International Immunopharmacology 77 (2019) 105961

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Sesquiterpene lactone potentiates the immunomodulatory, antiparasitic and cardioprotective effects on anti-*Trypanosoma cruzi* specific chemotherapy

Elda Gonçalves-Santos[a], Diego F. Vilas-Boas[a], Lívia F. Diniz[a], Marcia P. Veloso[b], Ana L. Mazzeti[a], Maria R. Rodrigues[b], Carla M. Oliveira[b], Victor Hugo C. Fernandes[d], Rômulo D. Novaes[c], Daniela A. Chagas-Paula[d], Ivo S. Caldas[a,*]

[a] Institute of Biomedical Sciences, Department of Pathology and Parasitology, Federal University of Alfenas, 37130-001 Alfenas, MG, Brazil
[b] Pharmaceutical Sciences Institute, Federal University of Alfenas, Alfenas 37130-001, MG, Brazil
[c] Institute of Biomedical Sciences, Department of Structural Biology, Federal University of Alfenas, 37130-001 Alfenas, MG, Brazil
[d] Chemistry Institute, Federal University of Alfenas, 37130-001 Alfenas, MG, Brazil

ARTICLE INFO

Keywords:
Chagas's disease
Benznidazole
Infectious myocarditis
Sesquiterpene lactone
Tagitinin C

ABSTRACT

We investigated the immunomodulatory, antiparasitic and cardioprotective effects of a sesquiterpene lactone (SL) administered alone or combined with benznidazole (Bz), in a murine model of Chagas' disease by *in vitro* and *in vivo* assays. Antiparasitic and cytotoxic potential of tagitinin C (SL) and Bz were tested *in vitro* against *T. cruzi* epimastigotes and cardiomyocytes. Swiss mice challenged with *T. cruzi* were also treated for 20 days with tagitinin C (10 mg/kg) alone and combined with Bz (100 mg/kg). Tagitinin C exhibited a higher antiparasitic (IC_{50}: 1.15 μM) and cytotoxic (CC_{50} at 6.54 μM) potential than Bz (IC_{50}: 35.81 μM and CC_{50}: 713.5 μM, respectively). When combined, these drugs presented an addictive interaction, determining complete suppression of parasitemia and parasitological cure in all infected mice (100%) compared to those receiving Bz alone (70%). Anti-*T. cruzi* immunoglobulin G, and pro-inflammatory cytokines IFN-γ and TNF-α levels were reduced in animals treated with tagitinin C combined with Bz, while IL-10 production was unaffected. Heart inflammation was undetectable in 90% of the animals receiving this combination, while only 50% of the animals receiving Bz alone showed no evidence of myocarditis. Together, our findings indicated that the combination of tagitinin C and Bz exerts potent antiparasitic, immunomodulatory and cardioprotective effects. Due to the remarkable suppression of parasitemia and high parasitological cure, this combination was superior to Bz monotherapy, indicating a high potential for the treatment of Chagas's disease.

1. Introduction

Chagas's disease is a neglected infection linked to poverty and caused by the parasite protozoan *Trypanosoma cruzi* [1]. About 6 million people are infected by this parasite and at least 50,000 cases of annual deaths are reported worldwide, especially in Latin American countries, where the disease is endemic [2,3]. Most of the fatal cases are consequence of a severe infectious cardiomyopathy associated with intense and unbalanced Th1 immunological polarization, as well as, immunomediated (i.e., autoimmunity) and reactive myocardial damage; which courses with cardiomyocytolysis, myonecrosis, heart fibrosis, electromechanical dysfunction and cardiac failure [4,5].

It is well established that the direct destruction of the host cells by the parasite, and the secondary immunomediated lesions of the target organs are the most serious pathological events of Chagas's disease [6]. Thus, eliminating the parasite and adjusting the immune response are the two pillars of Chagas's disease treatment [7,8]. Currently, this treatment is limited to benznidazole and nifurtimox, whose antiparasitic and immunomodulatory potential are more effective in acute infections [9,10]. However, these drugs do not guarantee cure even in the acute phase of infections [11–14], and the therapeutic failure is even more frequent in the chronic phase of Chagas's disease [15]. In addition, both references drugs require prolonged administration [16]. Because of this, they induce dose-dependent systemic toxicity and serious side effects (i.e., vomiting, anorexia, hepatotoxicity, bone marrow depression, dermatitis, polyneuropathy), which determined about 60% of treatment discontinuation [17,18]. Thus, the limited pharmacological efficiency, drug toxicity and low adherence to the chemotherapy protocol are recognized as direct causes of therapeutic failure for both and unique available references drugs [15,19].

* Corresponding author at: Department of Parasitology and Pathology, Federal University of Alfenas, Alfenas, Minas Gerais 37130-001, Brazil.
E-mail address: ivo.caldas@unifal-mg.edu.br (I.S. Caldas).

https://doi.org/10.1016/j.intimp.2019.105961
Received 18 September 2019; Received in revised form 1 October 2019; Accepted 2 October 2019
Available online 01 November 2019

The high prevalence, morbidity, mortality, increasing of infection in non-endemic countries, and money spent with the prevention of Chagas's disease worldwide, indicate the need and urgency for new and effective therapeutic strategies against *T. cruzi* infection [20,21]. In this sense, active molecules derived from natural products with potent immunomodulatory and anti-*T. cruzi* activities has been reported *in vitro* [22]. However, their biotechnological, antiparasitic and immunobiological potential *in vivo* remains poor explored for the treatment of the Chagas's disease.

Sesquiterpene lactones (SL) are a chemical group of molecules with marked pharmacological potential due to a broad spectrum of bioactive effects, especially potent anti-inflammatory, analgesic, anti-microbial, anti-diabetic, and anti-parasitic activities [23,24]. *In vitro* evidence suggests that the trypanocidal effect of SL is potentially mediated by the inhibition of trypanothione reductase, an enzyme essential to protect *T. cruzi* against molecular oxidative damage and death [25]. Sesquiterpene lactones also exerts potent anti-inflammatory effects [26], desirable properties in anti-*T. cruzi* drugs, since the sustained pro-inflammatory response is a major cause of excessive or uncontrolled inflammation, heart damage and death in patients with Chagas's disease [27,28].

As SL combine antiparasitic and anti-inflammatory potential, we used and *in vitro* and *in vivo* integrated approach to investigate the immunomodulatory, antiparasitic, cytotoxic and cardioprotective potential of the SL, tagitinin C (TC), administered alone or combined with benznidazole (Bz), a pro-oxidant drug used as a first-line treatment for Chagas's disease.

2. Materials and methods

2.1. Parasite strain and benznidazole

The *T. cruzi* Y strain (DTU II), which is well characterized as partially sensitive to experimental chemotherapy with Bz [29], was used in this study. The drug benznidazole (Bz) (compound N-benzyl-2- (2-nitroimidazole) acetamide) was supplied by the Pharmaceutical Laboratory of Pernambuco – LAFEPE (Recife, Pernambuco, Brazil).

2.2. Plant material and tagitinin C

The major sesquiterpene lactone TC was isolated from leaves of *Tithonia diversifolia* collected at 21°25′44.76″S 45°56′22.2″W. The voucher specimen D.A. Chagas-Paula 24 was deposited in the herbarium of Federal University of Alfenas. The access was registered on the National System of Genetic Resource Management and Associated Traditional Knowledge, access number A913C3F. The plant material was dried in a circulating air oven at 40 °C for seven days. Isolation of TC was performed as previously described [30] and the identification of TC was performed by ^{1}H and ^{13}C one-dimensional NMR spectroscopy and bidimensional HMQC and HMBC spectroscopy compared with those from authentic materials and data from the literature [30,31]. These spectra were obtained in a spectrometer (Bruker, 300 MHz) with samples diluted in $CDCl_3$ (Sigma Aldrich). The plant species and chromatogram profile of tagitinin C are shown in Fig. 1. Subsequently, TC was dried and stored at −20 °C, and kept covered from light until the time of the assays.

2.3. Trypanocidal activity in vitro

2.3.1. Anti-parasitic assays

Epimastigote forms were cultured in Liver Infusion Triptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) until the concentration of 1.5×10^6/mL. The natural substance TC were evaluated at eight decreasing concentrations (1:2), in triplicate, with the initial concentration being 10 μg/mL for TC and 150 μg/mL for Bz. For the evaluation of the combination of TC and Bz fixed proportions of both was applied: 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. A volume of 150 μL/well of each dilution was added to a 96-well sterile polystyrene plate. Negative controls (containing LIT medium), positive controls (containing the LIT medium and the parasite) and controls containing the medium and drug in the absence of the parasite were added to evaluate the potential of the compound to reduce the dye. After incubation for 72 h in a BOD oven, at 20 °C, 20 μL of resazurin was added; after further incubation for 12 h, the reaction was read on a spectrophotometer at 570 nm and 600 nm. From the percent inhibition of each concentration of the substances. The IC_{50} and IC_{50} FICs were calculated with the aid of the CalcuSyn program, and dose-response curves were constructed in Graph Pad Prism 5.0.

