Parasitology Research (2019) 118:369–376
https://doi.org/10.1007/s00436-018-6166-2

IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - SHORT COMMUNICATION

6,7-Dehydroroyleanone diterpene derived from *Tetradenia riparia* essential oil modulates IL-4/IL-12 release by macrophages that are infected with *Leishmania amazonensis*

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Received: 7 February 2018 / Accepted: 23 November 2018 / Published online: 5 December 2018

Abstract

The treatment of cutaneous leishmaniasis in associated with several adverse effects and therapeutic failure, resulting in patients' abandonment of treatment. Research on new drugs with leishmanicidal potential from medicinal plants is essential. The anti-*Leishmania* activity of *Tetradenia riparia* essential oil (TrEO) and its derivatives, such as the diterpene 6,7-dehydroroyleanone (TrROY), and the immunomodulatory effects of TrEO have been reported. However, few studies have investigated the effects of TrROY. The present study evaluated the modulation of cytokine production by murine macrophages that were infected with *Leishmania amazonensis* (6 parasites/macrophage) and treated with TrROY (0.1, 1, and 100 μg/ml). Cytokine levels were measured by flow cytometry. The results were analyzed using Student's *t* test at a 95% confidence interval. Microscopic counting was performed to evaluate the inhibitory effects of TrROY on intracellular infection. TrROY modulated the production of cytokines that are essential for the immune defense response to *Leishmania*, with a decrease in interleukin-4 (IL-4) levels and an increase in IL-12 levels. A TrROY concentration of 0.1 μg/ml was chosen for the subsequent experiments. This dose was chosen because it modulated IL-4/IL-12 release by murine macrophages that were infected with *Leishmania* and because it presented no cytotoxic effects. TrROY (0.1 μg/ml) induced a 31% reduction of the rate of infection in murine macrophages compared with untreated cells. TrROY may be a promising leishmanicidal agent. Further in vitro and in vivo studies should be conducted to evaluate the anti-*Leishmania* and immunomodulatory activity of TrROY.

Keywords 6,7-Dehydroroyleanone diterpene · *Tetradenia riparia* essential oil · *Leishmania amazonensis*

Introduction

Leishmaniasis is a noncontagious infectious disease. Its etiological agents are parasites of the genus *Leishmania*. Transmission occurs through bites from infected female phlebotomine sandflies of the genus *Lutzomyia* that introduce promastigote forms of *Leishmania*. Once in the host, the promastigote forms are phagocytosed by the cells of the phagocytic mononuclear system, and they become amastigotes, which are able to resist the host's immune defenses and establish infection. This disease has a broad spectrum of clinical forms, ranging from cutaneous and mucocutaneous lesions to visceral lesions (Brazil 2017). Cutaneous leishmaniasis affects approximately 1 million people worldwide and threatens 350 million people who live in high-risk areas, mainly in developing countries. One of the main species

Section Editor: Sarah Hendrickx

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that is responsible for cutaneous leishmaniasis is *Leishmania* (*Leishmania*) *amazonensis*, which is also the etiological agent of diffuse cutaneous leishmaniasis, the most severe and destructive clinical form of the disease (Marzochi and Marzochi 1994; World Health Organization 2010).

The intracellular localization of the parasite makes infection dependent on the immune response that is mediated by host cells. The main effector cell of the elimination of amastigotes is the macrophage. Macrophage activation by cytokines that are produced by immune cells (e.g., CD4+ helper T-lymphocytes) promotes adaptive responses that are appropriate to the specific pathogen (Gollob et al. 2014; Maspi et al. 2016). Macrophages also secrete cytokines, proteins that stimulate cellular activation, regulation, differentiation, and migration to combat the infection. Cytokines are classified into different families, based on the structural homology of their cellular receptors. The main cytokines belong to the families of interleukins (ILs), interferons (IFNs), and chemokines. These proteins are essential for both innate and adaptive immune responses and cellular growth, differentiation, death, angiogenesis, and repair processes (Argüello et al. 2015; Maspi et al. 2016).

