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Effect of *Tityus serrulatus* venom on cytokine production and the activity of murine macrophages

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The purpose of this study was to investigate the effects of ***Tityus serrulatus*** venom (TSV) on murine peritoneal macrophages evaluated in terms of activation. The effects of crude TSV were analysed by detection of cytokines, oxygen intermediate metabolites (H_2O_2) and nitric oxide (NO) in supernatants of peritoneal macrophages. Several functional bioassays were employed including an ***in vitro*** model for envenomating: cytotoxicity of TSV was assessed using the lyses percentage. Tumor necrosis factor (TNF) activity was assayed by measuring its cytotoxic activity on L-929 cells, and interleukin-6 (IL-6) and interferon-γ (IFN-γ) were assayed by enzyme-linked immunosorbent assay, whereas NO levels were detected by Griess colorimetric reactions in culture supernatant of macrophages incubated with TSV and subsequently exposed to either lipopolysaccharide or IFN-γ. Incubation of macrophages with TSV increased production of IL-6 and IFN-γ in a dose-dependent manner. TNF production was not detected in supernatants treated with TSV at any concentration. The increase in IL-6 secretion was not associated with concentration-dependent cytoxicity of TSV on these cells. These data suggest that the cytotoxicity does not appear to be the main cause of an increased cytokine production by these cells. Although NO is an important effector molecule in macrophage microbicidal activity, the inducing potential of the test compounds for its release was found to be very moderate, ranging from 125 to 800 mM. Interestingly, NO levels of peritoneal macrophages were increased after IFN-γ. Moreover, NO production had an apparent effect on macrophage activity. The results obtained here also shown that the TSV induces an important elevation in H_2O_2 release. These results combined with NO production suggest that TSV possesses significant immunomodulatory activities capable of stimulating immune functions ***in vitro***.

Key words: *Tityus serrulatus*, Cytokines, Nitric oxide, Macrophage

Introduction

Scorpion venoms, in terms of their biological properties, chemical composition, toxicity, biological actions, and pharmacokinetic and pharmacodynamic characteristics, vary enormously.

Tityus serrulatus is considered one of the most dangerous species for humans in Brazil, and is responsible for many clinical cases of envenomation in the southern region of this country. This venom is capable of exerting a variety of effects on excitable tissues, due to their action in the peripheral nervous system enhancing the release of neurotransmitters.[1] Victims of envenoming by a scorpion suffer a variety of pathologies, involving mainly sympathetic (tachycardia, hypertension, sweating and mydriasis) and parasympathetic (bradycardia, hypotension, secretions and miosis) stimulation as well as central manifestations such as irritability, hyperthermia, vomiting, tremor and convulsion. To address these issues, basic information obtained from representative experimental animal models using adequate venom samples as inflammatory inducers are necessary. Previous studies have shown different susceptibility to venom varies according to the strain of mice used.[2,3] It is well established that the predominant lethal action of scorpion venoms exerts a variety of effects on excitable tissues. For snake venoms, it is well known that lethality and toxicity possess an enormous variety according to the age, sex, nutritional state and geographic regions where the animal were captured.[4] To minimise the experimental bias, BALB/c mice and

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a mixture of *T. serrulatus* venom (TSV) obtained from 40 adult specimens from the same geographic region were used throughout all experiments.

Macrophages have been shown to be involved in different homeostatic mechanisms and pathological events, and may be engaged in complex interactions. Macrophages are involved in several areas of body function, such as phagocytosis, enzyme liberation, free radical generation, and as mediators of inflammatory processes. Cytokines release by macrophages has shown that excessive or insufficient production may significantly contribute to the pathophysiology of a range of diseases.[5–8] Generally, the treatment of macrophages with lead results in disregulation of the production of inflammatory cytokines, tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6, and preferential production of the TH1 type of cytokines interferon-γ (IFN-γ) and IL-2.

In this study, the effect of TSV on cytokines and nitric oxide (NO) production and macrophage activation were studied. Immunomodulatory effects induced by this venom for NO production and macrophage activation were compared with those obtained in the cells stimulated with lipopolysaccharide (LPS) or IFN-γ.

