Cytokine expression in the delayed-type hypersensitivity response of *Peromyscus yucatanicus* infected by *Leishmania (Leishmania) mexicana* that healed spontaneously

Expressão de citocinas na resposta de hipersensibilidade de tipo retardado de *Peromyscus yucatanicus* infectado por *Leishmania (Leishmania) mexicana* que curou espontaneamente

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**Karina Beatriz López-Avila**
Licenciatura en Químico Farmacéutico Biólogo por la Universidad Autónoma de Yucatán,
Facultad de Química
Institución: Universidad Autónoma de Yucatán, Centro de Investigaciones Regionales “Dr. Hideyo Noguchi”, Laboratorio de Inmunología
Dirección: Ave. Itzáes No. 490 x 59-A, Col. Centro, Mérida, Yucatán, México
Correo electrónico: karina.lopez@correo.uady.mx

**Erika Ivett Sosa-Bibiano**
Maestría en Ciencias por la Universidad Autónoma de Yucatán, Facultad de Medicina.
Institución: Universidad Autónoma de Yucatán, Centro de Investigaciones Regionales “Dr. Hideyo Noguchi”, Laboratorio de Inmunología
Dirección: Ave. Itzáes No. 490 x 59-A, Col. Centro, Mérida, Yucatán, México
Correo electrónico: erikasosa@correo.uady.mx

**Fernando Andrade-Narváez**
Doctorado en Ciencias por el Instituto Politécnico Nacional, Escuela de Ciencias Biológicas.
Institución: Universidad Autónoma de Yucatán, Centro de Investigaciones Regionales “Dr. Hideyo Noguchi”, Laboratorio de Inmunología
Dirección: Ave. Itzáes No. 490 x 59-A, Col. Centro, Mérida, Yucatán, México
Correo electrónico: anarvaez@correo.uady.mx

**Elsy Nalleli Loría-Cervera**
Doctorado en Ciencias de la Salud por la Universidad Autónoma de Yucatán, Posgrado Institucional en Ciencias de la Salud
Institución: Universidad Autónoma de Yucatán, Centro de Investigaciones Regionales “Dr. Hideyo Noguchi”, Laboratorio de Inmunología
Dirección: Ave. Itzáes No. 490 x 59-A, Col. Centro, Mérida, Yucatán, México
Corresponding author: nalleli.cervera@correo.uady.mx
ABSTRACT

*Peromyscus yucatanicus* has been employed as a model to study cutaneous leishmaniasis caused by *Leishmania (Leishmania) mexicana*. Nevertheless, there is no information about spontaneously healing and the associated immune response in these rodents. The objective of this work was to analyze the cytokine expression in the delayed-type hypersensitivity response of *Peromyscus yucatanicus* infected by *Leishmania (Leishmania) mexicana* that healed spontaneously. Mice (n=40) were inoculated with $2.5 \times 10^6$ parasites in the tail and evolution was recorded weekly until active lesions appearance (Group I: non-healed) and until spontaneous healing (Group II: healed). A control group was injected with RPMI-1640 medium. The delayed type hypersensitivity (DTH) response and cytokine (IFN-γ, IL-10, TNF) expressions were determined. Spontaneous healing was observed in 65% (13/20) of mice in Group II. The healed group developed a strong DTH reaction which was significantly higher than the control group. At 24 h, IFN-γ was highly expressed in the DTH reaction of both non-healed and healed mice. IL-10 was higher in healed mice in comparison with control group while TNF expression was higher in non-healed mice. At 48 h, INF-γ was highly expressed in non-healed mice. The spontaneous healing of cutaneous lesions in *P. yucatanicus* was associated with the expression of both immunoregulatory (IL-10) and effector cytokines (IFN-γ) in the DTH response.

