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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Heterogeneity of humoral immune response to *Leishmania tropica* in an experimental model

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Abstract

Humoral (antibody) response is an important part of immunity against pathogens. Despite the clear role of cell-mediated immune response in protection against leishmaniasis, the role of humoral responses is challenging. There is very limited data regarding humoral immune response against *Leishmania tropica* which is the causative agent of human cutaneous leishmaniasis in many parts of the world. Here, we have compared pathogenicity and antibody response against six Iranian *Leishmania tropica* isolates in BALB/c mice. A *Leishmania major* isolate was used for comparison. The parasites were injected into the mice followed by the evaluation of the lesion development, parasite load, and antibody responses (IgG1 and IgG2a). Our findings showed that some isolates caused the large lesions and high parasite load in the spleen and lymph node, while other isolates led to no lesion, no splenic parasitism, and low parasite load in the lymph node. The more pathogenic isolates induced higher antibody responses (IgG1 and IgG2a). Our results indicated that there is substantial heterogeneity among various *Leishmania tropica* isolates regarding the humoral immune response as well as the pathogenicity.

Keywords Antibody · Iran · *Leishmania tropica* · Lesion size · Parasite load

Introduction

Several *Leishmania (L.)* species can cause Leishmaniasis, a disease with different clinical manifestations from cutaneous leishmaniasis (CL), to a more severe form of visceral leishmaniasis (VL). *Leishmania tropica* is the causative agent of CL and rarely VL in humans in many parts of the world including Iran (World Health 2010).

It has been reported that *L. tropica* is very heterogeneous biochemically (Le Blancq and Peters 1986; Mebrahtu et al. 1992), serologically (Jacobson 2003; Schnur et al. 2004), and genetically (Schonian et al. 2001; Schwenkenbecher et al. 2006). However, in contrast to the well-known *L. major* (Sacks and Noben-Trauth 2002), there is limited data regarding immunology and pathogenicity of *L. tropica* infection in both humans and animal models (Rostamian and Niknam 2018). Studies on *L. tropica* infection in experimental models can increase our knowledge about this neglected species.

Humoral response is an important part of immunity against pathogens. These responses are mediated by antibodies which have evolved into different classes and subclasses. Antibodies have been shown to have several functions such as opsonization, cytotoxicity, and release of inflammatory mediators (Nimmerjahn and Ravetch 2005).

Four subclasses of immunoglobulin G have been identified in BALB/c mice: IgG1, IgG2a, IgG2b, and IgG3 (Fahey et al. 1964a, b; Nussenzweig et al. 1964; Grey et al. 1971). The expression of IgG subclasses is affected by many factors, including cytokines. Isotype switching to IgG2a and IgG1 is mediated by IFN-γ (as a TH1 cytokine) and IL-4 (as a TH2 cytokine), respectively (Snapper and Paul 1987; Finkelman et al. 1990). In many studies, these IgG isotypes have been applied as surrogate markers of TH1 and TH2 responses against many pathogens including *Leishmania* (Wang et al. 1994; Fox et al. 2014).

It is generally accepted that the protection against leishmaniasis is basically associated with cell-mediated immune response. However, it has been demonstrated that when B cells or antibodies are removed, the course and outcome of

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the disease are profoundly changed within genetically resistant and susceptible mouse strains (Sacks 1988). It is important to know the pathogenic or protective IgG isotypes of anti-*Leishmania* antibodies, to use these responses for the benefit of the host (Chu et al. 2010).

It has been reported that antibodies may have detrimental or beneficial roles in *Leishmania* infections. Further studies referred these different roles to the specific isotypes of antibodies. Some previous reports indicated that the susceptibility of BALB/c mice to leishmaniasis is associated with B cells and/or antibodies (Sacks et al. 1984). Furthermore, it has been reported that antibody contributes to leishmaniasis progression through IL-10 production from macrophages (Miles et al. 2005). The detrimental role of antibodies has been also reported in some other studies (Sutterwala et al. 1998; Padigel and Farrell 2005). Conversely, the beneficial role of humoral responses in *Leishmania* infection has been described in some studies. As an example, IgG production and the engagement of dendritic cell FcγR are required for control of CL in experimental mice (Woelbing et al. 2006). Moreover, it has been shown in several studies that IgG2a isotype is associated with controlling of leishmaniasis, while IgG1 isotype is associated with the disease progression (Bretscher et al. 1992; Zimmermann et al. 1998; Uzonna et al. 2001; Yamakami et al. 2001; Jaafari et al. 2006; Shimizu et al. 2007; Thakur et al. 2014).

