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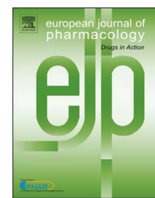

Full length article

Betulinic acid derivative BA5, a dual NF-kB/calcineurin inhibitor, alleviates experimental shock and delayed hypersensitivity

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ABSTRACT

Betulinic acid (BA) is a naturally occurring triterpenoid with several biological properties already described, including immunomodulatory activity. Here we investigated the immunomodulatory activity of eight semi-synthetic amide derivatives of betulinic acid. Screening of derivatives BA1-BA8 led to the identification of compounds with superior immunomodulatory activity than BA on activated macrophages and lymphocytes. BA5, the most potent derivative, inhibited nitric oxide and TNFα production in a concentration-dependent manner, and decreased NF-κB activation in Raw 264.7 cells. Additionally, BA5 inhibited the proliferation of activated lymphocytes and the secretion of IL-2, IL-4 IL-6, IL-10, IL-17A and IFNɣ, in a concentration-dependent manner. Flow cytometry analysis in lymphocyte cultures showed that treatment with BA5 induces cell cycle arrest in pre-G1 phase followed by cell death by apoptosis. Moreover, BA5 also inhibited the activity of calcineurin, an enzyme that plays a critical role in the progression of cell cycle and T lymphocyte activation. BA5 has a synergistic inhibitory effect with dexamethasone on lymphoproliferation, showing a promising profile for drug combination. Finally, we observed immunosuppressive effects of BA5 *in vivo* in mouse models of lethal endotoxemia and delayed type hypersensitivity. Our results reinforce the potential use of betulinic acid and its derivatives in the search for potent immunomodulatory drugs.

1. Introduction

Betulinic acid (BA) is a naturally occurring pentacyclic triterpenoid found in several plant species, which can be alternatively prepared from its metabolic precursor, betulin (Yogeeswari and Sriram, 2005). This natural product holds several biological activities, including anticancer, anti-HIV, antiparasitic and anti-angiogenic activities (Takada and Aggarwal, 2003; Dehelean et al., 2011; Ali-Seyed et al., 2016; Li et al., 2016; Meira et al., 2016). In particular, BA has been reported to possess cytotoxic effects on several tumor cell lines of different origins, as well as in animal models of cancer (Gheorgheosu et al., 2016).

BA has also been investigated as an immunomodulatory agent (Sultana and Saify, 2012). More specifically, BA inhibits the production of several inflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor alpha (TNFα), interleukin (IL)-6 and IL-1β (Jingbo et al., 2015; Kim et al., 2016). Most of these effects are related to the inhibition of nuclear factor-κB (NF-κB), a transcription factor involved in the regulation of several pro-inflammatory genes, in particular TNFα (Viji et al., 2010; Kim et al., 2016). BA is also known to suppress STAT3 signaling, which is essential transcription factor for differentiation of the $CD4^+$ Th17 cells in a variety of autoimmune diseases (Blaževski et al., 2013).

In addition, in a cecal ligation and puncture mouse model, it has been demonstrated that BA treatment reduces mortality and ameliorates lung and kidney function by down-regulating NF-κB (Lingaraju et al., 2015a, b). Furthermore, in lipopolysaccharide (LPS)-induced endotoxin shock, pre-treatment with BA significantly improves mice survival by modulating TNFα production by macrophages *in vivo*

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through a mechanism dependent on IL-10 (Viji et al., 2010; Costa et al., 2014). However, in a number of *in vitro* and *in vivo* models of inflammation, the anti-inflammatory activity of BA has been considered to be moderate (Cichewicz and Kouzi, 2004; Li et al., 2017).

In view of these findings, BA has been used as a prototype for the design and synthesis of more potent immunomodulatory agents. Chemical modifications of the carboxyl group have suggested that this part of BA molecule has potential for the production of derivatives with enhanced biological activity, including immunomodulatory agents (Sultana and Saify, 2012; Chen et al., 2017). Based on these facts, the purpose of our work was to evaluate the immunomodulatory potential of new semi-synthetic amide derivatives of betulinic acid *in vitro* and *in vivo*, as well as their mechanisms of action.

2. Materials and methods

2.1. Drugs

Betulinic acid (BA) was extracted from the bark of *Ziziphus joazeiro* Mart. (Rhamnaceae) by using a previously described method (Barbosa-Filho et al., 1985). Semi-synthetic compounds (BA1 to BA8; 94–98% purity by high performance liquid chromatography) were prepared from betulinic acid, as previously described (Meira et al., 2016). Gentian violet (Synth, São Paulo, SP, Brazil) was used as positive control in the cytotoxicity assays. Dexamethasone (Sigma-Aldrich, St. Louis, MO), a synthetic glucocorticoid, was used as positive control in immunomodulatory assays. Cyclosporin A (Sigma-Aldrich) was used as positive control in calcineurin activity assay and in DTH assay. Mifepristone (RU 486; Sigma-Aldrich), an antagonist of glucocorticoid receptor, was used in mechanism assays. All compounds were dissolved in Dimethyl sulfoxide (DMSO; PanReac, Barcelona, Spain) and diluted in cell culture medium for use in the assays. The final concentration of DMSO was less than 0.1% in all *in vitro* experiments and less than 5% in all *in vivo* experiments.

2.2. Animals

BALB/c and C57BL/6 mice 4–12 weeks old) were bred and maintained at the Gonçalo Moniz Institute (Oswaldo Cruz Foundation, Bahia, Brazil) in sterilized cages, under a controlled environment and receiving a balanced rodent diet and water *ad libitum*. All animal experiments and procedures were approved by the institution's committee on the ethical handling of laboratory animals (Approved number: L-IGM-018/15).

2.3. Cytotoxicity to mammalian cells

Cytotoxicity of the compounds was determined using the rat cardiomyoblast H9c2 and the monkey kidney LCC-MK2 cell lines. Cells were seeded into 96-well plates at a cell density of 1×10^4 cells/well in Dulbecco's modified Eagle medium (DMEM; Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 50 μg/ml of gentamicin (Life, Carlsbad, CA) and incubated for 24 h at 37 °C and 5% CO_2. The compounds tested were then added (10 - 0.04 μM), in quadruplicate, and incubated for 72 h. One μCi of ^{3}H-thymidine (Perkin Elmer, Waltham, MA) was added to each well, incubated for 18 h, frozen at −20 °C and subsequently thawed and the contents transferred to UniFilter-96 GF/B PEI coated plates (PerkinElmer) using a cell harvester. After drying, 50 μl of scintillation cocktail (MaxiLight, Hidex, Turku, Finland) was added in each well, sealed and plate read in a liquid scintillation microplate counter (Chameleon, Turku, Finland). Cell viability was measured as the percent of ^{3}H-thymidine incorporation for treated-cells in comparison to untreated cells. Gentian violet was used as positive control, at concentrations ranging from 0.04 to 10 μM. Cytotoxicity concentration at 50% (CC_{50}) values were calculated using data from three independent experiments.

