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Uncaria tomentosa extract increases the number of myeloid progenitor cells in the bone marrow of mice infected with *Listeria monocytogenes*

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Abstract

In this study, we demonstrated that *Uncaria tomentosa* extract (UTE) protects mice from a lethal dose of *Listeria monocytogenes* when administered prophylactically at 50, 100, 150 and 200 mg/kg for 7 days, with survival rates up to 35%. These doses also prevented the myelosuppression and the splenomegaly caused by a sublethal infection with *L. monocytogenes*, due to increased numbers of granulocyte-macrophage progenitors (CFU-GM) in the bone marrow. Non-infected mice treated with 100 mg/kg UTE also presented higher numbers of CFU-GM in the bone marrow than the controls. Investigation of the production of colony-stimulating factors revealed increased colony-stimulating activity (CSA) in the serum of normal and infected mice pre-treated with UTE. Moreover, stimulation of myelopoiesis and CSA occurred in a dose-dependent manner, a plateaux being reached with the dose of 100 mg/kg. Further studies to investigate the levels of factors such as IL-1 and IL-6 were undertaken. We observed increases in the levels of IL-1 and IL-6 in mice infected with *L. monocytogenes* and treated with 100 mg/kg of UTE. White blood cells (WBC) and differential counting were also performed, and our results demonstrated no significant changes in these data, when infected mice were pre-treated with 100 mg/kg of UTE. All together, our results suggest that UTE indirectly modulates immune activity and probably disengages *Listeria*-induced supression of these responses by inducing a higher reserve of myeloid progenitors in the bone marrow in consequence of biologically active cytokine release (CSFs, IL-1 and IL-6).

Keywords: *Uncaria tomentosa*; *Listeria monocytogenes*; Hematopoiesis; CFU-GM; CSA; IL-1; IL-6

1. Introduction

Uncaria tomentosa (Wild.) D.C. (Rubiaceae) commonly known as “cat’s claw” is a vine that grows in the Amazon rainforest. Traditionally its bark is

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prepared as a decoction and used for the treatment of many ailments, such as viral infections, cancer, gastric ulcers and inflammatory disorders [1]. This plant displays a diverse range of bioactive secondary metabolites, including quinic acid, tetracyclic and pentacyclic oxindole alkaloids, triterpenes, glycosides, flavonoids and procyanidins [2–4]. Studies have demonstrated its antiinflammatory [5–11], antioxidant [11–14], antimutagenic [15] and antiviral activities [16]. This plant also exerts antiproliferative effects against tumor cell lines [17,18]. Toxicological studies demonstrated that no acute or chronic signs of toxicity were observed in rats with the doses of 8 g/kg and 1 g/kg/28 days, respectively [12,19]. Considerable scientific interest in *U. tomentosa* has been generated by the demonstration of its potent immunostimulant action. In this respect, *in vitro* experiments have shown the ability of pentacyclic oxindole alkaloids to activate the production of regulatory factors by endothelial cells [20] and the phagocytic function of granulocytes [21]. In addition, increased production of interleukin-1 (IL-1) and IL-6 by rat alveolar macrophages was induced by extracts of *U. tomentosa* [22]. In vivo, a water extract of the plant was shown to increase blood leukocytes in normal rats [12] and splenic lymphocytes subsets CD4+, CD8+ and B cells in mice [4]. Doxorubicin-induced leukopenia in rats was also reversed after administration of *U. tomentosa* [23]. An increase in blood leukocytes was also observed in healthy volunteers [12] and patients with HIV-infection showed normalization in lymphocyte counts [19]. In order to better characterize the stimulatory activity of this plant on the cellular constituents of the immune system, in the present work, we evaluated the hematopoietic response in mice treated with *U. tomentosa* extract and infected with *Listeria monocytogenes*.

Listeria monocytogenes has been extensively used as an experimental model to study the mechanisms involved in innate and cell-mediated antimicrobial defense. This Gram-positive, facultative intracellular bacterium replicates primarily within macrophages and triggers a striking increase in innate immune responses that require interactions between neutrophils, macrophages, natural killer (NK) and T cells. As a food-borne human pathogen, it can cause significant disease in newborns, immunocompromised individuals and pregnant women [24–27].

During the early stages of infection, the production and migration of granulocytes and macrophages from bone marrow into infected tissues play a major role in limiting bacterial multiplication [28–30]. These phagocytes are derived from hematopoietic progenitors in the bone marrow, and the production and mobilization of these cells depend on the presence of specific growth factors called colony-stimulating factors (CSFs) [31–33]. Several studies have shown that primary *L. monocytogenes* infection of mice is associated with a sharp rise in serum CSF levels, an increase in differentiated monocytes and granulocytes at the site of infection and a decrease in the number of progenitor cells in the bone marrow [28–30,34]. In addition, there is some evidence that higher numbers of progenitor cells in the bone marrow before infection favour survival of mice after *L. monocytogenes* infection [35].