2.3.2. Cytotoxicity assay

Cells from the H9c2 line (American Type Culture Collection, ATCC: CRL 1446) from neonatal rat cardiomyoblasts were cultured at 37 °C and 5% CO_2 in DMEM medium ($NaHCO_3$, HEPES and Penicillin/Streptomycin) supplemented with 10% FBS. The concentration of $1x10^3$ cells/mL was added in duplicate in a 96-well plate and after incubation for 24 h at 37 °C, 5% CO_2, the medium was replaced with 200 μL/well of a new medium containing seven different concentrations of the drug Bz and substance TC (1:2 dilution starting at the concentration of 200 μg/mL). Negative controls (medium) and positive controls (medium + cells) were used in each plate. After 72 h of incubation, 20 μL/well of resazurin was added and after 12 h the reading was carried out using a spectrophotometer at an absorbance of 570 nm and 600 nm. After calculating the percent inhibition of each drug concentration and of the substances tested, the Graph Pad Prism program 5.0 was used to calculate CC_{50} (concentration of substances and drug reducing 50% of cell viability).

2.4. T. cruzi infection and chemotherapy strategy in vivo

2.4.1. Animals and infection

Five groups of 10 female Swiss mice were used, weighing approximately 37 g, from the Central Biotherm of the Federal University of Alfenas (UNIFAL-MG). Four groups of animals were inoculated intraperitoneally with 1000 blood trypomastigotes obtained from previously infected animals. Parasitemia and parasitic load were used and primary markers of infection [32]. The experiments were carried out in accordance with the principles of the Brazilian College of Animal Experimentation (COBEA) and approved by the Animal Research Ethics Committee of UNIFAL-MG (CEUA n° 646/2015).

2.4.2. Antiparasitic chemotherapy protocol

Treatment was started on the first day that the mice presented parasitemia confirmed by a fresh blood test. Three groups of animals received treatment orally for 20 consecutive days, once a day, using a metal cannula, according to the protocol described by Filardi and Brener [29]. A volume of 0.1 mL of Bz (at dose of 100 mg/kg body weight) was administered to each mouse of the drug-treated group, while 0.1 mL of TC (at dose of dosage of 10 mg/kg of body weight) was administered to each animal; to the group receiving treatment in combination, each animal was first given 0.1 mL of TC (10 mg/kg) and then 0.1 mL of the drug Bz (100 mg/kg). As positive and negative controls, a group of infected and untreated animals and one uninfected and untreated group, respectively, were used. The Bz was macerated and subsequently resuspended in water using 1% carboxymethylcellulose, and TC was solubilized in 5% cremophor (Merck, São Paulo, SP) for the *in vivo* experiments.

2.5. Toxicity assay

From the beginning of the treatment, the animals were weighed weekly and the weight change was calculated by subtracting the initial weight (corresponding to the first day of treatment) from the final

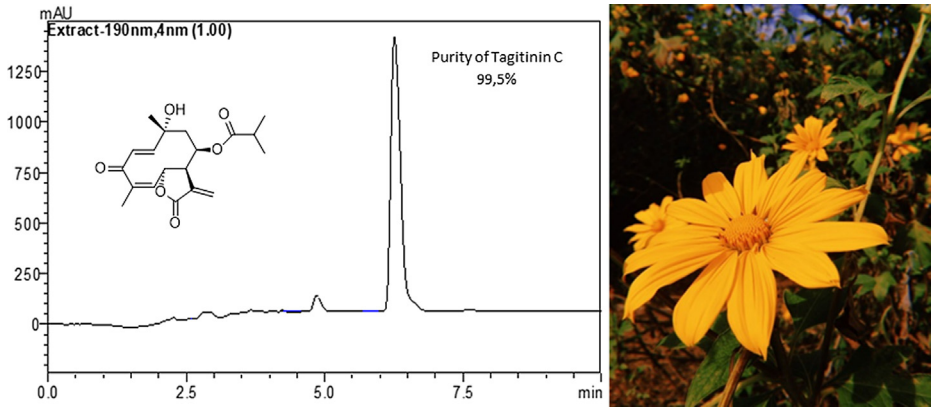

Fig. 1. Chromatogram of purity of the Tagitinin C (TC), its chemical structure and plant source image. Left image: Chromatographic profile of Tagitinin C-enriched *Tithonia diversifolia* extract fraction and molecular structure of TC. Right image: *T. diversifolia* plant species (Asteraceae family).

weight (corresponding to the thirtieth day after treatment). The levels of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified in the plasma of each animal using the Labmax Plenno automatic analyser following the manufacturer's guidelines (Labtest, Lagoa Santa, MG, Brazil) to evaluate if there is hepatic toxicity [33].

2.6. Analysis of parasitemia and parasite recrudescence: Fresh blood microscopic examination and hemoculture

Parasitemia was used as a direct marker of *T. cruzi* infection and to evaluate the progression of acute infection [32,34]. Parasitemia was evaluated daily by fresh blood examination according to the method described by Brenner [34]. Briefly, aliquots with 5 μL of blood collected from the tail tip of each animal were distributed in a glass slide and covered with a 22 mm × 22 mm (length × width) coverslips. Blood trypomastigotes was quantified in fifth random non-coincident microscopic fields, and the number of parasites by blood volume was estimated from the correction factor (CF = ×5000) of microscopic magnification. The number of parasites was registered and a time-dependent curve of parasitemia was plotted for all infected groups.

In animals with negative parasitemia, fresh blood examination was continued for 25 days after the end of treatment. In this period, the reappearance of blood trypomastigotes was considered an indicator of natural parasite recrudescence [11,12]. Parasite recrudescence was additionally evaluated by blood culture [35]. Briefly, moments before euthanasia, 200 μL of blood was collected in the retro-orbital venous sinus of each animal, added to 10 mL of LIT medium and maintained in a BOD incubator at 28 °C. For the detection parasite, aliquots of the blood culture (10 μL) was examined directly under a bright field microscope at 30, 45 and 60 days after the date of blood collection.

2.7. Screening for parasitological cure: serology and reverse transcription polymerase chain reaction quantitative (qPCR)

2.7.1. Serology

For detection of anti-*T. cruzi* antibodies of the IgG class by enzyme-linked immunosorbent assay (ELISA) [36]. The mean absorbance for ten negative control samples plus two standard deviations were used as the cut-off to discriminate positive and negative results. The reactivity index was calculated from dividing each sample by the cutoff value.

2.7.2. Real-time Polymerase Chain Reaction (PCR)

Quantitation of parasitic load by PCR was used as a direct marker of tissue parasitism by *T. cruzi,* as well as cure criteria [32,37]. Genomic DNA was purified from skeletal muscle fragments collected following euthanasia. DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega), with some modifications [37]. qPCRs were performed to amplify *T. cruzi* DNA using the TaqMan system (Applied Biosystems™) according to the manufacturer's instructions and using the primers *T. cruzi*, (CzFw 5′-CCACCATTCATAATTGGAAAC AAA-3′ and CzRv 5′-CTCGGCTGATCGTTTTCGA-3′) and murine TNF-α (TNF-F 5′-GCCCAGACCCTCACACTCA-3′ and TNF-R 5′-AACTGCCCTT CCTCCATCTTAAA-3′). It was also used the oligonucleotide probe for *T. cruzi* (5′-FAM-ACCACAACGTGTGATGC-3′-MGB-NFQ) and for TNF-α (5′-VIC-TAAGTGTTCCCACACCTC-3′-MGB-NFQ). Cycles of amplification were carried out in an ABI 7500 real-time PCR system from Applied Biosystems. The cycles consisted of an initial denaturation hold of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C with fluorescence acquisition. All samples were analyzed in duplicate, and negative samples and reagent controls were processed in parallel for each assay. The amplification efficiencies were determined automatically by the 7500 v2.3 software using the formula: Efficiency (E) = 10 (-1/slope), where slope corresponds to slope of the standard curve [38].

2.8. Anti-T. cruzi immunoglobulin assay

Blood samples (200 μL) were collected moments before euthanasia by cardiac puncture. Specific antibodies were detected according to Ref. [36]. Briefly, enzyme-linked immunosorbent assay plates were coated with *T. cruzi* antigen prepared from alkaline extraction of the *T. cruzi* Y strain at exponential growth in the LIT medium. Anti-mouse IgG and IgG1 peroxidase conjugated antibodies (Sigma Chemical Co., USA) were used.

2.9. Cytokines immunoassay

Cytokines immunoassays were performed using plasma from all experimental animals. Samples were evaluated in 96-well polystyrene plate by sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit and following the manufacturer's instructions (PeproTech, Rocky Hill, NJ, USA). The absorbance was analyzed in a microplate reader at 450 nm (ELx50, Bio-Tek Instruments, Winooski, Vermont, USA). Results were calculated using a standard curve of recombinant cytokines with known concentration.

