Induction of partial protection against *Leishmania major* in BALB/c mice by *Leishmania tropica*"

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Summary
Leishmaniasis is a parasitic disease of man and other mammals. Immunity against leishmaniasis appears to remain essentially species specific; however some cross-reactivity has been reported. The aim of this study was analyzing cross-protection induced by *Leishmania tropica* (*L. tropica*) against *Leishmania major* (*L. major*). BALB/c mice were infected with *L. tropica* in the footpad followed by a challenge infection in the contra lateral footpad by *L. tropica* or *L. major*. Footpad thickness and parasite load in the footpad, popliteal lymph node, and spleen were determined after challenge. The results demonstrate that *L. tropica* induces partial protection of BALB/c mice against *L. tropica* as well as *L. major*. The protection was more efficient against a homologous strain (*L. tropica*) than against a heterologous strain (*L. major*). The partial protection against *L. major* was detected in the footpad tissues as well as popliteal lymph node. No protection was observed against *L. major* in the spleen tissue. These findings have implications in vaccination strategies for Leishmaniasis based on the use of heterologous species of the parasite.

Introduction
Leishmaniasis is a disease resulting from infection by protozoan parasites of the genus *Leishmania*, which can affect man and several species of animals. In a mammalian host, *Leishmania* parasites are obligatory intracellular parasites. Infection by *Leishmania* parasites can manifest in different clinical forms, depending both on the infecting species and on the host factors that determine the immunological response to the infectious agent. Infection by *L. major* and *L. tropica* usually gives rise to an ulcerative cutaneous lesion developing at the site of the infecting insect bite, which tends to heal spontaneously within several weeks or months. The infection may involve the draining lymph nodes, but visceralization, which is observed in certain animal models (e.g. in mice of "non-healer" BALB/c strain infected with *L. major*), is highly uncommon in man. Self-cure is usually followed by solid immunity. *L. major*, when inoculated in the skin of BALB/c mice, produces large ulcers which fail to resolve; the parasite usually metastasizes, eventually leading to death of the animal (*Mauel, 2002*). In contrast, *L. tropica* infection in BALB/c mice results in long lasting, stable and non-healing disease (*Lira et al., 1998*).

Immunity to *leishmania* infections has been studied extensively. There is a remarkable level of immunological cross-reactivity between *leishmania* species at both the humoral and cellular levels (*Mauel, 2002*). This has been the rationale for using heterologous organisms of lower pathogenicity as vaccinating agents against the more virulent species (*Mauel, 2002*). In spite of this immunological cross-reactivity, however, there is some evidence that immunity against one *leishmania* species fails to confer protection against another one, i.e. immunity appears to remain essentially species specific (*Melby, 1991*). The "species-specific only" protection rule is not absolute, and several examples of cross-protection have been documented in

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the literature (*Adler & Gunders, 1964; Gicheru et al., 1997; Osorio et al., 1998; Lima et al., 1999; Veras et al., 1999; Alexander & Philips, 1978; Perez et al., 1979; Alexander, 1982; Howard et al., 1982; Rossel et al., 1987; Lujan et al., 1990*).

Further investigations are, therefore, required to determine the precise mechanisms that govern cross-protection and to what extent this can be utilized in vaccination strategies based on the use of heterologous species of low pathogenicity (*Mauel 2002*). *L. major* and *L. tropica* are endemic in the same biotope in many regions in Iran (*Ardehali et al., 2000*) and other countries. Therefore, it is important to know if people who have infection with one parasite are protected against infection with the other. Cross-protection studies in the murine model may help answer this question. Here we report partial heterologous protection conferred by *L. tropica* infection against severe, non-healing cutaneous leishmaniasis due to *L. major* in BALB/c mice.

Materials and Methods

Mice: Inbred BALB/c female, 6-8 weeks old mice were used throughout these experiments. These mice were obtained from the animal breeding facility of Pasteur Institute of Iran.