In addition, the cytotoxicity of compounds was also determined in peritoneal macrophages. Cells were obtained by washing, with cold phosphate buffered saline (PBS), the peritoneal cavity of BALB/c mice 4–5 days after injection of 3% thioglycolate (Sigma-Aldrich) in saline (1.5 ml per mice). Then, cells were seeded into 96-well plates at a cell density of 2×10^5 cells/well in DMEM supplemented with 10% FBS and 50 μg/ml of gentamycin and incubated for 24 h at 37 °C and 5% CO_2. After that time each test inhibitors were added at least in six concentrations (20 – 0,15 μM) in triplicate and incubated for 24 h. 20 μl/well of AlamarBlue (Invitrogen, Carlsbad, CA) was added to the plates during 10 h. Colorimetric readings were performed at 570 and 600 nm. CC_{50} values were calculated using data-points gathered from three independent experiments.

2.4. Macrophage cultures

Peritoneal exudate macrophages were seeded in 96-well plates at 2×10^5 cells/well in DMEM medium supplemented with 10% of FBS and 50 μg/ml of gentamicin for 2 h at 37 °C and 5% CO_2. Plates were washed to remove non-adherent cells, and the remaining cells were then stimulated with LPS (500 ng/ml, Sigma-Aldrich) and interferon gamma (IFNγ; 5 ng/ml, Sigma-Aldrich) in the absence or presence of compounds at different concentrations, and incubated at 37 °C. Cell-free supernatants were collected 4 h (for TNFα measurement) and 24 h (for IL-1β, IL-10 and nitrite quantifications), and kept at −80 °C until use.

2.5. NF-κB luciferase assay

The murine mouse leukemic monocyte macrophage cell line Raw 264.7 Luc cells bearing the pBIIX-luciferase (pBIIX-luc) targeting vector containing the firefly luciferase gene (luc) driven by two NF-kB binding sites from the kappa light chain enhancer in front of a minimal fos promoter (Zhong et al., 1997) were kindly provided by Maria Célia Jamur (University of São Paulo, Ribeirão Preto, Brazil). The cells were cultured in RPMI medium (Sigma) supplemented with 20% FBS and 50 μg/ml gentamicin in 24-well plates at 37 °C in a humidified environment containing 5% CO_2. For luciferase reporter assays, 5×10^5 cells/ml were pretreated with different concentrations of BA5 (20, 10 or 5 μM), BA (20 μM) or dexamethasone (20 μM) for 1 h prior to stimulation with LPS (500 ng/ml) and IFNγ (5 ng/ml) for 3 h. After washing with cold PBS, cells were lysed by adding TNT lysis buffer (200 mM Tris, pH 8.0, 200 mM NaCl, 1% Triton X-100) for 20 min at 4 °C. The determination of the luciferase activity was performed using the Promega luciferase assay system (Promega, Madison, CA), according to the manufacturer's instructions. The samples were analyzed in a Globomax 20/20 Luminometer (Promega). Data are expressed as relative light units.

2.6. Splenocyte cultures

For lymphoproliferation assays, BALB/c splenocyte suspensions were prepared in DMEM medium supplemented with 10% of FBS and 50 μg/ml of gentamicin. Splenocytes (1×10^6 cells/well) were plated in 96-well plates, in quadruplicate, and stimulated or not with concanavalin A (Con A; 2 μg/ml, Sigma-Aldrich) or Dynabeads® mouse T-activator CD3/CD28 (bead to cell ratio = 1:1; ThemoFisher Scientific, Waltham, MA). In some experiments, RU486, a glucocorticoid receptor antagonist (Sigma-Aldrich), was added to the cultures to investigate the mechanism of action of the most potent derivative. To evaluate the lymphoproliferation, splenocytes were activated in the absence or presence of various concentrations of derivatives (500–0.0005 nM). After 48 h of incubation, 1 μCi of ^{3}H- thymidine was added to each well, incubated for 18 h and read, as described above. Cell proliferation was measured as the percent of ^{3}H-thymidine incorporation for treated-cells in comparison to untreated cells. Dexamethasone was used as positive

control. Inhibitory concentration at 50% (IC_{50}) values were calculated using data from three independent experiments.

Splenocytes from BALB/c mice were also plated into 24-well plates at a cell density of 5×10^6 cells/ in DMEM medium supplemented with FBS containing or not 5 μg/ml of Con A in the absence or presence of different concentrations of BA5 (500, 50 and 5 nM), BA (500 nM) and dexamethasone (500 nM). After 48 h, cell-free supernatants were collected and kept at −80 °C until use for cytokine quantification.

2.7. Mixed lymphocyte reaction (MLR)

BALB/c mice were weekly immunized with intraperitoneal injections of 10^7 splenocytes obtained from C57BL/6, as previously described (Soares et al., 2006). After 3 weeks of immunization, mice were euthanized for spleen cell preparation in DMEM medium supplemented with 10% of FBS and 50 μg/ml of gentamicin. Spleen cells were plated in 96 well plates at a cell density of 1×10^6 cells/well in the absence or in the presence of irradiated C57BL/6 splenocytes at 10^6 cells/well (dose of 3 Gy in a ^{137}Cs source irradiator purchased from CisBio International, Cordolet, France) and different concentrations of drugs in testing, in quadruplicates. After 72 h of incubation, ^{3}H-thymidine was added to each well, and the plates were incubated for additional 18 h. The incorporation of ^{3}H-thymidine was determined as described above.

2.8. Assessment of cytokine and nitric oxide production

Cytokine concentrations in supernatants from peritoneal macrophages cultures collected at 4 h (for TNFα) and 24 h (for IL-1β and IL-10) and from splenocyte cultures (IL-2, IL-4, IL-6, IL-10, IL-17A and IFNγ) were determined by enzyme-linked immunosorbent assay (ELISA), using DuoSet kits from R & D Systems (Minneapolis, MN), according to the manufacturer's instructions. Nitric oxide production was estimated in macrophage culture supernatants harvested at 24 h using the Griess method for nitrite quantification (Green et al., 1982).