Using the listeriosis model we determined the effects of the oral administration of *Uncaria tomentosa* extract (UTE) on the growth and differentiation of bone marrow and spleen CFU-GM, in serum CSF levels, IL-1 and IL-6 production in mice. Our results demonstrated that UTE has a stimulating effect on the hematopoietic response in infected and normal mice, which may be responsible, at least in part, for the enhanced protection during a lethal infection.

2. Materials and methods

2.1. Animals

Male BALB/c mice, 6–8 weeks old, were bred at the University Central Animal Facilities (CEMIB, Universidade Estadual de Campinas, Campinas, SP, Brazil), raised under specific pathogen-free conditions and matched for body weight before use. Animal experiments were done in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Commitee [36].

2.2. Listeria monocytogenes infection

L. monocytogenes obtained at the Laboratory of Microbiology (Departamento de Patologia Clínica, Hospital das Clínicas, UNICAMP) was used to infect the animals. Bacterial virulence was maintained by

serial passages in BALB/c mice. Fresh isolates were obtained from infected spleens, grown in BHI medium and stored at −80 °C in 1-mL aliquots. Before use, each sample was thawed and diluted to appropriated concentrations in 0.9% NaCl. Mice were inoculated intraperitoneally (i.p.) with a sublethal dose of 1×10^3 viable *L. monocytogenes* per mouse for the study of the CFU-GM, CSFs levels, WBC, differential counting, IL-1 and IL-6 production. To evaluate survival rate, mice were inoculated i.p. with a lethal dose of 4×10^4 viable *L. monocytogenes* per mouse. In both cases, mice were infected at the end of a 7-day treatment with different concentrations of UTE.

2.3. Treatment regimen

Uncaria tomentosa extract (UTE) was provided as a powder, dosed in 1% of total alkaloids by Galena Química e Farmacêutica Ltda (Campinas, SP, Brazil).

UTE was dissolved in sterile water and diluted into appropriate concentrations immediately before use. Doses of 10, 50, 100, 150 and 200 mg/kg were administered orally to mice for 7 days prior to infection in a 0.2-mL volume/mouse. Mice were infected with a sublethal dose of bacteria 3 h after the end of the treatment and assays were performed 24, 48 and 72 h after bacterial inoculation. Each experiment included parallel control groups of normal and infected mice treated with an equivalent volume of the vehicle. Selection of these doses was based on previous literature [7,16].

2.4. Progenitor cell assay

Bone marrow cells were prepared aseptically from one complete femur shaft in RPMI 1640 medium (Sigma Chemical Co, St Louis, MO). Spleens were then removed aseptically and converted to dispersed cell suspensions in RPMI 1640 medium by gently pressing through a stainless-steel mesh net. Bone marrow and spleen cells were placed in duplicate 1 mL semisolid agar cultures in 35 mm Petri dishes using 1×10^5 bone marrow cells or 2.5×10^5 spleen cells per culture. The medium used was Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 20% fetal calf serum (FCS) and 0.3% agar. Colony formation was stimulated by the addition of 0.5 ng/mL of recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF; Sigma). The cultures were incubated for 7 days in a fully humidified atmosphere of 5% CO_2 in air and colony formation (clones>50 cells) was scored at ×35 magnification using a dissection microscope. Morphological analysis was performed in the whole cultures fixed in 2.5% glutaraldehyde and stained with Luxol fast blue/hematoxylin as previously described [37].

2.5. Assay for serum colony-stimulating activity (CSA)

Mice were bled from the heart under halothane anesthesia. Within each experimental group, blood was pooled in periods of 24, 48 and 72 h after the *L. monocytogenes* challenge. Pooled blood was left at 37 °C for 30 min and the clots were allowed to retract overnight at 4 °C. Following centrifugation, the serum was removed and stored at −20 °C. CSA was determined by the ability of serum obtained from control and experimental groups to induce the growth and differentiation of bone marrow progenitor cells (1×10^5 cells) from normal mice. Results are expressed as units of CSF/mL, where 1 unit/mL was defined as the lower amount of CSF that induces the formation of colonies [38].