**Keywords:** *Peromyscus yucatanicus; Leishmania*; localized cutaneous leishmaniasis; DTH response; cytokines.

RESUMO

*Peromyscus yucatanicus* tem sido empregado como modelo para estudar a leishmaniose tegumentar causada por *Leishmania (Leishmania) mexicana*. No entanto, não há informações sobre a cura espontânea e a resposta imune associada nesses roedores. O objetivo deste trabalho foi analisar a expressão de citocinas na resposta de hiper sensibilidade do tipo retardado de *Peromyscus yucatanicus* infectado por *Leishmania (Leishmania) mexicana* que cicatrizou espontaneamente. *Peromyscus yucatanicus* (n = 40) foram inoculados com $2.5 \times 10^6$ parasitas na cauda e a evolução foi registrada semanalmente até o aparecimento das lesões ativas (Grupo I: não cicatrizadas) e até a cicatrização espontânea (Grupo II: cicatrizadas). Um grupo controle foi injetado com meio RPMI-1640. A resposta de hiper sensibilidade do tipo retardado (DTH) e as expressões de citocinas (IFN-γ, IL-10, TNF) foram determinadas. A cura espontânea foi observada em 65% (13/20) dos *P. yucatanicus* do Grupo II. O grupo curado desenvolveu uma forte reação DTH que foi significativamente maior do que o grupo controle. Às 24 h, o IFN-γ foi altamente expresso na reação DTH de ambos os camundongos não curados e curados. IL-10 foi maior em camundongos curados em comparação com o grupo controle, enquanto a expressão de TNF foi maior em camundongos não curados. Em 48 h, INF-γ foi altamente expresso em camundongos não curados. A cicatrização espontânea de lesões cutâneas em *P. yucatanicus* foi associada à expressão de citocinas imunorreguladoras (IL-10) e efetoras (IFN-γ) na resposta DTH.

**Palavras-chave:** *Peromyscus yucatanicus; Leishmania*; leishmaniose cutânea localizada; Resposta DTH; citocinas.
1 INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by flagellated protozoans of the genus *Leishmania* and transmitted via infected female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Okwor et al., 2016). Approximately, 350 million people are at risk of suffering the disease and an estimated 0.9-1.6 million cases occur each year (Alvar et al., 2012). In the mammalian host, *Leishmania* is an obligate intracellular pathogen that replicates in cells of the mononuclear phagocytic system, mainly macrophages (Vannier-Santos et al., 2002). Depending on both parasite species and the immune response of the host, the infection can manifest through different clinical syndromes ranging from cutaneous disfiguring forms to visceral disease (Mcgwire and Satoskar, 2014).

*L. (L.) mexicana* is the main causal agent of LCL a wild zoonosis endemic in the Yucatan peninsula, Mexico (Canto-Lara et al., 1998). The infection may be subclinical but is predominantly presented as a single, painless and rounded ulcer with raised borders mainly located on the ear (Loría-Cervera et al., 2019). As it occurs with other *Leishmania* species, cutaneous lesions caused by *L. (L.) mexicana* can heal spontaneously within weeks to a few months (Melby et al., 1994).

It has been generally accepted that patients who resolve their lesions either spontaneously or after treatment develop a protective immune response that makes them resistant to reinfection (Bettaieb et al., 2020). This protection is evidenced through a delayed-type hypersensitivity (DTH) reaction that indicates the cellular immune response and memory developed after *Leishmania* infection. Nevertheless, little is known about the immunological mechanisms associated with protection in humans who resolve their lesions spontaneously or after treatment. Undoubtedly, this is an important knowledge for the rationale design of vaccines (Scott and Novais, 2016).

Most studies have been conducted in the murine model (*Mus musculus* inbred lines) infected by *L. major*, however, these mouse strains are highly susceptible to *L. (L.) mexicana* hindering the characterization of mechanisms associated with the control of this parasite species (McMahon-Pratt and Alexander, 2004).

