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ORIGINAL ARTICLE

Histopathological analysis of initial cellular response in TLR-2 deficient mice experimentally infected by Leishmania (L.) amazonensis

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Summary

Tegumentary leishmaniasis is an important public health problem in several countries. The capacity of the Leishmania species, at the initial moments of the infection, to invade and survive inside the host cells involves the interaction of surface molecules that are crucial in determining the evolution of the disease. Using C57BL/6 wild-type and TLR-$2^{-/-}$ mice infected with *L. (L.) amazonensis*, we demonstrated that TLR-$2^{-/-}$ mice presented eosinophilic granuloma in the ear dermis, different from C57BL/6 wild-type mice that presented a cellular profile characterized mainly by mononuclear cell infiltrates, besides neutrophils and eosinophils, during the two first week of infection. When the parasite load was evaluated, we found that the absence of TLR-2 lead to a significant reduction of the infection in deficient mice, when compared with C57BL/6 mice which were more susceptible to the infection. Using TLR-2 deficient mice, it was possible to show that the absence of this receptor determined the reduction of the parasite load and the recruitment of inflammatory cells during the two first weeks after *L. (L.) amazonensis* infection.

Keywords
C57BL/6 wild-type, cellular profile, histopathology, *Leishmania (L.) amazonensis*, TLR-2 deficient mice, Toll-'like' receptor-2

Introduction

Leishmaniasis is an anthropozoonosis widely distributed worldwide. As a result of a multiplicity of agents, of insect vectors and animal reservoirs, this disease occurs in different clinical modalities. In South America, Brazil is the country with the highest occurrence of American tegumentary leishmaniasis (ATL), with more than 25,000 cases annually (Ministério da Saúde 2007). Among various parasites of the genus *Leishmania*, *L. (L.) amazonensis* is the causative agent of cutaneous leishmaniasis and cutaneous diffuse leishmaniasis (Almeida *et al.* 1996), characterized by the appearance of chronic lesions and disseminated through the skin, being a rare and disabling disease and with difficult treatment. The severity and the clinical form of the illness are directly related with the parasite as well as with the genetic and immunological factors of the host (Kane & Mosser 2000). According to the genotype of the mouse, *L. (L.) major* infection leads to the development of polarized Th1 or Th2 responses: where BALB/c mice represent a susceptibility model with a Th2 response that results in increased injury and the number of parasites, and C57BL/6 mice represent a resistant model, with a Th1 response with inhibition of parasite proliferation and healing of the lesion (McMahon-Pratt

& Alexander 2004). In contrast, in the *L. (L.) amazonensis* infection the host can display an intermediate phenotype, where there is a balance between the Th1 and Th2 responses leading to the susceptibility of most mice strains (Ji *et al.* 2003).

So, the initial moments of the infection are crucial to determine the evolution of this disease (De Almeida *et al.* 2003). Skin is the main organ involved in the infection, where the resident and inflammatory cells play a crucial role in the initial immune response to the pathogens through the release of cytokines, chemokines and growth factors (Williams & Kupper 1996; Fuhlbrigge & Kupper 2004).

The capacity of the *Leishmania* species to invade and survive in the host cells involves complex mechanisms, with the participation of parasite and surface components of host cells. The TLR has been described as a family of Pattern Recognition Receptors (PRR), used by several cell types in the recognition, internalization and processing of antigens, acting as a molecule in the central link between innate and adaptive immune responses (Medzhitov & Janeway 2000; Akira & Hemmi 2003). Several studies have demonstrated the role of TLR in immunity against parasites through the recognition of molecules such as lysophosphatidylserine of *S. mansoni* (van der Kleij. 2002), anchor of GPI and GIPL of *T. cruzi* (Campos *et al.* 2001; Ouaissi *et al.* 2002).

Recent data have shown the involvement of TLR-2 by macrophages and NK cells in the recognition of the LPG of *Leishmania spp*. (Becker *et al.* 2003; De Veer *et al.* 2003). On the other hand, TLR-4 deficient mice infected by *L. (L.) major* presented an increase in the synthesis of IL-10 and the expression of the receptor for IL-4, besides the increased activity of arginase promoting parasite proliferation (Kropf *et al.* 2004a,b). Several questions about the initial response to infection caused by *L. (L.) amazonensis* should be elucidated. Thus, in this work we studied the influence of TLR-2 in cellular recruitment and parasite load during the initial stages of *L. (L.) amazonensis* infection.