2.6. Quantification of Interleukin-1 (IL-1) and IL-6 levels

Suspensions of spleen cells from all mice were prepared as described in the item 2.4. The cells were suspended in RPMI 1640 supplemented with 5% FCS and washed twice. Red blood cells were lysed with 0.17 M NH_4Cl and the remaining cells were again washed three times and counted. Viability was determined by trypan blue exclusion and consistently exceeded 90%. Splenocytes (1×10^6 cell/mL) were suspended in enriched RPMI 1640 culture medium supplemented with 5% FCS and seeded into 24-well culture plates (Corning) in the presence of 5 μg/mL Con A (Sigma). Cell-free supernatants were collected after 24 h of incubation at 37 °C in 5% CO_2, and cytokine levels were detected by ELISA. Cytokines (IL-1 and IL-6) were quantitated by sandwich ELISA using purified anti-mouse IL-1α mAB (BD Opteia™ ELISA Set Mouse Cat. 550347) and IL-6 mAB (BD Opteia™ ELISA Set Mouse Cat. 555240), purchased

from Pharmingen (San Diego, USA). Cytokine determinations were done according to Pharmingeńs cytokine ELISA protocol. Briefly, microtiter plates (96-well flat-bottom maxisorp microplate—NUNC, Roskilde, DM) were coated overnight with 2.5 μg/mL of anti-cytokine mAbs in a coating buffer of 0.1 M $NaHCO_3$, pH 9.5, at 4 °C. A blocking step was performed for 1 h at room temperature (RT) (Phosphate buffer saline/10% FCS). After washing, the recombinant standards and the supernatants were added to the coated plates and incubated for 2 h at RT. The plates were washed and then incubated with the biotinylated anti-cytokine detecting antibodies (2.5 μg/mL) for 1 h. After incubation with avidin-peroxidase for 30 min, the substrate consisting of tetramethylbenzidine (TMB) and H_2O_2 (BD Pharmingen Cat. 555214), was added. Reaction was determined by measure of optical density at 492 nm in a Labsystem Immunoreader (Finland) after stopping the reaction using 2 N H_2SO_4. Cytokine titers were expressed as picogram per milliliter, calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

2.7. Hematologic parameter

Peripheral blood was collected from retroorbital vein into EDTA tubes, in periods of 24, 48 and 72 h after the *L. monocytogenes* challenge. Blood samples were analyzed within 1 h by an automated cell counter Advia 120 Hematology System (Bayer, Dublin, Ireland). Differential morphological counting of blood smears stained with May–Grunwald Leishman's was performed.

2.8. Survival analysis

The protective effects of UTE was evaluated by determining the survival time of BALB/c mice treated for 7 consecutive days with oral daily doses of 10, 50, 100, 150 and 200 mg/kg of UTE. Animals were infected with a lethal dose of *L. monocytogenes*, 3 h after the end of treatment and observed for 30 days.

2.9. Statistical analysis

For statistical analysis of changes in progenitor cell assays, colony-stimulating activity and hematological parameters, a parametric method, the one-way analysis of variance (ANOVA) followed by the Turkey test, was used to compare data among all groups. Analysis of cytokine levels was done by Kruskal–Wallis nonparametric ANOVA with the Dunn's Multiple Comparisons test to compare single groups. The probability of survival among the experimental groups was calculated by the Kaplan–Meyer method and the logarithm-rank statistic was used to test differences between groups. Statistical significance was considered when $P<0.05$.

3. Results

3.1. Survival analysis

The antibacterial efficacy of the prophylactic administration of UTE was evaluated in mice infected with a lethal dose of *L. monocytogenes* (Fig. 1). All the untreated infected mice died within 5 days after infection. On the other hand, the prophylatic treatment of these animals with 50, 100, 150 e 200 mg/kg of UTE for 7 days, significantly increased survival ($P<0.001$). When the dose of 50 mg/kg was used, 15% of these animals survived, whereas with the doses of 100, 150 and 200 mg/kg there was a 35% of survival, and no difference was found. In contrast, the dose of 10 mg/kg did not present any protection against lethality.

3.2. Medullar and splenic hematopoiesis

The effects of the pre-treatment with 10, 50 and 100 mg/kg of UTE on bone marrow hematopoietic activity of normal and *Listeria monocytogenes* infected mice are presented in Fig. 2. Consistent with our previous results [39–45], the number of bone marrow CFU-GM was significantly reduced in infected mice at 48 and 72 h after bacterial challenge ($P<0.001$). The lower response was observed at 48 h, when the number of CFU-GM reached less than 60% of control values. On the other hand, at 24 h after the infection, no significant differences were found between the hematopoietic response of control and infected mice. Significant protection against the CFU-GM suppression induced by the infection was observed in the bone marrow of mice pre-treated with

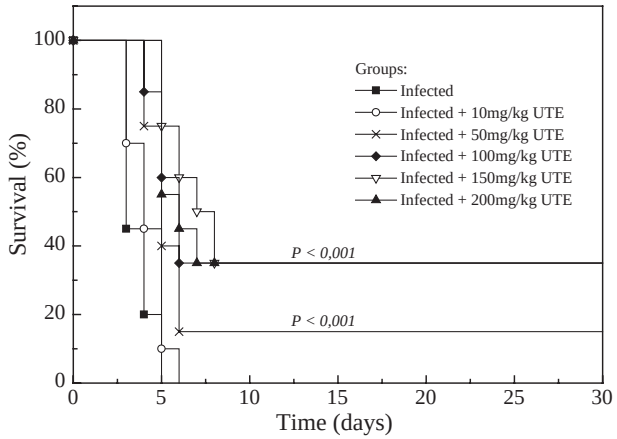

Fig. 1. Survival of infected mice pre-treated with *Uncaria tomentosa* extract (UTE) for 7 consecutive days. At the end of UTE administration, mice were inoculated i.p. with a lethal dose of 4×10^4 *Listeria*/mouse. Groups of 20 mice were checked daily for survival. $P < 0.001$ in relation to infected mice.