Materials and methods

Chemicals, reagents and buffers

Actinomycin D, orthophenyldiamine (OPD) and sodium nitrate (NO) were purchased from Sigma (St Louis, MO, USA), fetal calf serum (FCS) and RPMI-1640 medium were purchased from Cutilab (Campinas, SP, Brazil), murine anti-IL-6 (clones MP5–20F3 and MP5–32.C.11), recombinant IL-6, anti-IFN-γ (clones XGM1.2 and AN18) and recombinant IFN-γ were purchased from Pharmingen (Torreyana, San Diego, CA, USA), and recombinant TNF was purchased from Boehringer Mannheim (Mannheim, Germany).

Scorpion venom

T. serrulatus scorpions were provided by Laboratório de Artrópodos, Instituto Butantan (SP, Brazil). The venom was obtained by electrostimulation by the method previously described.[9] In brief, 15–20 V electrical stimuli were repeatedly applied to the scorpion and the venoms were collected with a micropipette, lyophilised and stored at –20°C. Venoms are pools from more than 40 adult specimens and referred to as TSV.

Animals

BALB/c female mice (18–20 g), obtained from Instituto Butantan, were used throughout the study. Mice were maintained and used according to animal welfare International reccomendations.[10]

Stimulation of mouse peritoneal macrophages

Groups of mice from BALB/c were sacrificed and their cells were harvested by peritoneal lavage.[11] The cells were seeded in 96-well microtiter plates at a concentration of 1×10^6 cells/ml and cultured in RPMI-1640 medium supplemented with 10% FCS. After incubation at 37°C for 2 h in humidified 5% CO_2, the plates were then washed twice with RPMI-1640 medium to remove non-adherent cells and the adherent cells were referred as macrophages. More than 95% of the cells were identified as macrophages by morphology as well as by their ability to ingest IgG-opsonised red cells.[12] These cells were exposed to different concentrations of TSV in RPMI-1640 containing 10% FCS. After incubation at 37°C for various intervals of time in a humidified atmosphere of 5% CO_2, the supernatants were collected and stored at –20°C until assayed for the presence of H_2O_2, NO and cytokines.

H_2O_2 determination

Peritoneal macrophages obtained as already described, where 1×10^6 cells/ml were seeded and incubated in a volume of 100 µl with no stimuli (control), or were stimulated with recombinant IFN-γ (10 ng/ml) or TSV (different amounts (µg/ml)). After incubation at 37°C for various intervals of time in a humidified atmosphere of 5% CO_2, the supernatants were then collected for NO determination and 100 µl of red phenol solution containing 140 mM NaCl, 10 mM K_2PO_4, 5.5 mM dextrose, and 5.5 mM horseradish peroxidase was added to the adherent cells for H_2O_2 determination. After incubation during 1 h at room temperature, 10 µl of 1 M NaOH were added and the absorbances were measured at 620 nm, using an automatic enzyme immunoassay reader.[13]

NO determination

The levels of NO in supernatants from macrophages control or stimulated with TSV or IFN-γ or TSV + IFN-γ were assayed by adding 100 µl of freshly prepared Griess reagent[14] to 100 µl of the sample in 96-well plates, and then reading the absorbance at 540 nm 10 min later by comparison with the absorbance curves of serial dilutions of sodium nitrate in complete culture medium. The minimum level of NO detectable under the assay conditions was 1 nmol.

Cytokine determination

The levels of cytokines IL-6 and IFN-γ in the culture supernatants were assayed by a two-site sandwich