Materials and methods

Mice

Female C57BL/6 mice were obtained from the Animal Facility (CECAL) of the Fundação Oswaldo Cruz (CECAL/FIOCRUZ). Toll-Like Receptor 2 deficient mice (TLR-$2^{-/-}$) in a homogeneous C57BL/6 background (Takeuchi *et al.* 1999) were kindly donated by Dr. Shizuo Akira (Osaka University, Japan). Animals were bred and maintained under standard conditions at the breeding unit of the Fundação Oswaldo Cruz, Brazil. The animals were used according to the rules set out by the Ethics Committee of FIOCRUZ for use of animals under the Protocol N^{o} p024705.

Parasite culture

Promastigote forms of *Leishmania (Leishmania) amazonensis* of MHOM/BR/77/LTB0016 strain, provided by Dr. Gabriel Grimaldi of the Center for Reference Laboratory of Leishmaniasis, Department of Immunology – IOC/FIOCRUZ, RJ, were used in all experiments. Parasites were incubated at 25 °C in BHI (Brain Heart Infusion) supplemented with 10% foetal bovine serum (FBS) and used in the stationary phase of growth until the third *in vitro* passage.

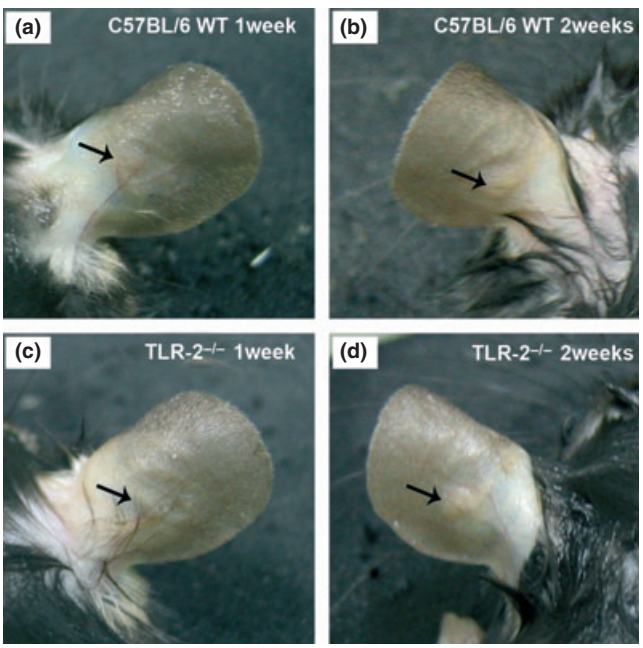

Figure 1 Ear lesions in C57BL/6 WT and TLR-$2^{-/-}$ mice 1 and 2 weeks following intradermal inoculation of 2.5×10^5 *Leishmania (L.) amazonensis* promastigotes. After the first week, C57BL/6 WT (a) and TLR-$2^{-/-}$ mice (c) present increased vascularization of the inoculation site. After the second week of infection, C57BL/6 WT mice (b) presented lesions with little ulceration, while in TLR-$2^{-/-}$ mice (d) the formation of small non-ulcerative nodular lesions were observed.

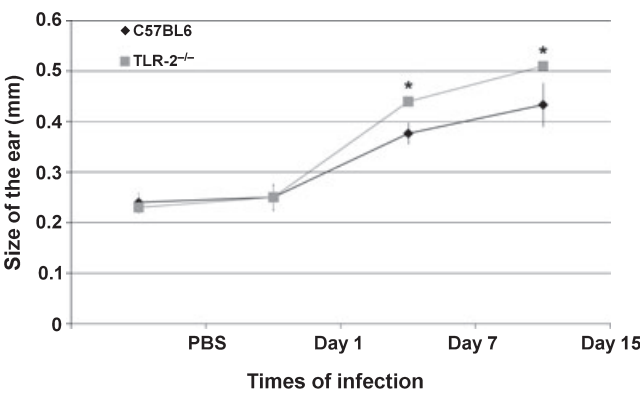

Figure 2 Diameter of induration following intradermal inoculation of 2.5×10^5 *Leishmania (L.) amazonensis* promastigotes in C57BL/6 WT (◆) and TLR-$2^{-/-}$ mice (■). Values represent the mean induration (millimetres) ±1 SD (10 mice/group). Kruskal–Wallis statistical test and a $^*P < 0.05$ was considered significant.