There is little information regarding the role of antibody in the immune response against *L. tropica*. In our previous work, we evaluated the role of *Leishmania* specific antibodies and their IgG isotypes in infection caused by one *L. tropica* isolate (KK27). We found that infection by *L. tropica* isolate KK27 leads to a lower IgG1 response than *L. major* infection, which is associated with protection against *L. tropica* infection (Rostamian et al. 2017). Here, we have determined the antibody response against six clinical isolates of *L. tropica* in BALB/c mice to more clarify the pattern of humoral response against different isolates of this parasite. Also, we have studied the heterogeneity of *L. tropica* pathogenesis and its visceralization.

Materials and methods

Mice, parasites, and species identification

Five to seven-week-old BALB/c mice were used. The mice were purchased from Animal Production Department of Pasteur Institute of Iran. Six isolates of *L. tropica* were used in this study (Table 1). These isolates have been collected from lesions of CL patients, as reported in our previous study (Mahmoudzadeh-Niknam et al. 2012).

Parasites with less than two in vitro passages were used for infection of mice in this study. The virulence of parasites was preserved by in vivo passage in BALB/c mice. Parasites were cultured from the lymph node of mice and stored in liquid nitrogen. The parasites were recovered from liquid nitrogen by thawing and culturing in Novye-MacNeale-Nicolle (NNN) medium for 3 days, followed by transferring to liquid RPMI 1640 medium appended by 10% fetal bovine serum (FBS). All the parasites were cultured at 24 °C. The stationary phase was determined by the equal numbers of parasites in the cultures in 2–3 subsequent days, counting by Neubauer chamber.

Table 1 Parasites used in this study

Species	International code	Abbreviation
L. tropica	MHOM/IR/02/IPI-MA6	Lt-MA6
L. tropica	MHOM/IR/02/IPI-MA10	Lt-MA10
L. tropica	MHOM/IR/02/IPI-MA14	Lt-MA14
L. tropica	MHOM/IR/03/IPI-SH29	Lt-SH29
L. tropica	MHOM/IR/06/IPI-JI1	Lt-JI1
L. tropica	MHOM/AF/88/KK27	Lt-KK27
L. major	MHOM/IR/03/IPI-DA27	Lm-DA27

The identification of the parasite species was done as reported elsewhere (Mahmoudzadeh-Niknam et al. 2011). Briefly, parasite genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, USA). The ITS1 region was amplified using ITS1 specific primers (Mahmoudzadeh-Niknam et al. 2011). The PCR product was further analyzed by restriction fragment length polymorphism (RFLP), using *Hae*III enzyme.

Infection and lesion measurement

Mice were infected by 2×10^6 stationary promastigotes/mouse subcutaneously in the footpad. The percentage of metacyclic promastigotes was approximately 25–35% for MHOM/AF/88/KK27 isolate, as counted by Ficoll enrichment (Spath and Beverley 2001). No evaluation of the percentage of metacyclic promastigotes was done for other isolates. Monitoring of the lesion development was done weekly by measuring footpad thickness by a dial-gauge caliper (Mitutoyo, Kawasaki, Kanagawa, Japan). The final lesion diameter was achieved from subtraction of the thicknesses of the infected footpad from the contra lateral un-infected one.

