promising cells for the treatment of malignant tumors, mainly because they can also activate the entire immune system against foreign and diseased cells (Alvey and Discher, 2017). Macrophages exist in a diverse collection of cell types and, under different external stimuli, can oscillate between different activation profiles (Denardo and Ruffell, 2019; Kowal et al., 2019). These macrophages are classically referred as activated (M1 – pro-inflammatory) or alternatively activated (M2 – anti-inflammatory), and although this simple concept of M1/M2 can explain the phenotypic heterogeneity of these cells,

in fact, macrophages appear to exist in different populations with an extensive variety of polarization states (Komohara et al., 2016).

Macrophages infiltrate the malignant tumor microenvironment (TME) in large numbers, being called tumor-associated macrophages (TAMs). Many studies in the last decade have revealed that TAMs play a protumor role and are related to the cancer progression (Revised by Komohara et al., 2016; Casseta et al., 2018). However, the role of macrophages in cancer has been described as complex and paradoxical (Petty et al., 2017). Usually, they are considered synonymous of M2-macrophages, but studies have shown that TAMs are a group of macrophages with transcriptional and phenotypic characteristics distinct from the M1 and M2 subsets (Petty et al., 2017). The main strategies to use TAMs as targets for immunotherapy are related to: 1) re-educating macrophages, from a pro-tumor profile, generally described as M2, to an anti-tumor profile, characterized as M1-like cell; 2) blocking the recruitment or survival of macrophages in the tumor; 3) promoting the activation of macrophage; and 4) using monoclonal antibodies or molecules that increase the killing activity and/or phagocytosis functions, promoting the destruction of cancer cells (Ruffell and Coussens, 2015; Mantovani, et al., 2017; Kowal, et al., 2019).

Since the first demonstration of cytoskeletal impairment in astrocytes treated with the venom of the South American wandering spider *Phoneutria nigriventer* (PnV) (Rapôso, et al., 2016), a sequence of studies by our group exhibited the effects of PnV on glioblastoma (GBM) cell lines *in vitro* and *in vivo*. The venom has cytotoxic and anti-migratory effects, arrested the cell cycle in G0 and reduced or eradicated tumor development in a xenogeneic model, in addition to increasing the infiltration of macrophages in the TME (Santos, et al., 2019; Bonfanti, et al., 2020; Barreto et al., preprint). Those previous findings led us to herein investigate the modulatory effect of PnV and its components on macrophages, and to find out whether and which of those molecules have the potential to act as immunoadjuvant in cancer treatment.

2 METHODOLOGY

2.1 Reagents and venom

The reagents were purchased from Sigma Aldrich (St. Louis, MO), unless otherwise specified. *P. nigriventer* venom samples were extracted from several adult spiders by electrical stimulation. The pattern of the crude venom was analyzed by high-pressure liquid chromatography (HPLC) and compared with other samples used and published. The fractionation of the venom was done by Amicon Ultra Centrifugal Filter (#UFC801008; Thermo Fisher Scientific, Suwannee, GA), following Santos et al. (2019). With this procedure, PnV was fractionated by molecular mass, resulting in three fractions: F1 = less than < 3 kDa; F2 = between

3 and 10 kDa; and F3 = above 10 kDa. Venom and fractions were lyophilized and stored at -80°C . The three fractions were used in experiments conducted to select the most significant among them, considering the macrophages modulation. After this first screening, F1 and F2 together were called Low Weight (LW) components, while F3 alone was named High Weight (HW) components. LW and HW were purified again by reversed phase HPLC, using a Shimadzu VP-ODS column, 0.1% trifluoroacetic acid (TFA) as mobile phase and 90 % acetonitrile 0.1% TFA as eluent. Twelve purified components were obtained from LW, named subfractions LW-1 – LW-12, and eleven from HW, named subfractions HW-1 – HW-11.

2.2 Cell cultures and treatments

2.2.1 Bone marrow derived macrophages

Murine bone marrow cells were cultured with the supernatant of fibroblasts (L929) cell line for differentiation in macrophages. Bone marrow precursors were collected from femurs of male C57BL6 mice at 7 to 10 weeks of age. Briefly, immediately after collecting from marrow, the cell suspension was cultivated in Petri dishes with 10 mL IMDM (Iscove's Modified Dulbecco's Media) supplemented with 20% fetal bovine serum (FBS) and 30% L929 cell supernatant (as a source of macrophage colony-stimulating factor – M-CSF). After four days, more 10 mL of fresh medium with the same proportions was added (Marim et al., 2010). After incubation of this culture for seven days at 37°C and 5% of CO_2 , the cells were washed with phosphate-buffered saline (PBS) and the adherent cells were reaped with a cell scraper for use in different assays, as described below.