Intradermal inoculation and lesion measurement

Mice were sedated by an intraperitoneal injection with Compaz® (Cristália, São Paulo, SP, Brasil) (Diazepam 5 mg/ml) at a dosage of 5 mg/kg and Fentanyl® (Janssen-Cilag, São Paulo, SP, Brasil) (Fentanyl citrate 78.5 μg/10 ml) at a dosage of 0.02 mg/kg. A total of 250,000 metacyclic promastigotes of *L. (L.) amazonensis* were inoculated intradermally into the ears of the animals in 10 μl of PBS. A group of mice of each strain was inoculated only with 10 μl of PBS as control. The lesion developments were measured with a calliper (Schnelltaster, HC Kröplin, GMBH, Hessen, Germany) and ear thicknesses given in millimetres. After 1, 7 and 15 days of intradermal inoculation, mice were killed in a CO_2 chamber and their ears were collected. Each experiment was carried out three times and the same results were obtained.

Histological Analysis

Ears of control and infected animals were washed in PBS and fixed in 10% buffered formalin. After fixation, samples were routinely processed for paraffin embedding in an Automatic Tissue Processor (Leica TP1020, Wetzlar, Germany). Five micrometre thick sections were obtained in a Rotary Microtome (Micron HM 360, Walldorf, Germany). The sections were stained with haematoxylin-eosin, differentiated into 1% hydrochloric alcohol, stained with alcoholic eosin 1%, assembled with Entelan and analysed in a light microscope (Zeiss Axioplan 2; Zeiss Inc., Thornwood, NY, USA).

Transmission Electron Microscopy

Ears were removed after 1, 7 and 15 days of infection, fixed in 2.5% glutaraldehyde in the buffer cacodylate sodium 0.1 M, pH 7.2 with 3.5% sucrose and postfixed with 1% of osmium tetroxide (OsO_4) for 1 h at 4 °C. Then, they were dehydrated in an acetone series and embedded in resin PolyBed 812. After polymerization, semi-thin sections (0.5 μm) were stained with toluidine blue and eosin and observed under a light microscope (Zeiss Axioplan 2). The quantification of cellular profile was made in five semi-thin sections with an area of 60 mm^2 per ear in an average of 3–5 mice/group. After the choice of