Parasite load assay

The parasite burden of mice lymph node and spleen were quantified by limiting dilution assay in biphasic medium, as previously described (Sacks and Melby 2015). Briefly, draining lymph nodes or spleens were homogenized to make single-cell suspensions. These suspensions were serially diluted (four- or tenfold) in RPMI-1640 and added to NNN medium in 96-well plates. Each dilution of cell suspension was

assayed in triplicate. Serial dilutions were performed to extinction of parasite growth. Highest dilution, at which parasites can be grown out, was considered as parasite load of the lymph node or spleen.

Preparation of soluble *Leishmania* antigen (SLA)

SLA was prepared as described previously (Rostamian and Niknam 2017; Rostamian et al. 2017) with some modifications. Briefly, parasites were cultured and stationary phase promastigotes were harvested, washed three times with PBS buffer, subjected to freeze-thaw cycles (five rounds), centrifuged (16,000×*g*, 20 min, 4 °C) and finally the supernatant was collected as SLA. The protein concentration of SLA samples was assayed by Bradford method (Simonian 1996). SLA was prepared from each isolate separately.

Blood samples

Eight weeks after infection, blood samples were obtained using retro-orbital bleeding procedure. All the experiments were approved by the ethics committee of the Pasteur Institute of Iran.

ELISA assay

Prepared SLA of each isolate was used for antibody assay of mice infected by the same isolate. ELISA was done as reported (Rostamian et al. 2017). Briefly, 0.2 μg/well of SLA of each isolate was coated in 96-well ELISA plates (Greiner, Microlon). The plates were incubated at 4 °C overnight. Then the plates were washed (three times by PBS +Tween 20), and blocked by BSA, and incubated at 37 °C for 2 h. After washing step (as above), the sera at optimized dilution (1/4000) were added to plates and incubated for 2 h at 37 °C. The plates were washed again, and 100 μl/well of 1/2000 HRP-conjugated anti-mouse IgG1 or IgG2a (BD Pharmingen) was added. After incubation and washing steps as above, tetramethylbenzidine (TMB) as horseradish peroxidase (HRP) substrate was added and the plates were incubated for 15 min at room temperature in a dark place. The enzymatic reaction was stopped by addition of stop solution (2N H_2SO_4), and the optical density (OD) of each well was read by a microplate reader (Anthos 2020; Eugendorf, Austria) at both 450 and 620 nm. Final OD values were calculated by subtracting values of 620 nm from values of 450 nm. Each sample was used in duplicate. The optimized dilution of sera was the dilution in which different experimental groups could be discriminated clearly. The optimized serum dilution was found through testing different dilutions of all sera (twofold dilutions from 1/500 to1/32,000). The optimal dilution was 1/4000 and used for all sera.

Study design

Species of the parasite were determined. Mice were randomly divided into seven groups of five mice each and were infected by the parasites in the footpad. The lesion development was monitored up to 8 weeks after infection. Mice were bled, sacrificed at 8 weeks after infection, and lymph nodes and spleens were removed. Antibody responses (IgG1 and IgG2a) in sera and parasite loads in lymph nodes and spleens were determined for each mouse.

Statistical analysis

One- or two-tailed Student's *t* test was applied for comparison of pathology and immune indexes between different groups using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). The *p* values ≤ 0.05 were considered significant.

Results

Species identification

The identification of the parasites was performed using ITS1 gene amplification by PCR as well as RFLP using *Hae*III digestion of the PCR products. PCR amplification of ITS1 region by specific primers resulted in a distinct single band of about 300 bp for all isolates. The presence of this band in all samples indicates that all isolates are *Leishmania* species. The digestion patterns of ITS1 PCR product with *Hae*III RFLP were concordant with *L. tropica* for Lt-MA6, Lt-MA10, Lt-MA14, Lt-SH29, Lt-JI1, and Lt-KK27 isolates and with *L. major* for Lm-DA27 isolate (Fig. 1).

