

Treatment with synthetic lipophilic tyrosyl ester controls *Leishmania major* infection by reducing parasite load in BALB/c mice

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(Received 14 January 2016; revised 11 May 2016; accepted 16 May 2016; first published online 17 June 2016)

SUMMARY

Synthesized lipophilic tyrosyl ester derivatives with increasing lipophilicity were effective against *Leishmania (L.) major* and *Leishmania infantum* species *in vitro*. These findings prompted us to test *in vivo* leishmanicidal properties of these molecules and their potential effect on the modulation of immune responses. The experimental BALB/c model of cutaneous leishmaniasis was used in this study. Mice were infected with *L. major* parasites and treated with three *in vitro* active tyrosyl esters derivatives.

Among these tested tyrosylcaprate (TyC) compounds, only TyC₁₀ exhibited an *in vivo* anti-leishmanial activity, when injected sub-cutaneously (s.c.). TyC₁₀ treatment of *L. major*-infected BALB/c mice resulted in a decrease of lesion development and parasite load. TyC₁₀ s.c. treatment of non-infected mice induced an imbalance in interferon γ /interleukin 4 (IFN- γ /IL-4) ratio cytokines towards a Th1 response. Our results indicate that TyC₁₀ s.c. treatment improves lesions' healing and parasite clearance and may act on the cytokine balance towards a Th1 protective response by decreasing IL-4 and increasing IFN- γ transcripts. TyC₁₀ is worthy of further investigation to uncover its mechanism of action that could lead to consider this molecule as a potential drug candidate.

Key words: *Leishmania major*, lipophilic tyrosyl ester, anti-leishmanial activity, parasite load.

INTRODUCTION

Leishmaniasis is a group vector borne infectious diseases caused by the protozoa *Leishmania (L.)*, and transmitted by sand flies. The population at risk is 310 million; overall prevalence being 12 million with 2 million new cases occurring annually (WHO, 2010; Alvar *et al.* 2012). Leishmaniasis' drugs are mainly based on pentavalent antimonial agents. These medicines are toxic and cause several side effects within the host (Ayatollahi and Halvani, 2011). Furthermore, large-scale parasite resistance against antimonials has also been described (Yasinzai *et al.* 2013).

Search for new active products against *Leishmania* parasites has received a considerable attention. In the ongoing search for better leishmanicidal treatments, investigation on plant-derived products and synthetic compounds, easily available and relatively

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cheap, is gaining ground (Nagle *et al.* 2014). Improving the anti-leishmanial activity of natural and synthetic compounds is one of the good drug alternatives (Sen and Chatterjee, 2011). It was for example reported that hydrolysis of ester functionality in amino acid subunit decreases the anti-leishmanial activity of this synthesized conjugate (Singh *et al.* 2010). Recently, a natural alkyl-phenol (gibbimol B), which is a phenol ring linked to an unsaturated alkyl chain, was evaluated for its potent activity against *Leishmania infantum* ('*Leishmania chagasi*') (De Oliveira *et al.* 2012). It was also reported that there is a link between lipophilicity and anti-parasitic activity of such components (Musonda *et al.* 2009). Cross screening of a library against cultured *Leishmania donovani* parasites revealed that compounds of this class are potent inhibitors of parasite development *in vitro*. We have recently synthesized a set of tyrosyl esters derivatives, with increasing lipophilicity, using lipase B from *Candida antarctica* (Novozyme 435). Among all the synthesized compounds, we showed that Tyrosylcaprylate (TyC₈), Tyrosylcaprate (TyC₁₀) and Tyrosyllaurate (TyC₁₂) were *in vitro* effective against *L. major* and *L. infantum* species, while

neither tyrosol nor short and long-chain derivatives have leishmanicidal activity (Aissa *et al.* 2012).

In this study, we have evaluated the sub-cutaneous (s.c.) *in vivo* anti-leishmanial activity of these three tyrosyl esters derivatives against *L. major* in experimentally infected susceptible BALB/c mice.