Parasite: *L. tropica* strain MHOM/AF/88/KK27 is a cutaneous *L. tropica* isolate from Afghanistan, and was initially described by Dr. R. Killick-Kendrick (*Lira et al., 1998*). 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, Maryland, 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). The parasites were grown at 25-27° C in 50-ml disposable centrifuge tubes containing 4 ml of culture medium. The culture medium consisted of 2 ml NNN and 2 ml RPMI-1640 medium (Catalogue No R-8005, Sigma Chemical Co, PO Box 14508, St Louis, MO 63178, USA). The NNN medium consisted of 12% rabbit blood (with sodium citrate as anticoagulant), 1.35% Glucose (Catalogue No G-5146, Sigma Chemical Co, PO Box 14508, St Louis, MO 63178, USA), 1.4% Agar (Agar-agar-ultra pure Cat No 1.01613, Merck K Ga A 64271 Darmstadt, Germany), and 0.6% (w/v) NaCl (Arasto Pharmaceutical Chemicals Co, Saveh, Iran). No antibiotics were added.

Infection: Promastigotes of *Leishmania* parasites were cultured in NNN media. Stationary promastigotes were harvested 6-8 days after beginning of culture. For *L. major* infection, $2x10^6$ stationary phase promastigotes were used. For *L. tropica* infection $5x10^6$ stationary phase promastigotes were used. Parasites were injected into the hind footpad of the mice in a final volume of 50 microliters of phosphate buffer saline (PBS). Primary infections were made in the left hind footpad and secondary infections in the right hind footpad. The same doses of parasite were used for primary and secondary infection.

Study design: Mice were divided into four groups. Each group included 4-8 mice. Two groups received *L. tropica* as primary infection. One group of primary *L. tropica* infected mice and one group of non-infected mice received *L. tropica* as secondary infection. One group of primary *L. tropica* infected and one group of non-infected mice received *L. major* as secondary infection. Disease evolution was monitored throughout the experiment. The experiment was carried out twice. Parasite load of footpad, popliteal lymph node and spleen were quantified at the end of the second experiment, that was 14-16 weeks after secondary infection.

Parasite load assay: Parasite load in footpad, draining lymph node, and spleen were quantified by dilution to extinction in biphasic medium (*Sacks & Melby, 1998*). The assay is briefly as follows. Single cell suspensions are made from draining lymph node or spleen. Footpad tissue was disrupted by a manual tissue homogenizer. Single cell suspension or disrupted tissues are diluted in the liquid phase

of NNN medium. NNN medium was the same as mentioned above. Two-fold serial dilutions were performed to extinction of parasite growth. Assays were performed in duplicate. Parasite load per total lymph node and spleen, or milligram footpad tissue were calculated from the highest dilution at which promastigotes can be grown out. Geometric mean of individual titers was used for comparison of parasite load in different groups.

Monitoring the course of infection: The course of infection was monitored by weekly measurment of footpad thickness by a dial-gauge caliper (Mitutoyu, Kawasaki, Kanagawa, Japan).

Statistical analysis: Student t-test was used for comparison of footpad thickness of mice between different groups. P values equal or greater than 0.05 were considered significant.

Results

Course of infection in naïve mice: *L. tropica* infection induces a non-healing, non-ulcerative infection in naïve BALB/c mice. As shown in Figure 1, footpad thickness increases after infection and reaches a stable state after about 2-3 months. *L. major* infection induces a rapidly progressive non-healing infection in footpad of BALB/c mice. Footpad thickness of *L. major* infected mice is shown in Figure 2. The ratio of number of parasites in footpad to number of parasites in lymph node for *L. major* infected naïve mice is about 1.9 (41344/21247=1.95). This shows that *L. major* grows similarly in footpad and lymph node tissues. The ratio of parasite number in footpad to lymph node for naïve mice infected with *L. tropica* is about 0.1 (108292/881744=0.123). This shows preferential growth of *L. tropica* in lymph node tissues in comparison to footpad tissues.

Course of secondary infection in primary *L. tropica* infected mice: A secondary *L. tropica* challenge in mice previously infected with *L. tropica* induces a stable, non-healing, non-ulcerative infection. This stable infection has statistically significant lower pathology in the footpad in comparison to primary *L. tropica* infection ($p<0.05$), as indicated by a slight increase in footpad thickness (Figure 1). Secondary *L. major* challenge induces a progressive infection in the footpad of previously *L. tropica*