2.10. Necropsy and heart microstructure

Cardiac fragments were collected at 50 days of infection and stored in 10% neutral formalin solution for 48 h, and subsequently dehydrated in ethanol. The fragments were diaphanized in xylene and embedded in paraffin. Serial sections (4 μm) were obtained with the aid of a microtome, which were later fixed on glass slides and stained with

hematoxylin and eosin (H&E). To assess the presence and severity of myocarditis, the counting of interstitial/inflammatory nuclei was performed in each image. Cell nuclei were quantified from 10 random non-coincident histological fields representative of all myocardial regions. The images were observed by using a 40 × objective lens (400× magnification) and scanned through the photomicroscope Axioscope A1 coupled with the image analysis software AxioVision (Carl Zeiss, Germany).

2.11. Statistical analyses

The fractional inhibitory concentrations (FICs) and the sums of FICs (ΣFICs) were calculated as follows: FIC of TC = 50% effective concentration (EC_{50}) of TC in combination/EC_{50} of TC pure (the same equation was applied to benznidazole) and ΣFICs = FIC TC + FIC benznidazole. An overall mean ΣFIC was calculated for each combination and used to classify the nature of the interaction. Isobolograms were constructed plotting the FIC of benznidazole against TC. Each curve represented the mean of two independent experiments. Data with parametric distribution were analyzed by unifatorial analysis of variance (ANOVA one-way) followed by the Tukey's test for multiple comparisons. Non-parametric results were compared by the Kruskal-Wallis test. Differences were considered significant if the $P \leq 0.05$.

3. Results

3.1. Tagitinin C has potent trypanocidal activity with additive effect when combined with benznidazole

First, we investigated the trypanocidal effect of TC against Y strain of *T. cruzi* epimastigotes and the cytotoxicity on H9c2 cells, compared to Bz (Table 1). The lactone TC showed a greater inhibition capacity against the epimastigote forms, with EC_{50} of 1.15 μM, an potency superior to that presented by the reference drug Bz, 35.81 μM. However, the cytotoxic concentration (6.54 μM) and the selectivity index (5.69 μM) of TC were not more favorable than Bz.

The TC and Bz interaction was assessed using a modified fixed-ratio isobologram method, and the data were analyzed at the 50% effective concentration (EC_{50}) level. The mean sums of fractional inhibitory concentrations (ΣFICs) of two independent experiments are presented in the Table 2, and representative isobolograms are shown in Fig. 2. The interaction was classified according to [39], where an FIC ≤ 0.5 indicates synergism; 0.5 < FIC ≤ 4 denotes additivity and FIC > 4 indicates antagonism. The interaction of TC with Bz was classified as an additive effect, based on the mean ΣFICs of 1.51 at the four drugs ratio Bz + TC tested (1:4, 2:3, 3:2, and 4:1).

3.2. Tagitinin C reduces blood parasitism and increases the cure rate when combined with benznidazole

Considering the additive effect observed *in vitro*, we assessed whether TC administered in combination with Bz is more effective than each drug alone in treating mice infected with *T. cruzi*. The fresh blood test performed from the fourth day after inoculation and until the 25th day after the end of the treatment allowed monitoring the natural suppression or reactivation of parasitemia to be observed. Thus, it was verified that the pre-patent period was six days in all groups (Fig. 3). Groups of animals treated with TC in combination with Bz and those treated with Bz in monotherapy were effective in inducing suppression of parasitemia in 100% of the mice by the second day of treatment, with no natural reactivation of parasites during the period of fresh blood examination (Fig. 3).

In the moment corresponding to the typical peak of parasitemia, which were observed on the infected control group, no parasites were observed in animals treated with TC alone or combined with Bz. Although TC-based monotherapy was not effective in suppressing parasitemia, a peak of parasitemia was observed with about 3500 blood trypomastigotes/0.1 mL, a much smaller number compared to

Table 1
In vitro antiparasitic, cytotoxic and selective potential of tagitinin C compared to the reference chemotherapy* against *Trypanosoma cruzi*.

	IC_{50} (μM)	CC_{50} (μM)	Selectivity index
Benznidazole* (Bz)	35.81 ± 6.95	713.5 ± 102	19.92
Tagitinin C (TC)	1.15 ± 0.20	6.54 ± 0.39	5.69

IC_{50}: Half maximal inhibitory concentration, CC_{50}: Half maximal cytotoxic concentration. Data are represented as mean and standard deviation.

Table 2
Anti-proliferative effects of benznidazole (Bz) and tagitinin C (TC) combined against epimastigotes of *Trypanosoma cruzi*.

Drug/TC ratio	Bz FIC	TC FIC	Σ FIC
Bz + TC (4:1)	1.42 ± 0.69	0.04 ± 0.04	1.46
Bz + TC (3:2)	1.28 ± 0.06	0.09 ± 0.06	1.37
Bz + TC (2:3)	1.52 ± 0.19	0.24 ± 0.19	1.76
Bz + TC (1:4)	1.03 ± 0.08	0.43 ± 0.28	1.46
Mean FIC in combination	1.31 ± 0.25	0.2 ± 0.14	1.51

FIC: fractional inhibitory concentrations at the IC50 level. Bz FIC and TC FIC: mean and standard deviation. Σ FIC: absolute values.

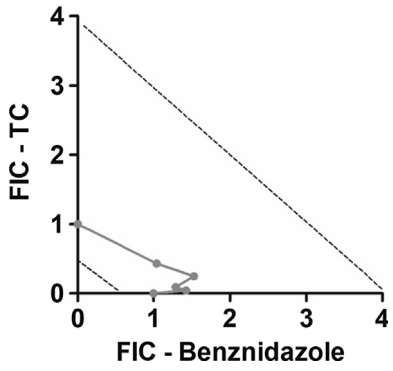

Fig. 2. Representative isobolograms for *in vitro* interactions between tagitinin C and benznidazole against *Trypanosoma cruzi*. Interactions are given at the EC_{50} level. Numbers on the axes represent normalized FICs (fractional inhibitory concentrations at the IC_{50} level) of Bz (X axis) and tagitinin C (TC, Y axis).

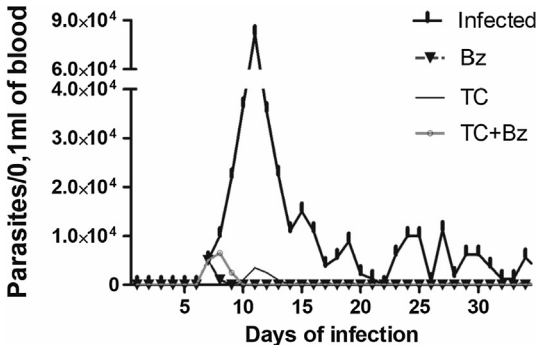

Fig. 3. Effect of combination therapy based on tagitinin C (TC) and benznidazole (Bz) or monotherapy on parasitemia in mice infected by *Trypanosoma cruzi*. Infected: untreated (n = 8*), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), TC + Bz: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). *Two animals died during the period of treatment. Data are represented as mean values. The lines represent the means values of parasitemia observed in mice infected by *T. cruzi*.

Table 3
Area under the curve of parasitemia and peak of parasitemia in *Trypanosoma cruzi*-infected mice treated with tagitinin C (TC) alone or combined with benznidazole (Bz).

Groups	AUC	Peak of parasitemia (Trypomastigotes/0.1 mL of blood)
Infected	3.41×10^5	8.15×10^4
Bz	6.0×10^3	*ND*
TC	1.85×10^4	3.5×10^3
Bz + TC	1.40×10^4	*ND*

ND: parasitemia not detected since was abolished by the treatment. Infected: animals infected and untreated. AUC: area under the curve of parasitemia. Infected: untreated (n = 8*), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), Bz + TC: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). *Two animals died during the period of treatment. Data are represented as mean values.

untreated infected animals (81,500 blood parasites/0.1 mL). Thus, although TC did not reach a complete parasitological negativation, its trypanocidal effect was confirmed by *in vivo* (Fig. 3 and Table 3). Thus, this corroborates the *in vitro* results (Table 1).

Post-therapeutic parasitological evaluation by fresh blood examination and/or hemoculture revealed that all infected untreated animals and those receiving TC presented active blood parasitism or positive hemoculture. Trypomastigotes were detected by hemoculture in 30% of Bz-treated mice, while no parasite recrudescence was detected in the blood of TC + Bz-animals. As expected, the absence of blood parasites was confirmed in uninfected animals (Fig. 4).

To confirm the efficacy of TC in monotherapy or combined with Bz in inducing parasitological cure in *T. cruzi*-infected mice, two independent tests were used: (i) PCR and (ii) serology for anti-*T. cruzi* IgG antibodies detection. The animals that showed negative results in both tests were considered cured (Table 4).