MATERIALS AND METHODS

Ethical statement and animals

Experiments were performed in compliance with the directive 86/609/EEC of the European parliament and the council on the protection of animals used for scientific purposes, in agreement with the guidelines of International Guiding Principles for Biomedical Research Involving Animals. The study protocol and procedures were reviewed and approved by the Institut Pasteur de Tunis Ethical Review Board (PV07/07). BALB/c mice were purchased from Janvier Company (Le Genet St Isle, France).

Reagents

N-hexane and Diethyl ether were purchased from Prolabo (Paris, France) and Pharmacia (Uppsala, Sweden) respectively. Acetonitrile and acetic acid were purchased from Pharmacia (Uppsala, Sweden). Caprylic, capric, lauric and oleic acids and 2-methyl-2-propanol were purchased from Fluka (Germany).

Esterification, purification and high-performance liquid chromatography (HPLC) analysis of tyrosyl esters

Tyrosyl lipophilic esters (TyC₈ for Tyrosylcaprylate; TyC₁₀ for Tyrosylcaprate; TyC₁₂ for Tyrosyllaurate and TyC_{18:1} for Tyrosyloleate) were synthesized by direct esterification of tyrosol with different fatty acids as previously described (Aissa *et al.* 2012). Briefly, purification of esters was achieved by chromatography on a silica gel columns (Merck) and purity of molecules was tested using HPLC system equipped with a pump, a column oven and a diode-array UV/VIS detector (Aissa *et al.* 2012).

Parasite culture and metacyclic form purification

Tunisian strain of *L. major* (MHOM/TN/95/GLC94) isolated from a cutaneous lesion of a Tunisian patient (Kébaier *et al.* 2001) was used in this work. Promastigotes were cultured in solid medium at 26 °C, and then progressively adapted to an RPMI 1640 medium containing 10% of fetal calf serum (FCS) and supplemented with 100 U penicillin mL⁻¹, 100 µg streptomycin mL⁻¹ and 2

mM L-glutamine. When the stationary phase was reached, the metacyclic promastigotes were then purified by a negative selection with peanut agglutinin (PNA) (Sigma, Saint-Quentin Fallavier, France). PNA⁻ and PNA⁺ fractions were separated by density gradient centrifugation. The PNA⁻ metacyclic promastigotes were then washed and diluted at the desired density.

Experimental infection of BALB/c mice

Six- to eight-week-old female BALB/c mice were infected with 2×10^6 metacyclic PNA⁻ *L. major* parasites by s.c. route in the left footpad in 50 µL of saline solution. Infection was assessed after 2 weeks by the appearance of a small lesion in the feet of infected mice. For ethical reasons, the experimental protocol lasts only 10 weeks during which infection was monitored once a week by measuring the inflammation volume of footpad lesions with a plethysmometer. The lesion size was defined as the increase in the footpad volume after subtracting the size of the contra-lateral uninfected one.

Treatment procedures

Infected animals were divided into several groups of six mice each. Four groups were treated s.c. either with Glucantime[®] [10 mg in 50 µL phosphate-buffered saline (PBS), TyC₈, TyC₁₀ or TyC₁₂ (2 mg in 50 µL PBS each)]. Doses and frequency of treatments were chosen from the extrapolation of *in vitro* results we described previously (Aissa *et al.* 2012). Two other infected groups were s.c. treated, either with PBS (50 µL) or TyC_{18:1} (2 mg in 50 µL PBS), and used as negative controls. Subcutaneous injections were administered within developed lesions twice a week for 8 weeks for all groups, including the control ones.

For experiments aiming to assess the direct effect of TyC₁₀ on mRNA cytokine levels in non-infected animals, mice were injected s.c. twice a week with either PBS or TyC₁₀ during 8 weeks. Animal experiments were repeated at least twice with reproducible results.

Parasite quantification

At the end of the experimental protocols, mice were sacrificed and infected footpads and popliteal lymph nodes draining the cutaneous lesions were removed. Parasite load was determined by a limiting-dilution method following a protocol we described elsewhere (Benhni *et al.* 2009). Briefly, excised tissues were weighted and homogenized. A serial 10-fold dilutions were plated in duplicate in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) containing Schneider's *Drosophila* medium (both from Gibco-BRL, Paisley,

Scotland) supplemented with 100 U penicillin mL⁻¹, 100 µg streptomycin mL⁻¹, 2 mM L-glutamine and 10% heat-inactivated FCS. Viable parasites were observed microscopically after seven to 10 days of incubation at 26 °C. Parasite load was then determined as the reciprocal of the highest dilution at which promastigotes could be grown in culture at 26 °C and detected by microscope (Benhni *et al.* 2009).