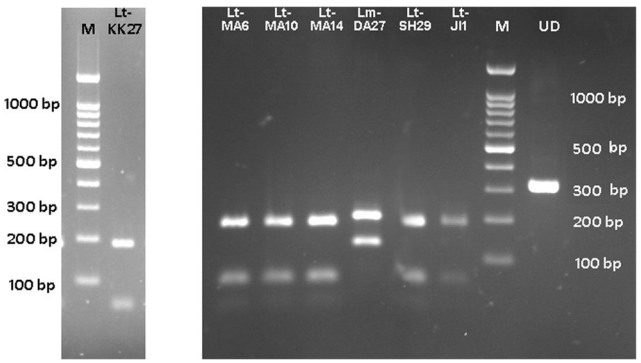


Fig. 1 Species identification of *Leishmania* parasites by RFLP. The ITS-1 PCR product was digested with *Hae*III restriction enzyme. The Lt-MA6, Lt-MA10, Lt-MA14, Lt-SH29, Lt-JI1, and Lt-KK27 isolates showed an RFLP concordant to *L. tropica*, while RFLP pattern of Lm-DA27 isolate was consistent with *L. major*. M, DNA marker; UD, un-digested ITS1 PCR product

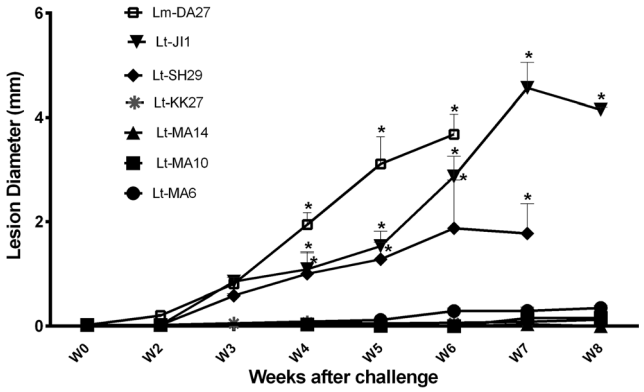


Fig. 2 Lesion development in mice infected by *Leishmania* parasites. Each *Leishmania* isolate (2×10^6 stationary promastigotes/mice) was injected to mice and their footpad thickness was measured weekly. Each point shows mean + SEM of 4–5 mice per group. Statistical differences ($p \leq 0.05$) were observed between isolates with lesion development (Lt-SH29, Lt-JI1, and Lm-DA27) and isolates without lesion development (Lt-MA6, Lt-MA10, Lt-MA14, and Lt-KK27) at week 4 post-infection onward (shown by *). The lesion measurement was stopped for isolates Lm-DA27, Lt-SH29, and Lt-JI1 after week 6, 7, and 8 post-infection, respectively due to severe necrosis in the footpad

Pathogenicity of the isolates

Pathogenicity of the isolates was measured by lesion development at the site of infection as well as parasite load in mice lymph nodes and spleens. As shown in Fig. 2, no lesion was observed in Lt-MA6, Lt-MA10, Lt-MA14, and Lt-KK27 isolates, while the Lt-SH29, Lt-JI1, and Lm-DA27 isolates showed a significant lesion development that was observed from week 4 post-infection onwards.

The Lt-SH29 and Lt-JI1 isolates of *L. tropica* as well as Lm-DA27 isolates of *L. major* showed higher parasite loads in the lymph node in comparison to isolates of Lt-MA6, Lt-MA10, Lt-MA14, and Lt-KK27 (Fig. 3a).

There was no parasite in the spleen sample of mice infected by *L. tropica* isolates of Lt-MA6, Lt-MA10, Lt-MA14, and Lt-KK27. However, mice infected by Lt-SH29 and Lt-JI1 isolates of *L. tropica* as well as Lm-DA27 isolates of *L. major* showed the presence of parasite in their spleen. The highest splenic parasite load was detected for Lt-JI1 isolates (Fig. 3b).