Spontaneous parasite recrudescence was observed during the fresh blood test in 5 animals treated with TC monotherapy, and the remaining animals showed positivity in the blood culture tests, evidencing some degree of therapeutic failure with TC-based monotherapy. The absence of natural reactivation in all animals treated with Bz was also verified, and the combination of TC and Bz was effective in inhibiting the parasite recrudescence in 100% of the animals. Thus,

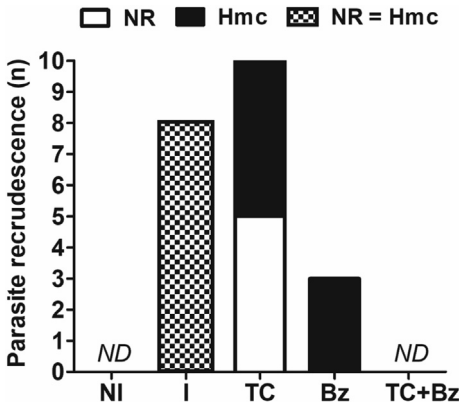

Fig. 4. Parasite recrudescence in *Trypanosoma cruzi*-infected mice treated with tagitinin C (TC) alone or combined with benznidazole (Bz). NR: Post-treatment natural recrudescence evaluated by fresh blood examination, Hmc: Recrudescence evaluated by hemoculture, NR + Hmc: Parasites equally detected by fresh blood examination and hemoculture. NI: uninfected untreated (n = 10), I: infected and untreated (n = 8*, recrudescence = 0), Bz: Benznidazole (n = 10, recrudescence = 7), TC: tagitinin C (n = 10, recrudescence = 10), TC + Bz: tagitinin C combined with benznidazole (n = 10, recrudescence = 10). *I: Two animals died during the period of treatment. ND: not determined. Data are represented as the absolute number of animals with positive parasite recrudescence in each diagnostic method.

Table 4
Parasitological cure in mice infected by *T. cruzi* and treated with benznidazole (Bz) and tagitinin C (TC) in monotherapy or in combination.

Three-step cure criteria	Bz	TC	Bz + TC	Infected
	n = 10	n = 10	n = 10	n = 10*
Dosage of IgG class antibodies (+)	03/10	10/10	0/10	8/8
Positive PCR	03/10	10/10	0/10	8/8
Cure rate	70%	0%	100%	0%

* Two infected and untreated animals died during the period of treatment. Infected: untreated (n = 8*), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), Bz + TC: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). Data are represented as the absolute number and the percentage of animals with parasitological cure in each diagnostic method.

following the curing criteria, all animals of these two groups were submitted to blood culture. Among the animals treated with Bz and submitted to blood culture, 30% (3/10) presented positive results. However, the association between TC and Bz presented the best result among all therapeutic strategies, and no positivity was observed among the 10 animals submitted to this test.

Thus, serology and PCR confirmed the results of the two previous tests of curing control, where 30% of the animals treated with Bz showed positive reaction for anti-*T. cruzi* IgG antibodies, indicating that 7 animals were cured. And, the combination TC and Bz presented more satisfactory results than Bz-based monotherapy, where serology and PCR results showed that all animals in this group (n = 10) were negative. These results evidenced the benefit of the treatment in combination, since 100% parasitological cure was obtained, through *in vivo* experiment to this treatment (TC + Bz).

3.3. Despite its lower selectivity index in vitro, tagitinin C was well tolerated in vivo

Throughout the experimental period, no behavioral and/or physical changes were detected in the experimental groups treated with all therapeutic regimens. As indicated in Fig. 5, the treatments did not induce a significant decrease in animals' body mass, since a similar profile was observed when compared to infected and untreated animals. There is a slight reduction in body mass in all infected groups from the second week of the experiment. Increased or stable body mass was observed at the end of treatment. This finding indicated that the body weight change is due to *T. cruzi* infection and not due to the treatment. No mortality was observed among the groups of infected and treated animals. The death of two infected mice was observed on the 14th and 18th days of infection only on the group that were not submitted to treatment.

Subsequently, serum levels of AST and ALT enzymes were assessed as markers of hepatic toxicity. There was a significant increase in AST level in all infected groups (Fig. 5). ALT serum levels was reduced in all *T. cruzi*-infected animals who received the different treatments. Only infected and untreated animals presented a statistical difference in relation to the group of uninfected animals, whereas the groups of animals treated with Bz, TC and TC combined with Bz showed lower levels of ALT when compared to infected and untreated animals. Together, the results demonstrate the good tolerability of TC treatments, either alone or in combination with Bz.

3.4. The antiparasitic activity of tagitinin C (TC) is accompanied by anti-inflammatory activity

The animals that underwent Bz and TC treatments in monotherapy showed high IFN-γ production, which was similar to the levels presented by infected untreated animals (Fig. 6). The levels detected in these animals were significantly higher in relation to uninfected

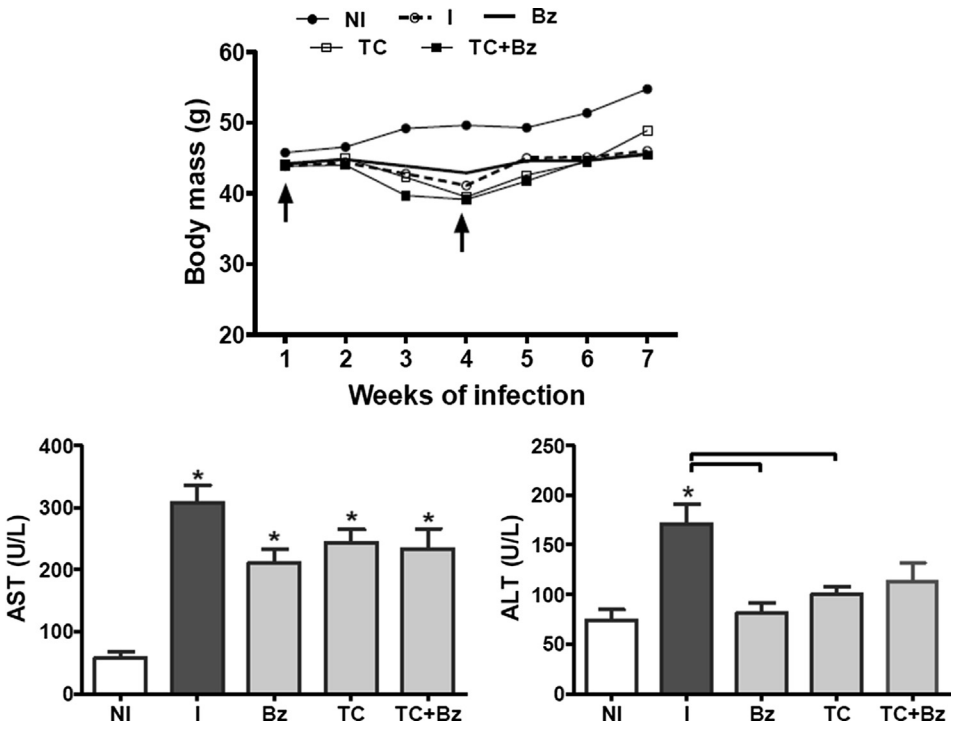

Fig. 5. Body weight and hepatic function enzymes in mice infected by *T. cruzi* and treated with benznidazole (Bz) and tagitinin C (TC) in monotherapy or in combination. AST: aspartate aminotransferase, ALT: alanine aminotransferase. Infected: untreated (n = 8*), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), TC + Bz: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). Body mass: mean values. AST and ALT: mean and standard deviation. * Statistical difference in relation to uninfected animals. The bars that connect the columns represent statistical differences between the groups (P ≤ 0.05).

animals. Interestingly, the combination of TC and Bz was the only treatment strategy effective in reducing IFN-γ production. In monotherapy, Bz was not effective in reducing TNF-α production, with values similar to those observed in infected and untreated animals. Reduced TNF-α levels were obtained when TC was used alone, and a similar result was obtained in animals treated with the combination of TC and Bz, which was the most satisfactory treatment strategy tested. These two groups were the only ones that present similar TNF-α production in relation to uninfected animals. In relation to the anti-inflammatory cytokine IL-10, animals that received TC treatments both in combination and in monotherapy had the highest IL-10 levels, and no significant difference was observed in relation to the group untreated.