UTE. With the 50 mg/kg dose, CFU-GM numbers reached control levels, whereas an stimulation over control values was produced by the 100 mg/kg dose, as soon as 24 h after bacterial challenge ($P < 0.001$), which was sustained throughout the experiment. Notably, a significant enhancement in colony formation was also observed after treatment of normal mice with the 100 mg/kg dose ($P < 0.001$), but not with the 50 mg/kg dose. As before, the dose of 10 mg/kg was not effective in the recovery of CFU-GM numbers of infected mice. The two higher doses studied (150 and 200 mg/kg), produced the same level of CFU-GM response observed with the 100 mg/kg dose (data not shown). Since *Listeria monocytogenes* infection is generally accompanied by higher numbers of progenitor cells in the spleen [35,45], we next evaluated the compartment of committed myeloid progenitors in this organ (Fig. 3). The dramatic

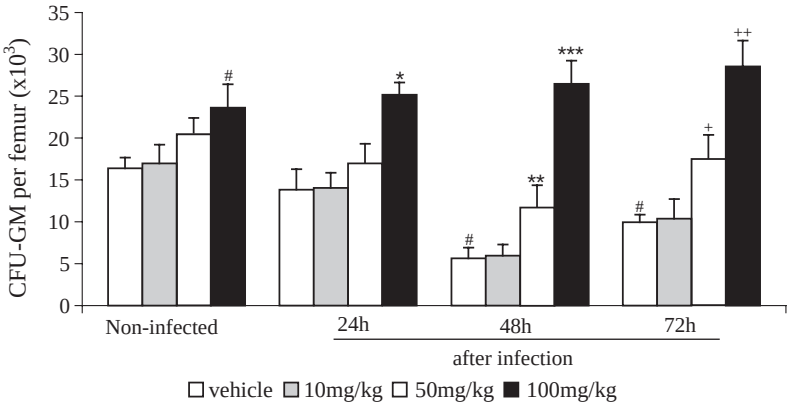

Fig. 2. Number of bone marrow granulocyte-macrophage progenitors (CFU-GM) in normal and *Listeria monocytogenes*-infected mice pre-treated with *Uncaria tomentosa* extract (UTE). UTE at 10, 50 and 100 mg/kg was given by gavage for 7 consecutive days and 3 h after the last dose, mice were infected i.p. with a sublethal dose of 1×10^3 *Listeria*/mouse. CFU-GM numbers were determined at 24, 48 and 72 h after infection. Control mice received vehicle only. Results represent the means ± SD of 6 mice/group. $^{\#}P < 0.001$ in relation to control non-infected group; $^{*}P < 0.001$ in relation to control non-infected, infected 24 h and infected 24 h + 50 mg/kg UTE groups; $^{**}P < 0.01$ in relation to infected 48 h group; $^{***}P < 0.001$ in relation to control non-infected, infected 48 h and infected 48 h + 50 mg/kg UTE groups; $^{+}P < 0.001$ in relation to infected 72 h group; $^{++}P < 0.001$ in relation to control non-infected, infected 72 h and infected 72 h + 50 mg/kg groups.

