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## Potent anti-inflammatory activity of betulinic acid treatment in a model of lethal endotoxemia

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## ABSTRACT

Betulinic acid (BA) is a lupane-type triterpene with a number of biological activities already reported. While potent anti-HIV and antitumoral activities were attributed to BA, it is considered to have a moderate anti-inflammatory activity. Here we evaluated the effects of BA in a mouse model of endotoxic shock. Endotoxemia was induced through intraperitoneally LPS administration, nitric oxide (NO) and cytokines were assessed by Griess method and ELISA, respectively. Treatment of BALB/c mice with BA at 67 mg/kg caused a 100% survival against a lethal dose of lipopolysaccharide (LPS). BA treatment caused a reduction in TNF- $\alpha$  production induced by LPS but did not alter IL-6 production. Moreover, BA treatment increased significantly the serum levels of IL-10 compared to vehicle-treated, LPS-challenged mice. To investigate the role of IL-10 in BA-induced protection, wild-type and IL-10<sup>-/-</sup> mice were studied. In contrast to the observations in IL-10<sup>+/+</sup> mice, BA did not protect IL-10<sup>-/-</sup> mice against a lethal LPS challenge. Addition of BA inhibited the production of pro-inflammatory mediators by macrophages stimulated with LPS, while promoting a significant increase in IL-10 production. BA-treated peritoneal exudate macrophages produced lower concentrations of TNF- $\alpha$  and NO and higher concentrations of IL-10 upon LPS stimulation. Similarly, macrophages obtained from BA-treated mice produced less pro-inflammatory mediators and increased IL-10 when compared to non-stimulated macrophages obtained from vehicle-treated mice. In conclusion, we have shown that BA has a potent anti-inflammatory activity *in vivo*, protecting mice against LPS by modulating TNF- $\alpha$  production by macrophages *in vivo* through a mechanism dependent on IL-10.

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### 1. Introduction

Betulinic acid (3 $\beta$ -hydroxy-lup-20(29)-en-28-oic acid; BA), a C-30 carboxylic acid derivative of the ubiquitous triterpene betulin, is a member of the class of lupane-type triterpenes. The molecule is abundant in the plant kingdom and has been isolated from several plant species, including *Zizyphus joazeiro* [1], *Syzygium clariflorum* [2], and *Doliocarpus schottianus* [3]. A number of reports have shown diverse biological activities of BA, such as anticancer [4], anti-HIV, anti-HSV-1 [5], anti-HBV [6], antihelmintic [6], antinociceptive [7], and antiplasmodial [1,8]. Of particular interest, in view of the large prevalence of chronic inflammatory-degenerative diseases, is the BA anti-inflammatory

activity [9,10]. However, in a number of *in vitro* and *in vivo* models of inflammation, the intensity of the BA anti-inflammatory activity has been considered only moderate (reviewed by [5]).

Sepsis is one of the most frequent complications in surgical patients and one of the leading causes of mortality in intensive care units. Severe sepsis is an important cause of mortality worldwide, and is estimated as directly responsible for 9% of all deaths in the United States [11]. It is defined as an infection-induced syndrome characterized by a generalized inflammatory state and can be caused by infection with Gram-negative or Gram-positive bacteria, fungi, or viruses. Sepsis can also occur in the absence of detectable bacterial invasion and, in these cases, microbial toxins (lipopolysaccharide; LPS) and endogenous cytokines have been implicated as initiators and mediators of the condition [12].

Macrophage activation by LPS results in the release of several inflammatory mediators, including proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1, IL-6, IL-8, and IL-12,

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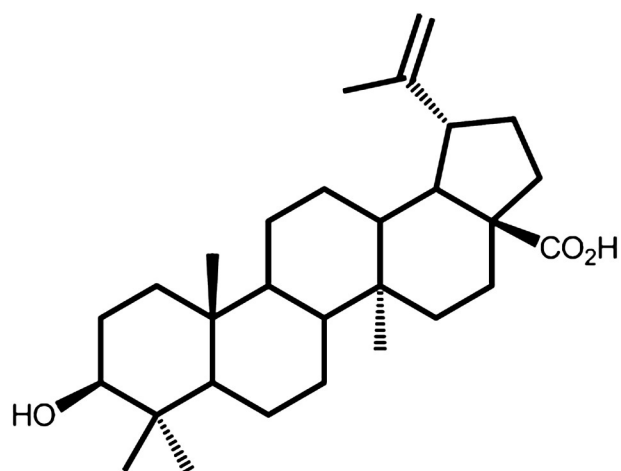