2.2.2 Human glioblastoma cell line (NG97)

For assays involving coculture with tumor cells, human glioblastoma (NG97) cells were used. The culture was maintained in IMDM supplied with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin, at 37°C and 5% of CO_2 . After confluence, the cells were detached using a scraper and placed in 48-well plates.

2.2.3 Treatments

After differentiation, the macrophages were pre-activated with 20 ng/mL of recombinant mouse IFN- γ (BD #554587) for 48 hours and separated into the following groups: M0-Control (untreated), M1-Control - 1 $\mu\text{g}/\text{mL}$ of lipopolysaccharide (LPS; E. coli O26:B6), PnV (14

µg/mL), F1, F2 , F3 (1 µg/mL), LW-subfractions (LW-1 to LW-12) and HW (HW-1 to HW-11) (1 µg/mL). After 24 hours of treatments, the macrophages were washed with PBS and used to perform the tests as described below.

2.3 Phagocytosis and killing assays

Phagocytosis tests were performed by using the fungus *Paracoccidioides brasiliensis* (Pb18) or pH Red *E. coli* Bioparticles (Invitrogen # P35361). For the Pb18 assay, macrophages were plated in 48 well plates at a density of 2×10^5 cells per well. After treatments, cells were incubated with the fungus for 4 hours in the proportion of 1 yeast:5 macrophages per well. After incubation, cells were washed and lysed with ice distilled water to release the internalized Pb18. Then, the cell lysate containing the fungus was seeded in Petri dishes with BHI (Brain Heart Infusion) supplemented with gentamicin (96 µg/mL) and FBS (4 %). After an incubation period of 7 days at 37°C, colony forming units (CFU) were manually counted. For image records, after incubation with the yeast, the wells were washed and the cells were fixed with cold methanol for 5 minutes and then stained with Giemsa solution for 15 minutes (Nicola and Casadevall, 2012). The wells were washed with PBS and the images were acquired using Cytation 5 - Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA). The killing assay was performed following the same procedure described above, however the incubation period with the fungus was 48 hours.

For the bioparticle assay, macrophages were seeded at a density of 2×10^5 cells in 24-well plates. After incubation for 2 hours with 50 µg of bioparticles, the macrophages were washed extensively and immunolabeled with anti-Iba-1, as described in the morphological analysis (2.4) (Naqvi et al., 2015). The florescence quantification (Integrated Density of Pixels) was carried out in 3 to 5 random photos, using the software Image J (Image Processing and Analysis in Java, version 1.39e).

2.4 Morphological analysis

After treatments, the cells were fixed with 4% paraformaldehyde for 15 minutes. Then, they were incubated with blocking buffer (PBS with 1% BSA, 0,3% Triton X100) for 2 hours, followed by incubation with anti-Iba-1 antibodies diluted in PBS (Wako #019-19741; 1:700, overnight). Cells were washed with PBS and incubated with secondary antibodies diluted in PBS (Cy2 Goat Anti-Rabbit IgG, Jackson ImmunoResearch #111225144; 1:1000, 1 hour). Then, nuclei were stained with DAPI (dilution 1:1000) (#D9542 Sigma) for 5 minutes and the plates were kept in a glycerol 3:1 PBS solution, at -25 °C. Images were acquired in the Cytation 5 Reader. In some wells, macrophages were seeded on a coverslip and, after treatments and immunolabeling, cells were washed with PBS and the slides were mounted using fluorescence

mounting medium (#F4680) to acquire qualitative images in a non-inverted microscope, in high amplification. The cell count was performed in 3 to 5 random photos, using the Image J software (Image Processing and Analysis in Java, version 1.39e).

2.5 Cell migration analysis

The cell invasion assay was performed using transwell inserts with polycarbonate membranes of 8 μm pore (Corning Inc., New York, NY). Macrophages in the FBS-free culture medium were added to the upper chamber of each insert (3×10^5 cells per well). The lower chamber was filled with medium supplemented with 20% FBS. Treatments were added in the upper chamber for 24 hours. After that, cells that failed to migrate were removed from the upper face of the membrane using a cotton swab and the macrophages present on the lower face were stained using May-Grünwald-Giemsa. Images were acquired in an inverted microscope (Nikon Eclipse TS100; Nikon, Tokyo, Japan). The cell count was performed in 3 to 5 random photos, using the Image J software (Image Processing and Analysis in Java, version 1.39e).

2.6 Cytokines measure by ELISA

After treatments, the supernatants of cell cultures were collected and the enzyme-linked immunosorbent assay (ELISA) was performed for the detection of TNF- α , IL-10 and IL-6. This characterization was performed using commercial cytokines dosage kits by R&D Systems (DuoSet ELISA), according to the manufacturer's instructions. The measurements were performed in a Thermo Scientific plate reader (Multiskan Go).