The evaluation of the humoral immune response showed the higher production of IgG antibodies in infected untreated group than treated groups (Fig. 7). Animals treated with TC in monotherapy had intermediate IgG levels compared to infected untreated animals and those detected in uninfected animals. However, they presented higher IgG levels than animals treated with the combination of TC and Bz. Animals treated with this combination also had a profile similar to animals treated with Bz alone. The untreated infected animals also had high IgG1 titers, which was higher than titers from the other groups. The results presented by the animals that were treated with TC caught the researcher's attention, since the IgG1 levels were considered similar to those presented by the animals treated with Bz. The three treated

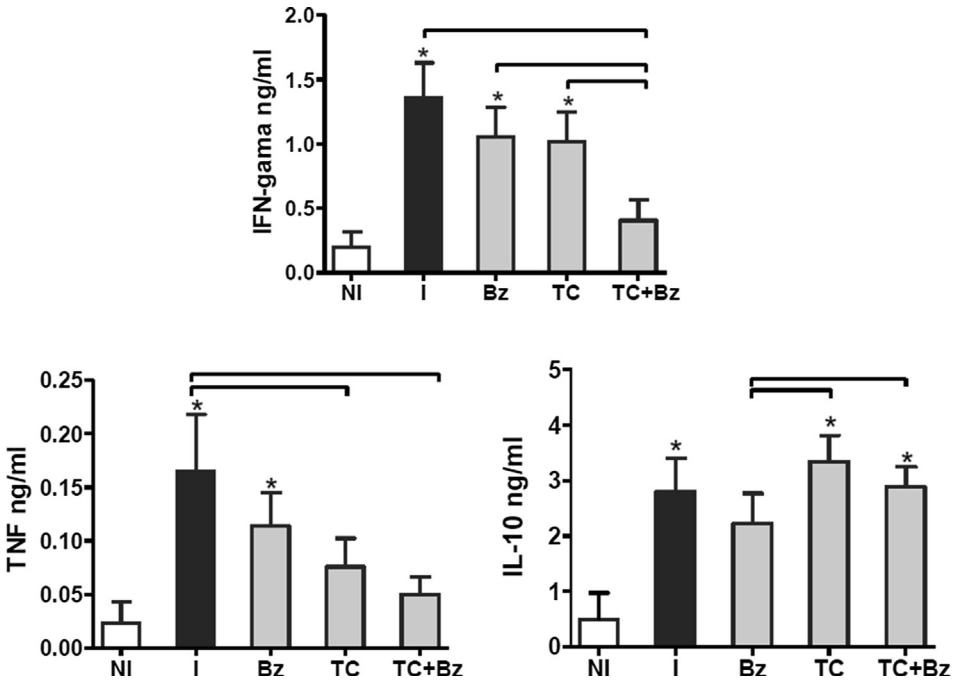

Fig. 6. Cytokines plasma levels in control and *T. cruzi*-infected mice treated with benznidazole (Bz) and tagitinin C (TC) in monotherapy or in combination. Infected: untreated (n = 8*), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), TC + Bz: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). Body mass: mean values. Data are represented as mean and standard deviation. *Statistical difference in relation to uninfected animals. The bars that connect the columns represent statistical difference between the groups (P ≤ 0.05).

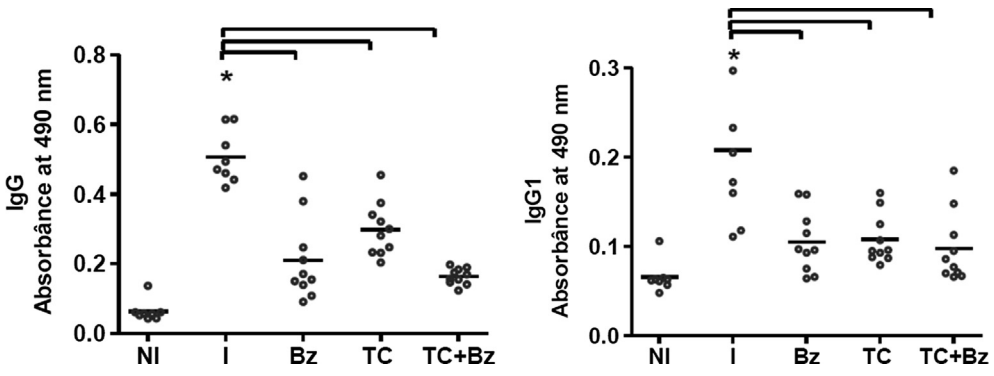

Fig. 7. Anti-*Trypanosoma cruzi* immunoglobulin G (IgG) plasma levels in control and *T. cruzi*-infected mice treated with benznidazole (Bz) and tagitinin C (TC) in monotherapy or in combination. Infected: untreated (n = 8), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), TC + Bz: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). Body mass: mean values. In each group, the points indicate the result obtained for each animal, and the horizontal lines indicate average values for the whole group. *Statistical difference in relation to uninfected animals. The bars that connect the columns represent statistical difference between the groups (P ≤ 0.05).

groups had similar IgG1 production when compared to the group of uninfected animals.

3.5. Tagitinin C is able to reduce myocarditis, especially when combined with benznidazole

All treatment regimens were effective in reducing the number of mononuclear and polymorphonuclear inflammatory cells, being significantly smaller when compared to infected untreated animals (Figs. 8 and 9). The lowest mean number of inflammatory nuclei were quantified among the TC treated animals alone or TC in combination with the Bz. However, these values were similar to those observed in Bz-treated animals. Figs. 8 and 9 show a marked inflammatory infiltrate in infected untreated animals, with a reduced number of inflammatory cells in all treated groups.

4. Discussion

Considering the immunomodulatory and antiparasitic evidence attributed to SL [26], we used *in vitro* and *in vivo* assays to explore the trypanocidal, immunomodulatory, and cardioprotective potential of TC. Direct antiparasitic effects of SL, including TC, were clearly demonstrated against different strains of *Leishmania* sp. [40], *Plasmodium falciparum* [41] and *L. braziliensis* [42]. As expected, we also identified a potent trypanocidal activity against *T. cruzi* in culture and in a murine model of Chagas' disease. From *in vitro* findings of EC50, we identified that TC had a trypanocidal effect about 31 times more potent than Bz. This remarkable efficacy was not surprising since potent anti-*T. cruzi* effects *in vitro* have been reported for other SL such as, helenalin, mexicanin, neurolenin B, psilostachyin C, goyazensolide, eremantholide C, and lychnopholide [43–45].

Through dose-response curves, our *in vitro* findings indicated that when used as monotherapy, Bz and TC induced the lowest percentage inhibition on epimastigote proliferation. However, as concentrations increased in the combinations of Bz and TC, the inhibition rates of parasite proliferation were also higher. This finding indicated an additive effect, which was confirmed using the FIC method [46]. Thus, the interaction between Bz and TC was positive, since the values of the sum of the FICs were close to the line of additivity and very distant from the line of antagonism.

By using an incremental approach *in vivo*, we developed a short pilot study to determine the lowest dose of TC effective in controlling parasitemia in animals infected with a virulent *T. cruzi* stock partially resistant to Bz. From this screening, 10 mg/kg/day TC was selected as the best dose, since induced greater trypanocidal activity than 5 mg/kg/day and a similar activity to 50 mg/kg/day. After this selection, we investigated if and to what extent the antiparasitic effect of TC alone and combined with Bz could also be obtained in animals infected with *T. cruzi*. For this, we used a standardized 20-day treatment protocol [11,12] administering TC (10 mg/kg) and Bz (100 mg/kg) as monotherapy and combined. Bz was used as the reference antitrypanosomal, since it is the drug of first choice for the treatment of Chagas's disease [20]. As TC showed to be more potent than Bz on the *in vitro* experiment, a smaller dose than Bz dose was adopted as rational approach to ensure the *in vivo* antiparasitic potential with a reduced risk of systemic toxicity.

Indeed, TC monotherapy was effective in reducing the number of circulating parasites in a dose 10 fold lower than Bz corroborating the *in vitro* results. The amount of parasites in the peak of parasitemia was very low after treatment with TC monotherapy, and a mean of 2.6 doses of TC were sufficient to completely suppress the parasitemia. However, TC was not effective in inhibiting the reactivation of the parasitemia, since fresh blood examination indicated that 50% of the animals presented natural post-therapeutic parasite recrudescence in hemoculture test. As expected, TC and Bz in combination induced a superior antitrypanosomal effect compared to all other groups, including Bz monotherapy. In addition, to the complete suppression of the parasitemia, this combination was effective in inhibiting parasites recrudescence in blood cultures. All animals submitted to this treatment also presented negative serological and PCR results, indicating 100% of parasitological cure. This result was superior to that achieved with the reference drug, Bz monotherapy, which induced cure in 70% of the animals, a result consistent with the cure rates reported in murine models of Chagas's disease treated with this drug 47,48].

Although Bz and SL can induce systemic toxicity, no adverse reactions were observed in this study. Besides, all groups receiving TC and Bz alone or combined had no weight loss and presented reduced ALT and AST levels at the end of the experiment compared to untreated animals. Thus, our chemotherapy protocol was well tolerated in all groups, indicating that the doses of Bz and TC was properly adjusted and did not induce morphofunctional liver damage [49]. All infected groups showed increased levels of AST compared to uninfected control animals, supporting evidence that organ damage is potentially linked to infection rather than to treatments. Since AST is also found in muscle tissue, higher levels of this enzyme could be related to myocarditis and not to hepatocyte lesions. This proposition is coherent with the classical tropism of *T. cruzi* strains, including Y, which is often associated with an intense parasitism of muscle cells [50,51]. Thus, those results indicated low or absent toxicity of the active doses of all treatments evaluated in mice, included the association of TC and Bz, which showed the best activity and cure. This corroborates study of Passoni et al. [52], which showed toxicological safety of low doses of SL extract in a sub chronic treatment in rats (10 mg/kg, 90 days).