Fig. 1. Molecular structure of betulinic acid.

as well as nitric oxide. The exacerbated production of these mediators by activated macrophages are the main cause of the deleterious consequences of septicemia or endotoxemia, and may lead to hypotension, disseminated intravascular coagulation, neutrophil extravasation to tissues, tissue hypoxia, and death. Conversely, IL-4, IL-10 and IL-13 may function as anti-inflammatory cytokines, modulating macrophage and monocyte responses and inhibiting the production of TNF- $\alpha$ , IL-1, and IL-8 [11–13]. The discovery of new agents capable of down-modulating the production of the inflammatory mediators that play key roles in the installation of sepsis is therefore of great interest for the development of effective treatments. Since BA has been shown to inhibit the TNF- $\alpha$ -induced activation of NF- $\kappa$ B [14], in this work we investigated the effects of BA in a mouse model of endotoxic shock and on the production of cytokines by activated macrophages.

## 2. Materials and methods

### 2.1. General experimental procedures

Melting points were determined using a Geahaka model PF1500 version 1.0 apparatus and were not corrected. The NMR spectra were acquired on a Varian System 500 spectrometer, equipped with a XW4100 HP workstation. High-resolution mass spectra were recorded on a microTOF spectrometer (LC-ITTOF model 225–07 100–34, Bruker) with positive ionization modes of the ESI source. Silica gel 60 (Merck) was used for column chromatography, and Si gel 60 PF254 (Merck)

was used for purification of compounds by preparative TLC. All solvents used were analytical grade (Merck).

### 2.2. Plant material

*Tabebuia aurea* (Manso) S. Moore was collected in March 2002 in the surroundings of São João do Cariri, State of Paraíba, Brazil, and identified by botanist Dr. Maria de Fátima Agra of the Universidade Federal da Paraíba. A Voucher specimen (Agra 2337) is deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), Universidade Federal da Paraíba.

### 2.3. Extraction and isolation of betulinic acid

Five kg of air-dried ground bark of *Tabebuia aurea* (Manso) S. Moore were exhaustively extracted with 95% ethanol. The solvent was evaporated to yield a dark syrup residue (167 g, 3.3%), which was partitioned with water and successively treated with hexane, chloroform, ethyl acetate and n-butanol, yielding 8.5 g (0.2%), 4.1 g (0.08%), 6.2 g (0.13%) and 74 g (1.5%), respectively. The chloroform residue was also subjected to column chromatography over silica gel, and eluted with a chloroform-hexane gradient. Seventy five 100-mL fractions were collected, after analysis by TLC silica gel-60 F254 developed with either I<sub>2</sub> reagent. Combined fractions 41–50 were rechromatographed on CC column silica gel (with a chloroform-methanol gradient) to afford pure betulinic acid (BA) (0.015 g, 0.0003%; Fig. 1). Identification of the betulinic acid was performed by analyzing <sup>1</sup>H e <sup>13</sup>C NMR spectral data and high-resolution mass spectra compared with those published in the literature [1,15].

### 2.4. Animals

Male 4- to 6-week old BALB/c, wild-type C57BL/6, and IL-10<sup>-/-</sup> C57BL/6 mice were used. All mice were raised and maintained at the animal facilities of the Gonçalo Moniz Research Center, Fundação Oswaldo Cruz, Salvador, Brazil, in rooms with controlled temperature (22 ± 2 °C) and humidity (55 ± 10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (6 am–6 pm) and provided with rodent diet and water *ad libitum*. All mice, when necessary, were subjected to euthanasia and treated according to the Oswaldo Cruz Foundation guidelines for laboratory animals. The work had prior approval by the institutional Ethics Committee in Laboratory Animal Use.

### 2.5. Endotoxic shock model

Groups of 11 BALB/c or C57BL/6 mice were used for the lethality assays and groups of 6 BALB/c mice were used for *in vivo* cytokine studies.