2.7 Analysis of antigen presenting parameters by flow cytometry

For the analyses of the costimulatory surface molecules, 2×10^5 macrophages/well were plated and, after 24 hours with the treatments (control without any treatment, LPS 1 $\mu\text{g}/\text{mL}$, GBM lysate, PnV 14 $\mu\text{g}/\text{mL}$, PnV plus GBM lysate and LPS plus GBM lysate), the cells were labeled with antibodies. The lysate was produced following Rainone et al. 2016. In brief, GBM NG97 cells were resuspended at 15×10^6 cells/mL and lysed by 3 cycles of freeze-thawing in liquid nitrogen. The preparation was centrifuged at 12,000 rpm for 15 minutes and stored in aliquots at -80°C until use. Tumor cell lysate was added at the ratio of 1 macrophage to five tumor cells. The immunostaining was performed with the following antibodies: anti-MHCII (clone M5/114.15.2, PE-Cy7, #25-5321-82, eBioscience), anti-CD80 (clone 16-10A1, FITC, #104706, Biolegend) anti-CD86 (clone GL-1, PE, #105008, Biolegend), anti-CD40 (clone 1C10, PE-Cy5, # 15-0401-82, e Bioscience), anti-CD11b (clone M1/70 , Alexa fluor 647, #17-0112-82, eBioscience),

incubated for 30 minutes (4°C, in a dark chamber). The cells were then fixed with paraformaldehyde 1% for 30 minutes and 10,000 events were acquired using the Flow Cytometer (BD FACSVerse). The data were analyzed by FlowJo VX software. For this experimental design, cytokines (IL-6, TNF- α and IL-10) were also dosed by ELISA, as described in the topic 2.7.

2.8 Macrophages and glioblastoma (NG97) coculture

Macrophages were plated at a density of 3×10^5 cells per well in 24-well plates; after 24 hours with treatments, the stimuli were removed and the macrophages were washed and incubated with 1 M glioblastoma (NG97) cells. The coculture was maintained for 24 hours and, after this period, the procedure for assessing cell viability was performed through the labeling of live cells with DAPI. The analyzes were made by flow cytometry or image acquisition in Cytation - 5. Anti-Iba1 was used to identify macrophages to differentiate from tumor cells. The fluorescence quantification (Integrated Density of Pixels) was carried out in 3 to 5 random photos, using the software Image J (Image Processing and Analysis in Java, version 1.39e).

2.9 Statistics

The data analysis was made by GraphPad Prism software (v. 5.0 GraphPad, San Diego, CA). The level of significance was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Unpaired Student's t-test was used to compare each treatment with the control. Data are presented as mean \pm standard error of the mean (SEM). $p < 0.05$ indicated statistical significance.

3 RESULTS

3.1 Verification of venom quality and purification in subfractions by HPLC.

Figure 1A shows that the standard and quality of the venom sample was as previously reported (Santos et al., 2019; Barreto et al., Preprint). The first procedure for separation of the crude venom into fractions was carried out by molecular mass criteria, using molecular filters with nominal separation at 10 and 3 kDa, according to Santos et al. (2019). Three main fractions were obtained: F1 (low weight, less than 3 kDa), F2 (intermediate weight, between 3 and 10 kDa) and F3 (high weight, above 10 kDa). These fractions are still complex mixtures and were evaluated as described below to select those with the most significant effects on macrophages.

Taking all the obtained results together, F2 and F3 were considered more effective in most parameters, however, F1 had a significant effect in the killing assay. Therefore, all fractions were purified again to continue the study. Figures 1B and 1C show the purification of F1 and F2 together (now referred as LW) and F3 (now referred as HW) by HPLC, respectively. This procedure generated 12 LW subfractions, named LW-1 to LW-12, and 11 HW subfractions, named HW-1 to HW-11. These PnV-components have been tested for macrophages immunomodulation, as reported below.

3.2 Crude venom and its fractions and subfractions activity on macrophage phagocytose.

In the phagocytosis assay with the fungus Pb18, fractions F2 and F3 significantly increased the number of colonies forming units (CFU) (Figure 2.1 A), while the number of yeast cells in suspension after lysis of macrophages was increased only in F2 (Figure 2.1 B), demonstrating that F2 and F3 were the fractions with higher potential to activate macrophages. Of note, all other treatments appeared to increase the phagocytic activity, but not significantly (Figure 2.1).

Figure 2.2 shows microscopic images of macrophages incubated with Pb18. It is possible to observe that macrophages treated with LPS, PnV and fractions (mainly F2 and F3) present more intracellular fungi (see detail in the highlighted panel G) compared to the control. These data confirm that groups treated with venom and fractions, similar to LPS (M1-control), induced higher phagocytosis capacity when compared to the untreated control (M0-control). An accurate observation reveals that F1 was very similar to M0-control, PnV and F3 induced more phagocytosis than F1 and M0, and LPS (M1-control) and F2 were greater than other groups.