RNA extraction and analysis of cytokine expression by RT-PCR

Total RNA was extracted from collected tissues homogenized in the Trizol Reagent. Messenger RNA (mRNA) enrichment was performed using the RNeasy mini kit (Qiagen) following the manufacturer's instructions, and the contaminating genomic DNA was removed using DNase I (Invitrogen, Carlsbad, CA, USA). mRNA content was measured by Nanodrop 1000 and transformed into cDNA by reverse transcription using a High Capacity cDNA Reverse Transcription kit (Applied Biosystem). Samples were then stored at -80 °C until use.

Real-time PCR (RT-PCR) amplification was performed using specific primers and probes targeting interferon (IFN) γ , interleukin (IL) 10, IL-4 and inducible nitric oxide synthase (iNOS) genes. *Porphobilinogendeaminase* (PBGD) was used as an endogenous control. The expression of this gene was described as much more stable than the expression of several other housekeeping genes (Radonić *et al.* 2004). All primers and probes were designed using the Primer Express 3.0 software provided by Applied Biosystem.

Sequence primers are as follow: PBGD: Forward-5' CGGCCACAACCGCGGAAGAA3', Reverse-5'GTCTCCCGTGGTGGACATAGCAATGA3'; IFN- γ : Forward-5'TCAAGTGGCATAGATGTGGAA GAA3', Reverse-5'TGGCTCTGCAGGATTTTC AT3'; IL-10: Forward-5'GGTTGCCAAGCCTTA TCGGA3', Reverse-5'ACCTGCTCCACTGCCTT GCT3'; IL-4: Forward-5'ACAGGAGAAGGGA CGCCAT3', Reverse-5'GAAGCCCTACAGACGA GCTCA3'; iNOS: Forward-5'CAGCTGGGCTGT ACAAACCTT3', Reverse-5'CATTGGAAGTGAA GCGTTTCG3'.

Each fold change in gene expression determination was performed in triplicate on samples obtained from the same groups of mice. Data were normalized by referring to the expression of the PBGD endogenous control. The RT-PCR universal cycle was run and mRNA fold change expression levels relatively to controls were estimated using the 2^{- $\Delta\Delta$ Ct} method. Genes' expressions were compared between TyC₁₀- and Glucantime®-treated *Leishmania*-infected groups or between PBS- and TyC₁₀-treated but non-infected mice groups.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5.0 software. The differences between groups were evaluated by different tests (parametric method or non-parametric method using Mann-Whitney test for the later). *P* values of 0.05 or lower were considered as statistically significant.

RESULTS

A region-selective procedure was used to synthesize lipophilic tyrosyl esters (TyC₈, TyC₁₀, TyC₁₂ and TyC_{18:1}). The conversion yields calculated after 72 h of incubation using lipase B from *C. antarctica* (Novozyme 435) were TyC₈: 85.55, TyC₁₀: 75.42, TyC₁₂: 73.33 and TyC_{18:1}: 57% as previously described (Aissa *et al.* 2012).

TyC₁₀ but not TyC₈ and TyC₁₂ *in vivo* effects correlate with *in vitro* anti-leishmania activities

The effect of synthesized molecules on the development of lesions induced by *L. major* infection was studied by the measurement in s.c. tyrosyl ester-treated and PBS-treated and *L. major*-infected mice of footpad lesion sizes. A set of three synthesized molecules was tested *in vivo*, and the lesion development was monitored during 10 weeks upon infection. These molecules were selected on the basis of their previously validated *in vitro* anti-leishmanial activity (Aissa *et al.* 2012). An *in vitro* non-active synthesized molecule i.e. TyC_{18:1} (Tyrosylolate) was used as an internal negative control, and showed no difference when compared with control group s.c. treated with PBS. Two molecules TyC₈ and TyC₁₂, having an *in vitro* activity against *L. major*, did not show any effect into lesion development (data not shown). In contrast, TyC₁₀ showed an inhibitory effect on lesion development compared with lesions in PBS-treated group. Decrease of the lesion size was observed starting at the fourth week of TyC₁₀ s.c. treatment.