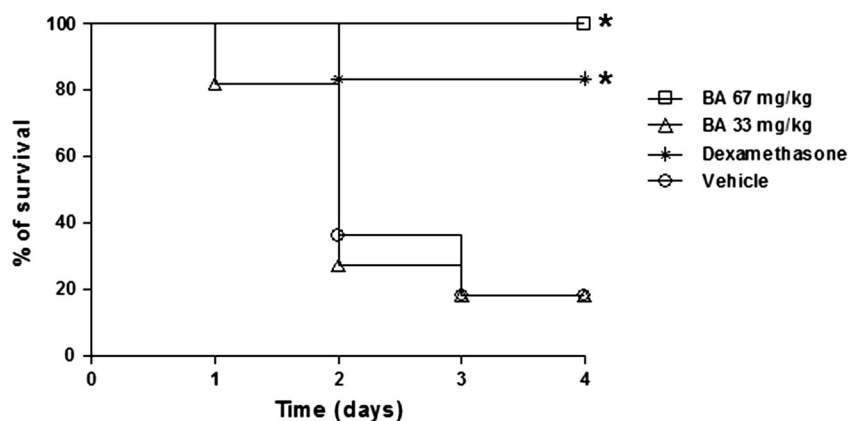
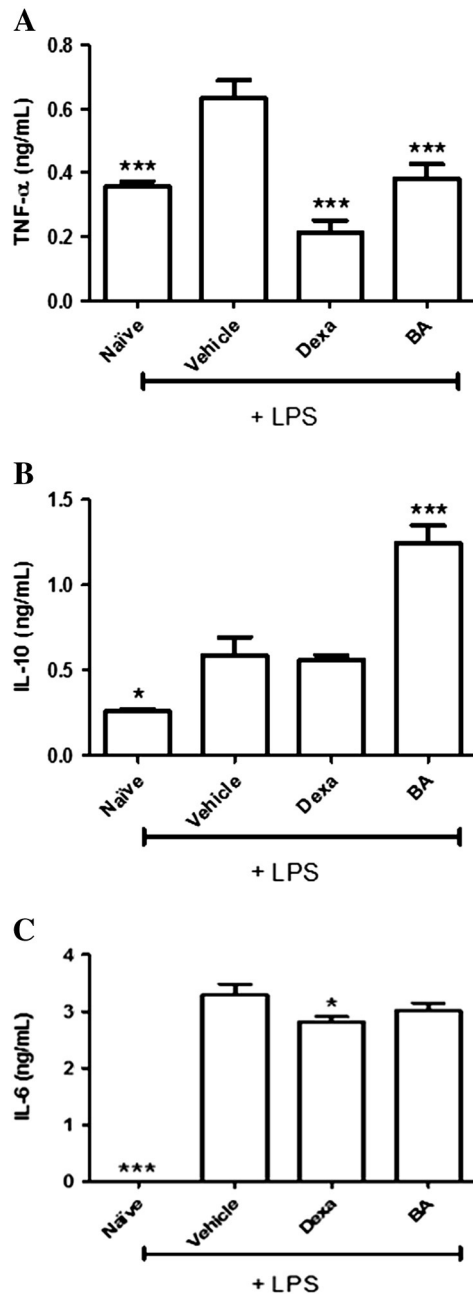


Fig. 2. Survival curve of mice treated with betulinic acid and submitted to endotoxic shock. Male BALB/c mice (n = 11) were treated with betulinic acid (33 and 67 mg/kg) or vehicle (5% DMSO in saline) and challenged with LPS 90 min later, intraperitoneally administered. Survival was monitored during 96 hours after LPS challenge. Results are from one experiment of two replicates performed. \*P < 0.05 compared to vehicle group. Statistical analysis was carried out using Logrank (Mantel Cox).



**Fig. 3.** Assessment of cytokine production after bacterial lipopolysaccharide (LPS) challenge. BALB/c mice (6 animals per group) were injected intraperitoneally with betulinic acid (BA, 67 mg/kg), dexamethasone (Dexa, 0.5 mg/kg) or vehicle (5% DMSO in saline), 90 min prior to intraperitoneal LPS administration. The mice were sacrificed 90 min later, to collect sera for assessment of TNF- $\alpha$  (A), IL-10 (B), and IL-6 (C), by ELISA. Values represent the means  $\pm$  SEM of six determinations obtained in one of two experiments performed. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared to untreated group stimulated with LPS. ANOVA followed by Newman-Keuls multiple comparison test.