As expected, animals receiving Bz alone or combined with TC presented a drastic reduction of IgG levels, which was consistent with a clear efficacy of both therapeutic strategies in reducing or abolishing parasitic load. However, TC-treated animals presented did not reduced IgG production completely. This finding reinforce the results of

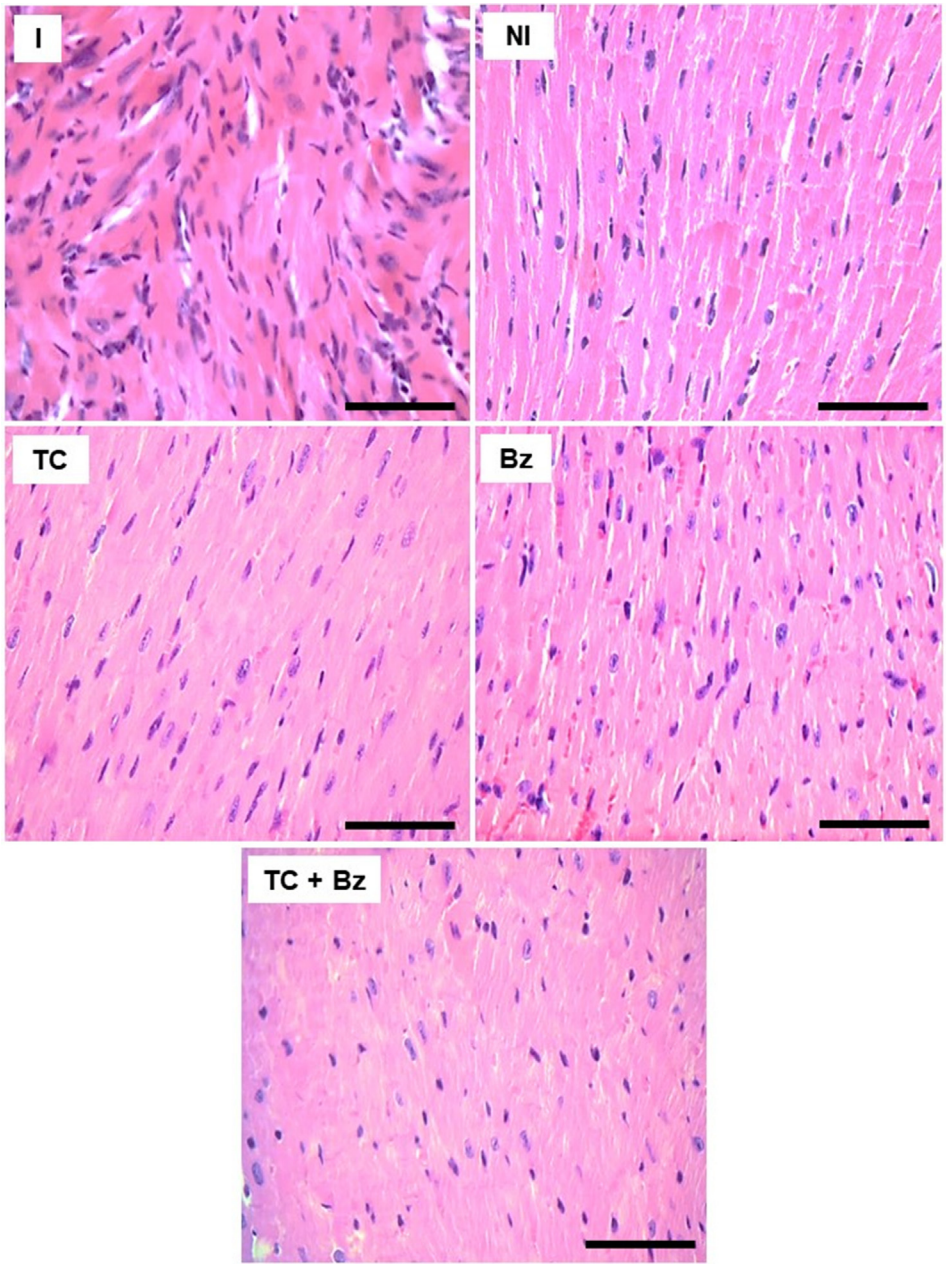

Fig. 8. Representative photomicrography's of the myocardium from uninfected and *T. cruzi*-infected mice treated with benznidazole (Bz) and tagitinin C (TC) in monotherapy or in combination (Hematoxylin and Eosin staining, scale bar = 60 μm). I: infected untreated (n = 8), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), TC + Bz: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10).

parasitological cure, indicating that TC alone was unable to assure sufficient parasitological control to abolish the humoral response.

In addition, all treated groups significantly decreased the level of IgG1. The IgG1 levels have been consistently used as predictors of the severity of inflammatory heart injury and relevant parasitological marker of cure. The, high IgG1 reactivity index has been closely correlated with intense myocarditis in dogs and mice infected with *T. cruzi* [8]. This relation is supported by autoimmune processes often identified in infected hosts, since cross-reaction of anti-*T. cruzi* antibodies with myocardial antigens (i.e., anti-adrenergic and muscarinic receptor; anti-myosin) has been linked to immunomediated heart damage [53,54]. Thus, in this sense, even in the absence of parasitological cure, the treatment with TC alone was beneficial in reducing heart inflammatory damage in *T. cruzi*-infected mice, which was clearly observed on myocardial histological results. Besides, this effect can be potentiated by the well known anti-inflammatory properties attributed to TC [26,30]. Thus, a cardioprotective effect linked to IgG downregulation is an issue that remains poorly understood and requires further investigation, but cannot be disregarded, and the results of this study, including from TC group, which had not parasitological cure, corroborates this linkage.

The imunomodulador effect of TC alone and especially combined with Bz was also observed on the differential production of cytokines such and IFN-γ, TNF and IL-10. The IL-10 levels and downregulation of IFN-γ and TNF was coherent with the anti-inflammatory potential of Bz [55]and TC [26,30,56]. As a potent anti-inflammatory mediator, IL-10 exerts an important counter-regulatory role in Chagas's disease, modulating the intensity of the inflammatory process triggered by *T. cruzi* [57]. In this sense, an adequate balance between IL and 10 and Th1 profile is essential to ensure effective parasitic control, minimizing the risk of immunomediated tissue damage [57]. As typical Th1 cytokines, IFN-γ and TNF-α exerts its antiparasitic effects mainly by stimulating

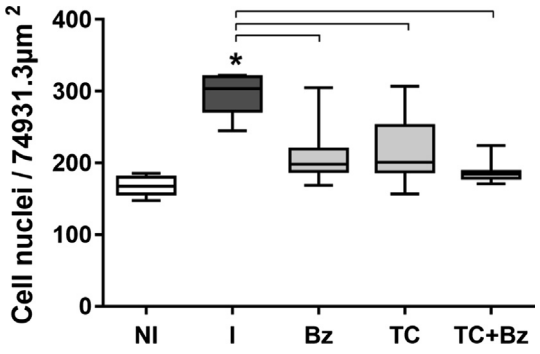

Fig. 9. Myocardial cellularity in *T. cruzi*-infected mice treated with benznidazole (Bz) and tagitinin C (TC) in monotherapy or in combination (Hematoxylin and Eosin staining, 400× magnification). I: infected and untreated, (n = 8), Bz: 100 mg/kg benznidazole (n = 10), TC: 10 mg/kg tagitinin C (n = 10), TC + Bz: 10 mg/kg tagitinin C combined with100 mg/kg benznidazole (n = 10). Body mass: mean values. Data are represented as median and interquartile intervals. *Statistical difference in relation to uninfected animals (P ≤ 0.05). In the graphic, the bars represent a statistical difference (P ≤ 0.05) between the groups.

macrophages activation and nitric oxide (NO) production, a non-radical nitrogen reactive specie highly toxic to *T. cruzi* [58–60]. However, it is recognized that exacerbated production of proinflammatory cytokines and NO are also correlated with marked tissue damage and disease severity in unbalanced inflammatory processes [61]. Thus, by attenuates IFN-γ and TNF-α levels without affecting IL-10 biosynthesis, Bz and TC probably can attenuate immunomediated heart damage, in addition decrease cardiomyocytes death caused by parasitism, since also eliminates the parasites. It is important to highlight that both parasitism and immune response play a central role in the pathophysiology of Chagas's cardiomyopathy [62]. As already demonstrated for Bz, attenuation of the myocarditis is expected when the parasitological control is obtained with antiparasitic chemotherapy, which reduce the antigen load that triggers the inflammatory process [63,64].

Taken together, our findings indicated that TC is potentially useful as an adjuvant strategy to the reference chemotherapy used in the treatment of Chagas's disease. From an additive effect, the trypanocidal, immunomodulatory and cardioprotective effects conferred by TC combined with Bz more prominent activity than those achieved when Bz was administered as monotherapy. This combination was well tolerated and reached 100% cure in *T. cruzi*-infected mice, a remarkable result compared with 70% cure obtained in the group treated with the reference drug Bz alone. The pharmacological and biotechnological potential of TC as an anti-*T. cruzi* drug is not entirely understood. Thus, a future step could be to seek strategies that aim to increase the therapeutic efficacy of this natural molecule in monotherapy from studies focused on pharmaceutical formulations, pharmacodynamics, pharmacokinetics and biological assays or even its structural modifications.

