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ORIGINAL PAPER

Leishmania tropica: suggestive evidences for the effect of infectious dose on pathogenicity and immunogenicity in an experimental model

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Abstract

Leishmania (*L.*) *tropica* is a causative agent of cutaneous and occasionally visceral or viscerotropic leishmaniasis in humans. The dose of parasites influences the course and outcome of disease in some *Leishmania* species. The effect of parasite dose on *L. tropica* infection in an experimental model was studied in the current paper. High and low doses of *L. tropica* were used for ear infection of BALB/c mice and lesion development, parasite load, and cytokine responses were assessed. *L. major* infection was used for comparison. Pre-infected mice were challenged in the footpad by a fixed high dose of *L. tropica*, and immune response and protection level were evaluated. High dose *L. tropica* infection in comparison to low dose results in higher lesion diameters, higher load of parasite in draining lymph node, higher levels of interferon-γ and interleukin-10, dissemination of parasite to spleen, and induction of protection against further *L. tropica* challenge. Comparison of *L. tropica* with *L. major* showed that *L. tropica* results in lower lesion diameters, more potential for growth in lymph nodes at early phases of infection, parasite dissemination to spleen, lower levels of IL-10, and a permanent lower cytokine response against low parasite dose in comparison to high dose. Our findings suggest that for *L. tropica* infection, only the high dose results in visceralization of the parasite and protection against further challenge of *L. tropica*. Therefore, the parasite dose may be an important factor in pathogenesis and immunity in *L. tropica* infection.

Keywords Pathogenicity · Infectious dose · *Leishmania tropica* · *Leishmania major* · Spleen · Cytokine · BALB/c mice

Introduction

Leishmaniasis is a disease that is caused by infection by *Leishmania* (*L.*) parasites. There are different forms of leishmaniasis. The most common forms are cutaneous leishmaniasis, which causes skin sores, and visceral leishmaniasis, which affects internal organs (spleen, liver, and bone marrow). *L. tropica* is a causative agent of cutaneous leishmaniasis in humans. It can occasionally be a causative agent of visceral or viscerotropic leishmaniasis in humans (Alborzi et al. 2006; Eroglu et al. 2015; Magill et al. 1993). The dose of parasites may influence the course and outcome of infection caused by some *Leishmania* species. The infectious dose is especially important in experimental models of *Leishmania* infection because the traditional infectious dose in experimental animal models has been about one million parasites while the infectious dose in natural transmission is much less, about < 600 parasites in 75% of sand fly bites (Kimblin et al. 2008; Warburg and Schlein 1986). Thus, the high infectious dose of millions may not represent what happens in the natural transmission of the disease. The effect of infectious dose of some *Leishmania* species has been studied in experimental models (Bastien and Killick-Kendrick 1992; Compton and Farrell 2002; Doherty and Coffman 1996; Kaur et al. 2008; Lira et al. 2000; Menon and Bretscher 1998; Oliveira et al. 2012; Ribeiro-Gomes et al. 2014; Ribeiro-Romao et al. 2014; Uzonna et al. 2004). Our previous report is the only study available for the effect of infectious dose in *L. tropica* infection, in which we mainly focused on antibody response of BALB/c mice (Rostamian et al. 2017). The present report is

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the continuation of our previous report which elucidates the effect of infectious dose in other aspects of *L. tropica* infection including parasite load, dissemination of the parasite to visceral organs, cytokine responses, and protection against further infection (challenge). In addition, *L. tropica* infection was compared to *L. major* in the current study to present a more comprehensive experimental model of *L. tropica* infection.

Materials and methods

Mice

Female BALB/c mice, 5–7 weeks old, were purchased from the Pasteur Institute of Iran and maintained under conventional conditions in the animal care facility. Mice were housed in cages in a ventilated room with unlimited access to food and water under a 12-h light and 12-h darkness cycle. Mice were euthanized by cervical dislocation before removal of the spleen and lymph nodes. All animal experiments were approved by Ethics Committee of the Pasteur Institute of Iran (license number 95/0201/20704). Caring for and using the mice in this study were done according to "Iranian national ethical guidelines: How to work with laboratory animals."