Mice were treated with BA at different doses (see figure legends), dexamethasone (0.5 mg/kg, Sigma-Aldrich, St. Louis, MO) or vehicle (5% of DMSO in saline), by the intraperitoneal (i.p.) route. Ninety minutes later, animals were challenged with 600  $\mu$ g of LPS (LPS from serotype 0111:B4 *Escherichia coli*, Sigma-Aldrich; previously determined  $LD_{90-100} = 42.8$  mg/kg) in saline by the i.p. route. Mice were monitored daily for 4 days. To evaluate the serum cytokine levels, mice were anesthetized with a combination of ketamine (100 mg/kg) and xilazine (10 mg/kg), 60 min after injection of 600  $\mu$ g of LPS, for blood collection via the brachial plexus. Serum samples were immediately used or stored at  $-80$  °C until use.

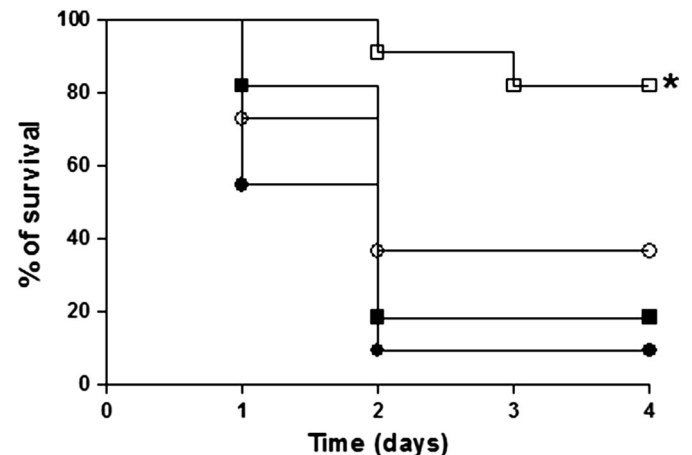
## 2.6. Macrophage cell cultures

Peritoneal exudate cells were obtained by washing, with cold Dulbecco's modified Eagle's medium (DMEM; Life Technologies, GIBCO-BRL, Gaithersburg, MD), the peritoneal cavity of mice 4–5 days after injection of 3% thioglycolate in saline (1.5 mL per mouse). Cells were washed twice with DMEM, resuspended in DMEM supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) and 50  $\mu$ g/mL of gentamycin (Novafarma, Anápolis, Brazil), and plated in 96-well tissue culture plates at  $2 \times 10^5$  cells per 0.2 mL per well. After 2 hours of incubation at 37 °C, non-adherent cells were removed by two washes with DMEM. Macrophages were then treated with LPS (500 ng/mL) in the absence or presence of BA (10 or 20  $\mu$ M) or dexamethasone (20  $\mu$ M), and incubated at 37 °C. Cell-free supernatants were collected 4 or 24 h after incubation and kept at  $-80$  °C for cytokine and nitric oxide determinations.

To assess cytokine production by resident macrophages, groups of male BALB/c mice were injected with BA (67 mg/kg), dexamethasone (0.5 mg/kg) or 5% of DMSO in saline, by the i.p. route. After 90 min, the mice were subjected to euthanasia for macrophage collection by means of peritoneal wash using cold DMEM. Cells were washed twice with DMEM, resuspended in DMEM supplemented with 10% fetal bovine serum and 50  $\mu$ g/ml of gentamycin, and plated in 96-well tissue culture plates at  $2 \times 10^5$  cells per 0.2 mL per well. After 2-hour incubation at 37 °C, non-adherent cells were removed by two medium changes. Macrophages were then stimulated or not with LPS (500 ng/mL), as indicated in the text and figure legends and further incubated at 37 °C and 5% CO<sub>2</sub>. Cell-free supernatants were collected 4 and 24 h after incubation for cytokines and nitric oxide measurement.