2.9. Flow cytometry analyses

Splenocytes from BALB/c mice were plated into 24-well plates at a cell density of 5×10^6 cells/ in DMEM medium supplemented with 10% FBS containing 5 μg/ml of Con A in the absence or presence of different concentrations of BA5 (500, 50 and 5 nM) for 24 and 48 h for cell death determination and cell cycle analysis respectively. For cell death determination, cells were centrifuged and then pellet washed twice with cold PBS and initially labeled with APC anti-mouse CD3 (Biolegend, San Diego, CA) diluted 1:100 for 15 min followed by stained with propidium iodide (PI) and annexin V using the annexin V-FITC apoptosis detection kit (Sigma-Aldrich), according to the manufacturer's instructions. For cell cycle analysis, cells were labeled with APC anti-mouse CD3 followed by a second stained with a solution of PBS with propidium iodide (2 μg/ml), RNAase (100 μg/ml) and 0.1% of Triton X-100 in the dark at 37 °C for 30 min. The cell preparations were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA). A total of 10,000 events positive for APC anti-mouse CD3 staining was acquired and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.10. Calcineurin activity

The calcineurin phosphatase activity was measured in cell extracts using a Calcineurin Cellular Activity assay kit (Enzo Life Sciences, Farmingdale, NY). In brief, splenocytes from BALB/c mice were plated into 24-well plates at a cell density of 5×10^6 cells/ in DMEM medium supplemented with 10% FBS containing 5 μg/ml of Con A in the absence or presence of BA5 (500, 50 and 5 nM), or cyclosporin A (500 nM) for 48 h. Then, cells were lysed in a buffer containing protease inhibitors, centrifuged and the supernatant was used for analysis. The same amount of protein (5 μg) per sample was used in the calcineurin activity assays. Colorimetric measurements were performed at 620 nm. The amount of phosphate released by calcineurin was calculated using a standard curve.

2.11. Drug combination assay

For *in vitro* drug combinations**,** doubling dilutions of each drug (BA5 and dexamethasone), used alone or in fixed combinations were incubated with splenocytes followed the protocol described above. The analysis of the combined effects was performed by determining the combination index (CI), used as cutoff to determine synergism, by using Chou-Talalay CI method (Chou and Talalay, 2005) and through the construction of isobologram using the fixed ratio method, as described previously (Fivelman et al., 2004).

2.12. LPS-induced endotoxic shock

Groups of six male BALB/c mice (4 weeks of age) were used for the LPS lethality assays. Mice were treated with BA5 (50 or 25 mg/kg), dexamethasone (25 mg/kg) or vehicle (5% of DMSO in saline), by intraperitoneal (i.p.) route. Ninety min later, animals were challenged with 600 μg of LPS (from serotype 0111:B4 *Escherichia coli*, Sigma-Aldrich) in saline, by i.p. route. Survival was then monitored daily, during 4 days.

2.13. Delayed type hypersensitivity (DTH) assay

BALB/c mice (8–12 weeks of age) were sensitized by injecting 50 μg of crystallized bovine serum albumin (BSA; Sigma-Aldrich) emulsified in 20 μl of complete Freund´s adjuvant (CFA; Sigma-Aldrich) subcutaneously into each side of the base of tail. Seven days later, animals were randomized into five groups and mice were treated with BA5 (50 or 25 mg/kg), cyclosporin A (25 mg/kg), dexamethasone (25 mg/kg) or vehicle (5% of DMSO in saline), by the intraperitoneal route 24 and 3 h before challenge. DTH was elicited by injection of 30 μl of a 2% suspension of heat-aggregated BSA in saline subcutaneously into the footpad, according to a previously reported method (Titus and Chiller, 1981). Before and 3 h post-challenge, footpad thickness was measured with calipers and swelling was calculated by subtracting the thickness of the footpad after challenge from that before challenge.

2.14. Statistical analyses

One-way analysis of variance and Newman-Keuls multiple comparison tests were employed by 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

3.1. *Cytotoxicity and modulation of macrophage function in vitro by BA derivatives*

In order to test the effects of the compounds *in vitro*, first we evaluated the cytotoxicity of compounds on peritoneal macrophages, LCC-MK2 and H9c2 cells. As shown in Table 1, all compounds had no cytotoxic effect in the concentrations tested, which were used in the following *in vitro* assays.

All the compounds were initially evaluated in cultures of macrophages activated with LPS and IFNγ. Dexamethasone, a standard glucocorticoid, was used as positive control. As shown in Table 1, betulinic acid inhibited NO production in 45.1% at 10 μM. In contrast, most of derivatives showed a weak inhibitory activity of nitric oxide production, with the exception of BA5. This derivative inhibited in 53.0% the production of NO, more potently than the prototype molecule (BA) and

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Table 1
Screening of cytotoxicity and immunomodulatory activity of semi-synthetic derivatives of betulinic acid.

Compounds	R	CC_{50} (μM) MØ[a]	CC_{50} (μM) MK2 cells[b]	CC_{50} (μM) H9c2 cells[b]	Inhibition of NO (%) production at 10 μM[c]	IC_{50} (nM) Mitogen Induced[d]
BA	OH	>20	> 10	> 10	45.1±1.9	284.5±32.8
BA1		>20	> 10	> 10	11.1±0.1	> 500
BA2		>20	> 10	> 10	7.2±0.9	> 500
BA3		>20	> 10	> 10	15.9±0.7	> 500
BA4		>20	> 10	> 10	7.7±0.1	> 500
BA5		>20	> 10	> 10	53.0±2.2	14.3±0.91
BA6		>20	> 10	> 10	25.9±2.7	27.6±2.1
BA7		>20	> 10	> 10	0±0.00	> 500
BA8		>20	> 10	> 10	16.7±1.4	58.7±4.9
Dexa	-	>20	> 10	> 10	57.6±3.2	6.9±0.4
GV	-	-	1.1±0.01	1.5±0.32	-	-

[a] Determined 24 h after incubation with compounds. [b] Determined 72 h after incubation with compounds. [c] Percent inhibition determined 24 h after incubation with compounds and LPS plus IFN-γ. [d] Determined 48 h after incubation with compounds and Con A. Values represents the mean ± S.E.M. and were calculated from three independent experiments performed. IC_{50} = inhibitory concentration at 50%. CC_{50} = cytotoxic concentration at 50%. MØ = peritoneal macrophages Dexa = Dexamethasone. GV = gentian violet. S.E.M. = standard error of mean.

close to that found for dexamethasone (57.6% of inhibition at 10 μM). BA5 caused a concentration-dependent inhibition of NO and TNFα production (Figs. 1**A and B**). Additionally, the production of IL-10 was significantly reduced by BA5 and dexamethasone, while it was increased by BA (Fig. 1**C**).