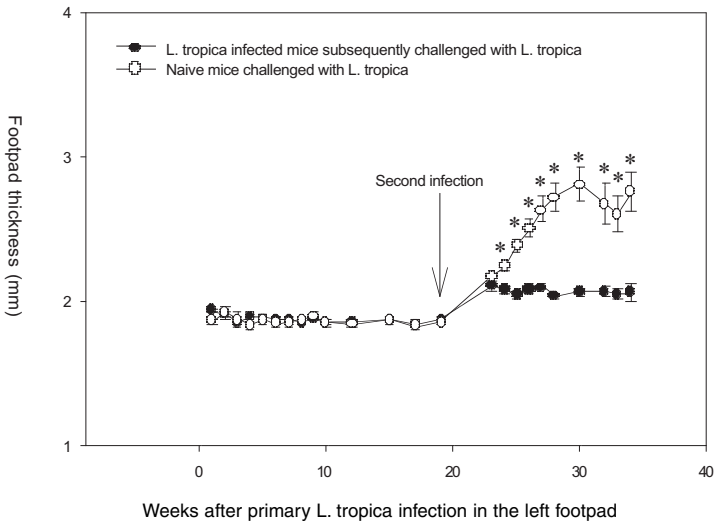

Figure 1. Effect of *L. tropica* infection on subsequent challenge with *L. tropica*. Data are mean ± SE of right footpad thickness (* = $p<0.05$).

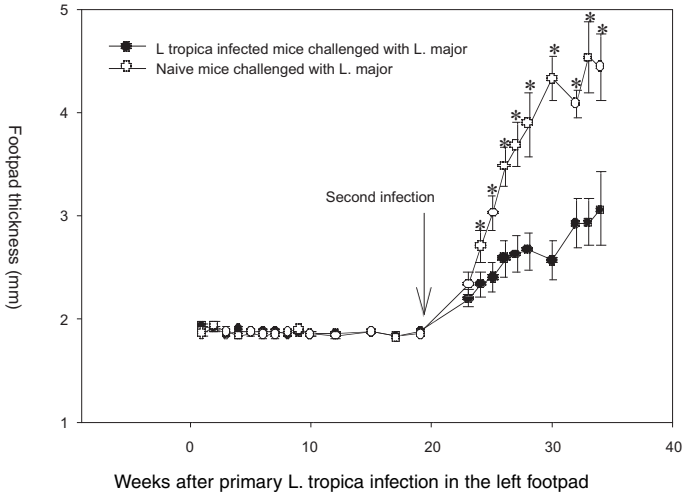

Figure 2. Effect of *L. tropica* infection on subsequent challenge with *L. major*. Data are mean ± SE of right footpad thickness (* = p <0.05).

infected mice. However, the rate of increase was statistically significantly lower than the rate of increase in naïve mice challenged with *L. major* (p<0.05) (Figure 2). These results show a partial protection against *L. major* as well as *L. tropica* challenge in previously *L. tropica* infected mice.

Parasite load in footpad: The number of parasites in the right footpad of *L. tropica* infected mice which received a secondary *L. tropica* challenge in the right footpad was lower than the number of parasites in the *L. tropica* challenged footpad of naïve mice (1862 and 108292 parasites respectively) (Table 1). Parasite load in the right footpad of *L. tropica* infected mice receiving secondary *L. major* challenge at the right footpad was lower than the parasite load of *L. major* challenged naïve mice (4899 and 41344 parasites respectively) (Table 2).

Parasite load in popliteal lymph node: The number of parasites in the right popliteal lymph node of *L. tropica* infected mice with secondary *L. tropica* challenge was lower than the same number in naïve mice receiving *L. tropica* challenge (99334 and 881744 respectively) (Table 1). Parasite load in the right lymph node of *L. tropica* infected mice with a secondary *L. major* challenge was lower than the same load of naïve mice receiving *L. major* challenge (3104 and 21247 respectively) (Table 2). These data indicate similar level of protection at lymph node tissues against both *L. tropica* and *L. major* induced by previous *L. tropica* infection.

The ratio of number of parasites in footpad to number of parasites in lymph node of *L. tropica* infected mice challenged with secondarily *L. tropica* or *L. major* were 0.019 and 1.58 respectively. This shows, similar to infection of naïve mice, preferential growth of *L. tropica* in lymph node tissues in comparison to footpad tissues.

Parasite load in spleen: In naïve BALB/c mice, *L. major* infection results in a higher parasite load in the spleen than *L. tropica* infection (4149 and 951 respectively). *L. tropica* infected mice challenged with *L. tropica* had a lower spleen parasite load than naïve mice challenged with *L. tropica* (18.55 versus 951). *L. tropica* infected mice challenged with *L. major* had a similar spleen parasite load as naïve mice challenged with *L. major* (4754 and 4149). (Tables 1 and 2).