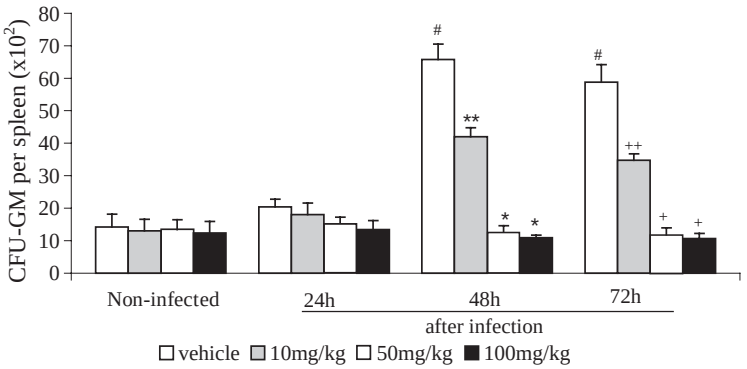

Fig. 3. Number of spleen granulocyte-macrophage progenitors (CFU-GM) in normal and *Listeria monocytogenes*-infected mice pre-treated with *Uncaria tomentosa* extract (UTE). UTE at 10, 50 and 100 mg/kg was given by gavage for 7 consecutive days and 3 h after the last dose, mice were infected i.p. with a sublethal dose of 1×10^3 *Listeria*/mouse. CFU-GM numbers were determined at 24, 48 and 72 h after infection. Control mice received vehicle only. Results represent the means ± SD of 6 mice/group. $^{\#}P<0.001$ in relation to control non-infected group; $^{*}P<0.001$ in relation to infected 48 h group; $^{**}P<0.001$ in relation to control non-infected and infected 48 h groups; $^{+}P<0.001$ in relation to infected 72 h group; $^{++}P<0.001$ in relation to control non-infected and infected 72 h groups.

increase in spleen CFU-GM numbers observed at 48 and 72 h after the infection ($P<0.001$) was fully reversed by treatment with 50 and 100 mg/kg of UTE for 7 days, prior to bacterial inoculation ($P<0.001$). With the 10 mg/kg dose, however, in spite of a significant decrease in the levels of these progenitors, compared to the infected/non-treated group, the levels of CFU-GM remained significantly high compared to controls ($P<0.001$). In normal, non-infected mice, UTE treatment produced no changes in number of CFU-GM in spleen.

3.3. Serum colony-stimulating activity

The effects of UTE in the serum colony-stimulating activity (CSA) are presented in Fig. 4. The treatment of normal, non-infected mice, with the doses of 10, 50 and 100 mg/kg of UTE produced a dose-dependent increase in CSA levels ($P<0.01$ and $P<0.001$, for the doses of 50 and 100 mg/kg, respectively). Similarly, the presence of the infection led to increased CSA titers, with a peak at the interval of 48 h after inoculation of bacteria. The treatment of

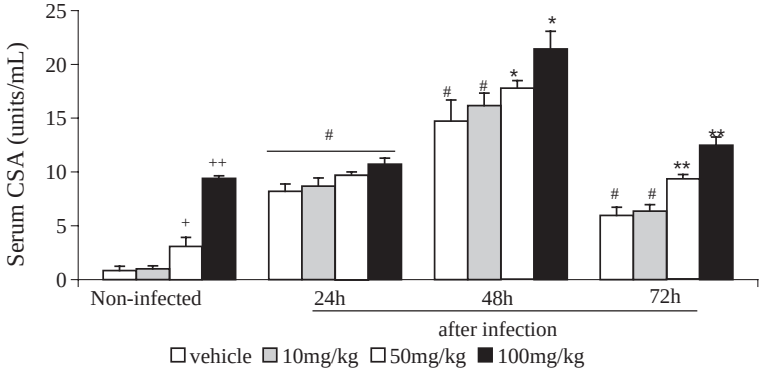

Fig. 4. Colony-stimulating activity (CSA) in serum samples of normal and *Listeria monocytogenes*-infected mice pre-treated with *Uncaria tomentosa* extract (UTE). UTE at 10, 50 and 100 mg/kg was given by gavage for 7 consecutive days and 3 h after the last dose, mice were infected i.p. with a sublethal dose of 1×10^3 *Listeria*/mouse. CSA was determined at 24, 48 and 72 h after infection. Control mice received vehicle only. Results represent the means ± SD of 6 mice/group. $^{+}P<0.01$ in relation to control non-infected; $^{++}P<0.001$ in relation to control non-infected and non-infected+50 mg/kg UTE groups; $^{\#}P<0.001$ in relation to control non-infected group; $^{*}P<0.001$ in relation to control non-infected and infected 48 h groups; $^{**}P<0.001$ in relation to control non-infected and infected 72 h groups.

infected mice with the plant extract further increased CSA titers, in a dose-dependent manner. In this regard, at 24 h after the infection, no changes in CSA response were produced by the UTE treatment. On the other hand, at the intervals of 48 and 72 h of bacterial inoculation, the doses of 50 and 100 mg/kg, but not the lower dose of 10 mg/kg, further increased the CSA response evoked by the infection ($P<0.001$). As before, a peak CSA response was observed at the interval of 48 h. It is interesting to mention that, no further increase in CSA titers was produced by the higher doses of 150 and 200 mg/kg of UTE (data not shown).

3.4. IL-1 and IL-6 production

The effects of the pre-treatment with 100 mg/kg of UTE on IL-1 and IL-6 levels of normal and *L. monocytogenes* infected mice are presented in Figs. 5 and 6. In the non-infected mice treated with 100 mg/kg of UTE, there was an increase of IL-1 ($P<0.05$) and IL-6 ($P<0.001$) production. The infection alone produced a rise in the levels of both cytokines, reaching a peak at 48 h for IL-1 ($P<0.001$) and 24 h for IL-6 ($P<0.001$), followed by an acute decrease. The treatment with 100 mg/kg of UTE prior to bacterial challenge increased to normal values ($P<0.01$) the decreased levels of IL-1 at 72 h period, and maintained the elevated levels of IL-6 throughout the experiment ($P<0.01$ at 48 h and $P<0.001$ at 72 h). The rise of both cytokines were consistent with higher numbers of CFU-GM in the bone marrow (Fig. 2).