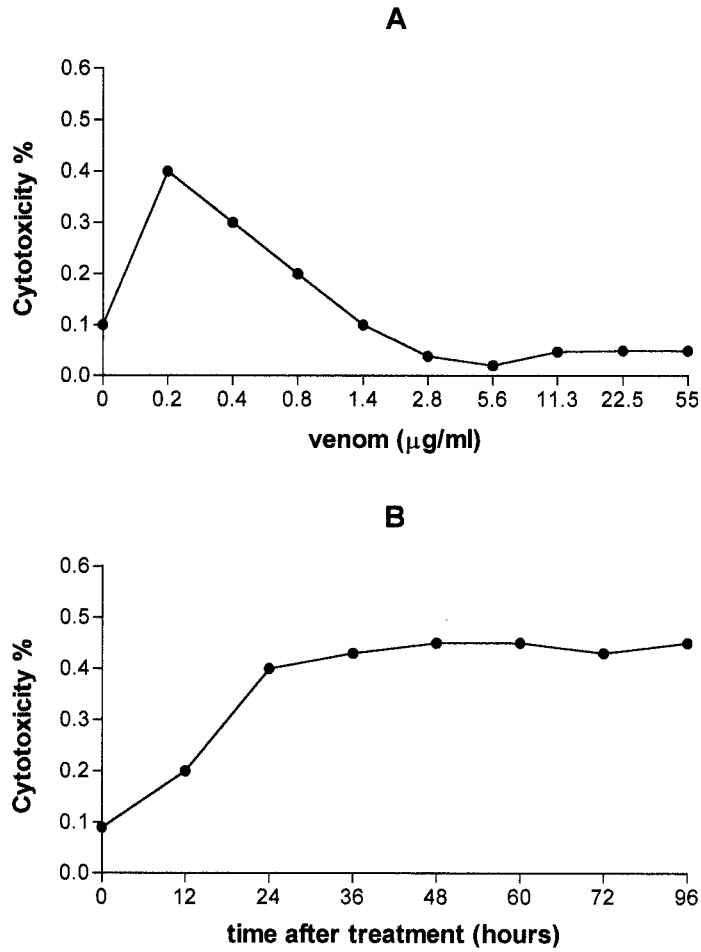

FIG. 1. Effect of TSV on macrophage cytotoxicity and viability. Groups of BALB/c mice were sacrificed and their peritoneal macrophages collected and stimulated *in vitro* with different amounts of TSV. (A) After different times of incubation of the cultured peritoneal macrophages, the cytotoxicity percentages were determined. (B) For the kinetics of the effect of TSV on cytotoxicity, the macrophages were obtained from mice and *in vitro* stimulated with 0.2 μg/ml of TSV. Each point represents the mean ± standard deviation value of samples from five experiments in different groups of five mice.

enzyme-linked immunosorbent assay (ELISA).[15] Briefly, ELISA plates were coated with 100 μl (1 μg/ml) of the monoclonal antibodies anti-IL-6 or anti-IFN-γ in 0.1 M sodium carbonate buffer (pH 8.2) and incubated for 6 h at room temperature. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100 μl of 10% FCS in PBS for 2 h at room temperature. After washing, duplicate supernatant macrophage culture samples of 50 μl were added to each well. After 18 h of incubation at 4°C, the wells were washed and incubated with 100 μl (2 μg/ml) of the biotinylated monoclonal antibodies anti-IL-6 or anti-IFN-γ as second antibodies for 45 min at room temperature. After a final wash, the reaction was developed by the addition of OPD to each well. Optical densities were measured at 405 nm in a microplate reader. The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokine (expressed in nanograms per milliliter). The minimum levels of each cytokine detectable in the conditions of the assays were 0.78 and 2.9 ng/ml for IL-6 and IFN-γ, respectively.

To measure the cytotoxicity of TNF present in the supernatants from the macrophages, a standard assay with L-929 cells, a fibroblast continuous cell line, was used as described previously.[16]

Cytotoxicity determination

In brief, macrophage cells maintained in RPMI-1640 medium supplemented with 10% FCS were seeded at 1×10^5 cells/well on to a 96-well plate, and control cells or treated cells were incubated at 37°C in a 5% CO_2 atmosphere. After different times of incubation, the supernatants were removed and the remaining live cells assessed by fixing and staining with crystal violet (0.2% in 20% methanol). Absorbance (A) was measured in each well by reading at 620 nm in a microplate reader. The percentage cytotoxicity was calculated as follows: $(A_{control} - A_{sample}/A_{control}) \times 100$. To determine the viability, in a separate group of