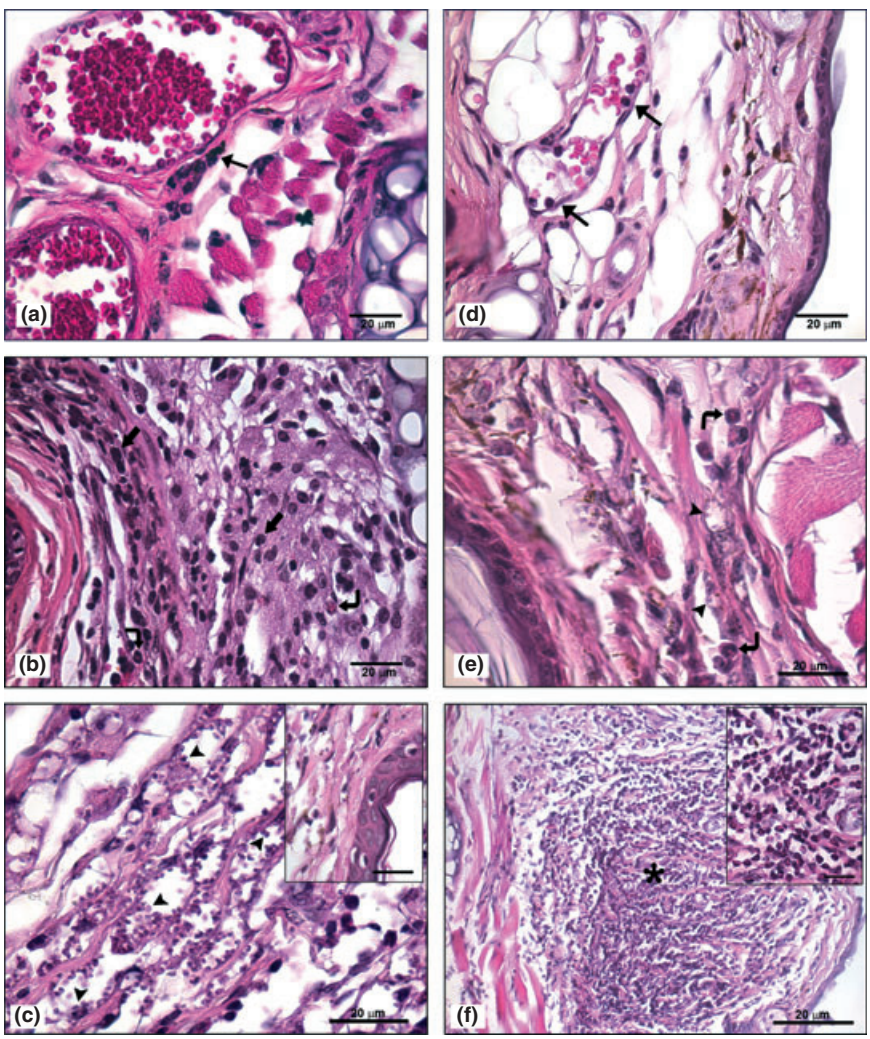

Figure 3 (a–f) – Histological analysis of sections from the ear region of C57BL/6 WT mice and TLR-2$^{-/-}$ mice after 1, 7 and 15 days of *Leishmania (L.) amazonensis* infection stained with haematoxylin-eosin (bar = 20μm). On the first day of infection the presence of congested blood vessels with marginalization and diapedesis of inflammatory cells (➞) in C57BL/6 WT (a) and TLR-2$^{-/-}$ mice (d) were seen. After first week of infection, the C57BL/6 WT mice showed mononuclear (➡) and polymorphonuclear (↱) cell infiltrations in ear dermis (b) and second week after infection the presence of epidermal alterations such as exocytosis (inset) and dermis with an increase of intracellular amastigotes (➤) (c). In TLR-2$^{-/-}$ mice during the first week of infection some polymorphonuclear cells (↱) were seen (e). After the second week of infection organized granulomas (*) formed by eosinophils and without parasites (inset) were observed (f). The experiment is representative of three separate experiments.