## 2.7. Measurement of cytokines and nitric oxide concentrations

TNF- $\alpha$ , IL-6, and IL-10 concentrations in serum samples or in supernatants from macrophage cultures, were determined by enzyme-linked immunosorbent assay (ELISA), using the DuoSet kit from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. After incubation with a streptavidin-peroxidase conjugate (Sigma-Aldrich), the reaction was developed using H<sub>2</sub>O<sub>2</sub> and 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) and the absorbance to 450 nm-wave length light read in a spectrophotometer. Quantification of nitric oxide was done indirectly through determination of nitrite concentrations 24 h after incubation, using the Griess method [16].



**Fig. 4.** Survival curve of IL-10<sup>+/+</sup> and IL-10<sup>-/-</sup> mice treated with betulinic acid and submitted to endotoxic shock. Groups of male IL-10<sup>+/+</sup> and IL-10<sup>-/-</sup> C57BL/6 mice (n = 11) were treated with betulinic acid (67 mg/kg) or vehicle (5% DMSO in saline) and challenged with intraperitoneal LPS administration 90 min later. Survival was monitored during 96 hours after LPS challenge. Results are from one of two experiments performed. \* $P < 0.05$  compared to IL-10<sup>+/+</sup> vehicle group. Statistical analysis was carried out using Logrank (Mantel Cox).

## 2.8. Statistical analyses

The statistical analysis of the differences in survival curves was made using the Logrank test. Comparisons among the experimental groups were performed by one-way analysis of variance and Newman-Keuls multiple comparison tests using Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, CA). Differences were considered significant when the values of  $P$  were  $< 0.05$ .

## 3. Results

To investigate the effects of BA on endotoxemic shock, groups of BALB/c mice were treated with the compound or vehicle (5% of DMSO in saline) and challenged with a lethal dose of LPS. Treatment with 67 mg of BA per kg induced protection to 100% of the animals (Fig. 2). BA at a lower dose (33 mg/kg) did not protect mice against the lethal LPS challenge and at the end of the observed period (four days), only 18% of animals were alive, similar to the vehicle-treated group. Dexamethasone, a control antiinflammatory drug, protected 83% at 0.5 mg/kg. Animals from all groups displayed signs of shock, such as piloerection, shivering, and lethargy.

To further investigate the effects of BA administration on endotoxemia, we measured the serum levels of cytokines in BA-treated animals after LPS challenge. Treatment with 67 mg of BA per kg decreased the TNF- $\alpha$  concentration significantly, when compared to treatment with vehicle, in LPS challenged mice, although less than dexamethasone, the reference drug (Fig. 3A). In contrast, the IL-10 concentration was significantly higher in animals treated with BA and challenged with LPS (Fig. 3B) than in vehicle-treated, LPS-challenged mice. The dexamethasone

treatment did not increase the production of IL-10 when compared to vehicle-treated mice (Fig. 3B). The concentration of IL-6 following LPS challenge was significantly reduced only by treatment with dexamethasone (Fig. 3C).

To evaluate the role of IL-10 in the BA-induced protection against LPS challenge, we carried out a lethality experiment using IL-10-deficient and wild-type C57BL/6 mice. IL-10 $^{-/-}$  mice treated with BA had an elevated mortality rate after LPS challenge, similar to vehicle-treated IL-10 $^{-/-}$  mice. In contrast, the IL-10 $^{+/+}$  control mice were significantly protected against the LPS challenge by the BA treatment (Fig. 4).

To assess the effects of BA on macrophages, we first measured nitric oxide levels in cultures of peritoneal exudate macrophages. Incubation of macrophages with BA (10 or 20  $\mu$ M), or with dexamethasone (20  $\mu$ M), significantly inhibited nitric oxide production induced by LPS stimulation, compared to LPS-stimulated untreated cultures (Fig. 5A). We next evaluated the effects of BA on TNF- $\alpha$ , IL-6 and IL-10 production. BA significantly decreased the production of TNF- $\alpha$  and modestly of IL-6, whereas dexamethasone significantly inhibited both cytokines (Fig. 5B and C). In contrast, a significant increase in IL-10 concentration was observed in BA-treated cultures (20  $\mu$ M) compared to LPS-stimulated untreated cultures (Fig. 5D) which was not observed in macrophage cultures treated with dexamethasone.