Table 1. Parasite load of secondary *L. tropica* infection of mice.

	Parasite load of *L. tropica* infection			
Specification of mice	Mouse number	Right footpad	Right lymph node	Spleen
Received primary *L.tropica* infection	1	No growth	32768	No growth
	2	4251	46341	283
	3	Not determined	262144	400
	4	1219	65536	No growth
	5	15984	Not determined	400
	6	145	370728	No growth
	Geometric Mean*	1862	99334	18.88
Naïve	1	147687	8388608	6400
	2	27522	11863283	200
	3	1906502	741455	9051
	4	723156	370728	800
	5	4611	92682	400
	6	62415	185364	200
	Geometric Mean*	108292	881744	951

*Parasite load is expressed as Geometric Mean of the final positive well of serial 2-fold dilution as described in Materials and Methods.

Table 2. Parasite load of secondary *L. major* infection of mice.

	Parasite load of *L. major* infection			
Specification of mice	Mouse number	Right footpad	Right lymph node	Spleen
Received primary *L.tropica* infection	1	18725	4	No growth
	2	216	463411	200
	3	17712	16384	289631
	4	8041	8192	144815
	Geometric Mean*	4899	3104	4754
Naïve	1	68089	8192	3200
	2	78840	65536	3200
	3	19205	16384	4525
	4	28340	23170	6400
	Geometric Mean*	41344	21247	4149

*Parasite load is expressed as Geometric Mean of the final positive well of serial 2-fold dilution as described in Materials and Methods.

Discussion

Protection against leishmaniasis can be evaluated in at least two ways in a murine model: measuring footpad thickness and enumeration of parasite load in infected tissue. Primary infection of BALB/c mice with *L. tropica* induced partial protection against secondary infection by *L. tropica* as well as by *L. major*.

Parasite load in infected footpad showed that footpad protection was more efficient against a homologous strain (*L. tropica*) than against a heterologous strain (*L. major*), 58 fold reduction of footpad parasite load in secondary infection by *L. tropica* and 8.4 fold reduction of footpad parasite load in secondary infection by *L. major*.

In the local draining lymph node, *L. tropica* infection induced similar protection in BALB/c mice against *L. tropica* and *L. major* (6.8 fold reduction in parasite load of secondary *L. major* infection and 8.9 fold reduction in parasite load of secondary *L. tropica* infection). What is the explanation for this pattern of protection? Our findings indicate that the tropism of *L. tropica* appears to be different from that of *L. major* in BALB/c mice: *L. tropica* grows preferentially in popliteal lymph node while *L. major* grows similarly in popliteal lymph node and footpad. This growth pattern was also seen in the secondary infection by *L. tropica* and *L. major*. These data suggest that *L. tropica* infection induces adaptive immune mechanisms that are expressed preferentially in the periphery to confer partial control over primary infection in the footpad and even stronger control over secondary challenge that was again preferentially expressed in the footpad compared to draining lymph node. Further analysis of local immune response is needed to clarify the underlying mechanisms of different levels of protection induced by *L. tropica* infection in different tissues.

L. tropica primary infection was efficient in reducing the parasite load of the spleen in secondary *L. tropica* infection (about 50 fold reduction). Interestingly, however, *L. tropica* primary infection was not efficient in reducing the parasite load of the spleen in secondary *L. major* infection. We can conclude that *L. tropica* and *L. major* do not have common immunomodulatory mechanisms involved in spleen based growth of the parasite; thus prior infection of *L. tropica* can not prevent growth of *L. major* in the spleen. These findings open new horizons for the study of different immunomodulatory mechanisms induced by infection with these two *leishmania* species and/or their different antigens.

An important finding in our data is the ability of *L. tropica* to infect the spleen after subcutaneous injection of the parasite into the footpad of BALB/c mice. This is the first report, as far as we are aware. In one study (*Lira et al., 1998*) the spleen was negative for *L. tropica* when cultured at 7 months after infection. However, culture of the spleen in our report was carried out 3-4 months post-infection, which may account for the different results, if the *L. tropica* was cleared from the spleen in later stages of the infection.