LPS-induced Toll-like receptor-4 (TLR4) activation leads to triggering of intracellular signaling pathways, including NF-kB activation, consequently causing the transcriptional regulation of several inflammatory genes, including *Tnf* and *Nos2* (Bartuzi et al., 2013). To investigate whether BA5 modulates NF-kB activation in macrophages, we performed an assay using RAW cells transduced with a reporter gene under the control of a promoter regulated by NF-κB. Addition of BA5

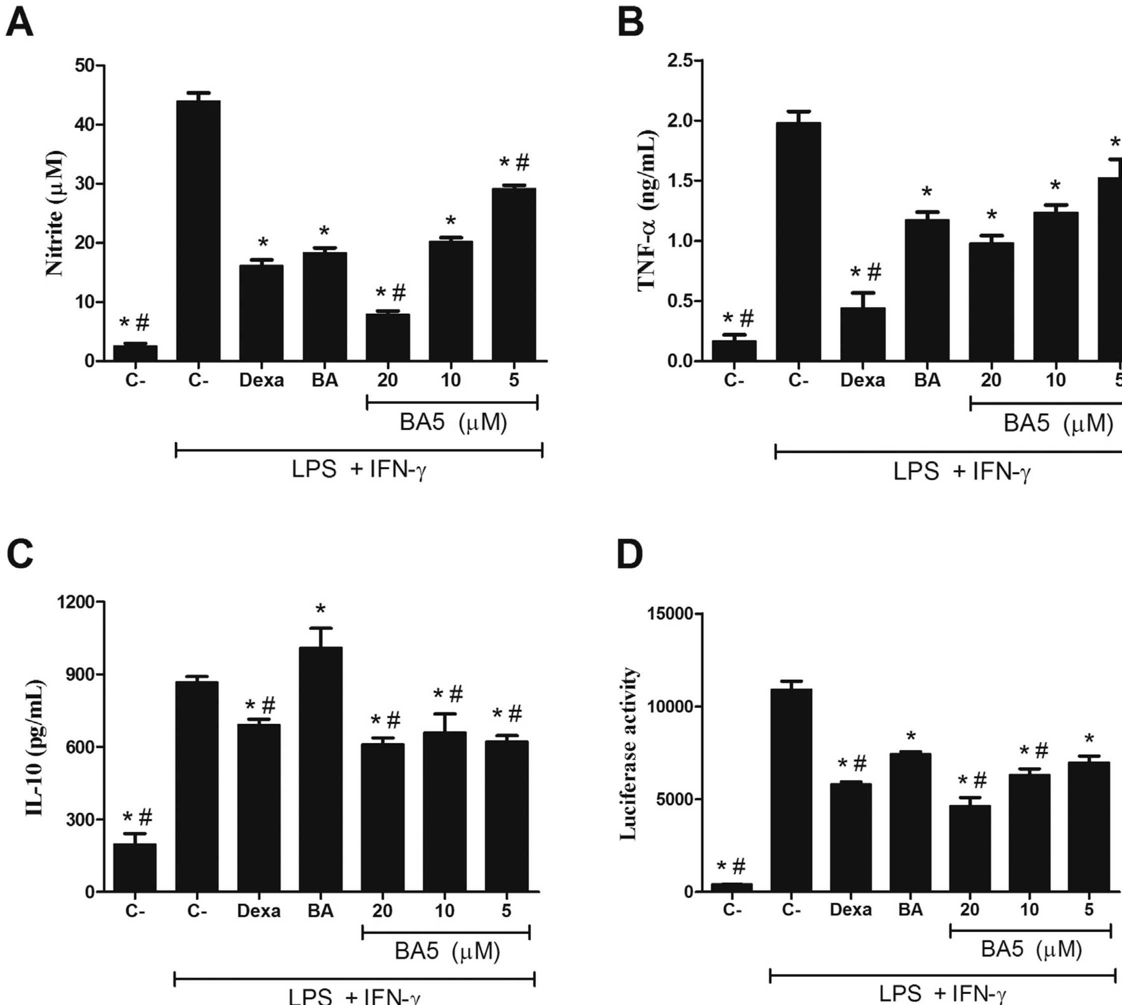

Fig. 1. Modulation of nitric oxide, cytokines and NF-κB activity in BA5-treated macrophages**.** Effect of BA5 (20, 10 or 5 μM), BA (20 μM) or dexamethasone (20 μM) on nitrite (**A**), TNFα (**B**), IL-10 (**C**) production and NF-κB activity (**D**). C- is untreated cells. Values represent the means ± S.E.M. of four determinations obtained in one of two experiments performed. *$P < 0.05$ compared to stimulated and untreated cells; #$P < 0.05$ compared to BA-treated cells.

caused the reduction of NF-kB activation, in a concentration-dependent manner, in RAW cells stimulated with LPS and IFNγ. BA and dexamethasone also caused a significant reduction of luciferase activity (Fig. 1**D**).

3.2. BA derivatives inhibit T cell proliferation

To evaluate the immunosuppressive effects of BA derivatives on lymphocytes, we first performed a Con A-induced lymphoproliferation assay. Screening of BA derivatives led to the identification of three compounds more active than BA (Table 1). The most active derivatives were BA5, BA6 and BA8, which present a morpholyl, thiomorpholyl and 4-methylphenylpiperidine groups, presented increased potency, especially the BA5 derivative, which had an IC_{50} value of 14.3 nM, close to that of dexamethasone (IC_{50} = 6.9 nM). Dexamethasone has a potent T cell proliferation inhibition activity (Table 1).

We further evaluated in splenocyte cultures activated with anti-CD3/anti-CD28 coated-beads to evaluate the effect of BA5 on the proliferation of $CD4^+$ T lymphocytes. As shown in Fig. 2**A**, BA5 caused a concentration-dependent inhibition of lymphoproliferation, with higher potency ($P <$ 0.05) than the prototype BA and similar to the activity observed for dexamethasone. Additionally, BA5 treatment inhibits *in vitro* alloreactive T cell response as demonstrated by a concentration-dependent reduction of proliferation in a mixed lymphocyte reaction (Fig. 2**B**).

3.3. BA5 modulates cytokine production and induces cell cycle arrest in activated T cells

As a potential immunotherapeutic agent, it was also important to determine the effect of BA5 on cytokine secretion. To this end, cytokine production by splenocytes stimulated with Con A and treated with BA5 was investigated (Fig. 3). Compared to untreated and stimulated cultures, treatment with BA5 decreased the secretion of IL-2, IL-4, IL-6, IL-10, IL-17A and IFN- γ in a concentration dependent manner. Under the same conditions, dexamethasone also promoted a significant decrease in cytokine production. BA also decreased the production of most cytokines but less efficient when compared with BA5. Once again, BA treatment increased IL-10 production (Fig. 3**D**).