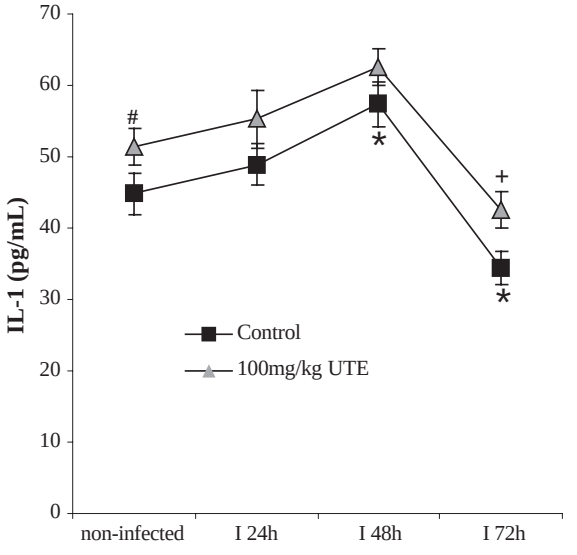

Fig. 5. IL-1 levels of normal and *Listeria monocytogenes*-infected mice pre-treated with *Uncaria tomentosa* extract (UTE). UTE at 100 mg/kg was given by gavage for 7 consecutive days and 3 h after the last dose, mice were infected i.p. with a sublethal dose of 1×10^3 *Listeria*/mouse. IL-1 was determined at 24, 48 and 72 h after infection. Control mice received vehicle only. Results represent the means ± SD of 10 mice/group. $^{\#}P<0.05$ in relation to control non-infected group; $^{*}P<0.001$ in relation to control non-infected group; $^{+}P<0.01$ in relation to infected (I) 72 h group.

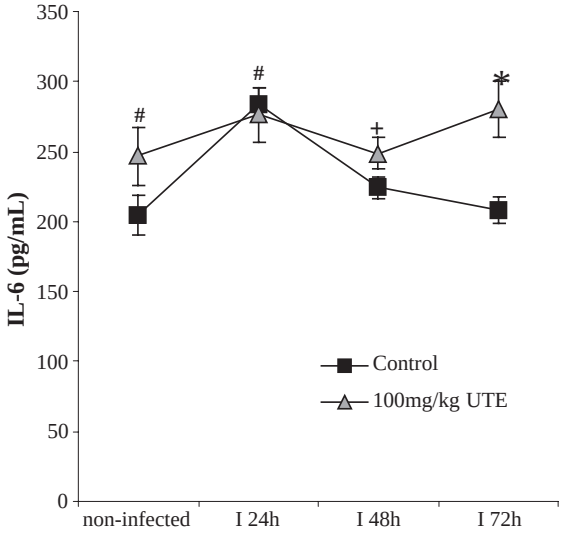

Fig. 6. IL-6 levels of normal and *Listeria monocytogenes*-infected mice pre-treated with *Uncaria tomentosa* extract (UTE). UTE at 100 mg/kg was given by gavage for 7 consecutive days and 3 h after the last dose, mice were infected i.p. with a sublethal dose of 1×10^3 *Listeria*/mouse. IL-6 was determined at 24, 48 and 72 h after infection. Control mice received vehicle only. Results represent the means ± SD of 10 mice/group. $^{\#}P<0.001$ in relation to control non-infected group; $^{+}P<0.01$ in relation to infected (I)/48 h group; $^{*}P<0.001$ in relation to infected/72 h group.

3.5. Hematologic parameter

The effects of the pre-treatment with 100 mg/kg of UTE on total and differential WBC counting of normal and *L. monocytogenes* infected mice are presented in Table 1. No significant changes were observed in total WBC counts among all samples examined. On the other hand, significant increases ($P<0.05$) of neutrophils and reduction ($P<0.01$) of lymphocytes and monocytes were observed at 48 and

Table 1
WBC and differential counting ($\times 10^3$ cells/μL) of normal and *Listeria monocytogenes* infected mice pre-treated with *Uncaria tomentosa* extract (UTE)