Acknowledgements

The authors are grateful to the support provided by Fundação do Amparo à Pesquisa do Estado de Minas Gerais, Brazil (FAPEMIG, processes APQ-01895-16 e PPM-00077-18), Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq, processes 303972/2017-3 e 423594/2018-4), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES, finance code 001).

Declaration of Competing Interest

None.

References

[1] A.R.L. Teixeira, C. Gomes, A.C. Rosa, P.F. Araujo, C.E. Anunciação, E. Silveira-Lacerda, et al., Prevention and control of Chagas disease-an overview, Int. STD Res. Rev. 7 (2) (2018) 1–15.
[2] E. Pinheiro, L. Brum-soares, R. Reis, Chagas disease: review of needs, neglect, and obstacles to treatment access in Latin America, Rev. Soc. Bras. Med. Trop. 50 (3) (2017) 296–300.
[3] World health organization: Chagas disease (American trypanosomiasis), (2019) [accessed 01 may 2019]. https://www.who.int/chagas/home_more/en/.
[4] A.R.L. Teixeira, M.M. Hecht, M.C. Guimaro, A.O. Sousa, N. Nitz, Pathogenesis of Chagas' Disease: Parasite persistence and autoimmunity, Clin. Microbiol. Rev. 24 (3) (2011) 592–630.
[5] K.M. Bonney, D.J. Luthringer, S.A. Kim, et al., Pathology and pathogenesis of Chagas heart disease, Annu. Rev. Pathol. 14 (1) (2019) 421–447.
[6] A. Rassi Jr, J.A. Marin, Neto, A. Rassi, Chronic Chagas cardiomyopathy: a review of the main pathogenic mechanisms and the efficacy of aetiological treatment following the Benznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT) trial, Memorias do Instituto Oswaldo Cruz 112 (3) (2017) 224–235.
[7] L.M. Bahia-Oliveira, J.A. Gomes, J.R. Cancado, et al., Immunological and clinical evaluation of chagasic patients subjected to chemotherapy during the acute phase of Trypanosoma cruzi infection 14–30 years ago, J. Infect. Dis. 182 (2) (2000) 634–638.
[8] I.S. Caldas, L. de F. Diniz, P.M. da M. Guedes, Á. F. da S. do Nascimento, L. M. da C. Galvão, W. G. de Lima, S. Caldas, M.T. Bahia, Myocarditis in different experimental models infected by *Trypanosoma cruzi* is correlated with the production of IgG1 isotype, Acta Tropica 167 (2017) 40–49.
[9] N. Ãnez, H. Carrasco, H. Parada, et al., Myocardial parasite persistence in chronic chagasic patients, Am. Soc. Tropical Med. Hygiene 60 (5) (1999) 726–732.
[10] C.A. Morillo, J.A. Marin-Neto, A. Avezum, S. Sosa-Estani, A. Rassi, F. Rosas, E. Villena, et al., Randomized trial of benznidazole for chronic chagas' cardiomyopathy, N. Engl. J. Med. 373 (2015) 1295–1306.
[11] I.S. Caldas, A. Talvani, S. Caldas, et al., Benznidazole therapy during acute phase of Chagas disease reduces parasite load but does not prevent chronic cardiac lesions, Parasitol. Res. 103 (2) (2008) 413–421.
[12] S. Caldas, F.M. Santos, M. Lana, L.F. Diniz, G.L. Machado-Coelho, V.M. Veloso, M.T. Bahia, *Trypanosoma cruzi*: acute and long-term infection in the vertebrate host can modify the response to benznidazole, Exp. Parasitol. 118 (2008) 315–323.
[13] P.M. Guedes, G.K. Silva, F.R. Gutierrez, et al., Current status of Chagas disease chemotherapy, Expert Rev. Anti-infective Ther. 9 (5) (2011) 609–620.
[14] H. Parada, H.A. Carrasco, N. Añez, C. Fuenmayor, I. Inglessis, Cardiac involvement is a constant finding in acute Chagas' disease: a clinical, parasitological and histopathological study, Int. J. Cardiol. 60 (1) (1997) 49–54.
[15] J.A. Pérez-Molina, A. Pérez-Ayala, S. Moreno, et al., Use of benznidazole to treat chronic Chagas' disease: a systematic review with a meta-analysis, J. Antimicrob. Chemother. 64 (6) (2009) 1139–1147.
[16] R. Paucar, E. Moreno-Viguri, S. Pérez-Silanes, Challenges in chagas disease drug discovery: a review, Curr. Med. Chem. 23 (2016) 3154–3170.
[17] A.M. Hasslocher-Moreno, P.E.A.A. do Brasil, A.S. de Sousa, S.S. Xavier, M.C. Chambela, G.M. Sperandio da Silva, Safety of benznidazole use in the treatment of chronic Chagas' disease, J. Antimicrob. Chemot. 67 (2012) 1261–1266.
[18] J. Bermudez, C. Davies, A. Simonazzi, J. Pablo Real, S. Palma, Current drug therapy and pharmaceutical challenges for Chagas disease, Acta Trop. 156 (2016) 1–16.
[19] M.O. Wiens, S. Kanters, E. Mills, A.A. Peregrina Lucano, S. Gold, D. Ayers, A. Krolewiecki, Systematic review and meta-analysis of the pharmacokinetics of benznidazole in the treatment of chagas disease, Antimicrob. Agents Chemother. 60 (12) (2016) 7035–7042.
[20] J.R. Coura, S.L. De Castro, A critical review on chagas disease chemotherapy, Mem. Inst. Oswaldo Cruz 97 (2002) 3–24.
[21] J.C.P. Dias, J.R. Coura, M.A.S. Yasuda, The present situation, challenges, and perspectives regarding the production and utilization of effective drugs against human Chagas disease, Rev. Soc. Bras. Med. Trop. 47 (2014) 123–125.
[22] N. Miranda, A.P. Gerola, C.R. Novello, T. Ueda-Nakamura, S. de Oliveira Silva, B.P. Dias-Filho, N. Hioka, J.C.P. de Mello, C.V. Nakamura, Pheophorbide a, a compound isolated from the leaves of Arrabidaea chica, induces photodynamic inactivation of *Trypanosoma cruzi*, Photodiagn. Photodyn. Ther. 19 (2017) 256–265.
[23] T.J. Schmidt, R. Brun, G. Willuhn, S.A. Khalid, Anti-trypanosomal activity of helenalin and some structurally related sesquiterpene lactones, Planta Med. 68 (8) (2002) 750–751.
[24] C. Di Giacomo, L. Vanella, V. Sorrenti, R. Santangelo, I. Barbagallo, G. Calabrese, C. Genovese, S. Mastrojeni, S. Ragusa, R. Acquaviva, Effects of *Tithonia diversifolia* (Hemsl.) A. Gray extract on adipocyte differentiation of human mesenchymal stem cells, PLoS One 10 (4) (2015) e0122320.
[25] V.P. Sülsen, V. Puente, D. Papademetrio, A. Batlle, Mode of action of the sesquiterpene lactones psilostachyin and psilostachyin C on *Trypanosoma cruzi*, *PLoS ONE* 11 (3) (2016) e0150526.1–14.
[26] D.A. Chagas-Paula, R.B. Oliveira, B.A. Rocha, F.B. Da Costa, ethnobotany, chemistry, and biological activities of the genus *Tithonia* (asteraceae), Chem. Biodivers. 9 (2) (2012) 210–235.
[27] C.S. Rosenberg, D.L. Martin, R.L. Tarleton, CD8+ T cells specific for immunodominant trans-sialidase epitopes contribute to control of *Trypanosoma cruzi* infection but are not required for resistance, J. Immunol. 185 (1) (2010) 560–568.
[28] P.M. de A. Vieira, A.F. Francisco, E.M. de M. Machado, N.C. Nogueira, K. da S. Fonseca, A.B. Reis, Different infective forms trigger distinct immune response in experimental chagas disease, Plos One 7 (3) (2012) e32912.