Flow cytometry analysis was carried out in splenocyte cultures in order to determine the effect of BA5 on cell cycle progression and cell death. Treatment with BA5 induced, in a concentration-dependent manner, cell cycle arrest on G0/G1 phase, accompanied by an increase in PreG1 phase (Figs. 4**A and B**). Moreover, the cell cycle arrest was accompanied by a significant and concentration-dependent increase in annexin V positive cells, which is a hallmark of apoptosis (Fig. 4**C**).

3.4. BA5 effects on lymphocytes is independent of glucocorticoid receptor, but affects calcineurin activity

To understand the mechanisms by which BA5 inhibits lymphocyte

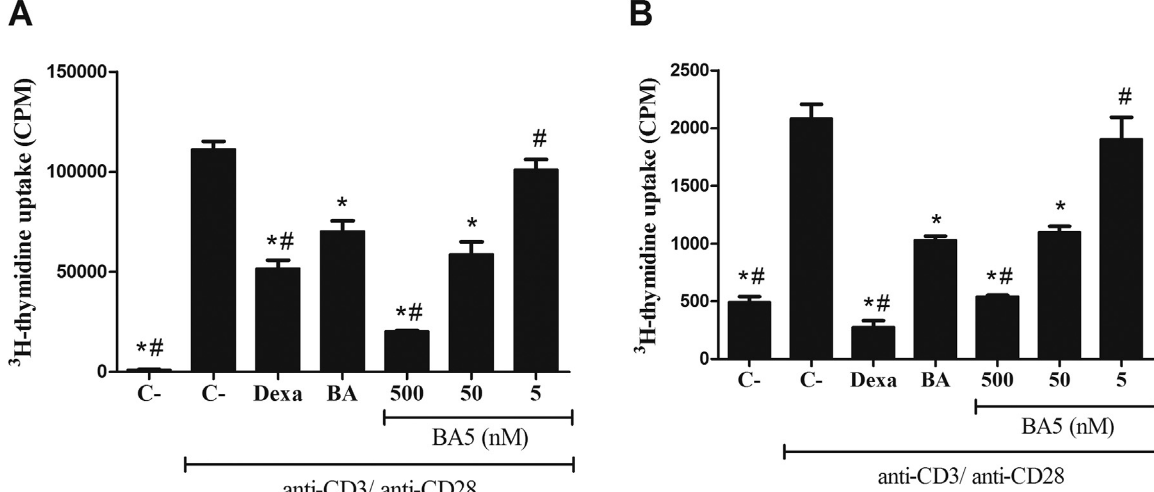

Fig. 2. Inhibition of lymphocyte proliferation by BA5. (**A**) Effect of BA5 (500, 50 and 5 nM), BA (500 nM) or dexamethasone (500 nM) on lymphoproliferation induced by anti-CD3 + anti-CD28 for 48 h. (**B**) Splenocytes from BALB/c mice sensitized with C57BL/6 cells were cultured in the absence (unstimulated) or presence (stimulated) of irradiated C57BL/6 spleen cells, with or without BA5 (500, 50 and 5 nM), BA (500 nM) or dexamethasone (500 nM) for 72 h. C- is untreated cells Proliferation rates were assessed through ^{3}H-thymidine incorporation. Values represent means ± S.E.M. of four determinations. Results shown were obtained in one representative of three experiments performed. *P < 0.05 compared to cells stimulated anti-CD3/anti-CD28 (**A**) or irradiated C57BL/6 spleen cells (**B**); #P < 0.05 compared to BA-treated cultures.

activation, we tested the ability of RU486, a glucocorticoid receptor antagonist, to reverse the effect of BA5 in Con A-stimulated splenocyte cultures. As shown in Fig. 5, addition of RU486 inhibited spleen cell proliferation. This is a glucocorticoid receptor (GR) antagonist which can block the effect of GR agonists, such as dexamethasone. However, it presents off-targets effects, which lead to an attenuation of T cell proliferation (Chien et al., 2009). When co-treated with dexamethasone, addition of RU486 promoted a partial block of dexamethasone activity. In contrast, the inhibition of spleen cell proliferation upon Con A activation by BA5 was not reversed under RU486 co-treatment.

We also investigated the effect of BA5 on calcineurin activity, which leads to the activation of Nuclear factor of activated T-cells (NFAT), a transcription factor involved in T cell activation (Macian, 2005). As shown in Fig. 6, treatment with BA5 decreased the activity of this