TyC₁₀ s.c. treatment of *L. major* infected mice decreases lesion development and parasite load

BALB/c mice were infected with *L. major* promastigotes as described above. After 2 weeks, they were s.c. treated, twice a week during 8 weeks, with TyC₁₀ or Glucantime® injections. Fig. 1A shows that there is a significant decrease in lesion size starting from the second week of treatment (fourth week post-infection) compared with PBS treatment. Although Glucantime® treatment had a more profound effect in decreasing the development of the size of the lesion, compared with TyC₁₀, both Glucantime®- and TyC₁₀-treatments decreased

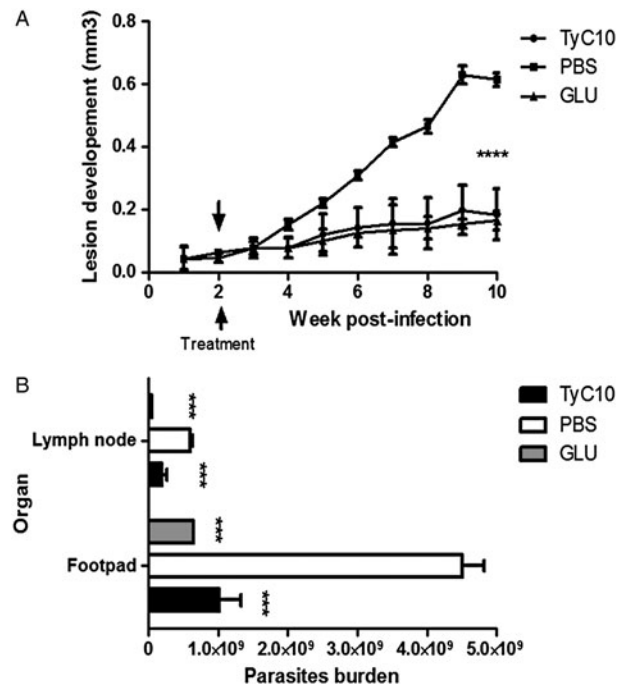


Fig. 1. Lesion size (A) and parasite burden (B) of BALB/c mice infected with *Leishmania major* parasites. Mice were infected in their hind footpads with 2×10^6 *L. major* metacyclic promastigotes. Two weeks later, mice were treated, twice a week, with synthesized tyrosol derivative TyC₁₀; Glucantime[®] and PBS during 8 weeks. Footpad swelling (A) was measured weekly and parasite burden (B) was measured after 10 weeks of infection by limiting dilution assay. Data are presented as mean \pm S.E.M. of six mice per group. * Indicates *P* values (***) indicate a *P* value < 0.001 and **** indicate a *P* value < 0.0001).

drastically the lesion size compared with PBS ($P < 0.0001$) by the end of the tenth week of infection. Parasite burden, quantified at the end of the s.c. treatment, was decreased both in the footpads ($P < 0.001$) and in the draining lymph nodes ($P < 0.001$) of TyC₁₀-treated mice compared with the PBS-treated control group (Fig. 1B). Similarly, parasite burden in footpads and lymph nodes was also decreased in mice s.c. treated with Glucantime[®] (Fig. 1B).

Impact of s.c. TyC₁₀ treatment of non-infected or infected mice

We assessed the effect of TyC₁₀-treatment alone on key Th1/Th2 cytokine mRNA fold change by measuring transcript levels of IFN- γ (Th1) and IL-4 (Th2) in non-infected mice s.c. treated or not with PBS and TyC₁₀. TyC₁₀-treatment induced high levels of IFN- γ mRNA in footpads ($P < 0.01$) and lymph nodes ($P < 0.0001$) comparatively to non-treated mice (Fig. 2A, left panel). Nevertheless, IL-4 mRNA fold change levels were slightly lower in both footpads and lymph nodes of TyC₁₀-treated mice ($P < 0.001$) compared with PBS-treated control group (Fig. 2C, left panel). Specifically, the measured levels of IFN- γ mRNA were higher in the proximity of injection site.