The Pb18-phagocytosis assay was also performed with the subfractions purified from F1+F2 (LW) and F3 (HW). Subfractions LW-2, -3, -5, -10, -11, -12 (Figure 2.3A) and HW-1, -2 and -3 (Figure 2.3B) induced a significant increase in phagocytosis compared to control. Although several subfractions have shown an effect on phagocytosis and killing (shown below), from overlapping these results, the following subfractions were chosen as the best to continue: LW-9, -11, -12, and HW-2, -3, -6; in addition, LW-6 was chosen as a control of fraction with no relevant effect.

The results obtained with *E. coli* Bioparticles (Figure 3) were compatible with those observed with the fungus. Figure 3.1 shows that PnV, F2 (not significant) and F3, as well as LPS, increased phagocytosis when compared to the M0-control, while F1 was very similar to untreated cells. Fluorescence quantification is shown in graph M. When the selected subfractions were tested (Figure 3.2), it was possible to observe that LW-9 (panels G and H) had the best effect, which was statistically significant (graph S).

3.3 Macrophage Pb18 killing ability by PnV, its fractions and subfractions stimuli.

In the killing assay, treated cells were incubated with the fungus Pb18 for a prolonged period (48h) to allow them to eliminate the pathogen. Then, cells were washed and lysed, and the supernatant was cultured to evidence alive fungi. The less Pb18, the increased killing capacity. In graph 4.1A, the results demonstrate that PnV, as well as LPS, increased intracellular death compared to control. The fractions also increased the killing of the fungus, especially F1 and F2.

The tests with subfractions (Figure 4B and C) showed that LW-9, -11, -12 (graph B), and HW-6, -8, -9 (graph C) were the subfractions with increased fungi killing capacity. Interestingly, LW-1, -2 and -3 decreased the ability of macrophages to kill the fungus.

3.4 Macrophage morphological alterations under venom, its fractions and subfractions treatment.

Cell morphology was analyzed by immunofluorescence label of Iba-1, a macrophage marker (green), and DAPI to highlight the nuclei (blue) (Figure 5). Iba1 is recognized as a pan-macrophage marker and its expression is increased when these cells are activated (Sasaki et al, 2001, Pierezan et al., 2014). Iba-1 staining demonstrated that the cells generated from bone marrow were successfully differentiated in macrophages (that was also confirmed by the CD11b staining detected by flow cytometry, as described later) (Figure 5).

Figure 5.1 shows that the cells treated with PnV had increased processes, were multinucleated and with increased cytoplasm, when compared to the M0-control, a profile characteristic of activated macrophages (more clearly visible in the panels with higher amplification – B, D, F, H, J, L). The same aspect was observed for the group treated with LPS, in which the cells were even larger. Fractions F2 and F3 (mainly F3) induced a morphology very close to PnV and LPS.

In addition, the images with lower amplification in Figure 5.1 (panels A, C, E, G, I, K) clearly suggest an increase in the number of cells, comparing all treatments with M0-control. Considering that in all groups there was initially the same number of cells, this result suggests that PnV and fractions could be stimulating cell proliferation. In fact, graph M shows that all treatments (mainly LPS, PnV and F1) significantly increased the cell count compared to M0-control.

Figure 5.2 shows the same analysis, using subfractions. LW-9 (panel D) and HW-3 (panel H) induced the most activated morphology of macrophages. LW-6, -11 and HW-2 induced the most discrete morphological alteration, being similar to M0. Graph J shows that there is no significant difference between the groups in cell count.

3.5 Stimulation of macrophage migration by the crude venom, its fractions and subfractions.

The cell migration assay was performed for 24 hours of exposure to treatments, using plates containing transwell inserts. Figure 6.1 shows that all treatments increased macrophage migration when compared to M0-control. Interestingly, LPS (M1-control) did not induce much migration, while PnV and fractions F2 and F3 increased it. It is also clear that in the groups treated with venom and fractions, especially PnV, cells have large and thin stretches, possibly the mechanism by which they can pass through the pores. This aspect was also observed in the morphological analysis by Iba-1 labeling.

Figure 6.2 demonstrates that all subfractions increased the migration of macrophages, but LW-9 and HW-2 were more efficient. The cell morphology was different depending on the subfraction; Treatment with LW-9 and HW-2 induced cells with long processes and thin body. On the other hand, HW-3 and, more pronounced, HW-6, showed cells with a large and rounded body, without (or with few) processes.

3.6 Venom, fractions and subfractions effects on cytokines release.

To better understand the functional profile of macrophages treated with PnV components, pro and anti-inflammatory cytokines were measured in the culture supernatant.