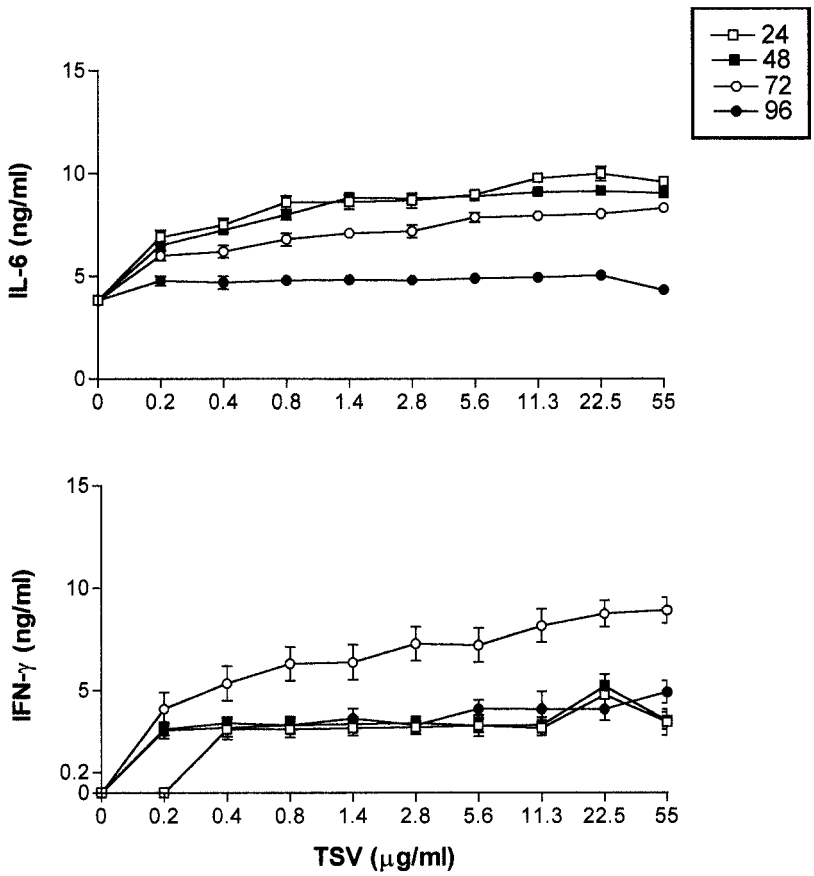

FIG. 2. Cytokine released by peritoneal macrophages from BALB/c mice. Peritoneal macrophages were obtained and stimulated *in vitro* as described in Fig. 1. IL-6 and IFN-γ were assayed by ELISA assay using monoclonal antibodies as the probe. Each point represents the mean ± standard deviation value of samples from five experiments in different groups of five mice.

macrophages stimulated with TSV, 8% Trypan blue solution was added to the wells. The percentage of the stained cells was determined in a hemacytometer.

Statistical analyses

Data are expressed as the mean ± standard deviation. Statistical analyses were performed by Student's *t*-test and the level of significant was set at $p < 0.05$.

Results

Effect of TSV on macrophage viability

Different amounts of TSV were added to peritoneal macrophage cultures. Stimulated and control cultures were incubated in the same conditions and, after 24 h, a dose-dependent change occurred in macrophages stimulated with TSV: macrophages treated with 0.2–0.4 μg/ml of TSV caused around 0.4% of the lyses in cells (Figure 1A); 0.8–1.4 μg/ml of TSV caused lyses of around 0.1 and 0.2%, respectively (Fig. 1A). Finally, concentrations of 2.8–55 μg/ml of TSV had little effect on cells, causing effect cytotoxic around 0.05% of macrophages lysed (Fig. 1A). The influence of the TSV on the viability of the treated macrophages was examined by trypan blue exclusion in a separate experiment in which the cells were incubated with different amounts of venom. TSV even at high concentrations did not induce staining of the macrophages by the dye, indicating that the cells remained viable (data not shown). To determine the kinetics of the effects of TSV on cytotoxicity, the macrophages were obtained from mice and stimulated with 0.2 μg/ml of TSV (Fig. 1B). The highest cytotoxicity percentage was observed 24 h post-treatment.

Effect of TSV on *in vitro* cytokine and NO production

To determine cytokine production, groups of mice were sacrificed and their macrophages, which were collected by peritoneal lavage, were stimulated *in vitro* with different amounts of TSV (Fig. 2). The *in vitro* stimulation of macrophages with TSV resulted in the production of IL-6 in a dose-dependent manner. The maximum production of IL-6 was detected in