In leishmaniasis, the cytokines that are produced by Th and other cells (e.g., macrophages) are essential to the course of the disease. Inflammatory cytokines (e.g., interferon γ [IFN-γ]) comprise a complex network of signaling pathways that stimulate antimicrobial activity that is directed toward intracellular parasites (Soulat and Bogdan 2017). This action is crucial for parasite death and clinical cure. Antiinflammatory cytokines, such as IL-10 and IL-4, are related to parasite resistance and treatment failure (Gollob et al. 2014; Maspi et al. 2016). In addition to the immune response, the specific parasite species influences disease development. *L.* (*L.*) *amazonensis* modulates the release of cytokines by immune cells and escape from microbicidal activity (Demarchi et al. 2016). Previous studies have investigated the effects of various drugs on cytokine activity in leishmaniasis as a new treatment strategy (Demarchi et al. 2015a, 2016; Okwor and Uzonna 2016).

Since 1940, the treatment of leishmaniasis has been mostly based on antimonials, such as Glucantime, but this therapy has severe adverse effects, such as cardiotoxicity. *L.* (*L.*) *amazonensis* has been associated with therapeutic failure and disease recurrence (World Health Organization 2010). For these reasons, investigations of natural products as novel treatment strategies for leishmaniasis have increased. Recently, Demarchi et al. (2015a, b) described the potential leishmanicidal and immunomodulatory effects of *Tetradenia riparia* (Hochstetter) Codd essential oil. The use of medicinal plants may be a strategy for modulating cytokine secretion, thus possibly offering new treatment approaches. Products with anti-*Leishmania* activity that are able to stimulate the release of cytokines through a protective cellular immune response and products that negatively regulate suppressive immunity could be alternatives for mitigating treatment resistance and failure and avoiding adverse effects (Sen and Chatterjee 2011). Research has been devoted to discovering new drugs with high anti-*Leishmania* potential, low toxicity, and beneficial immunomodulatory effects (Oliveira et al. 2011; Monge-Maillo and López-Vélez 2013).

The plant *T. riparia* is a shrub that is found in such tropical areas as Africa and Brazil. Gazim et al. (2010, 2014) identified a series of terpenoid compounds in *T. riparia* essential oil (TrEO), including 6,7-dehydroroyleanone diterpene (*T. riparia* 6,7-dehydroroyleanone [TrROY]). Gazim et al. (2010, 2014) isolated and characterized TrROY. Demarchi et al. (2015a, b) reported leishmanicidal and immunomodulatory activity of TrEO but not of TrROY in murine macrophages (Demarchi et al. 2015a, b). The antioxidant and antipyretic activity of TrROY was recently reported (Kusumoto et al. 2009; Gazim et al. 2014). To date, however, no studies have investigated the effects of TrROY on cytokine activity that is necessary for the immune response and the elimination of *Leishmania* parasites. Studies of this diterpene have been conducted in an effort to discover new leishmanicidal agents (Gazim et al. 2014; Demarchi et al. 2015a, b). The present study investigated the effects of TrROY on cytokine production by murine macrophages that were infected with *L.* (*L.*) *amazonensis*.

Material and methods

This study was approved by the Committee of Ethics in Animal Use in Experiments (CEAE) of the State University of Maringá (Paraná, Brazil; opinion nos. 133/2012 and 079/2012) to obtain cells from BALB/c mice. The *T. riparia* plant was identified by the Department of Pharmacy of the University of Paraná (UNIPAR; Umuarama, Paraná, Brazil) and deposited in the UNIPAR Herbarium (no. 2502; 23° 46′ 22″ S and 53° 16′ 73″ W, 391 m elevation). The essential oil was obtained from the leaves of *T. riparia* and extracted by hydrodistillation in a modified Clevenger-type apparatus as described by Gazim et al. (2010). Briefly, TrEO was characterized by gas chromatography coupled with mass spectrometry (GC-MS) using the Agilent 5973N GC-MS System. The compounds were identified by comparing retention times using different n-alkanes (C7-C25). The mass spectrometry data were compared with data from the literature and the Wiley spectra library (Gazim et al. 2010).