Parasite

The *L. tropica* strain MHOM/AF/88/KK27 is a cutaneous *L. tropica* isolate from Afghanistan and was initially described by Dr. R. Killick-Kendrick. It was provided for this study as a gift from Dr. D. Sacks (Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD, USA). The *L. major* strain MRHO/IR/75/ER is an *L. major* isolate from Iran and was a gift from Dr. M. Mohebali (School of Public Health, Tehran University of Medical Sciences, Tehran, Iran). Parasites were cultured and their species were confirmed as reported elsewhere (Mahmoudzadeh-Niknam et al. 2011a, b). Virulence of parasites was preserved by injection of the parasite to BALB/c mice and its retrieval from mice. The in vitro cultivation of the parasite in Novy Mac Neal Nicolle (NNN) media was kept to less than three passages after isolation from mice to maintain virulence. Stationary-phase promastigotes were used for infection.

Route of infection

Primary infection was injected by the intradermal route into the pinna of the ear and the challenge subcutaneously into the foot pad. The pinna was selected because this site is exclusively intradermal with no subcutaneous tissue.

Dose of parasites

Primary infection was performed by two doses of low (10^3 parasites/mouse) and high (10^6 parasites/mouse). The low dose of 10^3 promastigotes was used in order to simulate the natural transmission. The high dose of 10^6 was used to clarify possible differences between the high and low doses and also to make our data comparable to many published studies using the high dose. These two doses have been used in many studies involving *Leishmania* parasites. Challenging infection was performed by a fixed dose of 10^6 parasites/mouse.

Antigen preparation

Soluble *Leishmania* antigen (SLA) was prepared from *L. tropica* strain MHOM/AF/88/KK27. The parasite was cultured in liquid media consisting of RPMI-1640, 10% FBS, 200 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Stationary-phase promastigotes were harvested, washed three times with PBS, passed five cycles of freeze (in liquid nitrogen) and thaw (in 37 °C), and centrifuged 13,000×*g* for 5 min, and the supernatant was collected as SLA. Protein content was assayed by the Bradford method (Simonian and Smith 2006). SLA was aliquoted and stored in − 80 °C. A single preparation of SLA from *L. tropica* was used for all in vitro stimulation assays in our study. This approach was used with the assumption of sufficient similarity between epitope contents of SLA from *L. tropica* and *L. major*.

Lesion measurement

The course of infection was monitored by bi-weekly measurement of thickness of ear or footpad by a dial-gauge caliper (Mitutoyu, Kawasaki, Kanagawa, Japan). Increase in thickness was calculated through subtraction of thickness of infected ear or footpad from thickness of contra lateral uninfected ear or footpad.

Parasite load assay

Parasite load in draining lymph node and spleen were quantified by limiting dilution assay (Sacks and Melby 2015). The assay is briefly as follows: single cell suspensions are made from draining lymph node or spleen and diluted in the liquid phase of NNN medium. Two- or fourfold serial dilutions were performed to extinction of parasite growth. Assays were performed in triplicate. Parasite load per total lymph node and spleen was calculated from the highest dilution at which promastigotes can be grown out. Parasite load was determined for each individual mouse. Geometric mean titer of each experimental group was used for comparison between the groups.

Cytokine assay

Draining lymph nodes (auricular lymph node in ear of infected mice and popliteal lymph node in footpad infected mice) were removed from mice. Single cell suspensions were made from draining lymph nodes by passing them through a stainless steel mesh. Cells were grown in culture media and stimulated by SLA (10 μg/ml). The same SLA prepared from *L. tropica* was used for all in vitro stimulation. Culture media was composed of RMPI-1640, fetal bovine serum (10%), penicillin (100 IU/ml), streptomycin (100 μg/ml), L. glutamine (200 mM), and 2-mercapto ethanol (0.05 mM). Cell stimulated by concanavalin A (2 μg/ml) was used as positive control. Unstimulated cells were used as negative control. Culture supernatants were harvested after 72 h and aliquots were stored at − 80°C. Levels of cytokines of interferon-γ (IFN-γ) and interleukin-10 (IL-10) were assayed in the supernatant by commercial kits from e-bioscience and R&D according to manufacturer's instructions. The detection limit of the ELISA kits for both IFN-γ and IL-10 was 31.2–2000 pg/ml.