[29] L.S. Filardi, Z. Brener, Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease, Trans. R. Soc. Trop. Med. Hyg. 81 (1987) 755–759.
[30] A.E. Abe, C.E. de Oliveira, T.M. Dalboni, D.A. Chagas-Paula, B.A. Rocha, R.B. de Oliveira, T.H. Gasparoto, F.B. Da Costa, A.P. Campanelli, Anti-inflammatory sesquiterpene lactones from *Tithonia diversifolia* trigger different effects on human neutrophils, Revista Brasileira de Farmacognosia 25 (2) (2015) 111–116.
[31] R.N. Baruah, R.P. Sharma, K. Madhusudanan, G. Thyagarajan, W. Herz, R. Murari, A new melampolide from Sigesbeckia orientalis, Phytochemistry 18 (6) (1979) 991–994.
[32] I.S. Caldas, E.G. Santos, R.D. Novaes, An evaluation of benznidazole as a Chagas disease therapeutic, Expert Opin. Pharmacother. 20 (2019) 1797–1807.
[33] R.D. Novaes, M.V. Sartini, J.P. Rodrigues, R.V. Gonçalves, E.C. Santos, R.L. Souza, I.S. Caldas, Curcumin enhances the anti-*Trypanosoma cruzi* activity of benznidazole-based chemotherapy in acute experimental Chagas disease, Antimicrob. Agents Chemother. 60 (2016) 3355–3364.
[34] Z. Brener, Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*, Revista do Instituto de Medicina Tropical de São Paulo 4 (1962) 389–396.
[35] V.M. Veloso, P.M.M. Guedes, I.M. Andrade, I.S. Caldas, H.R. Martins, C.M. Carneiro, G.L.L. Machado-Coelho, et al., *Trypanosoma cruzi*: blood parasitism kinetics and their correlation with heart parasitism intensity during long-term infection of Beagle dogs, Memórias do Instituto Oswaldo Cruz 103 (6) (2008).
[36] A. Voller, D.E. Bidwell, A. Bartlett, Enzyme immunoassays in diagnostic medicine. Theory and practice, Bull. World Health Organ. 53 (1976) 55–65.
[37] S. Caldas, I.S. Caldas, L. de F. Diniz, W. G. de Lima, R. de P. Oliveira, A.B. Cecílio, I. Ribeiro, A. Talvani, M.T. Bahia, Real-time PCR strategy for parasite quantification in blood and tissue samples of experimental *Trypanosoma cruzi* infection, Acta Tropica 123 (3) (2012) 170–177.
[38] P. Stordeur, L.F. Poulin, L. Craciun, L. Zhou, L. Schandené, A. de Lavareille, S. Goriely, M. Goldman, Cytokine mRNA quantification by real-time PCR, J. Immunol. Methods 259 (1–2) (2002) 55–64.
[39] L.D.F. Diniz, J.A. Urbina, I.M. Andrade, A.L. Mazzeti, T.A.F. Martins, I.S. Caldas, A. Talvani, I. Ribeiro, M.T. Bahia, Benznidazole and Posaconazole in Experimental Chagas Disease: Positive Interaction in Concomitant and Sequential Treatments, PLOS Negl. Trop. Dis. 7 (8) (2013) 1–8 e2367.
[40] I.F. Wulsten, T.A. Costa-Silva, J.T. Mesquita, M.L. Lima, M.K. Galuppo, N.N. Taniwaki, S.E.T. Borborema, F.B. Da Costa, T.J. Schmidt, A.G. Tempone, Investigation of the anti-*Leishmania (Leishmania) infantum* activity of some natural sesquiterpene lactones, Molecules 22 (5) (2017) pii: E685.
[41] E. Goffin, E. Ziemons, P. De Mol, C. De Madureira, A.P. Martins, Ë. Cunha, G. Philippe, M. Tits, *In vitro* Antiplasmodial activity of *Tithonia diversifolia* and identification of its main active constituent: tagitinin C, Planta Med. 68 (6) (2002) 543–545.
[42] S.J. De Toledo, R.S. Ambrósio, H.C. Borges, V. Manfrim, G.D. Cerri, K.A. Cruz, B.F. Da Costa, *In Vitro* Leishmanicidal Activities of Sesquiterpene Lactones from *Tithonia diversifolia* against *Leishmania braziliensis* Promastigotes and Amastigotes, Molecules 19 (5) (2014) 6070–6079.
[43] V. Jimenez-Ortiz, S.D. Brengio, O. Giordano, C. Tonn, M. Sánchez, M.H. Burgos, M.A. Sosa, The trypanocidal effect of sesquiterpene lactones helenalin and mexicanin on cultured epimastigotes, J. Parasitol. 91 (1) (2005) 170–174.
[44] D. Chaturvedi, Sesquiterpene lactones: structural diversity and their biological activities, Opportunity Challenge Scope Natl. Prod. Med. Chem. 661 (2) (2011) 313–334.
[45] S. Saeidnia, A.R. Gohari, A. Haddadi, Biogenic trypanocidal sesquiterpenes: lead compounds to design future trypanocidal drugs – a mini review, DARU J. Pharm. Sci. 21 (1) (2013) 35.
[46] F.C. Odds, Synergy, antagonism, and what the chequerboard puts between them, J. Antimicrob. Chemother. 52 (1) (2003) 1.
[47] J.A. Urbina, R. Docampo, Specific chemotherapy of Chagas disease: controversies and advances, Trends Parasitol. 19 (11) (2003) 495–501.
[48] M. de F. Oliveira, A.T. Nagao-Dias, V.M.O. de Pontes, A.S. de S. Júnior, H.L.L. Coelho, I.C.B. Coelho, Tratamento Etiológico Da Doença De Chagas No Brasil, Revista de Patologia Tropical 37 (3) (2008) 209–228.
[49] J. Tarrant, D. Meyer, P. Katavolos, Use of optimized aminotransferase methods in regulated preclinical studies, Veter. Clin. Pathol. 42 (4) (2013) 535–538.
[50] M.T. Bahia, A.F.S. Nascimento, A.L. Mazzeti, et al., Antitrypanosomal activity of fexinidazole metabolites, potential new drug candidates for chagas disease, Antimicrob. Agents Chemother. 58 (8) (2014) 4362–4370.
[51] A.S. Souza, A.A. Alencar, On the tissular parasitism of *Trypanosoma cruzi* y strain in swiss mice, Revista do Instituto de Medicina Tropical de São Paulo 26 (6) (1984) 316–321.
[52] F.D. Passoni, R.B. Oliveira, D.A. Chagas-Paula, L. Gobbo-Neto, F.B. Da Costa, Repeated-dose toxicological studies of *Tithonia diversifolia* (Hemsl.) A. gray and identification of the toxic compounds, J. Ethnopharmacol. 147 (2) (2013) 389–394.
[53] F. Kierszenbaum, Chagas' disease and the autoimmunity hypothesis, Clin. Microbiol. Rev. 12 (2) (1999) 210–223.
[54] N. Gironès, M. Fresno, Etiology of Chagas disease myocarditis: autoimmunity, parasite persistence, or both? Trends Parasitol. 19 (1) (2003) 19–22.
[55] Á.C. Cevey, F.N. Penas, C.D. Alba Soto, G.A. Mirkin, N.B. Goren, IL-10/STAT3/SOCS3 Axis Is Involved in the Anti-inflammatory Effect of Benznidazole, Front. Immunol. 10 (2019) 1267.
[56] Abraham e Delgado Garcia, Guillermo, Constituents from Tithonia diversifolia: Stereochemical Revision of 2α-Hydroxytirotundin, J. Mexican Chem. Soc. 50 (4) (2006) 180–183.
[57] E. Cunha-Neto, L.G. Nogueira, P.C. Teixeira, R. Ramasawmy, S.A. Drigo, A.C. Goldberg, S.G. Fonseca, A.M. Bilate, J. Kalil, Immunological and non-immunological effects of cytokines and chemokines in the pathogenesis of chronic Chagas disease cardiomyopathy, Mem. Inst. Oswaldo Cruz 104 (2009) 252–258.
[58] G.N.R. Vespa, F.Q. Cunha, J.S. Silva, Nitric oxide is involved in control of *Trypanosoma cruzi*-induced parasitemia and directly kills the parasite in vitro, Infect. Immun. 62 (11) (1994) 5177–5182.
[59] A. Talvani, M.O.C. Rocha, L.S. Barcelos, Y.M. Gomes, A.L. Ribeiro, M.M. Teixeira, Elevated concentrations of CCL2 and tumor necrosis factor-alpha in chagasic cardiomyopathy, Clinical Infect. Dis.: Off. Publ. Infect. Dis. Soc. Am. 38 (7) (2004) 943–950.
[60] Tania C. de Araújo-Jorge, S.L.de Castro, Doença de chagas: manual para experimentação animal, Editora Fiocruz 20 (2012) 368.
[61] P.E.A. Souza, M.O.C. Rocha, E. Rocha-Vieira, C.A.S. Menezes, A.C.L. Chaves, K.J. Gollob, W.O. Dutra, Monocytes from patients with indeterminate and cardiac forms of Chagas' disease display distinct phenotypic and functional characteristics associated with morbidity, Infect. Immun. 72 (9) (2004) 5283–5291.
[62] P.R.L. Machado, L. Carvalho, M.I.A.S. Araújo, E.M. Carvalho, Mecanismos de resposta imune às infecções, Anais Brasileiros de Dermatologia 79 (6) (2004) 647–664.
[63] S. Revelli, C.L.E. Page, E. Piaggio, J. Wietzerbin, O. Bottasso, Benznidazole, a drug employed in the treatment of Chagas' disease, down-regulates the synthesis of nitrite and cytokines by murine stimulated macrophages, Clinical e Exp. Immunol. 118 (2) (1999) 271–277.
[64] W.O. Dutra, Z.M. DaLuz, J.R. Cançado, M.E. Pereira, R.M. Brigido-Nunes, L.M. Galvão, D.G. Colley, et al., Influence of parasite presence on the immunologic profile of peripheral blood mononuclear cells from chagasic patients after specific drug therapy, Parasite Immunol. 18 (11) (1996) 579–585.