Finally, the effects of *in vivo* treatment with BA on macrophages were evaluated. For this purpose, nitric oxide and TNF- $\alpha$ , IL-6 and IL-10 were measured on resident macrophages from animals previously treated with BA. As revealed in Fig. 6, treatment with 67 mg/Kg of BA significantly reduced nitric oxide (Fig. 6A) and TNF- $\alpha$  (Fig. 6B) relative to the LPS-stimulated vehicle-treated cultures. A reduction of IL-6 levels

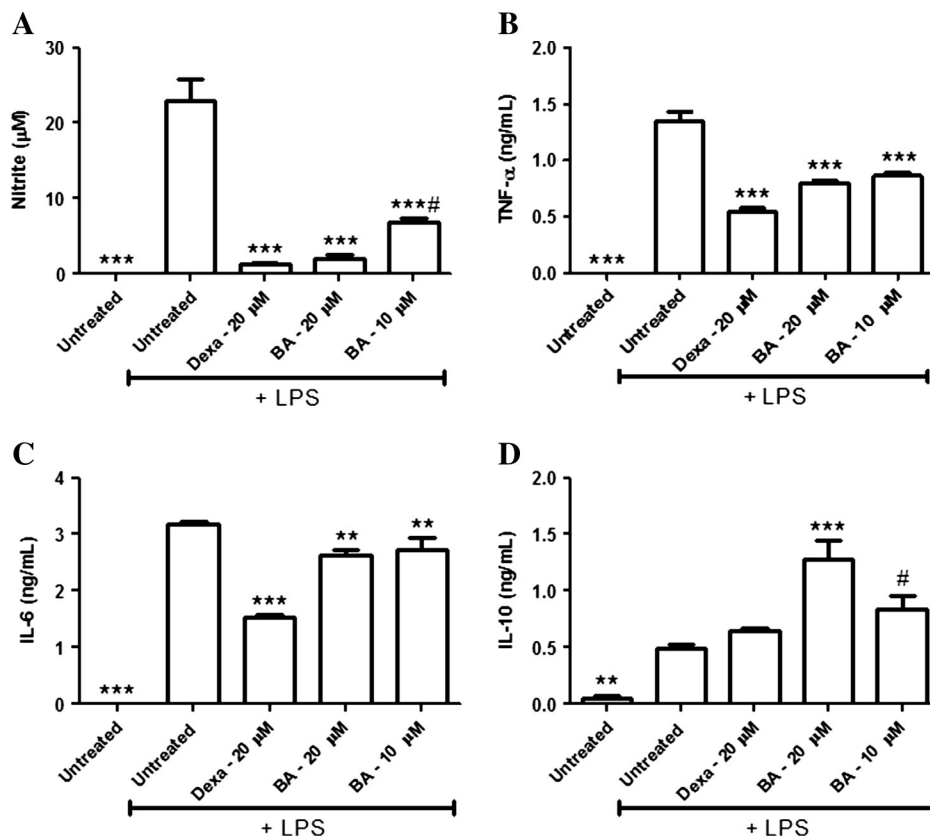
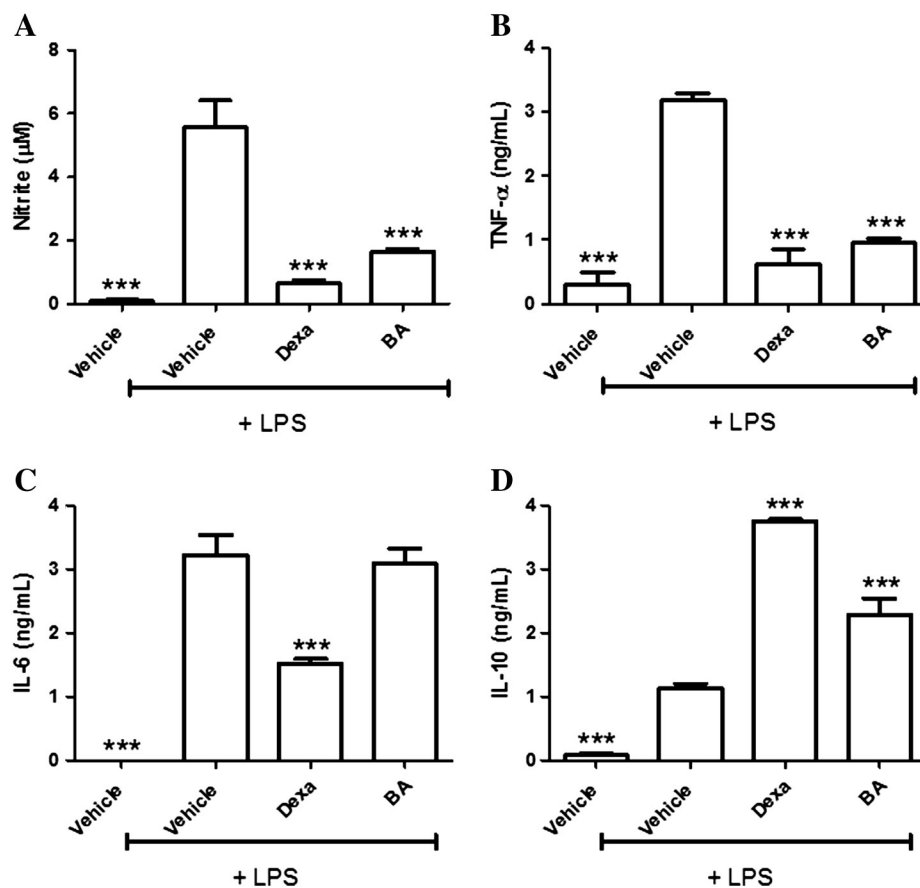