Prof. Zilda Cristiane Gazim from UNIPAR performed the extraction, isolation, and analysis of the diterpene TrROY

(Gazim et al. 2014; Baldin et al. 2018). Briefly, the essential oil underwent column chromatography on a silica gel carrier and was eluted in pentane, pentane-dichloromethane (9:1; 8:2; 7:3, and 1:1), dichloromethane-pentane (3:7), dichloromethane, dichloromethane-methanol (9:1; 7:3, and 1:1), and methanol, resulting in 29 fractions. Fraction 17 (11.7 mg; pentane-dichloromethane [80:20]) gave an [M−H]− at *m/z* 313, and the isolated molecule (not racemic mixture) was identified by electrospray ionization mass spectrometry and nuclear magnetic resonance as 6,7-dehydroroyleanone (TrROY) (Gazim et al. 2014; Baldin et al. 2018). The spectral data corresponded to the data that were previously reported by Kusumoto et al. (2009).

The modulation of cytokine production by TrROY was evaluated. Promastigotes of *Leishmania* (*Leishmania*) *amazonensis* (MHOM/BR/1977/LTB0016) were maintained in Medium 199 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin). Peritoneal macrophages were obtained from BALB/c mice according to Lonardoni et al. (2000). Briefly, mice (30–40 days of age) were sacrificed by 40% CO_2 inhalation in a chamber at a moderate fill rate (AVMA Guidelines on Euthanasia, 2007). The peritoneal cavity was washed with 5–8 ml of sterile RPMI 1640 culture medium. The cell suspension was adjusted to 1×10^6 macrophages/ml (considering 60% of the cells as macrophages), and 1 ml of the suspension was added to each well of a 12-well plate for cell culture (TPP, Sigma-Aldrich, Trasadingen, Switzerland). The plates were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere, followed by washes with sterile phosphate-buffered saline (PBS) to remove nonadherent cells (Lonardoni et al. 2000). Afterward, the macrophages were subjected to the following conditions: (*i*) untreated and uninfected macrophages (negative control), (*ii*) macrophages infected with *Leishmania* (*Leishmania*) *amazonensis* promastigotes (6 parasites/macrophage; positive control of infection), (*iii*) macrophages infected with *Leishmania* (1:6) and treated with 0.1, 1, and 100 μg/ml TrROY, and (*iv*) uninfected macrophages treated with 0.1, 1, and 100 μg/ml TrROY. The cultures were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. The supernatant was removed and stored in aliquots at − 80 °C. The assays were performed in triplicate and in two independent experiments.

The supernatant was centrifuged, and a 50-μl aliquot was used to determine cytokine levels (in pg/ml) using the Cytokine Magnetic 10-Plex Panel Multiplex Mouse Kit (Invitrogen, Carlsbad, CA, USA). The dosage was performed in duplicate and according to the manufacturer's standards. This method is based on a suspension that contains beads, in which encoded fluorescent beads have cytokine capture antibodies to bind the proteins. The final analysis was performed by flow cytometry using a Luminex 200 apparatus. The levels of the following cytokines were determined: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), INF-γ, and tumor necrosis factor alpha (TNF-α). The results were analyzed using Statistic 7 software and Student's *t* test at a 95% confidence interval.

Microscopic counting was conducted to analyze the inhibitory effects of TrROY on amastigote infection. A cell suspension (500 μl, 1×10^6 macrophages/ml) was distributed on 13-mm-diameter sterile glass coverslips (Glastecnica, São Paulo, SP, Brazil) and placed in 24-well culture plates (TPP, Sigma-Aldrich, Trasadingen, Switzerland). The plates were incubated in a 5% CO_2 atmosphere for 2 h at 37 °C, followed by washes with sterile PBS to remove nonadherent cells. Adherent macrophages were incubated in RPMI 1640 culture medium. The conditions mentioned above (groups *i–iv*) were again tested. After incubation at 37 °C for 24 h in a 5% CO_2 atmosphere, the cells on the coverslips were fixed in 95% ethanol and dyed with hematoxylin and eosin. The infection index was determined as the percentage of infected cells multiplied by the mean number of parasites per cell. At least 200 cells were counted using an optical microscope. All of the conditions were tested in triplicate and in two independent experiments.

Results and discussion

The quantification of cytokine production by murine peritoneal macrophages that were treated with TrROY and infected or uninfected with *Leishmania* sp. was performed after 24 h of incubation (Table 1). We considered cytokine production by uninfected and untreated macrophages (negative control) for the analysis and comparison to the other conditions tested. The uninfected and TrROY-treated macrophages presented no significant difference in cytokine levels compared with the negative control (Table 1).