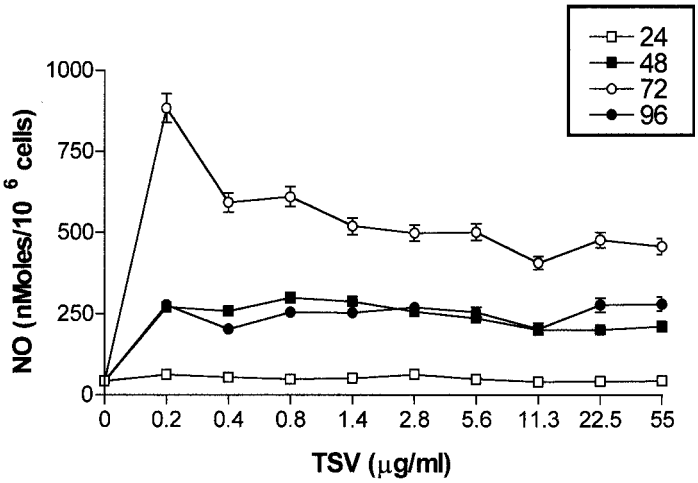

FIG. 3. NO production. Peritoneal macrophages were obtained and stimulated *in vitro* as described in Fig. 1. NO levels were detected by the Griess colorimetric reaction. Each point represents the mean ± standard deviation value of samples from five experiments in different groups of five mice.

cultures stimulated for 24 h (Fig. 2). The levels of IL-6 in groups of macrophages stimulated *in vitro* with TSV were higher when compared with those obtained in non-stimulated cultures ($p > 0.001$). Figure 2 shows that the levels of IFN-γ started to appear after 24 h in all cultures. The levels of IFN-γ increased in a dose-dependent manner until 72 h in all macrophage groups treated *in vitro* with TSV (Fig. 2). The levels of IFN-γ in groups of macrophages stimulated *in vitro* with TSV were significantly higher when compared with those obtained in control cultures ($p > 0.001$). TNF levels were not detected in these supernatants (data not shown).

The levels of NO increased until 72 h in all macrophage groups stimulated *in vitro* with TSV, decreasing thereafter (Fig. 3). The levels of NO in groups of macrophages stimulated with TSV were significantly higher when compared with those obtained in non-stimulated cultures ($p > 0.001$).

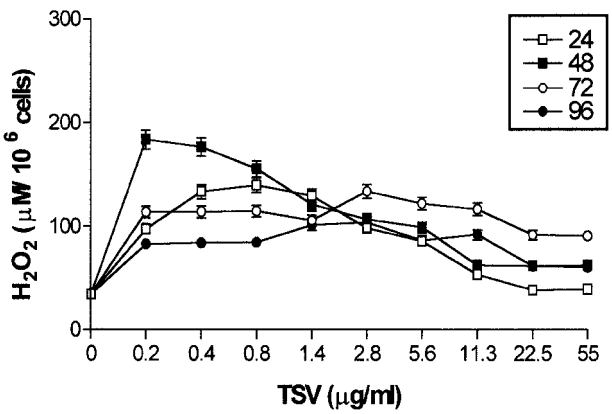

FIG. 4. Macrophage activation. Peritoneal macrophages were obtained and stimulated *in vitro* as described in Fig. 1. The activation of the cultured peritoneal macrophages was determined by measuring the oxygen intermediate metabolites (H_2O_2). Each point represents the mean ± standard deviation value of samples from five experiments in different groups of five mice.

Effect of TSV on *in vitro* activation

To compare macrophage activation, mice were sacrificed and their macrophage collected by peritoneal lavage and stimulated with different amounts of TSV. The effects of venom on macrophage were determined by measuring oxygen intermediate metabolites. TSV was capable of increasing macrophage activation. As shown in Fig. 4, in groups of macrophages treated with 0.2–1.4 μg/ml of TSV, the highest levels of activation were observed 48 h post-treatment. Similar levels of activation for all groups of macrophages stimulated with 2.8–11.3 μg/ml of TSV were observed 72 h after this treatment. The lowest levels of activation were observed in groups of macrophages treated with 22.5–55 μg/ml.