Study design

The study consisted of the following experimental groups (12–15 mice per group): *L. tropica* high dose, *L. tropica* low dose, *L. major* high dose, *L. major* low dose, and naive mice. Primary infection was performed by intradermal injection into ear pinna (Sacks and Melby 2015). The low dose of 10^3 parasites/mouse and the high dose of 10^6 parasites/mouse were used for primary infection. *L. tropica* infection was compared to *L. major* infection in the ear pinna using the same low and high parasite doses and the following criteria were assessed: lesion development, parasite load of lymph node and spleen, and cytokine levels. The study was carried out in two independent experiments and results of one representative experiment are shown. Pre-infected mice (low and high doses of *L. tropica* and low dose of *L. major*) as well as naive mice were challenged in the footpad by 10^6 *L. tropica* stationary promastigotes 6 months after primary infection. Protection induced by primary infection of *L. major* or *L. tropica* against *L. tropica* challenge was evaluated by assessing lesion diameter, parasite load, and cytokine levels.

Statistical analysis

One- or two-tailed Student's *t* test was used for comparison of thicknesses, parasite loads, and cytokine amounts between different experimental groups using the GraphPad Prism 6.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). *P* values equal or lower than 0.05 were considered significant.

Results

Effect of infectious dose on pathogenicity

As published in our previous report (Rostamian et al. 2017), lesions developed from a high dose of *L. major* or *L. tropica* were significantly larger than those caused by a low dose from the same species ($P < 0.05$) and at equivalent infectious doses, *L. major* infection in comparison to *L. tropica* results in higher lesion diameter showing more pathogenesis ($P < 0.05$) (Fig. 1).

Our results (Fig. 2a) show that high dose in comparison to low dose of either *L. major* or *L. tropica* results in significantly higher load of parasite in draining lymph node at all intervals studied (1 week, 1 month, and 4 months after infection) ($P < 0.05$). An interesting finding of our study is that the high dose of *L. tropica*, in comparison to the high dose of *L. major*, resulted in significantly higher parasite load in lymph nodes at 1 week as well as at 1 month after infection ($P < 0.05$). The difference between *L. tropica* and *L. major* became significant for both high and low doses at 1 month after infection ($P < 0.05$). However, there is no significant difference between *L. tropica* and *L. major* of both low and high doses at 4 months after infection. Our study showed that only *L. tropica* at 4 months after infection disseminates to spleen. As shown in Fig. 2b, there is no parasite growth in the spleen of all experimental groups except for the high-dose group of *L. tropica* at 4 months after infection.

Effect of infectious doses on immune response

IFN-γ response The data for *L. major* showed that high dose in comparison to low dose results in higher levels of IFN-γ at 1 week and 1 month after infection (Fig. 3a). However, there is no significant difference between high and low doses of *L. major* at 4 months (Fig. 3a). Comparison of IFN-γ response against high and low doses of *L. tropica* showed that higher levels of IFN-γ is produced against high dose in comparison to low dose at all intervals post-infection (Fig. 3a).

There was no significant difference between IFN-γ levels in response to *L. major* and *L. tropica* infection at similar infectious doses (Fig. 3a).

IL-10 response Higher levels of IL-10 were produced in response to high dose of *L. major* in comparison to low dose at 1 week and 1 month post-infection (Fig. 3b). However, at 4 months after infection, there are similar levels of IL-10 against high and low doses of *L. major* (Fig. 3b). Similar late-phase IL-10 response to low and high doses of *L. major* is concordant with similar late-phase IFN-γ response to low and high doses of this parasite as mentioned above.