Figure 7.1 shows that LPS (M1-control) significantly increased the release of pro- (IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines, corroborating the literature (Stenvinkel et al., 2005). On the other hand, the venom and all fractions did not alter any of those molecules when compared to the control. These data suggest that the venom activates macrophages for a different profile rather than the classic M1. This atypical and specific profile was referred as M-PnV.

Figure 7.2 demonstrates that a similar result was observed for the subfractions, where none of them, induced TNF- α expression. However, LW-1 and LW-9, as well as LPS, significantly increased IL-10 release (graph B).

3.7 Action of PnV, fractions and subfractions on molecules involved in antigen presentation by macrophages.

Considering that macrophages present antigens, this ability was assessed to verify whether the venom and its components could alter that function. For that, macrophages were pulsed with GBM cell lysate, concomitantly to the treatment with LPS or PnV. Only lysate or the treatments alone were also evaluated. Figure 8 (A – E) shows the main molecules involved in the

antigen presentation. In general, LPS (mainly) and lysate alone were able to induce this function in macrophages, while PnV had no effect.

CD11b expression was significantly increased by LPS and lysate (graph A), but not by PnV. When concurrent exposures of PnV plus lysate or LPS plus lysate were performed, this molecule was increased, allowing to infer that the stimulation was not due to the venom components, but to the presence of GBM antigens in the lysate. Similar results were observed for CD80 and MHCII (graphs B and C). For the co-stimulatory molecules CD40 and CD86 (graphs D and E), only LPS induced a significant increase.

Cytokines release in the supernatant was also evaluated in this experimental design (Figure 8 graphs F – H). The results confirmed that the PnV and fractions did not induce cytokines release. Graphs F and G show that only LPS increased the levels of TNF- α and IL-10, while the lysate, PnV and PnV plus lysate had no effect. IL-6 release (graph H) was increased by LPS, lysate and LPS plus lysate, while PnV had no effect. The treatment with PnV plus lysate induced an increase in this cytokine, but this is due to the lysate stimulation *per se*.

3.8 PnV and its fractions were efficient in activating macrophages to kill tumor cells.

Finally, it was necessary to confirm whether the specific macrophage profile induced by PnV components, which can phagocyte and kill Pb fungus, presents an activated morphology, can migrate more efficiently, but does not release cytokines and does not present antigens, would be able to kill tumor cells. For this, a co-culture with macrophages and GBM cells was performed. The macrophages were previously stimulated with LPS, PnV, F1, F2 or F3.

The viability of tumor cells was assessed by DAPI labeling in living cells. The principle of the method is that DAPI is predominantly impermeable to living cells, what allows it to be used as a viability dye in unfixed cells to discriminate intact cells and cells with compromised membrane.

Figure 9 demonstrates that all treatments increased the DAPI fluorescence, suggesting that there is a greater number of dead cells, when compared to the control. Macrophages treated with LPS, F2 and F3 (panels B, E and F) were apparently slightly more efficient in killing tumor cells than PnV and F1. Graph G shows the difference observed when fluorescence quantification was performed.

It is clear, therefore, that PnV and, even more expressively, fractions F2 and F3 have components capable to activating macrophages, increasing their antitumor ability.

4 DISCUSSION

Natural products are an important tool for the development of drugs represented by 35% of medicines that come from natural sources. The greatest biodiversity of the world is located in Brazil and Brazilian scientists have published scientific evidences of the potential of plants and venoms in the development of drugs. The development of new therapies to treat cancer is challenging; specifically for GBM, which about 85% of therapies fail in early clinical trials (Ledford, 2011). Natural products can represent an alternative for the development of new antineoplastic drugs. Since the 1980s, 174 new compounds were approved to be commercialized for cancer treatment and, among them, 93 (53%) were natural products or derivatives directly or based on natural sources (Amaral et al., 2019).

A series of studies by our group have shown that the venom of *P. nigriventer* (PnV) has anti-tumor proprieties. PnV components had a direct effect on GBM cells *in vitro*, reducing viability and proliferation, arresting the cell cycle in G0, inducing cell death (Santos et al., 2019), and decreasing cell migration and adhesion (Barreto et al., preprint). In addition, PnV impaired GBM progression in an *in vivo* murine model, with a concomitant modulation of circulating immune cells, increasing monocytes in the blood; also, the venom induced an augment in the macrophages infiltrated in the tumor (Bonfanti et al., 2020). However, some questions remained open: whether the infiltrated macrophages were being modulated by the components of the venom and if these immune cells were contributing to the effect of PnV on tumor progression.