Fig. 5. Assessment of nitric oxide and cytokine production by peritoneal macrophages treated *in vitro* with betulinic acid. Concentrations of nitrite (A), TNF- $\alpha$  (B), IL-6 (C) and IL-10 (D) were determined in peritoneal macrophages treated or not with BA (10 or 20  $\mu$ M) or dexamethasone (20  $\mu$ M) in the presence of LPS (500 ng/mL) during 4 or 24 h. Cell-free supernatants were then collected for nitrite quantification by the Griess method and cytokine measurement by ELISA. Values represent the means  $\pm$  SEM of four determinations obtained in one of two experiments performed. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to untreated cultures stimulated with LPS. #  $P < 0.01$  compared to BA 20  $\mu$ M; ANOVA followed by Newman-Keuls multiple comparison test.





**Fig. 6.** In vivo treatment with betulinic acid (BA) decreases nitric oxide and TNF- $\alpha$  production and increases IL-10 production by LPS-stimulated macrophages. BALB/c mice (6 animals per group) were treated with BA (67 mg/kg), dexamethasone (Dexa; 0.5 mg/kg) or vehicle (5% DMSO in saline) and, after 90 min, were euthanized to harvest resident peritoneal macrophages. Cells were cultured in 96-well plates in the presence or absence of LPS (500 ng/mL) and, after 4 or 24 h, cell-free supernatants were collected. Concentrations of nitrite (A), TNF- $\alpha$  (B), IL-6 (C) and IL-10 (D) were obtained through Griess method and cytokines measurement by ELISA respectively. Values represent the means  $\pm$  SEM of six determinations obtained in 1 of 2 experiments performed. \*\*\*  $P < 0.001$  compared to macrophages stimulated with LPS from vehicle-treated group; ANOVA followed by Newman-Keuls multiple comparison test.

was observed in macrophages derived from dexamethasone-treated, but not from BA-treated mice (Fig. 6C). In contrast, IL-10 production was significantly increased in macrophages obtained from mice treated with BA or dexamethasone.

#### 4. Discussion

Sepsis, a life-threatening clinical condition, is associated with an overproduction of cytokines, including tumor necrosis factor and interleukins, as well as increased expression of adhesion molecules, as a result of stimulation by pathogenic agents or endotoxins [17]. Interaction between these various cytokines results in the activation of a cascade reaction that triggers an excessive inflammation, being a major cause of organ failure [18]. Recent studies have made considerable progress on antibiotic therapies and critical care techniques for sepsis treatment [19]. However, little progress was done about the anti-inflammatory treatment of sepsis.