As shown in Fig. 3b, higher levels of IL-10 are produced against high dose of *L. tropica* in comparison to low dose in all

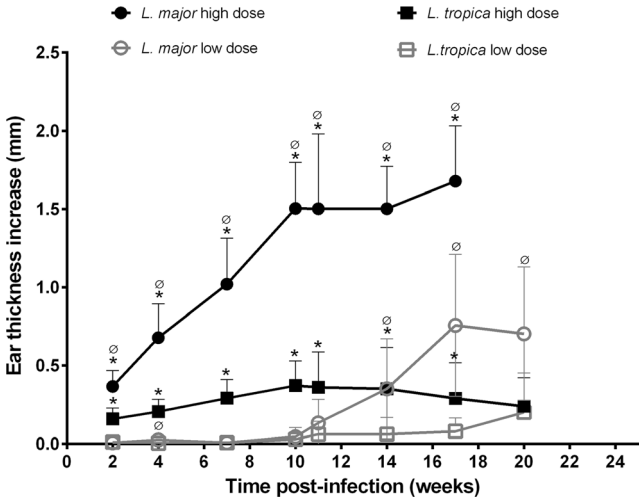

Fig. 1 Effect of infectious dose on lesion development of BALB/c mice. Each point shows mean + SD of ear lesion diameter of 5–10 mice per group at indicated time point after infection. Lesion measurements were discontinued after 17 weeks post-infection in high-dose *L. major* group due to ear necrosis. Single asterisk shows statistical significant difference between high and low doses of the same parasite species (*L. tropica* or *L. major*). Symbol ø shows statistical significant difference between *L. tropica* and *L. major* of the same dose. Data of this figure has been already published in our previous report (Rostamian et al. 2017) and is reused here with permission from the publisher

post-infection intervals. This pattern of IL-10 response against *L. tropica* is different from that of IL-10 response against *L. major* as mentioned above in which similar levels of IL-10 are produced against low and high doses at 4-month post-infection interval.

Considering high-dose infection, higher level of IL-10 is produced in response to *L. major* in comparison to *L. tropica* at 1 month post-infection ($P < 0.05$) and this difference wanes at 4 months post-infection. Regarding low-dose infection, no significant difference was observed between the two *Leishmania* species (Fig. 3b).

Effect of infectious dose on protection against *L. tropica* challenge

Mice infected by different doses of *L. tropica* were challenged by *L. tropica* 6 months after primary infection, in order to assess the homologous protective effect of primary infection of *L. tropica* against the secondary infection of the same species. Mice infected by low dose of *L. major* were used as a control group which already had had a Th2 response and challenged by *L. tropica* in order to assess how the primary *L. major* infection affects the secondary *L. tropica* infection. Primary infections were in the ear by injection of high (10^6) and low (10^3) doses of *L. tropica* and low (10^3) dose of *L. major*. The secondary (challenge) infection was in the footpad by injection of 10^6 *L. tropica*. Footpad diameter increase was measured up to 10 months after challenge. The results showed that footpad lesion diameter of *L. major* pre-infected mice were significantly higher than lesion diameter of naïve mice showing that previous infection by *L. major* induces an exacerbation in BALB/c mice as was anticipated (Sacks and Noben-Trauth 2002) (Fig. 4a). On the other hand, lesions of mice pre-infected with high (10^6) dose of *L. tropica* were significantly lower than naïve mice showing that a protective immunity was induced at the site of injection by high dose of *L. tropica* pre-infection (Fig. 4a). Mice pre-infected by low (10^3) dose of *L. tropica* showed lesion diameter similar to the naïve mice (Fig. 4a) showing that the immune response of low-dose pre-infected mice has not been sufficiently shifted to a protective response. Lymph node parasite load after *L. tropica* challenge was significantly lower only in mice pre-infected by high dose of *L. tropica* in comparison to naïve mice (Fig. 4b). It is noteworthy that during the 10-month period after *L. tropica* challenge in the footpad, the ear lesions of primary infections of high and low doses of *L. tropica*