Infected and untreated macrophages presented a decrease in IFN-γ levels and increase in IL-4 levels (Fig. 1). In cutaneous leishmaniasis, the host's immune response is one of the most important factors for the establishment of infection and cure of the disease (World Health Organization 2010). However, the *Leishmania* parasite can modify its innate and adaptive immune response, thus inhibiting the antimicrobial mechanisms of host cells (Gollob et al. 2014). The activation of cells of the cellular immune response, such as TH_1 cells and macrophages that produce IFN-γ and IL-12, promotes the activation of microbicidal mechanisms and parasite death by macrophages (Tripathi et al. 2007). However, the intracellular localization of the pathogen may influence the effect of

Table 1 Determination of cytokines (pg/ml) by flow cytometry produced by murine macrophages infected and not infected with *Leishmania* (*L.*) *amazonensis* and treated with *T. riparia* 6,7-dehydroroyleanone (TrROY)

Mean of quantification of cytokines (pg/ml) in 24 h ± standard deviation										
	IFN-γ	GM-CSF	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	TNF-α
MO (control−)	176.89 ± 0	6.08 ± 2.51	7.13 ± 0	25.00 ± 23.77	67.18 ± 0	94.16 ± 0	11.00 ± 10.99	44.72 ± 0	17.82 ± 2.44	0.86 ± 0
MO LLa (control+)	141.555 ± 0.01*	4.307 ± 0	3.989 ± 4.44	25.00 ± 23.77	338.354 ± 0*	47.462 ± 66.04	3.2305 ± 0	44.719 ± 0	11.279 ± 1.76	7.093 ± 4.49
MO LLa TrROY (1)	98.5455 ± 0*#	2.3415 ± 2.78	0.848 ± 0	4.224 ± 5.61	1.6355 ± 0*#	47.462 ± 66.04	3.2305 ± 0	44.719 ± 0	24.974 ± 7.68	0.864 ± 0
MO LLa TrROY (2)	31.265 ± 0.01*#	2.3415 ± 2.78	7.13 ± 0	4.224 ± 5.61	67.1825 ± 0#	0.767 ± 0	3.2305 ± 0	44.719 ± 0	17.1535 ± 6.55	0.864 ± 0
MO LLa TrROY (3)	64.908 ± 47.57	4.307 ± 0	7.13 ± 0	21.0335 ± 29.38	187.6005 ± 0*#	47.462 ± 66.04	3.231 ± 0	44.719 ± 0	20.4065 ± 11.15	2.3895 ± 2,16
MO TrROY (1)	31.265 ± 0.01*#	4.307 ± 0	0.848 ± 0	41.81 ± 0	100.3145 ± 0*#	94.157 ± 0	3.231 ± 0	44.719 ± 0	30.402 ± 0#	0.864 ± 0
MO TrROY (2)	31.27 ± 0.01*#	4.307 ± 0	0.848 ± 0	8.191 ± 0	130.9285 ± 0*#	94.157 ± 0	15.724 ± 17.67	617.455 ± 809.97	22.777 ± 7.80	0.864 ± 0
MO TrROY (3)	64.908 ± 47.57	2.3415 ± 2.78	3.989 ± 4.44	4.224 ± 5.61	29.1425 ± 0*#	47.462 ± 66.04	59.6575 ± 79.80	44.719 ± 0	36.052 ± 5.04*#	2.3895 ± 2.16

The experiment was made in duplicate. The incubation period was 24 h. *MO*, macrophages (negative control); *LLa*, *Leishmania* (*Leishmania*) *amazonensis* (6 *Leishmania* by macrophage—positive control); *TrROY* (1) treatment with 100 μg/ml, (2) treatment with 1 μg/ml, and (3) treatment with 0.1 μg/ml. *p value < 0.05 by Student's t test for comparison with uninfected macrophages (negative control). #$p < 0.05$ for comparison with macrophages infected with *L.* (*L.*) *amazonensis* (positive control)