L. tropica and *L. major* result in different forms of non-healing infections in BALB/c mice. *L. tropica* results in long lasting chronic infection, while *L. major* cause non-healing infection, which results in rapid death. This murine model of leishmaniasis has fundamental differences with infections of humans by these two *leishmania* species. Both species result in self-healing infections in the majority of human individuals. So we cannot extrapolate easily our findings from this murine model to human infection. However, the murine model can reveal common as well as distinct epitopes, immunomodulatory mechanisms, and pathological pathways that are presented by these two different species of *leishmania* parasites.

Our findings confirm that protection between *leishmania* species is mainly species-specific. *L. tropica* infection induces more protection against a homolgous strain (*L. tropica*) than a heterolgous strain (*L. major*). Results of this study revealed common as well as different immunomodulatory mechanisms between *L. tropica* and *L. major*. Further studies in this field can unravel the pathophysiology

as well as immunology of leishmaniasis due to these two parasite species in relation to prevention and treatment of these diseases.

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References

Adler S & AE Gunders: Immunity to *Leishmania mexicana* following spontaneous recovery from oriental sore. Trans R Soc Trop Med Hyg. 1964, *58* : 274-277.

Alexander J & RS Philips: Leishmania tropica and *Leishmania* mexicana: cross-immunity in mice. Exp Parasitol 1978, *45*, 93-100.

Alexander J: A radioattenuated *Leishmania major* vaccine markedly increases the resistance of CBA mice to subsequent infection with *Leishmania* mexicana. Trans R Soc Trop Med Hyg. 1982, *76*, 646-649.

Ardehali S, A Moattari, GR Hatam, SMH Hosseini & I Sharifi: Characterization of *Leishmania* isolates in Iran: 1. Serotyping with species specific monoclonal antibodies. Acta Trop 2000, *75*, 301-307.

Gicheru MM, JB Olobo & CO Anjili: Heterologous protection by *Leishmania Donovani for Leishmania major* infections in the vervet monkey model of the disease. Exp Parasitol 1997; *85*, 109-116.

Howard JG, S Nicklin, C Hale & FY Liew: Prophylactic immunization against experimental leishmaniasis: I. Protection induced in mice genetically vulnerable to fatal *Leishmania tropica* infection. J Immunol 1982; *129*, 2206-2212.

Lima HC, GK DeKrey & RG Titus: Resolution of an infection with *Leishmania braziliensis* confers complete protection to a subsequent challenge with *Leishmania major* in BALB/c mice. Mem Inst Oswaldo Cruz 1999; *94 (1)*, 71-76.

Lira R, S Mendez, L Carrera, C Jaffe, F Neva & D Sacks: Leishmania tropica: the identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. Exp Parasitol 1998; *89*, 331-334.

Lujan R, WL Chapman, WL Hanson & VA Dennis: Leishmania braziliensis in the squirrel monkey: Development of primary and satellite lesions and lack of cross-immunity with *Leishmania donovani*. J Parasitol 1990; *76,* 594-597.

Mauel J: Vaccination against *Leishmania* infections. Curr Drug Targets Immune Endocr Metabol Disord 2002; *2,* 201-206.

Melby PC: Experimental Leishmaniasis in Humans: Review. Rev Infect Dis 1991; *13,* 1009-17.

Osorio Y, SJ Gonzalez, VL Gama & BL Travi: Reinfection of American Cutaneous *Leishmaniasis*: Evaluation of clinical outcomes in the hamster model. Mem Inst Oswaldo Cruz 1998: *93 (3),* 353-356.

Perez H, B Beverly Arredondo & R Mavhado: Leishmania mexicana and *Leishmania tropica*: cross-immunity in C57BL/6 mice. Exp Parasitol 1979; *48,* 9-14.

Rossel RAD, RS Bray & J Alexander: The correlation between delayed type hypersensitivity, lymphocyte activation, and protective immunity in experimental murine leishmaniasis. Parasite Immunol 1987; *9,* 105-115.

Sacks DL & P Melby: Animal's models for the analysis of immune responses to leishmaniasis. In: Coligan, Kruisbeek, Margulies, Shevach, Strober, editors. Current Protocols in Immunology, Vol 4, John Wiley & Sons, New York, 1998; 19.2.1- 19.2.20.

Veras P, C Brodskyn, F Balesteri, LD Freitas, A Ramos, A Queiroz, A Barral, S Beverley & M Barrel-Netto: A dhfr-ts-*Leishmania major* knockout mutant cross-protects against *Leishmania amazonensis*. *Mem Inst Oswaldo Cruz* 1999; *94 (4),* 491-496.