*Peromyscus yucatanicus* is a cricetid rodent endemic from the Yucatan peninsula and one of the three known reservoirs of *L. (L.) mexicana*. This mouse reproduces the clinical features of LCL, and the immune response associated with both the pathogenesis and control of *L. (L.) mexicana* like those observed in humans (Loría-Cervera et al., 2018). After inoculation with $1 \times 10^6$ promastigotes in the tail, ulcerated lesions and crusts were induced in 19.4% (7/36) of *P. yucatanicus* (Sosa-Bibiano et al. 2012). Later, when a higher inoculum ($2.5 \times 10^6$ parasites) was injected, 92.3% (12/13) of mice developed one or more suggestive signs (edema, induration, and ulcers) of LCL at two weeks’ post-infection (Loría-Cervera et al., 2013). Although spontaneous healing of lesions was
documented in two mice inoculated with $1 \times 10^6$ promastigotes (Sosa-Bibiano et al. 2012), neither the proportion of animals that achieve the clinical cure with a larger inoculum nor the immune response associated with the spontaneous healing of lesions has been investigated. Therefore, this study aimed to analyze the cytokine expression in the delayed-type hypersensitivity response of *Peromyscus yucatanicus* infected by *Leishmania (Leishmania) mexicana* that healed spontaneously.

2 MATERIALS AND METHODS

2.1 ANIMALS

*Peromyscus yucatanicus* from 6 to 18 months of both sexes were obtained from a colony derived from wild progenitors captured in the state of Campeche, which has been maintained for experimental studies in our institution since 1998 (Van Wynsberghe et al., 2020). Animals were kept at $22^\circ$C $\pm$ $2^\circ$C with a 12/12 h light/dark cycle, fed with rodent chow (1018S Harlan, Wisconsin) and water *ad libitum*, and handled according to the Mexican Law for the use of laboratory animals (Norma Oficial Mexicana: NOM-062-ZOO-1999). Physical enrichment was provided weekly in form of cardboard tubes for hiding and paper for nesting. Experiments were approved by the Ethics Research Committee of the Regional Research Center “Dr. Hideyo Noguchi” (Reference: CEI/16/15).

2.2 PARASITES AND EXPERIMENTAL INFECTION

Promastigotes of *L. (L.) mexicana* (strain MHET/MX/97/Hd18) were maintained by serial passage in the footpad of Syrian golden hamsters to retain its virulence. Parasites were obtained by aspirate and cultivated in M199 media supplemented with 10% fetal bovine serum (FBS), 0.1% β-mercaptoethanol, 10 mM L-Glutamine, 20 mM sodium pyruvate, and 100 U/mL penicillin-streptomycin for seven days at $23.5^\circ$C. Stationary phase promastigotes were washed three times with phosphate-buffered saline (PBS) 1X before being counted and adjusted to the concentration needed for inoculation. Mice were inoculated subcutaneously in the tail with 40 μL of parasite suspension containing $2.5 \times 10^6$ promastigotes. The evolution of the infection was monitored weekly to record signs of *Leishmania* infection, such as edema, erythema, nodule, and ulcers for Group I (n=20) and until the healing of lesions for Group II (n=20). A lesion was considered healed when a complete re-epithelization of the tissue was observed without the presence of edema, erythema, nodule, and crust. A control Group III (n=20) was injected with 40 μL of RPMI-1640 medium (Gibco).
2.3 DELAYED TYPE HYPERSENSITIVITY (DTH) REACTION

To compare the DTH response between mice with non-healed and healed lesions, animals were injected in the footpad with 40 μL of a phenolized suspension containing 1 x 10⁷ promastigotes of *L. (L.) mexicana* (leishmanin). The DTH reaction was determined by measuring footpad thickness at 24 and 48 h post-injection using an electronic caliper. The change in the size of footpads was recorded as the increase in footpad thickness after its injection relative to the size of the same footpad just before injection.