The present findings showed that the venom induced greater phagocytosis and killing activity, promoting an activated morphological profile, and increased migration. On the other hand, interestingly, the venom did not alter the release of cytokines and could not induce the expression of molecules involved in the presentation of antigens. Finally, macrophages pre-activated with PnV were able to kill cancer cells more efficiently than M0-control. The results also showed that purified fractions and subfractions of the venom have different effects on macrophages. This is expected, since the venom is a very complex mixture of heterogeneous molecules. F2 and F3 were better than F1 in immunomodulatory effects and were chosen to be purified again. A total of 23 subfraction were obtained and 7 of them (LW-9, -11, -12, and LW-2, -3, -6) were selected as the most effective to continue the tests. The results suggest that LW-9 demonstrated the most efficient immunomodulatory effects and can be an effective immunoadjuvant, improving the response of macrophages in the treatment of GBM. LW-6 was chosen from the first screening as a control of a subfraction with no effect; the next experiments confirmed that this subfraction was not able to immunomodulate macrophages.

The data of the present study revealed that M0 cells had a slight capacity for phagocytosis and killing, while M1 demonstrated a high capacity to do so. The results showed that components

present in the venom were able to modulate macrophages to an activated profile capable of phagocytosing bioparticles and Pb18 fungus. In addition, PnV components could kill the yeast more than M0 cells. This is an important test to assess the functional activity of macrophages, considering that this is one of the main functions performed by these cells (Kapellos et al., 2016). The findings also show that the PnV components that induced phagocytosis were not necessarily the same as those that induced death. Furthermore, it is possible that molecules from PnV have synergistic and antagonistic effects, considering that, when separated, the fractions and subfractions were less or more efficient than the whole venom.

Taking these results and considering that the use of molecules that increase the functions of killing and/or phagocytosis can be a strategy to fight cancer (Ruffel and Coussens, 2015; Mantovani, a. et al., 2017; Kowal, et al., 2019), other experiments were carried out to confirm whether the components of the venom could be strategic tools to modulate macrophages. The analysis of morphology has been recognized as a robust and important biomarker for assessing cell function (Rodell et al., 2018). The activated profile of macrophages treated with PnV-components was confirmed by the morphological observation, which shows that the cells were larger, with multiple nuclei and with long and slender processes, similar to the M1-control phenotype. Apparently, PnV-components, as well as LPS, induced macrophages proliferation. Another important parameter analyzed was the macrophage migration capacity. The migration test is defined as the movement of cells through a membrane in order to analyze chemotactic responses (Kramer et al., 2013). Results published by our group demonstrated that the tumors (GBM) from mice treated with PnV have more infiltrated macrophages (Bonfanti et al., 2020). The present findings corroborate those results, showing that PnV-components, mainly LW-9, induced greater macrophages migration. Interestingly, Santos et al. 2019 and Barreto et al. (Preprint) showed that PnV decreased the migration of GBM tumor cells in vitro, and F1 was the main fraction implicated in this effect. In the present work, we showed that F2 and F3 were able to increase macrophages migration, indicating that PnV-components have different actions that may depend on the type of cell (target), as well as the venom molecule.

Although all so far discussed parameters discussed so far suggest that the components of the venom induce a macrophage profile very similar to M1, the release of cytokines results demonstrated that in fact it is not the same phenotype. The M1 macrophage (LPS-control) increased the three cytokines analyzed: IL-6 and TNF- α (pro-inflammatory) and IL-10 (anti-inflammatory). On the other hand, PnV, its fractions and subfractions did not alter them, except LW-1 and LW-9, which increased IL-10. It is well known that M1-macrophages, classically activated by LPS and IFN- γ , are characterized by the production of pro-inflammatory cytokines, such as IL-6 and TNF- α , and also iNOS, in murine macrophages (Genin et. al, 2015). Thus, we can infer that the profile of the cells treated with the venom is not an M1, but a specific phenotype,

named M-PnV macrophages. The specific features and consequences of such phenotype will be investigated in further studies.

Even though M1 is described as an antitumor macrophage, there is a consistent literature that clarifies that, regardless of its polarization, TAMs with anti or pro-inflammatory phenotype are related to an worst prognosis in many types of cancer (Helm et al., 2014). Recent literature has shown that M1 macrophages may be associated with a poor tumor prognosis, and this effect is specifically related to the release of TNF- α (Chou et al., 2018). Moreover, another important point is that therapies with pro-inflammatory cytokines or that induce an increase in their release have the potential to promote autoimmune diseases as an adverse effect, such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes and inflammatory bowel disease (Chávez-Galán et al., 2015). Therapies with TCR-T and CAR-T cells mediate the release of cytokines, with the consequent risk of developing cytokine release syndrome (Harris et al., 2018; Xu et al., 2018). Also, immunotherapy in general, including checkpoint blockers, may cause autoimmune diseases, for example, pneumonia and colitis (Barjaktarevic et al., 2013). Therefore, LW-9 induces a macrophage that has the ability to phagocyte, kill and migrate more, but has caused no pro-inflammatory cytokine release, while releasing IL-10, which can be an important balance of immune modulation. This specific macrophage phenotype appears to be an important tool for the development of a new immunoadjuvant not based on cytokines or antibodies, with less chance of autoimmune reactions.