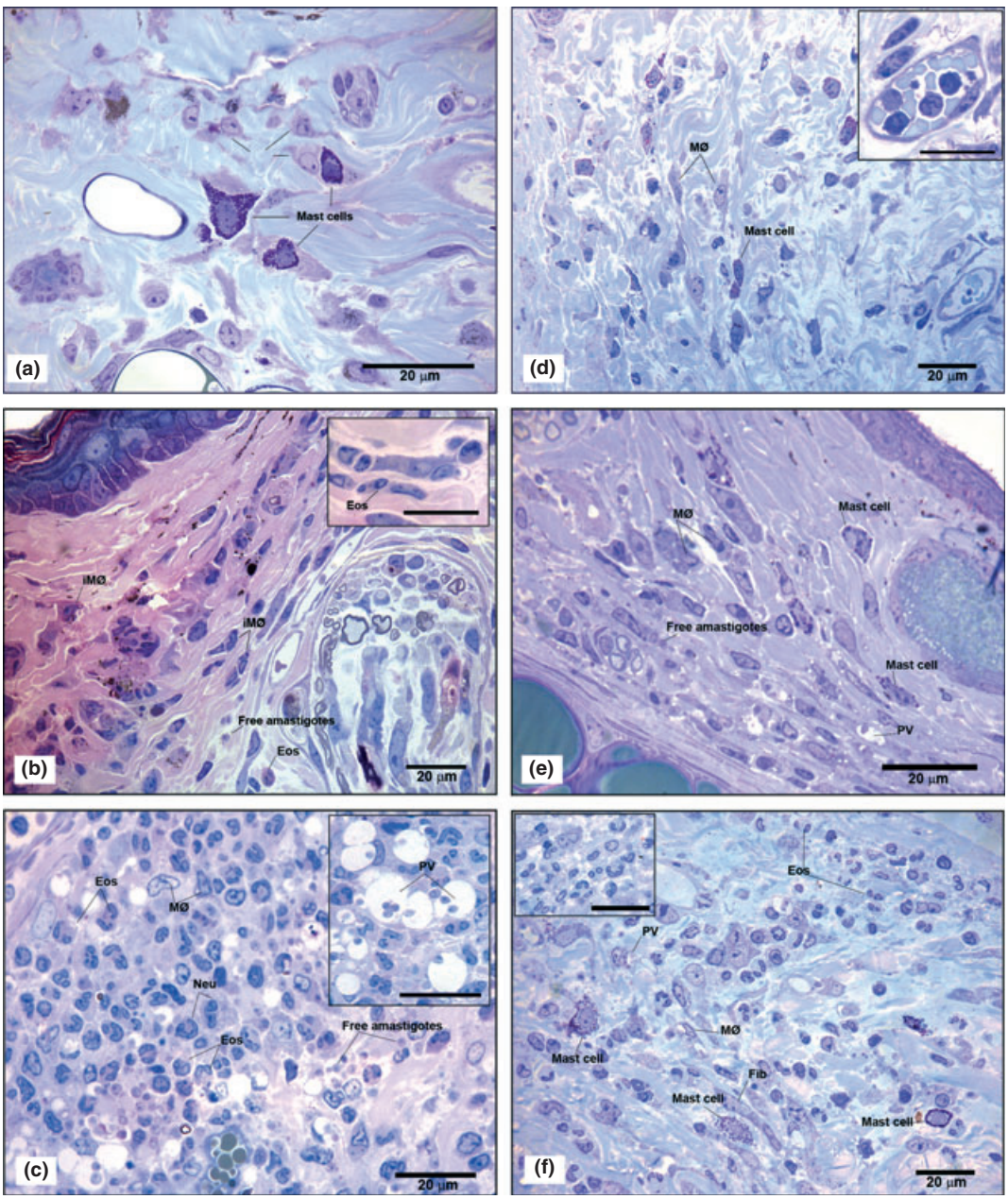

Figure 4 (a–f) – Semi-thin sections from the ear lesion of C57BL/6 WT mice and TLR-2$^{-/-}$ mice after 1, 7 and 15 days of *Leishmania (L.) amazonensis* infection, stained with toluidine blue and eosin (bar = 20μm). On the first day of infection inflammatory infiltrates composed of neutrophils (inset), macrophages (MØ), degranulated mast cells in C57BL/6 WT (a) and TLR-2$^{-/-}$ mice (d) were observed. In the first week of infection, in C57BL/6 WT mice (b) showed immature macrophages (iMØ) and eosinophils (Eos) composing the inflammatory infiltrate (inset) and free parasites in dermal ear (b). In the second week (c) an increase of inflammatory infiltrate predominantly composed of macrophages (MØ), as well neutrophils (Neu) and eosinophil (Eos) populations were seen. In addition to the presence of free amastigotes and a large amount of macrophages (MØ) containing amastigotes within large parasitophorous vacuoles (PV) (inset) and numerous free amastigotes in the matrix were observed. In TLR-2$^{-/-}$ mice, during the first week of infection (e), mast cells were observed between parasitized macrophages (MØ) and some free amastigotes in the matrix. In the second week of infection (f), organized granulomas formed predominantly of eosinophils (Eos), a large amount of macrophages (MØ), some mast cells and fibroblasts were seen. Also, few macrophages (MØ) with amastigotes in parasitophorous vacuoles (PV) were found. The experiment is representative of three separate experiments.

areas, the ultra-thin sections were prepared (ultramicrotome Reichert OmU3), collected on copper grids of 300 mesh, contrasted with 5% uranyl acetate and lead citrate and observed by a transmission electron microscope (Zeiss EM10C) of the Oswald Cruz Institute electron microscopy Platform. Data were obtained from three independent experiments.

Statistical analysis

The significance of the results was calculated by a non-parametric one-way analysis of variance test (Kruskal–Wallis) and a *P*-value of <0.05 was considered significant.