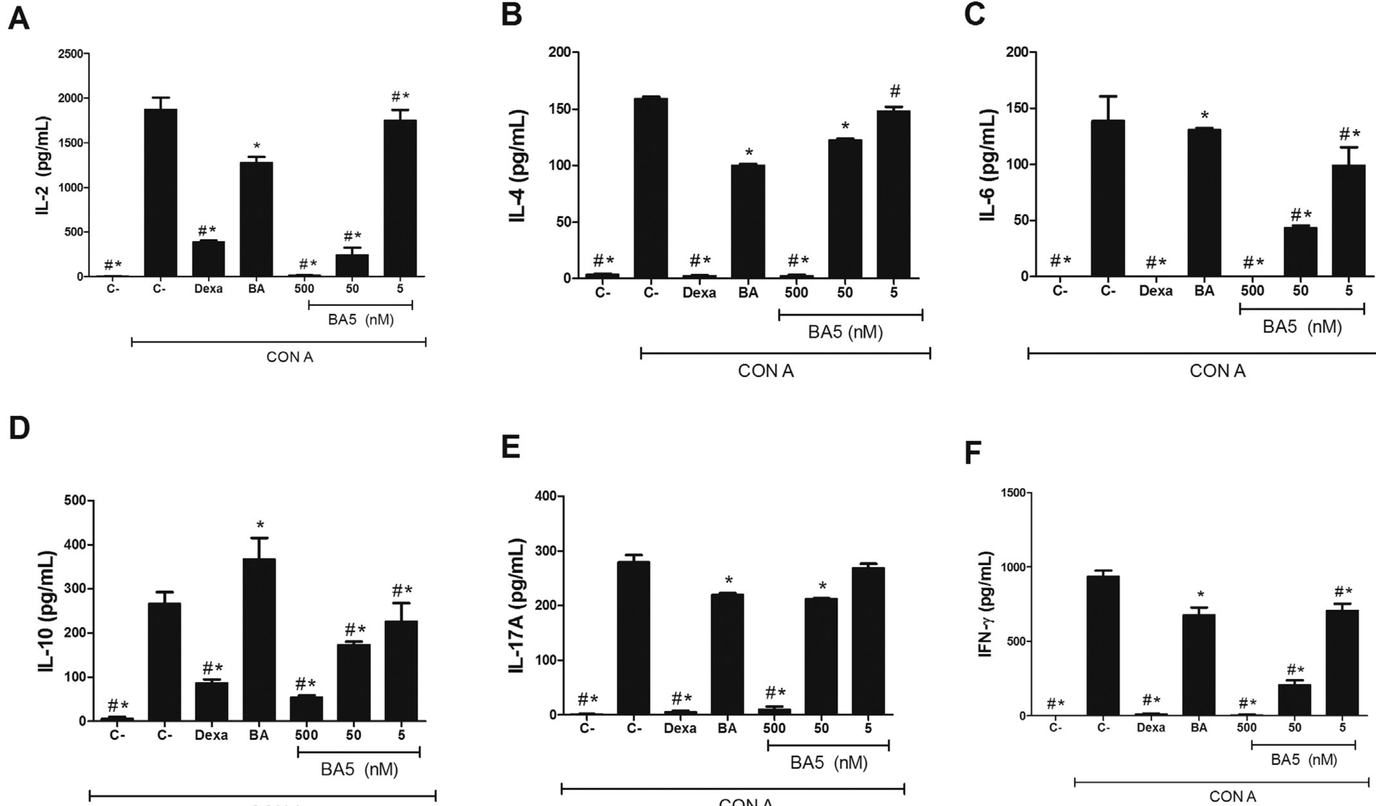

Fig. 3. Assessment of cytokine production by splenocytes treated with BA5. Concentrations of IL-2 (**A**), IL-4 (**B**), IL-6 (**C**), IL-10 (**D**), IL-17A (**E**) and IFN- γ (**F**) were determined in splenocytes treated or not with BA (500 nM), dexamethasone (500 nM) or BA5 (500, 50 and 5 nM) in the presence of concanavalin A (Con A; 5 μg/ml) for 48 h. C- is untreated cells Cell-free supernatants were then collected for cytokine measurement by ELISA. Values represent the means ± S.E.M. of four determinations obtained in one of three experiments performed. *P < 0.05 compared to stimulated and untreated cells; #P < 0.05 compared to BA-treated cells.

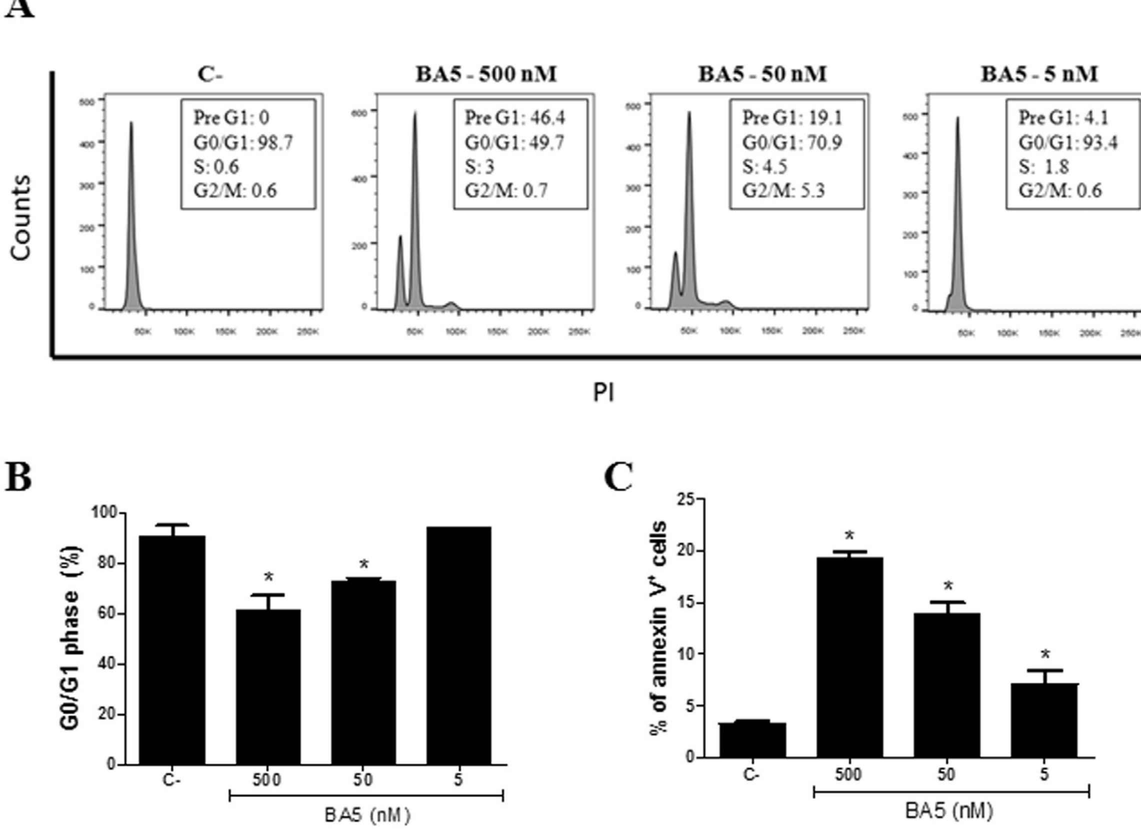

Fig. 4. Analysis of cell cycle progression and cell death after treatment with BA5. (**A** and **B**) Splenocytes were treated or not with BA5 (500, 50 or 5 nM) for 24 and 48 h for cell death determination and cell cycle analysis respectively. After staining with propidium iodide (2 µg/ml), DNA content was analyzed by flow cytometry. The distribution and percentage of cells in pre-phase, G0/G1, S and G2/M phase of the cell cycle are indicated. (**C**) Percentage of stained cells for annexin V after 24 h of treatment with BA5. Values represent the means ± S.E.M. of four determinations obtained in one of two experiments performed. $^*P < 0.05$ compared to stimulated and untreated cells.

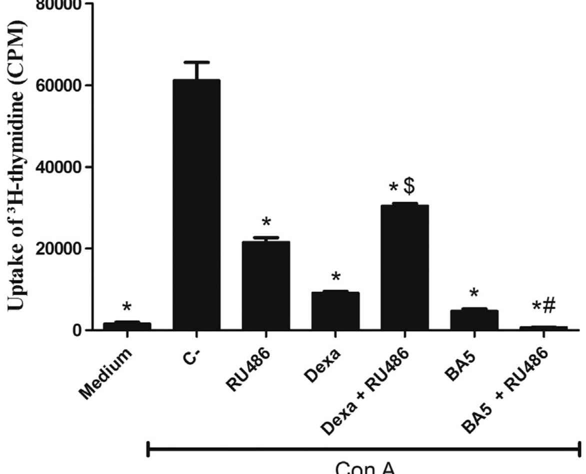

Fig. 5. Effects of RU486 on BA5 activity on Con A-induced lymphoproliferation. BALB/c splenocytes were stimulated with Con A alone or in the presence of dexamethasone (Dexa; 50 nM), BA5 (50 nM) and RU486 (10 µM). C- is untreated cells Proliferation was assessed 48 h later by ^{3}H-thymidine incorporation. Values represent the means ± S.E.M. of 4 determinations. $^*P < 0.05$ compared to Con A; $\#P < 0.05$ compared to BA5 + Con A; $\$P < 0.05$ compared to Dexa + Con A.