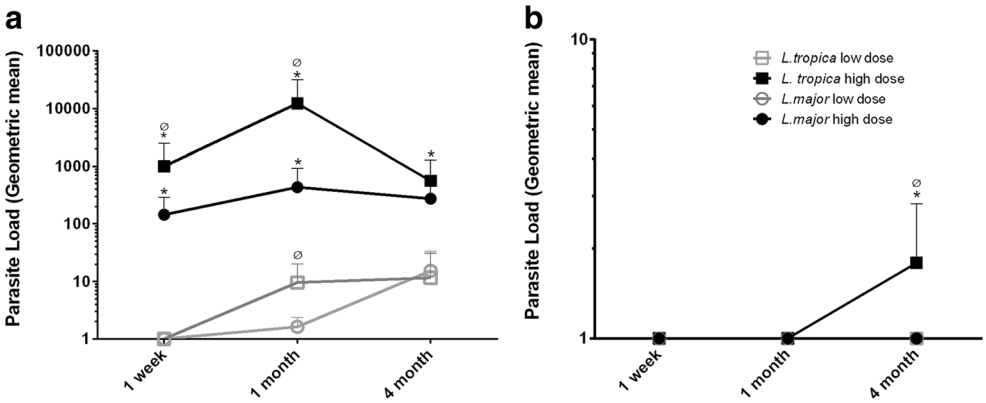

Fig. 2 Effect of infectious dose on parasite load. BALB/c mice were infected in the ear by high (10^6) and low (10^3) doses of *L. tropica* and *L. major* and parasite loads were assayed. **a** Parasite load of lymph node. **b** Parasite load of spleen. Parasite loads were determined at intervals of 1 week, 1 month, and 4 months post-infection. Each point shows geometric mean + SD of parasite load of 3–4 mice per group. Single asterisk shows statistical significant difference between high and low doses of the same parasite species (*L. tropica* or *L. major*). Symbol ø shows statistical significant difference between *L. tropica* and *L. major* of the same dose