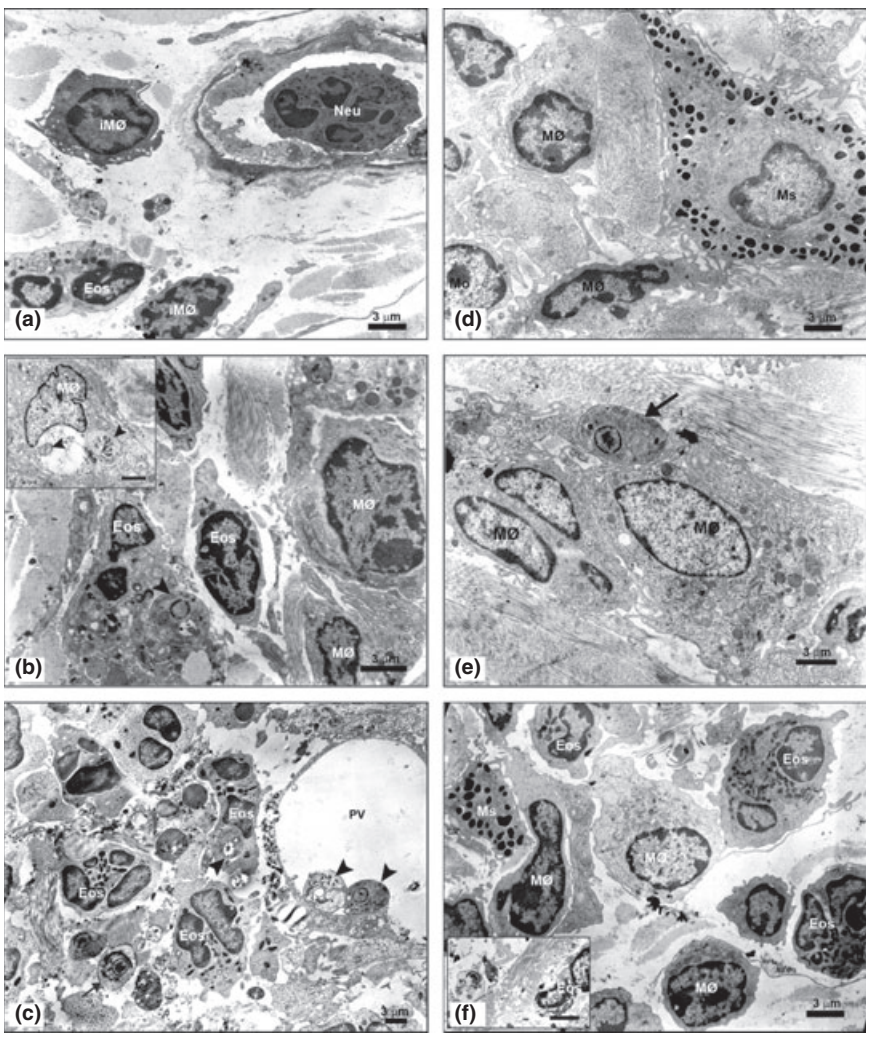

Figure 5 (a–f) – Ultrastructural analysis of the ultra-thin sections from the ear lesion of the C57BL/6 WT and TLR-2$^{-/-}$ mice after 1, 7 and 15 days of *Leishmania (L.) amazonensis* infection contrasted with 5% uranyl acetate and lead citrate (bar = 3µm). Electron micrography of the first day infection (a) showed neutrophils (Neu) adhered to the endothelium of blood vessel, immature macrophages (iMØ) and eosinophils (Eos) in dermal ear of the C57BL/6 WT. After first week of the infection (b), eosinophils (Eos) containing one amastigotes within parasitophorous vacuoles close (➤) and immature macrophages characterized by few organelles and a nucleous with electron-dense chromatin were seen. Also, mature macrophages (MØ) containing amastigotes within large parasitophorous vacuoles were observed (inset). After the second week (c) mature macrophages (MØ) presented amastigotes (➤) attached at the large parasitophorous vacuoles membrane (PV), many eosinophils (Eos) parasitized contained only one amastigote within parasitophorous vacuoles close and free amastigotes (⟶) in extracellular matrix were found. Furthermore, several biconvex granules, proceeding from eosinophils, were observed free in the extracellular matrix. In the first day of TLR-2$^{-/-}$ mice infection (d) were found mast cells (Ms) with electron-dense granules characteristic of this cell, distributed in the cytoplasm and macrophages (MØ). In the first week (e), presented some free amastigotes (⟶) in the extracellular matrix showed entire plasma membrane and nucleous with chromatin attached to the nuclear envelope and central nucleolus being phagocytized by mature macrophages (MØ). And, in the second week (f), mature macrophages (MØ), eosinophils (Eos) and mast cells (Ms) in ear dermis were observed. In this time, rare free amastigotes in the extracellular matrix presented cytoplasm rarefied and disruption at the plasma membrane and nuclear envelope, indicating cell death of these parasites (inset). The experiment is representative of three separate experiments.

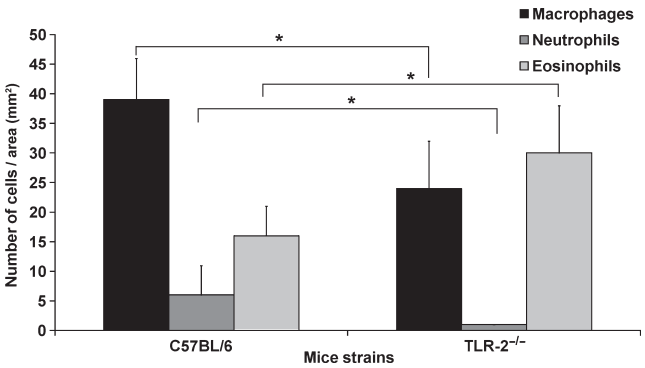

Figure 6 Cellular profile presented in the ear lesion of the C57BL/6 WT and TLR-$2^{-/-}$ mice after the second week of infection with metacyclic promastigotes of *Leishmania (L.) amazonensis*. The quantification was performed in five semi-thin sections with an area of 60 mm^2 per ear. Each bar represents mean ± SEM from three experiments (3–5 mice/group). Kruskal–Wallis statistical test and a $*P < 0.05$ was considered significant.