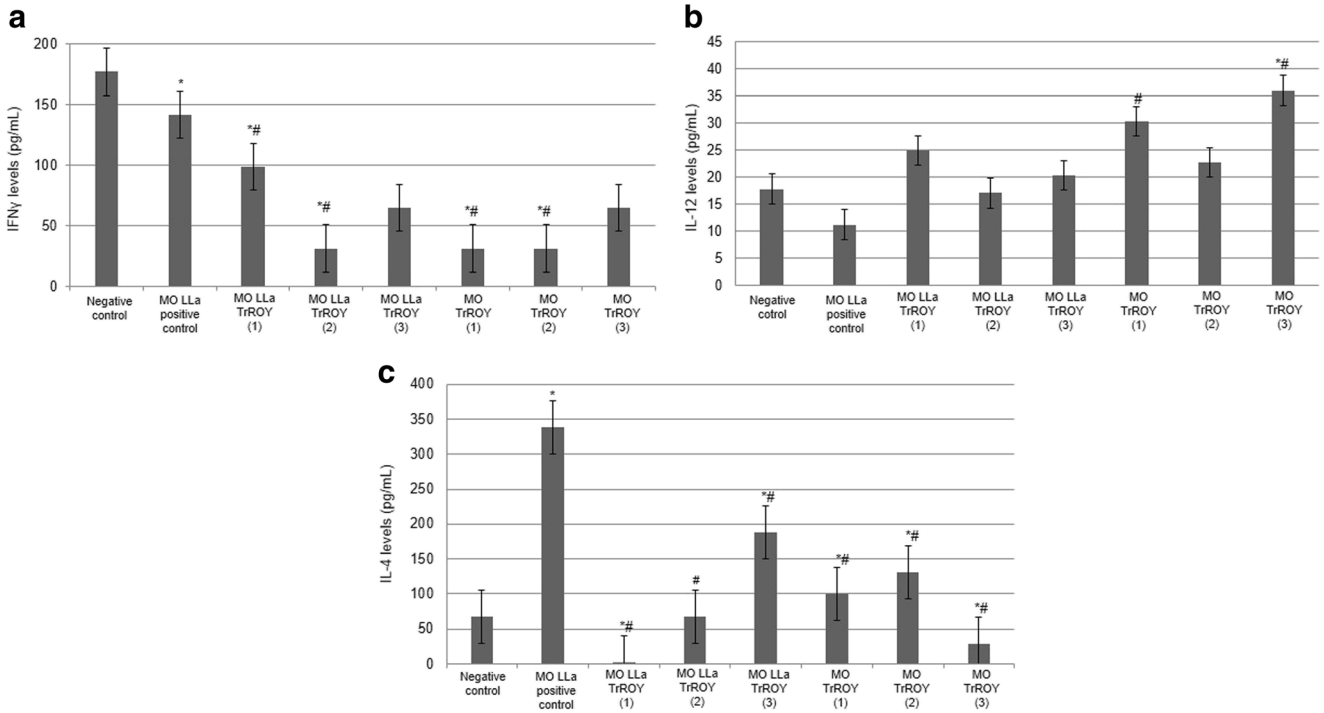

Fig. 1 Immunomodulation of production of the cytokine interferon-γ (IFN-γ, **a**), interleukin-12 (IL-12, **b**), and IL-4 (**c**) in murine macrophages treated with 6,7-dehydroroyleanone (TrROY) derived from *Tetradenia riparia* and uninfected or infected with *Leishmania* (*Leishmania*) *amazonensis*. The experiment was performed in duplicate. The incubation period was 24 h. Flow cytometry was performed to determine cytokine levels (in pg/ml). MO, macrophages (negative control); LLa, *Leishmania* (*L.*) *amazonensis* (6 parasites/macrophage); (1) treatment with 100 μg/ml TrROY; (2) treatment with 1 μg/ml TrROY; (3) treatment with 0.1 μg/ml TrROY. $^{*}p < 0.05$, compared with uninfected macrophages (negative control); $^{\#}p < 0.05$, compared with macrophages infected with *L.* (*L.*) *amazonensis* (positive control; Student's *t* test)

leishmanicidal drugs. Furthermore, the parasite can resist intracellular microbicidal mechanisms and prevent cell death. Infection with *L.* (*L.*) *amazonensis* induces the release of IL-10, IL-4, and IL-5, which are involved in the TH_2-type immune response and considered nonprotective and directly related to disease progression, and decreases IFN-γ and IL-12 levels to evade the protective cellular immune response (Arango Duque et al. 2014).