Considering that the macrophage is known to be a multifunctional antigen presenting cell (Li et al., 2018), this ability was also evaluated to understand the immunomodulatory mechanisms of PnV-components. Results showed that, while LPS and tumor cell lysate improved this function, the venom was unable to modulate.

Finally, even though PnV is capable of modulating macrophages for an active phenotype, it was important to assess whether this specific profile induced by PnV-components could kill tumor cells, since the evasion of the immune system by tumor cells is well known as one of the key points in the failure of immunological response (Muenst et al., 2016). The coculture of macrophages, pre-activated with the venom and fractions, with tumor cells showed that M-PnV macrophages decreased the viability of tumor cells more efficiently than M0 macrophages, as demonstrated by the entry of DAPI into GBM cells. The M-PnV function was very similar to the expected effect of M1 macrophages (LPS-Control) (Genard et al., 2017; Zhou et al., 2014).

Therefore, it is possible to conclude from the present findings that PnV-components, mainly LW-9, are capable of activating macrophages against tumor cells, and the mechanisms behind this effect appear to be the modulation of phagocytosis without altering cytokines release and antigen presentation. One question that remains unanswered is how the components of the venom do it. Some mechanisms for inhibiting phagocytosis in cancer have recently been taken into account in the literature. For example, the inhibition of the engulfment by macrophages

through cluster of differentiation 47 (CD47) (Jaiswal et al., 2009). CD47 is a transmembrane molecule, probably expressed in all types of cancer cells, which has a role in macrophages and dendritic cells, through the signal regulatory protein- α (SIRP α) (Jiang et al., 1999). Through overexpression of CD47 on its surface, cancer cells defend themselves against macrophages phagocytosis (Zhang et al., 2016; Li et al., 2018). Blocking the CD47-SIRP α interaction by monoclonal antibodies vigorously awakens the innate immunity in mice (Majeti et al., 2009). Furthermore, the leukocyte immunoglobulin-like receptor B1 (LILRB1) on the surface of TAMs has been shown to bind to a portion of MHC-I in cancer cells, which inhibited the ability of macrophages to engulf them. In vitro and in vivo analysis showed that blocking the MHC-I and LILRB1 pathways stimulated macrophages, increasing the engulfment, and significantly delayed tumor growth in mice (Barkal et al., 2018). Therefore, the inhibitory signals of phagocytosis by CD47-SIRP α and MHC-I-LILRB1 can be potential targets for the components of the venom, and these mechanisms will be evaluated in future studies.

5 CONCLUSION

In recent years, our group has focused on understanding and characterizing the effects of the *Phoneutria nigriventer* spider venom (PnV) on tumor cells. Continuing the sequence of studies, this work describes important findings about the action of PnV and its purified fractions in the activation of macrophages, inducing a specific profile. Taken together, these results suggest that PnV-components, specifically present in LW-9, may be new immunoadjuvants to modulate macrophages, increasing phagocytosis without inducing the release of pro-inflammatory cytokines. The inhibition of macrophage-mediated phagocytosis is an essential mechanism for the immune evasion of the tumor; therefore, agents targeting macrophages, which have recently received attention and been developed, can be important tools for cancer treatment. Further investigation into the detailed mechanisms of LW-9 is needed to advance a new non-cytokine and non-antibody immunoadjuvant to treat cancer.

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LEGENDS OF FIGURES

Figure 1. Venom quality and purification in subfractions by HPLC. Graph A shows the standard and quality of the venom sample. Graphs B and C show the purification of F1+F2 (LW) and F3 (HW) by HPLC, respectively, generating 12 LW-subfractions (LW-1 to LW-12), and 11 HW-subfractions (HW-1 to HW-11).

Figure 2. Phagocytosis of *Paracoccidioides brasiliensis* (Pb18) fungus by macrophages. Figure 2.1 shows the first screening with PnV, F1, F2 and F3. Macrophages were more phagocytic when pretreated with LPS (M1), PnV, F2 or F3 (less significantly with F1), compared to untreated cells (M0). Figure 2.2 represents microscopic images of macrophages incubated with Pb18; PnV and fractions (mainly F2 and F3) showed more intracellular fungi (highlighted in panel G), compared to the control. Figure 2.3 demonstrates that phagocytosis was increased with the subfractions LW-2, -3, -5, -10, -11, -12 and HW-1, -2, -3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared to the control (M0).