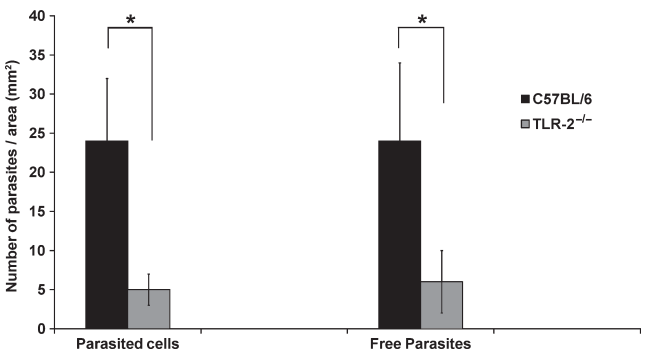

Figure 7 Parasite load in the ear lesion of the C57BL/6 WT and TLR-$2^{-/-}$ mice intradermally inoculated with 250,000 metacyclic promastigotes of *Leishmania (L.) amazonens*is after the second week of infection. The quantification was performed in five semi-thin sections with an area of 60 mm^2 per ear. Each bar represents mean ± SEM from three experiments (3–5 mice/group). Kruskal–Wallis statistical test and a $*P < 0.05$ was considered significant.

Results

Evolution of the dermal lesion in TLR-$2^{-/-}$

Macroscopic analysis of C57BL/6 wild-type (WT) and TLR-$2^{-/-}$ mice showed increased vascularization of the inoculation site after the first week of *L. (L.) amazonensis* promastigote infection. After the second week of infection, C57BL/6 WT mice presented lesions with little ulceration, while in TLR-$2^{-/-}$ mice the formation of a small nodule at the inoculation site was observed (Figure 1). The evaluations of the thickness of the inoculation site, after different times of infection, are represented in the Figure 2.

Evolution of the cellular profile of dermal lesion in TLR-$2^{-/-}$

Analysis of the control mice ears showed the presence of resident cells of the dermis, whereas in PBS inoculated mice only a mild inflammatory infiltrate was observed (data not shown). On the first day of infection the presence of congested blood vessels with marginalization and diapedesis of inflammatory cells (Figure 3a,d) were seen in both C57BL/6 WT and TLR-$2^{-/-}$ mice. In the dermal ear, inflammatory infiltrates composed of neutrophils, macrophages, degranulated mast cells were observed in both C57BL/6 WT (Figures 4a and 5a) and TLR-$2^{-/-}$ mice (Figures 4d and 5d).

In the first week of infection, the analysis of the inoculation site in the C57BL/6 WT mice showed inflammatory infiltrates composed of mononuclear and polymorphonuclear cells in the dermal ear (Figure 3b). In this infiltrate, many eosinophils and neutrophils with a preponderance of monocytes, immature and mature macrophages were observed. Moreover, in these mice large amounts of parasitized cells and free parasites in the extracellular matrix were observed (Figure 4b). Ultrastructural analysis showed the presence of eosinophils containing generally one amastigote in parasitophorous vacuoles close in the dermal ear. Also, Immature macrophages characterized by cytoplasm with few organelles and nucleus with electron-dense chromatin and macrophages containing several amastigotes within large parasitophorous vacuoles were observed (Figure 5b). Moreover, in the TLR-$2^{-/-}$ mice a reduced infiltrate of inflammatory cells composed mostly of immature and mature macrophages and large amount of mast cells and fibroblasts were observed (Figure 3e). Also a small amount of parasitized cells such as macrophages, fibroblasts and rare free amastigotes in extracellular matrix (Figures 4e and 5e) were seen.