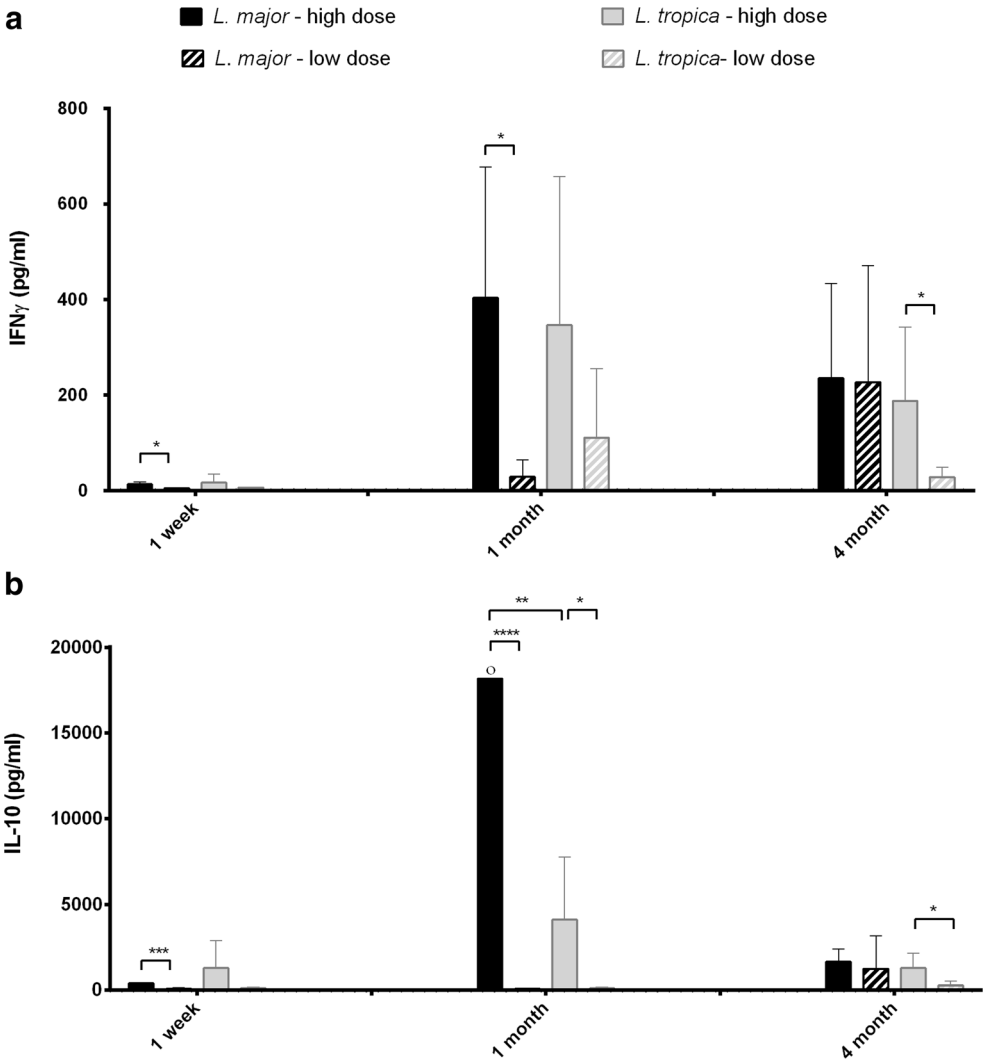

Fig. 3 Effect of infectious dose on cytokine responses. Mice were infected in the ear by high (10^6) or low (10^3) dose of *L. tropica* and *L. major*. Lymph node cells of mice were collected at intervals of 1 week, 1 month, and 4 months post-infection. Lymph node cells were cultured and stimulated by *L. tropica* SLA and cytokine levels of culture supernatants were assayed. **a** IFN-γ response. **b** IL-10 response. Each bar shows mean + SD of cytokine levels of 3–4 mice per group. Asterisks show statistical significant difference between experimental groups ($*P \leq 0.05$; $**P \leq 0.01$). Symbol "o" shows that IL-10 levels are higher than assay limit

reduced to baseline level but the ear lesion of primary infection of *L. major* increased and resulted in necrosis of the ear.

Levels of IFN-γ response after challenge showed that while all pre-infected groups have significantly higher

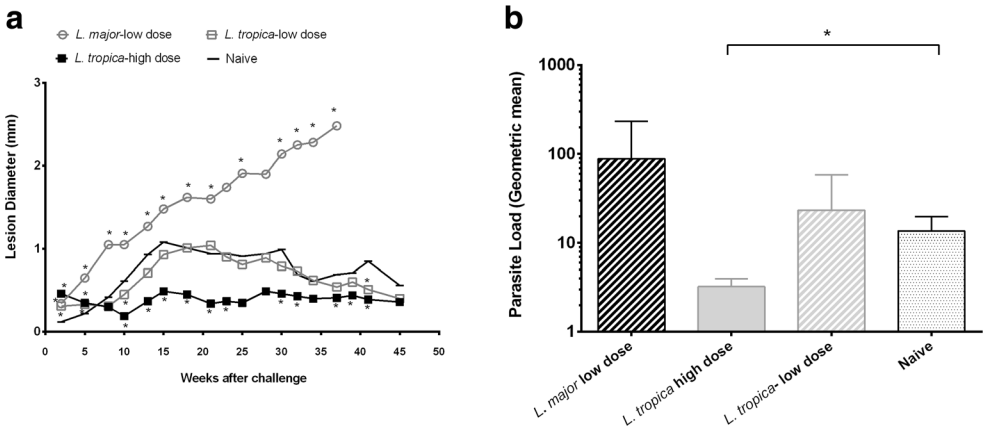

Fig. 4 Effect of infectious dose on induction of protection against *L. tropica* challenge. Mice pre-infected in the ear by high (10^6) and low (10^3) doses of *L. tropica*, low dose of *L. major*, and naïve mice were challenged by 10^6 *L. tropica* in the footpad and lesion diameter and parasite loads were determined. **a** Lesion diameter. Footpad diameter was measured up to 10 months after challenge. Asterisk shows statistical significant difference between pre-infected group and naïve group. **b** Parasite load. The parasite load of lymph nodes was determined 1 week after challenge. Asterisks show statistical significant difference between the two groups

IFN-γ in comparison to the naïve group, there was no significant difference between all pre-infected groups (Fig. 5a). Results of IL-10 response after challenge showed significantly higher levels of this cytokine in the "low-dose *L. major*" group in comparison to all other experimental groups (Fig. 5b).