Figure 3. Phagocytosis assay using *E. coli* bioparticles. The green fluorescent label on macrophages corresponds to Iba-1, and the bioparticles are the red fluorescence. The pictures above on each panel show the double labeling and the pictures below show only the phagocytized bioparticle. Figure 3.1 shows that LPS, PnV, F2 (not significant) and F3 induced greater phagocytosis capacity when compared to the M0-control, while F1 was very similar to untreated cells (quantification demonstrated in the graph M). Figure 3.2 shows that LW-9 (panels G and H) had the best effect (quantification demonstrated in the graph S). *P<0.05, **P<0.01, ***P<0.001, compared to the control (M0).

Figure 4. Killing assay of *Paracoccidioides brasiliensis* (Pb18) fungus by macrophages, where reduced CFU number refers to the increased killing capacity and *vice versa*. Graph A shows that PnV, LPS, F1 and F2 significantly increased the killing of fungi. Graphs B and C demonstrate the results of subfractions isolated from F1+F2 (LW) and F3 (HW), respectively. LW-9, -11, -12, and HW-6, -8, -9 had the best effects, inducing a greater ability of macrophages to kill fungi. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, compared to the control (M0).

Figure 5. Macrophage morphology analysis: Immunolabeling for Iba-1 (green) and DAPI to highlight the nuclei (blue). Panel 5.1 shows that macrophages treated with PnV and fractions F2 and F3 exhibited a morphological profile similar to M1-control (LPS), with long processes, increased cytoplasm and multinucleated cells. The cell count (graph M) revealed that all treatments significantly increased the number of cells compared to M0. Panel 5.2 demonstrates that, among subfractions, LW-9 and HW-3 induced the most activated morphology; however, there was no significant difference when cells were counted (graph J). Scale bars: 5.1 upper panel, 200 μ m; 5.1 lower panel, 100 μ m; 5.2, 200 μ m. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, compared to the control (M0).

Figure 6. Cell migration assay using transwell. Panel 6.1 shows that all treatments significantly increased macrophage migration compared to M0-control (A); F2 (E) and F3 (F) had the best effect (cells count showed in graph G). Panel 6.2 demonstrates that, among subfractions, LW-9 (D) induced significantly increased migration (cell count presented in graph J). The pictures also show that LW-9 and HW-2 induced cells with long processes and thin body, while HW-3 and, more pronounced, HW-6, showed cells with a large and rounded body, without (or with few) processes. The arrows point to the cells. *P<0.05, **P<0.01, ****P<0.0001, compared to the control (M0).

Figure 7. Quantification of cytokines released in the culture supernatant by ELISA. Graphs in 7.1 shows that LPS strongly increased IL-6 (A), TNF- α (B) and IL-10 (C), while PnV, F1, F2 and F3 did not alter the release of any evaluated cytokines. Graphs in 7.2 demonstrate that LW (A and B) and HW-subfractions (C and D) did not induce cytokines, excepting LW-1 and LW-9 (B), which increased IL-10. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared to the control (M0).

Figure 8. Flow cytometry analysis of costimulatory molecules involved in antigen presentation (graphs A – E) and cytokines dosage in the supernatant by ELISA (F – H). For this experiment, macrophages were pulsed with glioblastoma (NG97) cells lysate, concomitantly to the treatment with LPS or PnV. The treatments alone were also evaluated. Lysate and LPS, alone or combined, increased CD11b (A), CD80 (B) and MHCII (C). PnV plus lysate also increased the molecules, but this effect is obviously due to the lysate since PnV alone had no effect. CD40 (D) and CD86 (E) were modulated only by LPS. IL-6 (F) was upregulated by LPS and lysate, alone or combined with LPS, and PnV plus lysate also increased this cytokine, but not PnV alone. TNF- α (G) and IL-10 (H) were increased only by LPS with or without lysate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared to the control (M0).

Figure 9. Glioblastoma (NG97) cells viability with DAPI labeling in unfixed cells. A co-culture was performed with macrophages and GBM cells. The macrophages were previously stimulated with LPS, PnV, F1, F2 or F3. Panels A – F demonstrate the DAPI fluorescence (blue nuclei), corresponding to dead cells. All treatments (B – F) increased the fluorescence intensity compared to the control (A). Macrophages treated with LPS, F2 and F3 (panels B, E and F, respectively) were apparently more efficient in killing tumor cells than PnV (C) and F1 (D). Graph G shows the statistical difference in fluorescence quantification. Graph H demonstrates the analysis by flow cytometry, confirming the results (however, by this method, the differences were not statistically significant). The picture in detail (I) shows the macrophages labeled with Iba-1 (green; indicated by arrows), demonstrating that the nuclei labeled with DAPI are mainly GBM cells and not macrophages. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared to the control (M0).