Macrophages that were infected with *L.* (*L.*) *amazonensis* and treated with TrROY did not present significant changes in the production of IL-1β, GM-CSF, IL-2, IL-5, IL-10, or TFN-α. Differences were only found in the production of IFN-γ (Fig. 1a), IL-12 (Fig.1b), and IL-4 (Fig. 1c). Murine macrophages that were infected and treated with TrROY at all concentrations tested (0.1, 1, and 100 μg/ml) exhibited a decrease in IFN-γ production compared with the negative control. Infection with *L.* (*L.*) *amazonensis* in the absence of treatment also decreased IFN-γ levels, with no difference between treated and untreated macrophages. The decrease in IFN-γ levels likely occurred because of parasitic infection, and treatment with TrROY was unable to subvert this action. Other cytokines that were not investigated in the present study, such as IL-17 and IL-18, may play a role in the modulation of IFN-γ production. These cytokines are involved in protective immunity against *Leishmania*. IL-17 participates in the recruitment of neutrophils that activate the inflammatory process. IL-18 participates in induction of the Th_1-type immune response and IFN-γ production. *T. riparia* essential oil and *L.* (*L.*) *amazonensis* infection negatively regulated the mRNA expression of IL-18 (Demarchi et al. 2016), which could explain this reduction of IFN-γ in the present study. No studies of which we are aware have investigated the modulation of this cytokine by TrROY.

Previous studies showed that TrEO modulates both the mRNA expression and production of various cytokines that are produced by murine peritoneal macrophages that are infected with *L.* (*L.*) *amazonensis* (Demarchi et al. 2016). The essential oil of the diterpene 6,7-dehydroroyleanone reduced the production of some cytokines that are involved in disease progression. However, it did not stimulate the production of IFN-γ, which is critical for resolution of the disease, to induce TH_1-producing cells that are involved in the activation of macrophages in the host (Oliveira et al. 2014).

In infected and uninfected macrophages that were treated with TrROY, an increase in IL-12 production was observed (Fig. 1b). This cytokine promotes cell-mediated immunity by stimulating TH_1 lymphocytes and controlling inflammation. Both IFN-γ and IL-12 are critical for controlling the diffusion

of intracellular infectious agents, such as *Leishmania*, and can be a target for leishmaniasis therapy (Iniesta et al. 2005; Shweash et al. 2011; Oliveira et al. 2014). The production of IL-12 induces the activation of macrophages that produce reactive oxygen species and nitric oxide (NO), microbicidal substances that promote the death of *Leishmania* (Murray and Nathan 1999; Oliveira et al. 2014). TrROY did not alter nitrite production during *Leishmania* infection. TrROY may possibly modulate other immune mechanisms, such as arginase, but this requires further investigation.

At a noncytotoxic concentration (0.1 μg/ml) in murine macrophages, TrROY decreased IL-4 levels in both infected and uninfected macrophages (Fig. 1c). At concentrations of 1 and 100 μg/ml in infected macrophages, TrROY also decreased IL-4 levels, but cytotoxicity at these concentrations was observed. This inhibitory effect on the production of this cytokine may be related to cytotoxicity that is promoted by this diterpene (Demarchi et al. 2015b). TrROY treatment (1 and 100 μg/ml) increased IL-4 levels in the absence of infection (Fig. 1c). Thus, this diterpene was able to block the increase in IL-4 production that was induced by *L.* (*L.*) *amazonensis* infection when used at low concentrations (Demarchi et al. 2016). IL-4 is also a critical cytokine for the production of other cytokines, such as IL-5 and IL-10. IL-4 promotes negative regulation of the immune response and stimulates the TH_2-like cellular response, thus inhibiting the mechanisms of macrophage-induced death and promoting the progression of infection (Oliveira et al. 2014). With IL-4 inhibition, TH_2 and TH_{17} cells are inhibited, and protective cytokines (e.g., IL-12) are stimulated by the diterpene, thus contributing to *Leishmania* death and infection control.

TrROY modulated IL-4 and IL-12 release by infected cells but did not alter nitrite production or IFN-γ levels, the main leishmanicidal substance and cytokine, respectively, that are involved in infection resolution. TrROY did not activate NO release, but other microbicidal mechanisms were likely modulated. Two distinct types of macrophage activation are observed during infection with *Leishmania*, which leads to different outcomes. These two pathways use L-arginine as a common substrate for enzymatic activity. L-Arginine is a crucial amino acid that is required for both NO-mediated parasite death and polyamine-mediated parasite replication. During the classic macrophage activation process (M1), IFN-γ induces NO synthase 2 (NOS2) production by macrophages. NOS2 converts L-arginine into OH-arginine and then into NO. In alternatively activated (M2) macrophages, the release of IL-4, IL-10, or IL-13 induces arginase I production, which degrades arginine into urea and ornithine, which are then subsequently metabolized into proline and polyamines and enhance parasite replication and persistence (Mantovani et al. 2002; Gordon 2003; Noël et al. 2004).