Discussion

In this research, we have concentrated on a variable of the experimental model of diseases caused by *L. tropica*: "infectious dose." The dose of antigen is an important factor that influences the type of immune response. Results of primary culture of naïve $CD4^+$ T cells showed that development of Th1 or Th2 type of $CD4^+$ T cells strictly depends on the dose of antigen (Hosken et al. 1995).

This study aimed to determine whether the infectious dose of *L. tropica* affects the pathology of this infection. It elucidates the effect of the infectious dose on parasite load, dissemination of the parasite to visceral organs, cytokine responses, and protection against further infection (challenge).We used *L. major* as a control species because pathogenicity and the immune response against this *Leishmania* species have been already studied in detail (Sacks and Noben-Trauth 2002). Our results related to *L. major* infection were as predicted and were completely consistent with previous report (Sacks and Melby 2015).

L. tropica infections (both low and high doses) displayed a chronic self-healing infection and are in agreement with the previous reports (Lira et al. 1998; Mahmoudzadeh-Niknam et al. 2007). Results of lesion diameter and parasite load show, as predicted, that decreasing the infectious dose of *L. tropica* decreases the pathogenicity of this parasite species in BALB/c mice.

Dissemination of *L. tropica* into the spleen (visceralization) is an indication of pathology of this parasite. Our results showed that dissemination of the parasite to the spleen occurs only in the high-dose infection at 4 months after infection. This result indicates that increasing the dose of *L. tropica* increases the parasite pathogenesis. Dissemination of the parasite into the spleen was observed only for *L. tropica* and not for *L. major*. This suggests that *L. tropica* is biologically more suited to dissemination than *L. major*. This is reflective of the fact that dissemination of human *L. tropica* but not human *L. major* has been described (Alborzi et al. 2006; Eroglu et al. 2015).

An important finding of our study is that immune response against the low dose of *L. tropica* is always lower than immune response against high dose of this parasite and this difference remained up to the end of our study. In other words, regarding the immune response, there is a significant difference between low-dose and high-dose infections which is maintained in all phases of infection. Higher immune response against higher dose of *L. tropica* is concordant with our previous report that showed antibody titer against high dose of *L. tropica* infection is significantly higher than the antibody titer against the low dose (Rostamian et al. 2017). Comparison of *L. tropica* data with those of *L. major* shows that the difference between immune responses against low and high doses of *L. tropica* is not valid for *L. major* infection, because low- and high-dose infections of *L. major* result in similar cytokine levels at later phase of infection.

IL-10 is a cytokine that has potent deactivating effects on IFN-γ-mediated killing by macrophages (Bogdan and Nathan 1993; Sacks and Anderson 2004). Higher levels of IL-10 in *L.*