Groups (normal values)		WBC (3.2–12.7)	Neutrophil (0.5–2.0)	Monocyte (0–0.3)	Lymphocyte (3.8–8.9)
Control		9.65 ± 1.54	1.11 ± 0.15	0.17 ± 0.03	8.02 ± 1.40
UTE 100 mg/kg		7.97 ± 1.19	1.10 ± 0.20	0.15 ± 0.02	6.49 ± 0.97
Infected	24h	7.85 ± 0.51	1.75 ± 1.03	0.17 ± 0.02	5.63 ± 0.79
	48h	7.62 ± 1.68	3.52 ± 0.77*	0.08 ± 0.03$^{\&}$	4.06 ± 1.23$^{\#}$
	72h	6.52 ± 0.57	2.80 ± 0.59*	0.08 ± 0.03$^{\&}$	3.65 ± 0.76$^{\#}$
Infected +UTE 100 mg/kg	24h	7.02 ± 2.59	1.85 ± 1.31	0.11 ± 0.04$^{\&}$	4.78 ± 1.32$^{\#}$
	48h	6.54 ± 1.61	3.23 ± 0.56*	0.04 ± 0.01$^{\&}$	3.38 ± 0.64$^{\#}$
	72h	6.59 ± 2.02	2.83 ± 0.60*	0.04 ± 0.02$^{\&}$	2.38 ± 1.28$^{\#}$

UTE at 100 mg/kg was given by gavage for 7 consecutive days and 3 h after the last dose, mice were infected i.p. with a sublethal dose of 1×10^3 *Listeria monocytogenes*/mouse. Hematologic parameters were determined at 24, 48 and 72 h after infection. Results represent the means ± SD of 6 mice/group. *, $^{\#}$, $^{\&}$ $P<0.05$ in relation to control non-infected group.

72 h after bacterial challenge. Interesting to note that UTE treatment produced no changes in the profile of response observed in all groups.

4. Discussion

Herbal medicines with immunomodulating activities play a relevant role in the search of new pharmacologically active compounds, since they are capable of enhancing host defense mechanisms to provide protection against opportunistic infections [46]. In the present study, experiments were undertaken to quantify the effects of *Uncaria tomentosa* extract on myelopoiesis after the experimental infection caused by *Listeria monocytogenes* in mice.

Granulocytes and macrophages are vital to the defense against bacterial infection [30,33]. Several works have demonstrated that in listeriosis the ability of bone marrow to produce and mobilize granulocytes and macrophages to the site of infection is an essencial component of the host defense during the early inflammatory response and consequently survival [28–31,47]. Studies from our laboratory have defined listeriosis as a useful experimental model to study the influence of various natural compounds and toxicants in the many components of the host immune responses involved in defense against *L. monocytogenes*, with special emphasis on the ability of hematopoietic tissues to produce and mobilize phagocytes [39–45]. It has been well demonstrated in the literature that during the early phase of *Listeria* infection there is a decrease in the number of granulocyte-macrophage colony-forming cells (CFU-GM) in bone marrow related with a massive migration to the spleen, showing an extramedullar hematopoiesis with consequently splenomegaly and an increase in the serum colony-stimulating factors (CSF) concentration [35–37].

Our results corroborate previous data showing that the reduced frequency of progenitor cells in the bone marrow of BALB/c mice infected with *Listeria* determines a deficient inflammatory response and an impaired natural resistance [39–45]. The oral administration of 50 and 100 mg/kg of *U. tomentosa* extract (UTE) for 7 days prior to a sublethal dose of bacteria prevented myelosuppression and concomitantly restored spleen CFU-GM numbers. The dose of 100 mg/kg caused stimulation of CFU-GM production in the bone marrow of normal and infected mice as soon as 24 h after bacterial inoculation. Moreover, this effect was sustained at least up to 3 days. Of major importance is the fact that the dose of 100 mg/kg UTE upregulated the production of colony-stimulating factors (CSFs) in the same manner as for CFU-GM. It is well known that the persistent elevation of CSF levels in infected mice serve as a continuing stimulus that support the survival, proliferation, differentiation and end cell function of granulocytes and monocytes, once the levels return to normal when bacterial counts in the liver and spleen approach zero [29–31,47–49]. Therefore, it seems likely that UTE administered prophylactically at 100 mg/kg could contribute to increase the pool of progenitor phagocytic cells able

to control infection. The highly significant percentage of survivors detected in mice challenged with a lethal dose of *Listeria* (35%) after the treatment with 100 mg/kg UTE, corroborates this assumption. Similarly, the dose of 50 mg/kg induced high levels of CSA irrespective of infection and increased to 15% the number of survivors. Treatment with 150 and 200 mg/kg of UTE did not raise additional colony numbers, serum CSA and survival rate. This fact suggests that all available progenitor cells could be induced to proliferate and differentiate by treatment with the dose of 100 mg/kg of UTE, reaching a plateau.