Here we demonstrated the protective effects of BA, a triterpenoid found in many plant species, in a mouse model of endotoxemia. Administration of BA (67 mg/kg) prevented the mortality against a lethal dose of LPS. This protection followed the suppression of TNF- $\alpha$  production, one of the main soluble mediators involved in several aspects of the pathophysiology of endotoxemia. Altogether, our data demonstrate a potent *in vivo* anti-inflammatory activity of BA.

IL-10, a cytokine with potent anti-inflammatory properties, inhibits LPS-induced TNF- $\alpha$  release *in vitro* [20] and *in vivo*, and protects mice against lethal endotoxemia [21]. An increase in the levels of serum IL-10 was observed in mice treated with BA, suggesting an important role of

this cytokine in BA-induced protection. To confirm this hypothesis, we carried out an experiment using wild-type and IL-10<sup>-/-</sup> C57BL/6 subjected to LPS-induced endotoxemia. Differing from wild-type mice, IL-10<sup>-/-</sup> mice treated with BA had an elevated mortality rate after LPS challenge, suggesting a role for IL-10 in the protection against lethal endotoxemia conferred by BA. However, the fact that BA modulates I $\kappa$ B $\alpha$  phosphorylation [22] suggests that its action on TNF- $\alpha$  inhibition may also be related to alterations in signaling pathways, such as NF- $\kappa$ B activation.

A previous report has shown that BA inhibits the expression of cyclooxygenase 2 (COX-2) expression in cultures of human peripheral blood mononuclear cells, leading to a decrease in PGE<sub>2</sub> production [22]. The increase in IL-10 induced by BA described in our study may be related to the inhibition of PGE<sub>2</sub> production, another inflammatory mediator in LPS-challenged mice, since IL-10 is known to inhibit COX2 expression by LPS-stimulated monocytes [23]. Thus, we are establishing the role of another molecule involved in the anti-inflammatory activity produced by BA.

Since lethal endotoxemia results from an undesirable overproduction of cytokines by activated mononuclear phagocytes, we carried out *in vitro* experiments aiming to observe the effects of BA treatment in LPS-activated macrophages. Thus, addition of BA to macrophage cultures inhibited the production of inflammatory mediators, such as NO and TNF- $\alpha$ , and increased IL-10 production, in agreement with our *in vivo* findings. These data were also confirmed using macrophages harvested from mice previously treated with BA. Altogether, our data indicate that BA-induced IL-10 production by macrophages suggests a role of this anti-inflammatory cytokine in the protection against lethal

LPS challenge. The mechanisms leading to the increase in IL-10 production induced by BA remain still to be determined.

In contrast to the inhibition of TNF- $\alpha$ , BA did not significantly affect IL-6 production induced by LPS. IL-6 is a pleiotropic cytokine with both pro and anti-inflammatory actions. Although a number of agents, including dexamethasone, cause the down-modulation of both TNF- $\alpha$  and IL-6, selective inhibition of TNF- $\alpha$  may occur. In fact, a previous report has shown that a vasoactive sand fly peptide (maxadilan) exerts its anti-inflammatory action by a mechanism dependent on IL-6 [24].

A number of natural compounds have immunomodulatory activity in experimental models of LPS-induced endotoxemia, where TNF- $\alpha$  plays an important effector role [25,26]. Plant-derived polyphenols, alkaloids, terpenes, sterols, and fatty acids are reported in literature as inhibitors of TNF- $\alpha$  signaling [26], and may exert protective effects in endotoxemia. Physalins B, F and G, which are seco-steroids isolated from *Physalis angulata*, are examples of natural compounds with TNF- $\alpha$  suppressing activity which are able to prevent LPS-induced lethality [13]. BA, however, is a well-studied molecule that has originated a new class of antiretroviral drugs, named inhibitors of maturation and which has been evaluated in phase II clinical studies [27]. In addition, BA is a very well tolerated drug and its use is safe in doses higher than 500 mg/kg, according to studies on antineoplastic activity [5,28,29]. Indeed, the anti-inflammatory activity of BA should be taken into consideration in the context of the antineoplastic therapy.

#### 4.1. Conclusion

The present findings reinforce the potential of BA, a natural compound as an anti-inflammatory drug candidate consider the role of IL-10 as another important mediator involved in the immune regulation produced by the drug and indicate the carrying out of future clinical evaluations involving BA effect on severe sepsis.

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