In vitro inflammatory cytokines, H_2O_2 and NO production on TSV stimulation

To compare the effects of TSV on the cytokines, H_2O_2 and NO production with LPS stimulation, groups of mice were sacrificed and their macrophages were collected by peritoneal lavage. TSV (55 μg/ml) or LPS (5 μg/ml) were added to macrophage cultures and IL-6, IFN-γ, H_2O_2 and NO were measured in these supernatants after various times. As shown in Fig. 5, similar levels of IL-6 for all groups of macrophages stimulated with TSV or LPS were observed after 24 h using both treatments. For all groups of macrophages treated with TSV or LPS, IL-6 levels decreased with an increase in the time of stimulation. The levels of IFN-γ started to appear after 24 h in both groups, increasing

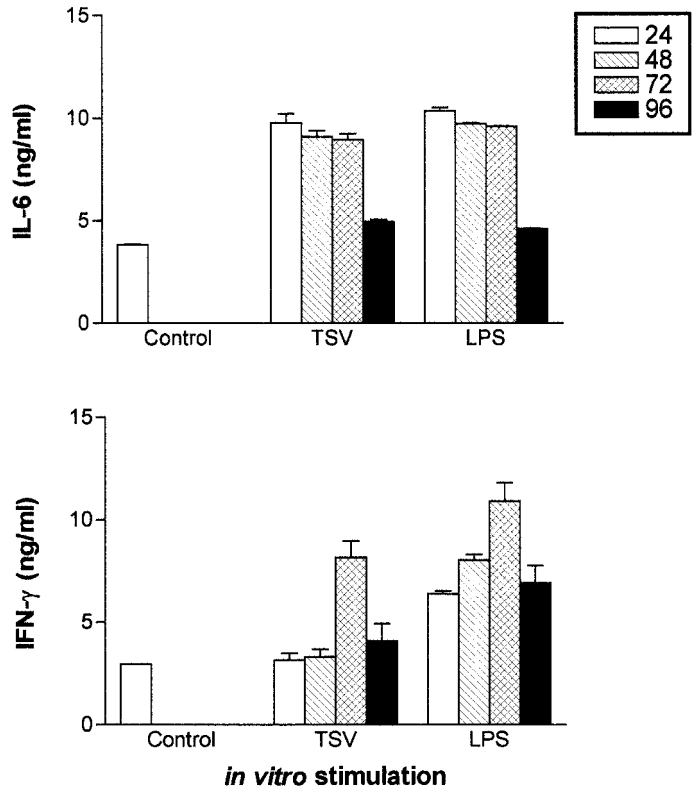

FIG. 5. Comparision of cytokine released after TSV or LPS stimulation. Groups of mice were sacrificed and their peritoneal macrophages were collected. Peritoneal macrophages were stimulated *in vitro* with 55 μg/ml of TSV or 5 μg/ml of LPS and, after different time intervals, the supernatants were collected. IL-6 and IFN-γ were assayed as described in Fig. 2. Each point represents the mean value of samples from five experiments ± standard deviation in different groups of five mice.

until 72 h and decreasing thereafter (Fig. 5). Figure 6 shows the levels of H_2O_2 in macrophages stimulated with TSV or LPS *in vitro* for 72 h. The lowest levels of H_2O_2 ($p < 0.05\%$) were observed in cultures stimulated with LPS. Figure 6 shows that in the macrophages cultured with 0.4–55 μg/ml of TSV the levels of NO were lower than the macrophages stimulated with LPS.

Effect of exogenous cytokine on macrophages stimulated with TSV

To verify whether exogenous cytokine, such as IFN-γ, showed an effect on macrophages treated with TSV, the levels of H_2O_2 and NO were determined. As shown in Figure 7, both groups of macrophages treated with TSV + IFN-γ showed a marked increase in H_2O_2 release and NO production when compared with those obtained in control cultures. The maximum H_2O_2 release and NO levels were detected in cultures stimulated for 72 h.

Discussion

T. serrulatus is an aggressive scorpion found in Southeast Brazil, where it is responsible for most scorpion accidents in humans. A better understanding of the host response may contribute to improved treatment strategies of the envenomating. To establish the optimal conditions ruling macrophage–venom interactions, the effect of TSV on cytotoxicity and viability percentage were studied. This study shown that TSV (0.2–55 μg/ml) did not induce lyses in peritoneal macrophages from BALB/c mice.

With respect to immune response, recent studies regarding the roles that cytokines may play in host defenses also indicate the importance of this aspect of the host response to envenomating. The ability of TH1-type and TH2-type cytokines to bias the development of immune responses to stimulus is known.[17,18] A number of stimuli such as microbial infection, tissue injury, and tumor cells can active macrophages or mononuclear phagocytes. These immune cells have complex and multifactorial regulatory activities exerted through the production of various cytokines, reactive oxygen metabolites and NO.