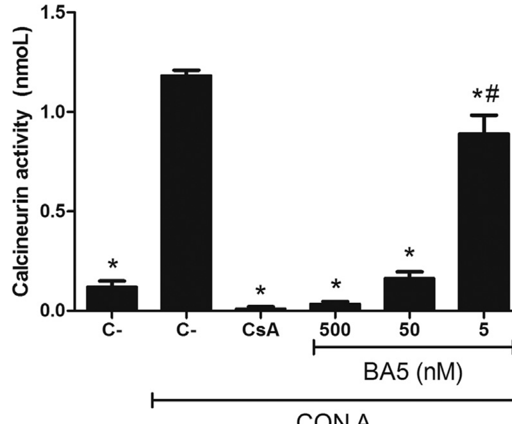

Fig. 6. BA5-based treatment inhibits calcineurin activity in a concentration-dependent manner. Spleen cells stimulated or not with Con A (5 µg/ml) were treated or not with different concentrations of BA5 (500, 50 and 5 nM) or cyclosporin A (Csa; 500 nM) for 48 h. C- is untreated cells Then, cells were lysed and cellular calcineurin phosphatase activity was measured in cell extracts, as described in the methods Values represent the means ± S.E.M. of four determinations obtained in one of two experiments performed. $^*P < 0.05$ compared to stimulated and untreated cells; $\#P < 0.05$ compared to cyclosporin A-treated cells.

enzyme in a concentration-dependent manner, when compared to control untreated cultures. At 500 nM, BA5 caused the inhibition of calcineurin activity comparable to that of cyclosporin A, a reference inhibitor of calcineurin (Fig. 6).

3.5. Synergistic effects of BA5 and dexamethasone

Drug combinations are often employed in the clinical setting for treatment of immune-mediated diseases. Thus, we investigated the immunosuppressive effects of BA5 and dexamethasone in combination on lymphocyte proliferation induced by Con A. Compared to each drug

Table 2
Concentration reductions and combination indexes for immunossupression by BA5 and dexamethasone.

Compounds	IC_{50} ± S.E.M. (nM)[a]		IC_{90} ± S.E.M. (nM)[a]		CI[b]	
	Drug alone	Combination	Drug alone	Combination	IC_{50}	IC_{90}
BA5	13.7 ± 0.9	0.4 ± 0.05	205 ± 7.7	10 ± 2.5	0.13 ± 0.01	0.14 ± 0.03
Dexa	4.2 ± 0.1	0.5 ± 0.06	76 ± 8.7	8.1 ± 2.8		

[a] IC_{50} and IC_{90} values were calculated using concentrations in quadruplicates and two independent experiments were performed.
[b] Combination index (CI). Cutoff: CI value of 0.1–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism; 0.9–1.1, additivity; > 1.1, antagonism. S.E.M. = standard error of the mean.

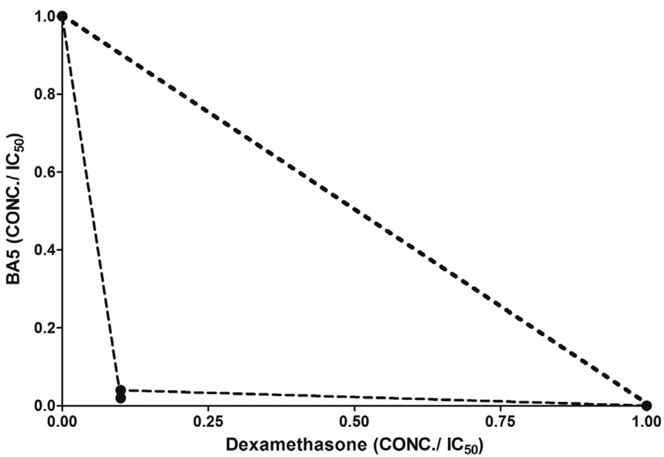

Fig. 7. Isobologram describing the synergistic effects of BA5 and dexamethasone on lymphocyte proliferation induced by Con A. Broken lines correspond to the predicted positions of the experimental points for additive effects.

alone, the combination of BA5 and dexamethasone reduced both IC_{50} and IC_{90} values. In fact, the IC_{50} and IC_{90} of both drugs decreased at least eight times (Table 2). The combination index values associated with a concave isobologram revealed that BA5 and dexamethasone have synergistic effects (Table 2**;** Fig. 7).

3.6. BA5 protects mice against a lethal LPS challenge and reduces edema in delayed-type hypersensitivity reaction

To investigate the effects of BA5 in a model of endotoxic shock, groups of BALB/c mice were treated with BA5 or vehicle (5% of DMSO in saline) and challenged with a lethal dose of LPS. Treatment with 50 mg/kg BA5 induced protection to 83% of the animals (Fig. 8). Mice treated with BA5 at a lower dose (25 mg/kg) had, at the end of the observed period (four days), a survival rate of 33%, similar to the vehicle-treated group. Administration of dexamethasone protected 100%

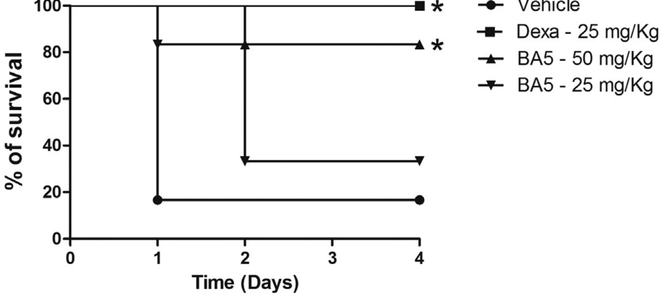

Fig. 8. Survival curve of mice treated with BA5 and submitted to endotoxic shock. Male BALB/c mice (n = 6) were treated with BA5 (25 and 50 mg/Kg) or dexamethasone (25 mg/Kg) or vehicle (5% DMSO in saline) and challenged with LPS 90 min later, intraperitoneally administered. Survival was monitored during 4 days after LPS challenge. Results are from one experiment of two experiments performed. *$P < 0.05$ compared to vehicle group. Statistical analysis was carried out using Logrank (Mantel Cox).