2.4 CYTOKINE mRNA EXPRESSION BY REAL-TIME RT-PCR

After euthanasia with a pentobarbital overdose, a skin fragment from the DTH reaction was taken at both 24 h (n=10) and 48 h (n=10) post-injection for each group. Total RNA was extracted from 20 to 30 mg of tissue using TRIzol Reagent (SIGMA), followed by a precipitation step with isopropanol. Samples were washed in 75% and 100% ethanol, air-dried at room temperature, and eluted in sterile water. RNA integrity was checked by electrophoresis in 2% agarose gel containing ethidium bromide (5 μg/mL). RNA samples were DNase treated (1 U/μl) and quantified using a Thermo Scientific NanoDropTM Spectrophotometer. The RNA (500 ng) was reverse transcribed using the Improm-II™ Reverse Transcription system kit (Promega), following the manufacturer’s instructions. The cDNA was diluted 1:5 in a final volume of 100 μl, aliquoted, and stored at -20 °C until use. The expression of IFN-γ, TNF, and IL-10 was determined using the iTaq Universal SYBR Green Supermix (Biorad) and 500 nM each of forward and reverse primers in a StepOne equipment (Applied Biosystems). The primer sequences and PCR conditions used were reported previously (Loría-Cervera et al., 2016). The 18S rRNA was used as an endogenous control for the relative amount of mRNA in each sample. The relative quantification of products was determined by the number of cycles over endogenous control and results are expressed as ΔCt (Ansari et al. 2011; Katara et al., 2011).

2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Normality tests were applied to determine either the parametric or non-parametric distribution of quantitative data. Then, the Kruskal-Wallis test with the Dunn’s post hoc test and one-way analysis of variance followed by Tukey’s multiple comparison test were used to evaluate statistical significance between groups. Data are presented as means ± SD. *P* values of less than 0.05 were considered significant.
3 RESULTS

Upon inoculation of $2.5 \times 10^6$ promastigotes into the tail, 100% of *Peromyscus yucatanicus* developed signs of *Leishmania* infection. The onset of edema, observed between one and nine weeks’ post-infection was followed by an ulcer with crust which appeared between weeks three and 13 after inoculation. Spontaneous healing of lesions leading to hypochromic scars very like those observed in patients after treatment with meglumine antimoniate was recorded in 65% (13/20) of mice between weeks six and 31 (mean= 16.5 weeks) post-infection. The mean of time between the onset of lesions and its healing was lower in females (8.18) than in males (10.44), however, the difference was not significant (p=0.3736).

At 24 h post-injection of the leishmanin suspension, both non-healed (p=0.0019) and healed (p=0.0119) mice mounted a significantly higher DTH response in comparison with uninfected mice (Figure 1A). At this point, no differences were found between non-healed and healed mice (p=0.6856). At 48 h, the DTH response of the non-healed group was significantly lower (p=0.0419) than the response observed at 24 h post-injection. The healed mice maintained a high DTH response at 48 h which was significantly different (p=0.0015) to that observed in uninfected mice (Figure 1B).

![Figure 1. DTH response in *P. yucatanicus* at 24 (A) and 48 h (B) post-injection of leishmanin suspension. Data are expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA.](image-url)
Regarding the cytokine response in the DTH reaction at 24 h (Fig. 2), the mRNA expression of IFN-γ was significantly higher in both non-healed and healed mice (p=<0.0001) in comparison with the control group. The IL-10 mRNA expression at 24 h was higher (p=0.0164) in healed mice than in uninfected ones. At that time, non-healed animals with cutaneous active lesions had a higher mRNA expression of TNF (p=0.0217) in comparison with uninfected mice. At 48 h (Fig. 3), only the expression of IFN-γ between uninfected mice and the non-healed was significantly different (p=0.0441).

Figure 2. Relative cytokine mRNA expression levels in the DTH response at 24 h post injection of leishmanin suspension. Results represent the number of cycles over 18 s and are expressed as ΔCt. Data are expressed as the mean ± SD. Statistical analyses were performed using either one-way ANOVA (IFN-γ and TNF) or the Kruskal-Wallis test (IL-10).
Figure 3. Relative cytokine mRNA expression levels in the DTH response at 48 h post injection of leishmanin suspension. Results represent the number of cycles over 18 s and are expressed as ΔCt. Data are expressed as the mean ± SD. Statistical analyses were performed using either one-way ANOVA (IL-10 and TNF) or the Kruskal-Wallis test (IFN-γ).

4 DISCUSSION

In this work, we presented a non-conventional mouse model to study the immune response associated with self-healing of LCL caused by L. (L.) mexicana. This is of particular relevance since following the infection with this Leishmania species most inbred mice develop large lesions that do not cure (Sosa et al., 2001). Even when a low-inoculum (1 × 10^3) of parasites is injected into the ear of Mus musculus different degrees of disease severity are observed depending on the genetic background (Rosas et al., 2005).