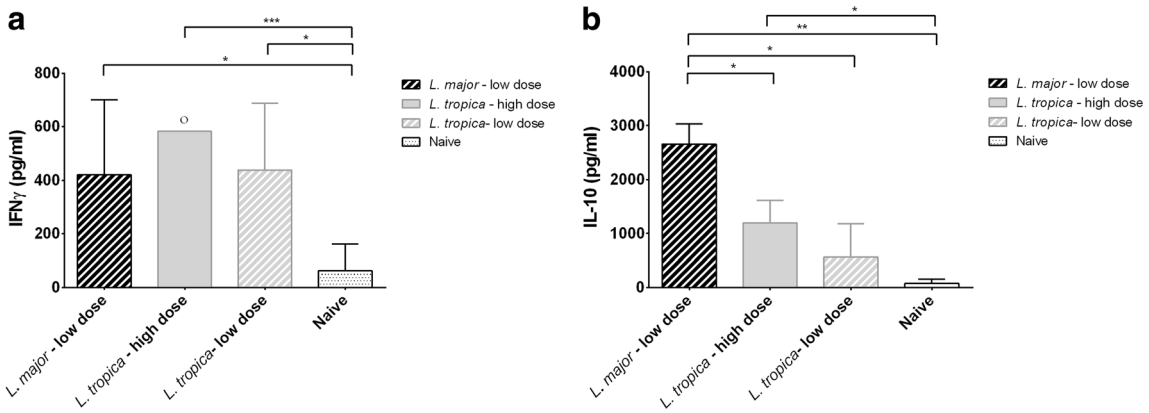

Fig. 5 Effect of infectious dose of primary infection on cytokine responses against *L. tropica* challenge. Mice pre-infected in the ear by high (10^6) and low (10^3) doses of *L. tropica*, low dose (10^3) *L. major*, and naïve mice were challenged by 10^6 *L. tropica* in the footpad and cytokine levels were determined. Lymph node cells were collected 1 week after challenge, cultured, and stimulated by *L. tropica* SLA, and levels of IFN-γ and IL-10 in the culture supernatants were assayed. **a** IFN-γ levels. **b** IL-10 levels. Each bar shows mean + SD of cytokine levels of 3–4 mice per group. Asterisks show statistical significant difference between the two indicated groups (*$P \leq 0.05$;**$P \leq 0.01$; ***$P \leq 0.001$). Symbol "o" shows that IFN-γ levels in the samples are higher than assay limit

major-infected mice in comparison to *L. tropica* suggests that this cytokine may be associated with the higher pathology of *L. major* in comparison to *L. tropica* in BALB/c mice. These findings confirm previous reports (Chatelain et al. 1999; Kane and Mosser 2001; Mahmoudzadeh-Niknam et al. 2011b; Noben-Trauth et al. 2003; Stober et al. 2005) that IL-10 is associated with exacerbation of *L. major* infection in BALB/c mice. However, in contrast to *L. major*, it is not clear how IL-10 affects pathogenesis and immunity against *L. tropica*, although higher levels of IL-10 were observed in response to high dose in comparison to low-dose *L. tropica*. The role of IL-10 in pathogenesis and immunity in *L. tropica* infection needs further studies.

Here, we showed the effect of primary *L. major* infection on exacerbation of secondary *L. tropica* infection. These results show that pre-infection by *L. major* induces a disease-promoting response as was anticipated (Sacks and Noben-Trauth 2002). On the contrary, high dose of *L. tropica* results in protection against further *L. tropica* challenge. However, pre-infection by low dose of *L. tropica* did not result in the protection against *L. tropica* challenge as shown by similar lesion diameters in this group in comparison to naïve mice. Results of lesion diameter, parasite load, and IL-10 response showed general concordance: higher lesion diameter was associated with higher parasite load and higher IL-10 response. These results confirm the validity of our findings. These results may show that the immune response of low-dose *L. tropica* pre-infected mice is not sufficiently powerful to alter the lesion development of *L. tropica* challenge, but more scrutinizing studies are needed in this case. Our results regarding the sharp difference between high and low doses in induction of protective immunity are concordant with another report, in which a high dose, but not a low dose, of *L. chagasi* promastigotes induces protective immunity against challenge infection with *L. chagasi* (Streit et al. 2001).

The less protective immunity induced by a low dose in comparison to a high dose of *L. tropica* suggests that the infectious dose has an important role in *L. tropica* infection. On the other hand, only the high dose of *L. tropica* showed the visceral growth in our study that again underscores the important role of the infectious dose in certain phenomenon associated with pathogenicity of *L. tropica*.

It is noteworthy that sand flies become infected in nature with different doses of *Leishmania* parasites. Also, transmission of different *Leishmania* doses (from high to low doses) to experimental animals after sand fly bites has been reported (reviewed in Courtenay et al. (2017)). Due to these varying initiating doses of sand fly bites, here, we used needle injection to more accurately determine the amount of parasites, although the sand fly bite procedure is more similar to natural infection. In spite of this limitation, the results of the present study may help to broaden the view of dose effect on cutaneous leishmaniasis.

Conclusion

Results of this study reveal that some phenotypes of *L. tropica* infection in BALB/c mice experimental models depend on the infectious dose. These phenotypes include visceralization of parasite to spleen, induction of high levels of cytokine, and protection against challenging infection. Our results also reveal substantial differences between *L. tropica* and *L. major* infections which may be helpful for elucidation of the complex profile of *L. tropica* infections.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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