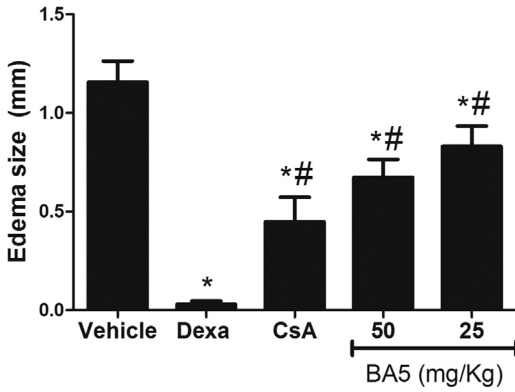

Fig. 9. BA5 reduces BSA-induced DTH in mice. Male BALB/c mice (n = 6) were treated with BA5 (25 and 50 mg/Kg) or dexamethasone (Dexa; 25 mg/Kg) or cyclosporin A (CsA; 25 mg/Kg) or vehicle (5% DMSO in saline) and challenged with 30 µl of a 2% suspension of heat-aggregated BSA in saline administrated in the footpad. At 3 h post-challenge, footpad thickness was measured with calipers and the extent of swelling was calculated by subtracting the thickness of the footpad after challenge from that before challenge. Values represent the means ± S.E.M. of six determinations obtained in one of two experiments performed. *$P < 0.05$ compared to vehicle group; #$P < 0.05$ compared to dexamethasone group.

of animals at 25 mg/kg. Animals from all groups displayed signs of shock, such as piloerection, shivering, and lethargy.

Finally, we investigated the effect of BA5 on BSA-induced DTH reaction in BALB/c mice. Following sensitization, groups of mice were treated with different doses of BA5 and thicknesses of paw measured before and after challenge were used as clinical signs of hypersensitivity. As shown in Fig. 9, treatment with BA5 at 50 and 25 mg/kg caused a reduction of paw edema of 41.7% and 27.8% respectively. Treatment with dexamethasone or cyclosporin A (25 mg/kg) caused a reduction of 97.4% and 61.7%, respectively.

4. Discussion

Natural products are still a promising source for drug discovery through the continuous process of prospection of new compounds or synthesis of derivatives based on natural products (Newman and Cragg, 2016). This is exemplified by betulinic acid, a natural product with pleiotropic biologic effects and used successfully to generate more active compounds, such as antitumor and anti-HIV agents (Smith et al., 2007; Willmann et al., 2009; Ali-Seyed et al., 2016). In the present study, we investigated the immunomodulatory activity of amide semi-synthetic betulinic acid derivatives containing substituents attached in the lupane backbone. We showed that the incorporation of an amide on C-28 enhanced the immunomodulatory effects on macrophage activation and lymphocyte function. The investigation led to the identification of a potent immunomodulatory agent, the semi-synthetic derivative BA5.

It is well-known that TNFα and NO play critical roles in inflammatory conditions. TNFα recruits and activates macrophages and T cells and modulates the secretion of other pro-inflammatory cytokines

(Elenkov et al., 2005). Nitric oxide, due to its over production on inflammatory conditions, causes vasodilation (Xu et al., 2013). Here, we demonstrated a potent inhibitory activity of BA5 on TNFα and NO production in a concentration-dependent manner without affect cell viability in the tested concentrations. Consistent with these findings, BA5 also suppressed NF-κB activity, an important transcription factor that regulates several pro-inflammatory genes, such as TNFα (Lawrence et al., 2002). Moreover, the anti-inflammatory mechanism of BA5 differs from that of BA, the prototype molecule, which is IL-10 dependent (Fiorentino et al., 1991; Costa et al., 2014).

Although there are several reports about the anti-inflammatory activity of betulinic acid and its derivatives, little is known about the effects of these molecules on lymphocyte function. In the present work, we showed that betulinic acid is a potent inhibitor of lymphocyte proliferation and production of key cytokines in the proliferation and activation of lymphocytes, such as IL-2 and IFNγ (Kohno et al., 1997; Boyman and Sprent, 2012). In addition, we showed that these properties are enhanced in BA5 derivative. In fact, the antiproliferative effect of BA and other terpenoids is well recognized in different tumor cell lines, especially in leukemia lineages, such as Jurkat cells, in which BA treatment induced cell cycle arrest in pre-G1 phase followed by cell death by apoptosis (Chen et al., 2008). Interestingly, a similar pathway of cell death was observed in lymphocytes treated with BA5. In addition, BA5 also potently inhibited calcineurin activity, an enzyme that plays a critical role in the progression of cell cycle and T lymphocyte activation through activation of NFAT (Rusnak and Mertz, 2000; Lipskaia and Lompré, 2004; Cen et al., 2013).

Despite the large number of immunosuppressive drugs available, most have side effects associated to a prolonged use and are not efficient in certain conditions (Garcia et al., 2004; Malvezzi et al., 2015). Combined drug therapy may be a valuable tool to improve treatment efficacy and reduce dose levels and toxicity immune disorders, as well as to prevent the potential development of resistance (Vedtofte et al., 2017; Kavishe et al., 2017). In our study, BA5 had synergistic effects with dexamethasone on the inhibition of lymphocyte proliferation, showing a promising profile for drug combination.

Previous studies detected the potential of betulinic acid and others triterpenoids in inflammatory mouse models (Costa et al., 2014; Niu et al., 2014; Lingaraju et al., 2015b). In the present work, we observed the effects of BA5 in a mouse model of endotoxemia induced through intraperitoneally LPS administration. In this model of lethal shock, LPS binds to CD14 receptors of macrophages and triggers the release of proinflammatory cytokines, including IL-1 and TNF-α that can in turn induce lethal shock (Blank et al., 1997). Despite having protective effects in T-cell mediated shock models, classic calcineurin inhibitors such as cyclosporin A didn´t have protective effects in LPS mediated shock models (Gonzalo et al., 1993). In contrast, BA5 proven to be a potent inhibitor of calcineurin and also able to protect animals from a lethal dose of LPS, showing a advantage when compared to others calcineurin inhibitors. We also observed a reduction of paw edema in a mouse model of BSA-induced DTH, a expected feature for a calcineurin inhibitor. Our results reinforce the potential use of betulinic acid and its derivatives in the searching for more potent and selective immunomodulatory drugs

In conclusion, betulinic acid can be successfully used for the generation of more active compounds, such as the derivative BA5, identified herein with a potent immunomodulatory *in vitro* and *in vivo*. BA5 also proved to be a suitable partner for drug combination, evidencing its potential use for the treatment of immune-mediated disorders.

Conflict of interest

All authors have no conflict of interest to disclose.

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