Although, the precise mechanism whereby *U. tomentosa* improved the survival rate of mice infected with *L. monocytogenes* is not completely elucidated, our results clearly demonstrated that the increased reserve of bone marrow CFU-GM induced by this plant extract plays an important role in resistance to infection. This is in agreement with literature data showing that higher numbers of myeloid progenitors in the bone marrow of genetically resistant mice is associated with an earlier influx of phagocytes to the site of infection and increased resistance [34,35,50–52]. C57BL/10 mice which are genetically resistant to *L. monocytogenes*, present higher numbers of CFU-GM in the bone marrow both before and after the infection, when compared to BALB/c mice. In addition, an earlier influx of phagocytes to the site of infection is observed in the resistant C57BL/10 strain, probably as a consequence of the higher numbers of hematopoietic precursor cells in the bone marrow and spleen observed in these animals [30,34,50–52].

Another important observation was that the increase in myeloid progenitors in the bone marrow of *L. monocytogenes*-infected mice mediated by the pre-treatment with the extract occurs concomitantly with a reduction in the splenic CFU-GM. It is known that alterations in the distribution of hematopoietic activity in the tissues may occur as a result of increased hematopoietic stress, like those provoked by experimental murine infections [35,53,54]. In addition, hematopoietic stem cells and progenitor cells can migrate from the bone marrow in blood to other tissues like the spleen as part of the host defenses against intracellular bacteria, sustaining a second level of compensatory hematopoietic response [28,55,56]. Therefore, the reduced hematopoietic activity found in the spleen of treated mice presumably reflected a lower number of bacteria as a result of the improved protective mechanisms induced by the plant.

With the purpose to demonstrate some additional mechanisms by which UTE modulates the immune response, we investigated its effects in white blood cells (WBC) and differential counting. Contradicting previous results in literature that *U. tomentosa* increase blood leukocytes in normal rats [12], our results demonstrated no significant changes in WBC and differential counting when mice were pre-treated with 100 mg/kg of UTE for 7 days prior *L. monocytogenes* inoculation. A possible explanation could be the prolonged period of treatment applied in previous studies since rats were treated for 16 until 60 consecutive days, but our results are corroborated by recent findings in the literature, once no increase in WBC, blood lymphocytes or red blood cells was observed in mice treated with *U. tomentosa* extract during 21 days [4].

It was proposed that this plant may have a direct myelostimulating effect through the induction of endogenous cytokine production [23]. In this respect, it was demonstrated that *U. tomentosa* stimulates IL-1 and IL-6 production by rat alveolar macrophages [22]. In agreement with previous data, we observed in this study, an increase of IL-1 and IL-6 production in non-infected mice treated with 100 mg/Kg of UTE. The infection alone produced a rise in citokines levels, reaching peak at 48 h to IL-1 and 24 h to IL-6, followed by an acute decrease. The treatment with 100 mg/kg of UTE prior to bacterial inoculation produced higher levels of IL-1, at the 72 h period, supporting titers of normal mice. In a similar way, the increased levels of IL-6 were maintained by the pre-treatment with UTE during infection.

Both IL-1 and IL-6 are required for resistance to listeriosis, appearing during early stages of infection. IL-1 induces chemokines or activate adhesion molecules to promote neutrophil trafficking and accumulation at sites of infection [57–60]. IL-1 also synergizes with IL-12 for the production of interferon-γ (IFN-γ), which is essential to macrophage activation [61,62]. IL-1 neutralization showed a decreased number of neutrophils in infective foci and unresponsiveness macrophages to IFN-γ, exacer-

bating the infection [61]. IL-6 has a multiple roles, including synergistic stimulation of hematopoiesis with CSFs, contribution to the maintenance of mature neutrophil viability, stimulation of T-cell proliferation, differentiation and activation [58,63,64]. IL-6-deficient mice exhibit increased bacterial growth in the liver and spleen [61]. IL-1 is reported to be a potent inducer of IL-6, suggesting that these cytokines may regulate each other [59]. In line with these findings, our results are showing that a possible interaction of IL-1, IL-6 and CSFs in mice treated with UTE, contributes as an additional stimulus to the production and migration of neutrophils and macrophages during infection, since higher numbers of CFU-GM were also found in bone marrow at the same time.

All together, the results presented in this work suggest that UTE indirectly modulates immune activity and probably disengages *Listeria*-induced supression of these responses by upregulating the production of colony-stimulating factors and inducing a higher reserve of myeloid progenitors in the bone marrow favouring an efficient and sustained response of phagocytes in the control of infection, in consequence of biologically active cytokine release (CSFs, IL-1 and IL-6).

Today, the multiple effects of growth factors open up new opportunities for treatment, not only by enhancing body defense in fighting infections and perhaps certain tumors but also for the different forms of hematopoietic diseases and hematopoietic disorders following radio/chemotherapy and bone marrow transplantation. In this regard, the ability of UTE to restore physiological balance in the disturbed cytokine network with apparent lack of toxicity and low cost might open a field of interest concerning its possible use in clinical aplications.

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