Leishmania expresses the L-arginine transporter and other enzymes of the L-arginine metabolic pathway, including arginase and ODC, an enzyme that converts the arginase-enzymatic product L-ornithine into polyamine (Wanasen and Soong 2008). Vendrame et al. (2007) suggested that parasite-derived arginase plays a critical role in enhancing parasite growth in vivo (Vendrame et al. 2007). Therefore, the parasite and host cell compete for the same nutrients, and the parasite's ability to compete for L-arginine appears to be favored, depending on the microenvironment (Wanasen and Soong 2008). Arginase is an immunomodulatory protein that can be induced by leishmanial infection, and its expression has been linked to the inhibition of IL-12. IL-12 is well known to be essential for maintaining the TH_1 response (Iniesta et al. 2005; Herbert et al. 2010; Shweash et al. 2011). TrROY induced IL-12 production and inhibited IL-4 production, which could negatively regulate the stimulation of arginase and favor infection control. The arginase pathway should be investigated further to elucidate the possible immunomodulatory effects of TrROY on this pathway. The effects of TrROY should also be tested in other parasitic diseases that are related to TH_2 bias.

Although TrROY presents no toxicity in human cells (Demarchi et al. 2015a), cytotoxicity of this compound was observed in murine macrophages at concentrations ≥ 1 μg/ml. Gazim et al. (2014) showed that TrROY is noncytotoxic to melanoma cells of the nervous system and human colon. The 50% cytotoxic concentration (CC_{50}) of TrROY was reported to be 0.53 μg/ml. The selectivity index and therapeutic index for leishmaniasis were 0.22 and 0.03, respectively (Demarchi et al. 2015a). Thus, TrROY appears to be more selective for these cells than for the parasite. The inhibitory concentration of TrROY that reduced parasite growth by 50% was 2.45 μg/ml, and the concentration that was able to kill 50% of the parasites was 16.9 μg/ml (Demarchi et al. 2015a). These results suggest that the best concentration of TrROY that is able to exert an immunomodulatory effect on cytokine production for the control of *L.* (*L.*) *amazonensis* infection is 0.1 μg/ml. At this concentration, TrROY inhibited the production of IL-4 and induced the production of IL-12, a cytokine that is essential for disease control, thus promoting the TH_1-type immune response and controlling inflammation.

With regard to cytotoxicity and the immunomodulation of IL-4 and IL-12 production, we conducted the experiments with the intracellular form of *Leishmania* (amastigote) using 0.1 μg/ml TrROY. The infection index was reduced by approximately 37% (infection index = 114) compared with infected and untreated macrophages (i.e., positive control; infection index = 182; Fig. 2). These effects of TrROY on amastigote forms were consistent with Demarchi et al. (2015a). These authors did not observe significant cytotoxicity and found that TrROY promoted a 31% reduction of the