RT-PCR was also used in infected mice to assess the expression levels of key molecules in the establishment of Th1/Th2 balance. Treatment with TyC₁₀

induced significantly higher levels of iNOS in both footpads and lymph nodes compared with treatment with Glucantime[®] ($P < 0.0001$; Fig. 2B). Concomitantly, IFN- γ was induced in footpads of infected-mice and s.c. treated with TyC₁₀, but at a lesser extent ($P < 0.05$; Fig. 2A) to the levels measured in infected mice and treated with Glucantime[®]. Synthesis of iNOS transcripts was decreased in lymph nodes of TyC₁₀-treated mice, comparatively to what was observed in Glucantime[®]-treated ones ($P < 0.05$; Fig. 2B, right panel).

It is also interesting to note that IL-4 and IL-10 mRNA levels were less expressed in lymph nodes of TyC₁₀-treated mice compared with those of Glucantime-treated mice ($P < 0.0001$ and $P < 0.001$, respectively; Fig. 2C, right panel and 2D). A statistically significant difference was also observed between the fold change levels of IL-10 mRNA measured in the footpads of s.c. TyC₁₀-treated mice compared with those measured in the footpads of Glucantime[®]-treated mice ($P < 0.05$; Fig. 2D).

Although measured only at the end of the experiment, the detected shift in IFN- γ /IL-4 cytokine balance in non-infected mice (Fig. 2A and C, left panels) might be the reason why we saw reduction in lesion size and parasite burden in infected mice treated with TyC₁₀ synthetic molecule at the infection site.