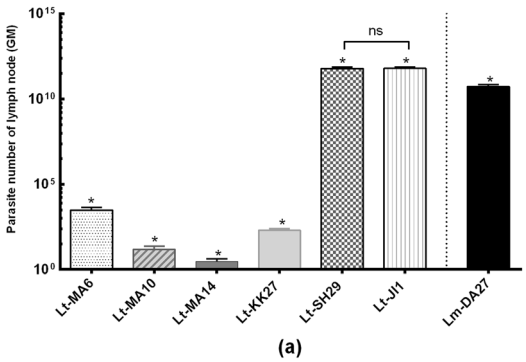
Humoral responses to the isolates

The level of antibody subclasses of IgG1 and IgG2a was determined using ELISA for each experimental group. In order to reduce the effects of different variables of ELISA (e.g., ELISA plate, chromogenic substrate, and incubation time) on results of different ELISA runs, all sera were assayed in a single run with the same ELISA setting (with the same plates, same reagents, same time, etc.).

Mice infected by *L. major* isolate of Lm-DA27 showed higher IgG1 response than other groups. There was no significant difference between IgG1 responses of other groups (Fig. 4a). Furthermore, the highest IgG2a response was seen in the group of *L. major* isolate of Lm-DA27. Mice infected by Lt-SH29 and Lt-JI1 showed significantly higher IgG2a response than other *L. tropica* isolates (Fig. 4b).

Discussion

It has been reported that the *L. tropica* infection of BALB/c mice results in no or small lesions, while *L. major* causes severe lesions in this inbred mice (Lira et al. 1998; Kobets et al. 2012; Rostamian et al. 2017). Our results showed that *L. tropica* isolates of Lt-MA6, Lt-MA10, Lt-MA14, and Lt-KK27 did not cause evident lesion in BALB/c mice that is concordant with previous reports (Lira et al. 1998; Rostamian et al. 2017, 2018a). Conversely, *L. tropica* isolates of Lt-SH29 and Lt-JI1 developed large lesions in the injection site resulting in severe necrosis similar to the



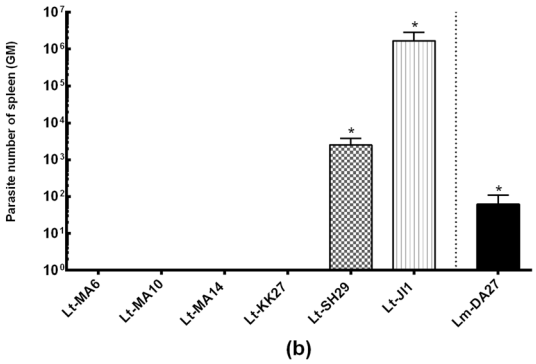


Fig. 3 *Leishmania* parasite load of lymph node and spleen. Two months after *Leishmania* infection in mice, their lymph nodes and spleens were removed and assayed for parasite load. **a** Parasite load of lymph node. **b** Parasite load of spleen. Statistically significant differences were observed between lymph node parasite load of each group with other groups (shown by "*"), except the parasite loads of mice infected with Lt-SH29 and Lt-JI1 which showed no significant difference (shown by "ns"). The parasite loads of spleens were significantly different between mice infected with Lt-SH29, Lt-JI1, and Lm-DA27 with each other (shown by "*"). Each bar shows geometric mean + SD of parasite load of 4–5 mice per group. GM, geometric mean