Here, we showed that upon the injection of 2.5 × 10^6 promastigotes, 65% of P. yucatanicus spontaneously healed their cutaneous lesions leaving a depressed, hypochromic, smooth-surfaced scar. Such as our results, a high spontaneous cure rate (44%, ranging from 19 to 72%) has been
observed in *L. (L.) mexicana*-infected patients who did not receive treatment or received a placebo in treatment trials (Cota et al., 2016).

Although the resolution of cutaneous lesions caused by some *Leishmania* species is common, the persistence of parasites in either scars or blood from cured patients has been documented suggesting that clinical cure is rarely associated with the complete elimination of parasites (Mendonça et al., 2004). Similarly, the retention of parasites in the tail of *P. yucatanicus* naturally infected with *L. (L.) mexicana* has been previously demonstrated confirming its role as a reservoir (Van Wynsbergh et al., 2000). However, further studies to investigate the presence and quantity of viable parasites in different tissues, including blood, are needed and could be very useful to understand the implications of parasite persistence in the *Leishmania* transmission cycle.

The resolution of cutaneous lesions caused by *Leishmania* spp., either spontaneously or after treatment with meglumine antimoniate, has been associated with the development of a protective immune response (Bettaieb et al., 2020). This protection is characterized by a strong DTH reaction, assessed by the leishmanin skin test (LST), that indicates a cellular immune response and memory developed after *Leishmania* infection (Alimohammadian et al., 2012). In this work, we demonstrated that *P. yucatanicus* developed a DTH response from the time of lesion appearance until its healing, being stronger at 48 h after injection of the leishmanin suspension in the healed group. This result indicates that these mice generate a specific secondary immune response, however, whether this response is long-lasting and could be associated with resistance to a subsequent *Leishmania* infection needs to be investigated.

Although it was demonstrated that individuals with previous episodes of CL exhibit resistance to reinfection that increases in proportion to the size of the LST, epidemiological evidence revealed that protection in individuals with typical CL scars are neither lifelong nor absolute and that resistance to reinfection is lost with time, particularly in the absence of continuous antigen stimulation (Ben Salah et al., 2005; Bettaieb et al., 2020). Despite there is vast information about the memory immune response developed against *Leishmania* infection, to date there remains no vaccine for use in humans (Scott et al., 2004). This is partly because most of the studies have been carried out on inbred strains of the murine model that mimic none the wide clinical outcomes nor the immune response observed in human leishmaniasis (Loría-Cervera et al, 2014). The knowledge of the events involved in the development of an effective protective immune response is critical for the development of an effective vaccine, however, a model who mimic natural infection and exhibit pathology, immune response and clinical evolution comparable with the disease in humans is urgently needed (Scott et al., 2004; Uliana et al., 2018).
Here, we propose that *P. yucatanicus* is an appropriate model to study the host-pathogen interactions associated with the clinical cure and the immune response related to protection against *L. (L.) mexicana*. Our findings regarding the cytokine response in the DTH reaction at 24 h suggested that the expression of IFN-γ and IL-10 could be involved in the resolution of lesions, while high expressions of pro-inflammatory cytokines such as IFN-γ and TNF are associated with the pathology of the disease. Similarly, studies carried out in humans with leishmaniasis revealed that although the production of IFN-γ and TNF-α might be involved in the control of parasites, their production is not sufficient and could also be involved in the tissue damage (Ribeiro-de-Jesus et al., 1998; Melby et al., 1994). On the contrary, a balance between immunoregulatory (IL-10) and effector cytokines (IFN-γ) has been associated with resistance to the infection and control of pathology (Bittar et al., 2007).

We demonstrated that *P. yucatanicus* can spontaneously heal the cutaneous lesions caused by *L. (L.) mexicana* and that spontaneous healing was associated with the expression of both immunoregulatory (IL-10) and effector cytokines (IFN-γ) in the DTH response.

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