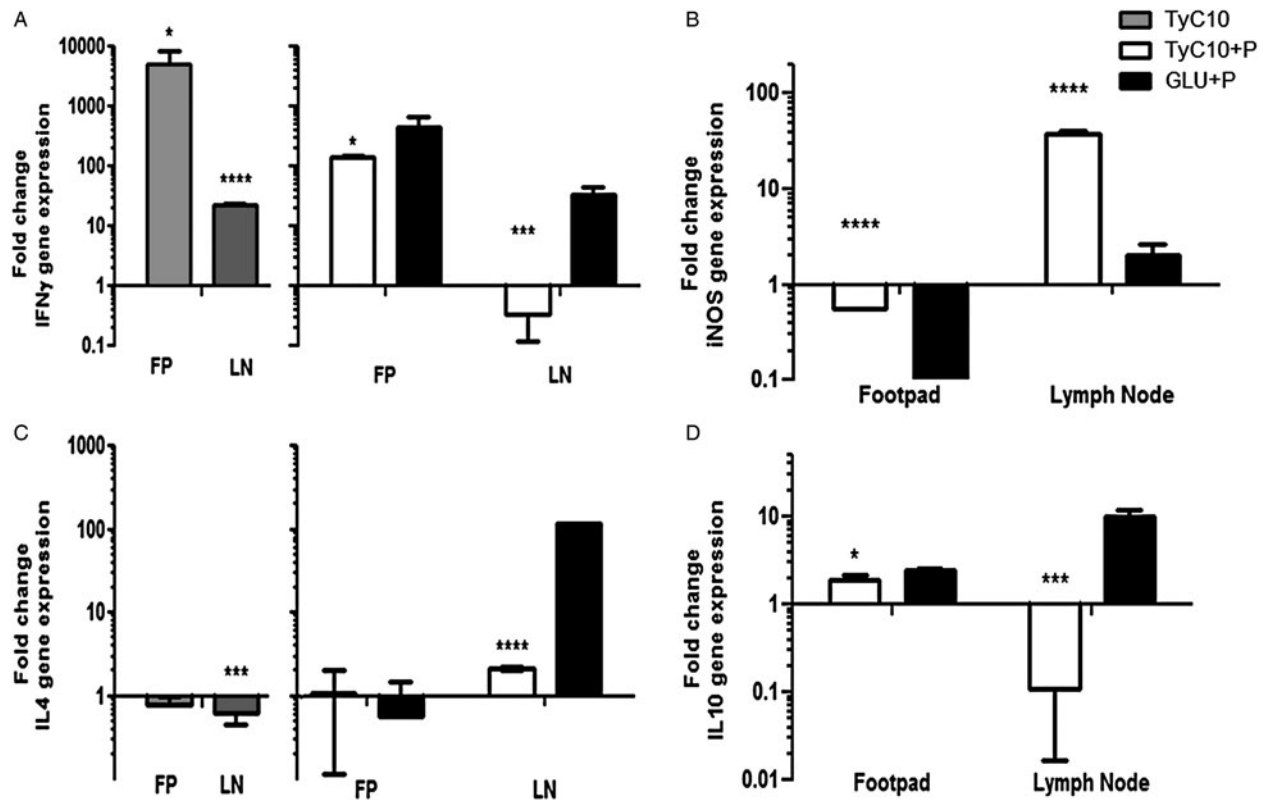


Fig. 2. Fold change mRNA expression of IFN- γ (A), iNOS (B), IL-4 (C) and IL-10 (D) in the footpad (FP) and the draining lymph nodes (LN) after *Leishmania major* infection and 8-week s.c. treatment with TyC₁₀ (white) or Glucantime[®] (black). Infected and PBS-treated mice are used as controls. Results were normalized to PBGD fold expression in comparison to infected and PBS-treated control mice. Left panels in A and C shows fold change IFN- γ and IL-4 transcripts respectively in the FP and the draining LN of non-infected TyC₁₀-treated in comparison with PBS-treated mice. Results were normalized to the same housekeeping gene and compared with the non-infected and PBS-treated control mice. All results were expressed using the $2^{-\Delta\Delta C_t}$ method. Statistical significance between TyC₁₀- and Glucantime[®]-treated and infected mice (or between TyC₁₀- and PBS-treated and non-infected mice) were indicated by number of * (*when P value < 0.05; *** when P value < 0.001 and **** when P value < 0.0001).

DISCUSSION

The current treatments of leishmaniasis, usually based on the use of pentavalent antimonials, often induce parasite resistance, high toxicity and high cost for patients from tropical endemic countries, and highlight the increased need for new treatments (Minodier and Parola, 2007; Siqueira-Neto *et al.* 2010). The search for new products active against *Leishmania* parasites has received a considerable attention. The *in vitro* effect against *Leishmania* promastigotes described in our previous report raised our interest in investigating lipophilic tyrosol derivatives as a potential anti-*Leishmania* component *in vivo* (Aissa *et al.* 2012). Tyrosol has been previously described to have antioxidant and anti-inflammatory properties (Wahle *et al.* 2010). This study was conducted to assess the *in vivo* activity of tyrosol derivatives in the s.c. treatment of BALB/c experimental mice model. Screening results showed that only TyC₁₀ molecule had the capacity to reduce lesion size in infected mice (Fig. 1A). Further experiments using TyC₁₀ synthesized molecule s.c. treatment showed that, in addition to lesion size reduction, a

decreased parasite load in the footpad and in the draining lymph nodes of infected animals was observed (Fig. 1B). A structurally similar molecule to synthesized tyrosyl esters, especially to TyC₁₀, gibbilibol B [extracted from the leaves of *Piper malacophyllum* (Piperaceae)], was also described to be active against *L. infantum* (*L. chagasi*) *in vitro* (De Oliveira *et al.* 2012). In addition, tyrosol has been previously described to have antioxidant and anti-inflammatory properties (Wahle *et al.* 2010).

Besides, the protective effect observed *in vivo* is coupled with a high induction of iNOS expression, one of the catalysing enzymes of NO molecule previously used as a potential treatment of *Leishmania* infection (Gradoni and Ascenzi, 2004; Müller *et al.* 2013). Although no increase of IFN- γ mRNA was observed in lymph nodes of infected mice (Fig. 2A), iNOS mRNA fold change increased (Fig. 2B). While IFN- γ signalling is critical for iNOS induction, it is also known that other signals might act directly on macrophages to induce iNOS expression or indirectly by favouring the development of Th1 cells (Rottenberg *et al.* 1996).