Previous studies have shown that cytokines such as TNF[19] and IL-1[20] are key intermediates of an over-responsive host–response reaction. TNF exerts a wide spectrum of biological activities and contributes to the pathophysiology of septic shock. At conditions used in the present study, TNF release from murine peritoneal macrophages was not observed after treatment with TSV.

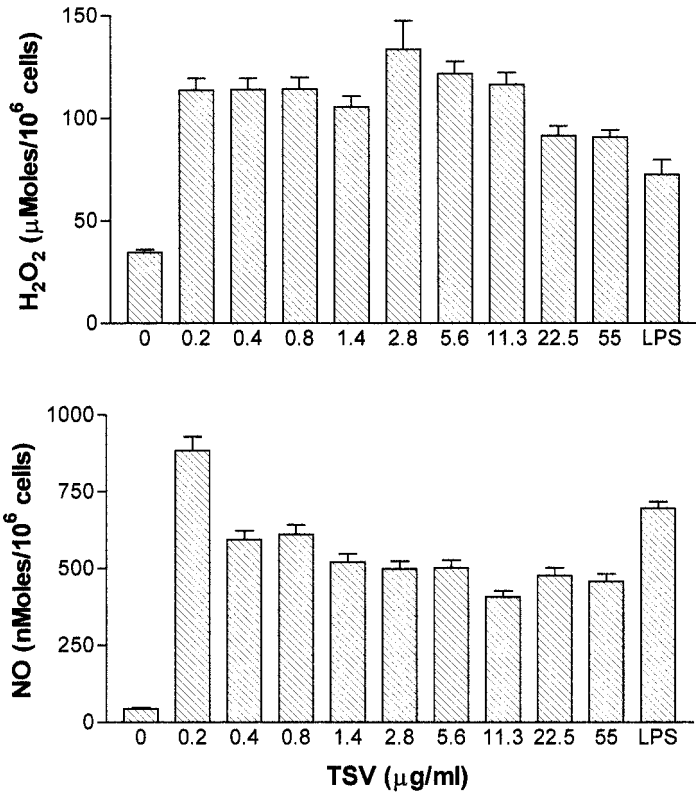

FIG. 6. H_2O_2 and NO production. Peritoneal macrophages were obtained and stimulated *in vitro* as described in Fig. 1. H_2O_2 levels and NO levels were determined 72 h post-stimulation. Each point represents the mean ± standard deviation value of samples from five experiments in different groups of five mice.