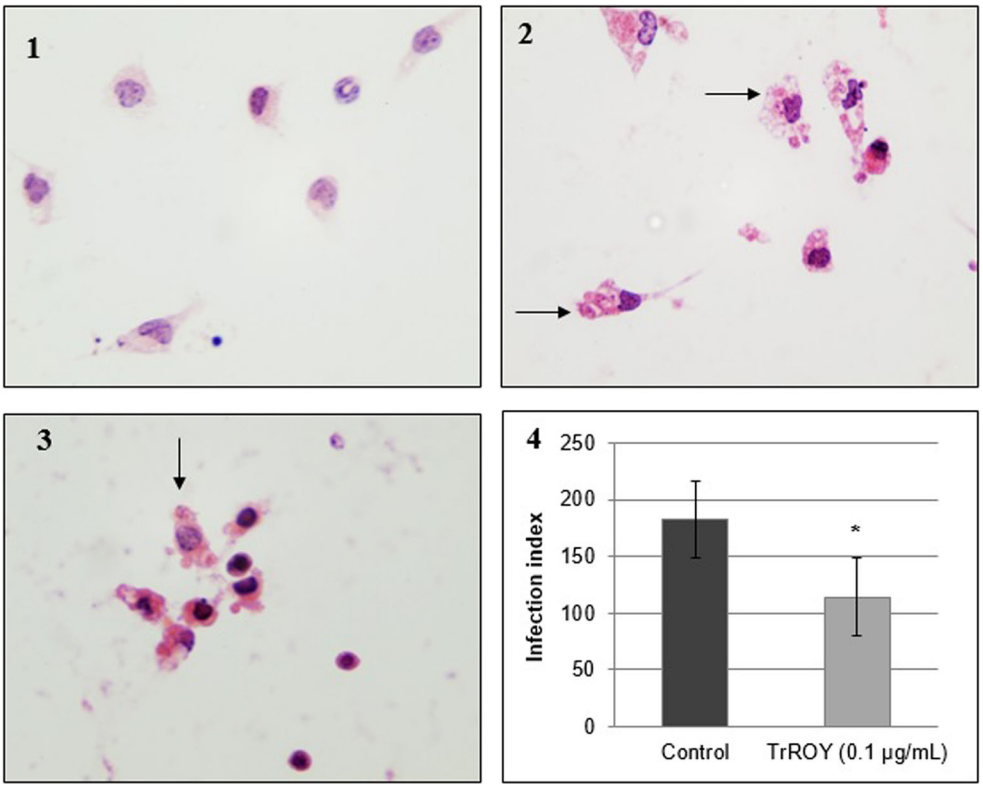

Fig. 2 Effects of 6,7-dehydroroyleanone (TrROY) derived from *Tetradenia riparia* on intracellular forms of *Leishmania* (*Leishmania*) *amazonensis*. Peritoneal macrophages from BALB/c mice were infected and treated with TrROY. (1) Uninfected and untreated macrophages (negative control); (2) macrophages infected with *L.* (*L.*) *amazonensis* (6 parasites/macrophage); (3) macrophages infected and treated with 0.1 μg/ml TrROY. The cells were stained with hematoxylin and eosin (× 100 objective). The arrow indicates amastigote intracellular forms. (4) Infection index of macrophages infected and treated with 0.1 μg/ml TrROY compared to the control (macrophages infected and untreated). All of the conditions were tested in triplicate and in two independent experiments. The analysis was performed after 24 h of incubation at 37 °C in a 5% CO_2 atmosphere. *$p < 0.05$, compared with uninfected macrophages (negative control)

rate of infection in infected and untreated murine macrophages. Comparing with previous studies (Demarchi et al. 2015a, 2016) and considering the cytotoxicity and the anti-*Leishmania* activity doses, TrROY has less leishmanicidal activity than its parent essential oil. Since the TrEO (0.03 μg/ml) modulates some cytokines produced by murine macrophages (Demarchi et al. 2016) and TrROY modulates only IL-12/IL-4 release at 0.1 μg/ml, we could suggest that essential oil is better at modulating cytokine release. It can be attributed to the interactions between compounds of TrEO which may lead to synergistic, antagonistic, or additive effects (Demarchi et al. 2015a). Thus, is necessary studies about the derivates from TrEO, as TrROY, to evaluate which compounds have biological effects.

Overall, the present findings indicate that TrROY may be a promising anti-*Leishmania* agent that can stimulate the protective immune response against intracellular pathogens and inhibit undesirable immune reactions. TrROY may be an alternative therapeutic agent for leishmaniasis. Also, considering the modulation of IL-4 release by TrROY, this diterpene should be evaluated in other parasitic diseases that induce TH_2 bias. The expression and activity of arginase should also be investigated to elucidate the immunomodulatory effects of TrROY on this pathway. Additional in vitro and in vivo tests should be performed to confirm the anti-*Leishmania* and immunomodulatory potential of TrROY.

Funding information Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil, supported this work.

Compliance with ethical standards

This study was approved by the Committee of Ethics in Animal Use in Experiments (CEAE) of the State University of Maringá (Paraná, Brazil; opinion nos. 133/2012 and 079/2012) to obtain cells from BALB/c mice.

Conflict of interest The authors declare that there is no conflict of interest.

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