However, the mechanisms by which these signals act *in vivo* in conjunction with IFN- γ to induce iNOS on macrophages still remain unknown.

The question whether the presence of host pro-inflammatory molecule production could be important or not for the control of parasite infection has been addressed by a study conducted on *Leishmania amazonensis*-infected macrophages treated *in vitro* with 17-AAG, an inhibitor of heat-shock protein 90 (Petersen *et al.* 2012). Indeed, the authors clearly showed that treatment of infected macrophages by 17-AAG directly induces parasite killing, independently of macrophage activation, and that O $_2^-$ and NO play no role in the induction of intracellular *Leishmania* death. These results are different from ours showing an activation of infected host cells within the lymph nodes.

Efficient killing of two intracellular pathogens, i.e. *Salmonella* and *Mycobacteria*, required co-localization of iNOS with pathogen-containing compartments (Chakravorty *et al.* 2002; Davis *et al.* 2007). It was elegantly shown that during *L. major* infection, cells expressing iNOS are unable of controlling parasite load (Olekhnovitch *et al.* 2014). The collective production and diffusion of NO create an anti-parasitic environment that allows parasite killing. Hence, to control the intracellular pathogens, a co-operative mechanism at the tissue level is needed whereas direct cell infection is not required for iNOS induction (Olekhnovitch *et al.* 2014). We hypothesize that the absence of induced iNOS in the footpads of infected mice might result from the low burden of intracellular parasites by the end of our experimental protocol, as the cells previously infected might have already cleared the majority of these parasites in the infection site by the tenth week of infection. It was also shown that CD4 $^+$ T cells can induce iNOS in infected cells at distance from their site of antigen recognition, most likely by cytokine diffusion and that direct interaction with these cells is not required for iNOS induction (Müller *et al.* 2013; Olekhnovitch *et al.* 2014).

In addition, s.c. treatment of non-infected mice with TyC $_{10}$ molecule has the capacity to increase IFN- γ mRNA levels in both lymph nodes and footpads, constituting an important driving effect towards Th1 response. Although this effect was demonstrated only at the end of the experimental protocol and not during different time points, it is likely to be important towards a Th1 profile. Similarly, it was shown that garlic extracts, which act as modulators of immune response, have protective effects on BALB/c mice infected with *L. major* parasites (Ghazanfari *et al.* 2000). These extracts reduce macrophage infection through the induction of NO production (Gamboa-León *et al.* 2007), and shift the cytokine pattern towards a Th1 profile (Ghazanfari *et al.* 2000). Our results indicate that s.c. TyC $_{10}$ treatment shows a dual effect: (i) an *in*

vitro leishmanicidal properties (Aissa *et al.* 2012), and (ii) a potent inducer of IFN- γ transcripts' synthesis in non-infected mice. They also show that TyC $_{10}$ mode of action, towards parasite load reduction, may differ from the Glucantime $^{\text{®}}$ one.

Further investigations are required to explore the synergistic dual leishmanicidal and immune-modulating effects of TyC $_{10}$ in *L. major* experimental model, and to elucidate its mode of action.

Concluding remarks

In summary, we have showed that tyrosylcaprate ester (TyC $_{10}$) may be explored further as a potential alternative treatment against *L. major* infection. Indeed, this molecule, according to its direct action towards parasite clearance but also to its immune-modulatory effect in terms of shifting the cytokine IFN- γ /IL-4 balance towards a Th1-type pattern, may be exploited to counteract parasite development.

ACKNOWLEDGEMENTS

We thank Mr A. Marouani and Dr Z. Ben Lasfer (Animal Facilities of the Institut Pasteur de Tunis) for their help conducting this study. We are also grateful to Ms S. Amouri for her help and thankful to Dr K. Ben-Aissa (Washington, USA) and Dr S. Gritli (Department of Oto-Rhino-Laryngology, Charles Nicolle Hospital, Tunis, Tunisia) for their critical reading and grammar corrections.

FINANCIAL SUPPORT

This work received financial support from the Tunisian Ministry of Higher Education and Scientific Research to the Transmission, Control and Immunobiology of Infections Laboratory (LR11IPT02, D. Laouini). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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