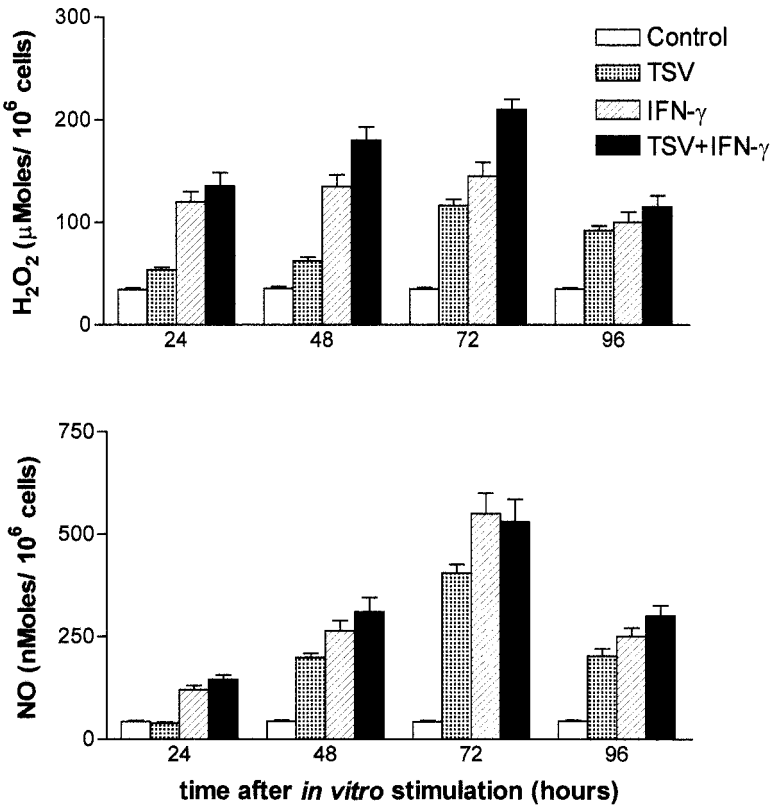

FIG. 7. H_2O_2 and NO release by peritoneal macrophages. Peritoneal macrophages were stimulated by 55 μg/ml of TSV or IFN-γ (10 ng/ml) or TSV + IFN-γ. H_2O_2 and NO were determined as described in Fig. 6. Each point represents the mean ± standard deviation value of samples from five experiments in different groups of five mice.

Other studies carried out in inflammatory models have indicated that pro-inflammatory substances and some cytokines are deeply involved in the activation of endothelial cells and leukocytes, leading them to express on their cell surface adhesion molecules.[21] The results obtained in this study showed that TSV enhances IL-6 release from murine peritoneal macrophages. These results agree with previous studies that showed IL-6 release from sera mice injected with venom,[22] victims envenomated by a snake bite,[23] and murine peritoneal macrophage stimulated with snake venom.[24]

IFN-γ also plays an important role in the lethal response to stimuli such as LPS, particularly in mediating the lethal Shwartzman reaction.[25] On the contrary, IFN-γ is a key cytokine in host defenses against intracellular organisms[26] and enhances the ability of peritoneal macrophages and Kupffer cells to phagocytosis and to kill virulent *Escherichia coli*.[27] Thus, IFN-γ may exert and participate in both the beneficial and detrimental effects of LPS. The present study shows that TSV is capable of stimulating murine peritoneal macrophages to release IFN-γ. Cytokines are powerful modulators of murine macrophage reactive oxygen intermediates such as H_2O_2, and O_2^-, and reactive nitrogen intermediates such as NO and NO_2^-.

The present study describes an experimental model designed to test the hypothesis that TSV could exert action in macrophage activation. To examine this possibility, different amounts of TSV were added to cultured macrophages, and the activation of these cells was determined. All amounts of TSV used for this study were capable of activating macrophages to release H_2O_2.

Reactive nitrogen intermediate production by murine macrophages is an important effector mechanism against a variety of pathogens.[28] In macrophages, NO and other reactive nitrogen intermediates are derived from L-arginine via an enzymatic pathway controlled by an inducible NO synthase (iNOS),[29] and their synthesis are modulated by cytokines. While TNF and IFN-γ are potent activators of iNOS, IL-4 and IL-10 suppress it.[30–32] NO is known to be involved in multiple biologically important reactions, including those with transition metal ions, thiols, and redox forms of oxygen.[33] The present study showed that TSV is capable of enhancing NO production from peritoneal macrophages (Fig. 3).

The effects of TSV on cytokines, H_2O_2 and NO production were compared with those caused by LPS stimulation. In the present study, the results showed similar levels of IL-6 for both groups of macrophages (Fig. 5). In contrast, lower levels of IFN-γ were observed for groups of macrophages stimulated with TSV. With respect to *in vitro* stimulated macrophages, it was observed that TSV induced an increase in H_2O_2 production when compared with those obtained in macrophages stimulated with LPS, while the NO production was higher in groups stimulated with LPS (Fig. 6). Interestingly, on the contrary, these results evidenced correlation of H_2O_2 and NO produced by macrophages stimulated with TSV.

The effects of TSV combined with IFN-γ were also investigated. Peritoneal macrophages stimulated with TSV fail to produce TNF either alone or in combination with IFN-γ as a co-stimulus. The levels of NO and H_2O_2 were significantly higher for groups of macrophages stimulated with TSV combined with IFN-γ. These results suggest that one compound or a synergism of several TSV constituents presented immunomodulatory activity via the activation of macrophage function with consequent oxygen metabolite liberation. These results agree with previous reports showing that priming of murine macrophages with IFN-γ significantly enhances the NO production.[34–36] However, the exact mechanism in which these molecules interplay is not completely understood.

TSV inhibited the production of TNF and synergised with IFN-γ to stimulate the production of NO by macrophages. In conclusion, this study suggested that TSV possesses immunomodulatory activities capable of stimulating immune functions *in vitro*.

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