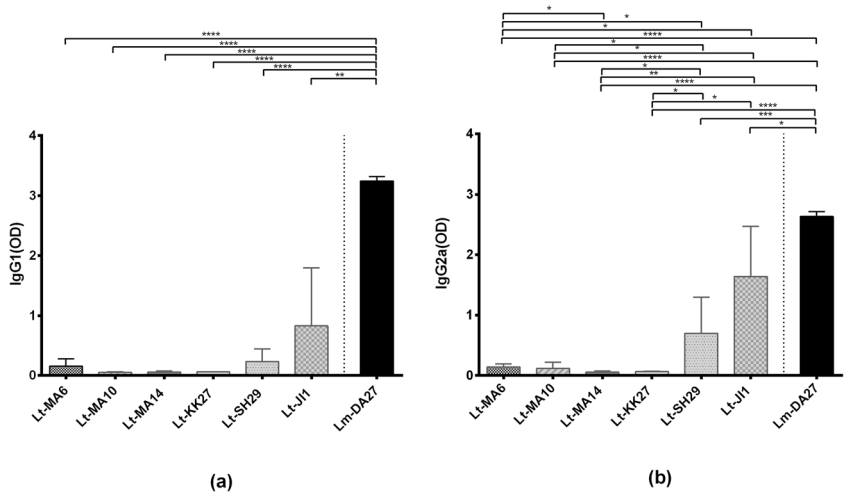


Fig. 4 Levels of antibody response against *Leishmania* isolates. Two months after infection by *Leishmania* parasites, mice were bled and antibody responses were assayed. **a** IgG1 responses, **b** IgG2a responses. Each value is average optical density (OD) + SEM of values of 4–5 mice per group. Asterisks show statistically significant difference between groups (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$)

lesions of *L. major* isolate of Lm-DA27. The results of parasite loads in lymph node confirm the lesion results, i.e., higher parasite loads are associated with higher lesion diameter, revealing that *L. tropica* isolates of Lt-SH29 and Lt-JI1 are more pathogenic than other *L. tropica* isolates used in this study. These results indicate that with regard to pathogenicity in mice, there are significant differences among *L. tropica* isolates.

Visceralization of the *Leishmania* parasites is a process that depends on many factors including infectious dose, host sex, and infectious route (Mahmoudzadeh-Niknam et al. 2007; Kobets et al. 2012; Rostamian et al. 2018b). Here, we found that different strains of *L. tropica* have different potentials for visceralization in BALB/c mice. The more pathogenic strains disseminate to spleen while less pathogenic strains do not. The *L. major* Lm-DA27, as a control species, showed visceralization that confirmed optimization of our experimental settings. Visceralization of *L. major* in our study is consistent with other reports (Nasseri and Modabber 1979; Sacks and Melby 2015). However, there are conflicting reports regarding visceralization of *L. tropica*: visceralization in some studies (Mahmoudzadeh-Niknam 2004; Mahmoudzadeh-Niknam et al. 2007; Kobets et al. 2012), but no visceralization in others (Lira et al. 1998; Rostamian et al. 2018a). Our findings provide an explanation for the controversies regarding visceralization of *L. tropica* in BALB/c mice: the heterogeneity between different strains of *L. tropica*. It is noteworthy that the parasite strain is not the only factor responsible for these controversies about visceralization of *L. tropica*, since one strain (Lt-KK27) showed visceralization in some reports (Mahmoudzadeh-Niknam 2004; Mahmoudzadeh-Niknam et al. 2007) but no visceralization in another one (Lira et al. 1998). This data suggests that visceralization of *L. tropica* in BALB/c mice is a complicated phenomenon and needs more investigation.

Our results indicated that higher levels of antibodies (IgG1 and IgG2a) in BALB/c mice are produced against more pathogenic isolates of *L. tropica* (Lt-SH29 and Lt-JI1) in comparison to less pathogenic ones (Lt-MA6, Lt-MA10, Lt-MA14, and Lt-KK27). There is no comprehensive research on antibody response of BALB/c mice infected by *L. tropica*; however, it is clear that *L. tropica* cause completely different patterns of pathology and immune responses in BALB/c mice compared to *L. major* (Lira et al. 1998; Kobets et al. 2012; Rostamian et al. 2017). Our results revealed that in terms of immune response in mice, in particular, antibody levels, there is heterogeneity among *L. tropica* isolates.

In the present study, we observed that IgG2a responses are associated with severe lesion and high parasite loads for some *L. tropica* isolates. However, it should be noted that the antibody response may not be the main factor of protective immunity against *L. tropica* infection in BALB/c mice and other factors of immune response (e.g., regulatory cells, cytotoxic cells, and cytokines) may be involved. More studies are needed to better clarify the impact of antibody response on *L. tropica* infection.

In summary, our results indicate that there is a substantial heterogeneity among various strains of *L. tropica* that causes different patterns of pathogenicity and humoral immune responses in BALB/c mice.

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Compliance with ethical standards

All the experiments were approved by the ethics committee of the Pasteur Institute of Iran.

Conflict of interest The authors declare that they have no conflict of interest.

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