During the second week of infection there was a significant increase in the influx of inflammatory cell infiltrates in the dermal ear in both mice strains, thus the cellular profile for the initial response to infection by *L. (L.) amazonensis* could be defined. In the C57BL/6 WT mice, we observed the presence of disorganized granuloma predominantly composed of macrophages, as well as the presence of neutrophils and eosinophils (Figures 3c and 4c). Also, a large amount of macrophages containing amastigotes in large parasitophorous vacuoles and eosinophils with only one amastigote in the parasitophorous vacuoles close were observed. Furthermore, free amastigotes and biconvex eosinophils granules in extracellular matrix were seen (Figure 5c). In these mice the number of parasitized cells was significantly higher when compared with the number of free amastigotes in extracellular matrix (Figure 6). In the ear dermis of TLR-$2^{-/-}$ mice there were organized granulomas (Figure 3f) formed predominantly of eosinophils and a large amount of macrophages and mast cells (Figures 4f and 5f). Differently to what was found in WT mice, the TLR-$2^{-/-}$ mice showed a low parasite load, few eosinophils and parasitized macrophages and rare amastigotes free in extracellular matrix (Figures 4f and 6). An important observation was the presence

of eosinophils near the infected macrophages in both mice strains.

A quantitative evaluation of the cellular profile and the parasite load in the second week of *L. (L.) amazonensis* infection in C57BL/6 and TLR-2$^{-/-}$ mice is represented in Figures 6 and 7 using the Kruskal–Wallis statistical test and a *P*-value of <0.05 was considered significant.

Discussion

The first moments of infection by *Leishmania* are crucial to drive the progress of the disease. There is evidence that the phenotype of the leishmaniasis can be determined in the first hours after infection, starting with the recognition of PAMPs of the parasite by PRRs present on the surface of host cells, such as TLRs (Launois *et al.* 1995; Sacks & Noben-Trauth 2002). The involvement of TLRs in the initial response to infection with *Leishmania* have been described in recent years, through various *in vivo* and *in vitro* studies (Hawn *et al.* 2002; De Veer *et al.* 2003; Debus *et al.* 2003; De Trez *et al.* 2004).

So, to evaluate the involvement of TLR-2 during *in vivo* *L. (L.) amazonensis* infection TLR-2 deficient mice were used. The ear dermis was chosen as the inoculation site because, in addition to being a common transmission site in rodent reservoirs, it offers the advantage that all the dynamic events occur at the infection site, facilitating the study of the initial inflammatory response (Belkaid *et al.* 1996, 1998).

The control group of mice, inoculated only with PBS, showed a small inflammatory infiltrate composed of neutrophils, increase of vascularization and the presence of oedema at the inoculation site. This initial inflammatory response, generated by mechanical disruption of cells in the epidermis and dermis was consistent with the results presented by Grimaldi and Moriearty (1981). Nonetheless, the histological analysis of inoculation site after the first day of *L. (L.) amazonensis* infection showed that the inflammatory infiltrate in the ear dermis was higher when compared with mice inoculated only with PBS. This fact is related to the presence of free parasites in the extracellular matrix of the ear dermis leading to activation of resident cells, through recognition of parasite surface molecules by receptors present in these cells (De Almeida *et al.* 2003).

Since the first days of infection, we observed the neutrophils infiltration at the inoculation site in both mice strains, being able to occur because of the disruption of the skin caused by either the insect vector or the needle. This mechanical injury induces the recruitment of these cells to participate in the process of tissue repair, even in the absence of the parasite (Peters *et al.* 2008).

The initial recruitment of neutrophils to the inoculation site is supported by several studies that demonstrate the role of these cells in the first line of defence against infection by protozoa of the *Leishmania* genus, acting directly on the endocytosis and destruction of the parasite by proteolytic enzymes, production of reactive oxygen intermediates, inflammatory mediators and cellular recruitment to the infection site (Chang. 1981; Pimenta *et al.* 1987; Awasthi *et al.* 2004).

C57BL/6 WT mice showed significant increase of immature and mature macrophages at the infection site in first week of infection. This high concentration of macrophages at the inoculation site may be corroborated by Soong *et al.* (1996, 1997) who showed that mice infected with *L. (L.) amazonensis* are able to recruit a large quantity of immature macrophages, but they are unable to eliminate the parasite facilitating the spread of infection. Thus, the increased number of immature and mature macrophages found in C57BL/6 mice during the first week of infection may be related to the maturation of recruited monocytes in the early stages of the infection. These observations could explain the presence of many macrophages parasitized with numerous amastigotes within large parasitophorous vacuoles and the presence of free amastigotes in the tissue from the disruption of these cells.

Using TLR-2$^{-/-}$ mice, we showed that the absence of this receptor leads to an alteration of the cellular profile and an expressive reduction in the susceptibility of these animals to *L. (L.) amazonensis* infection, which are capable of controlling the parasite load during the first 2 weeks of infection. TLR-2$^{-/-}$ mice presented a lower infiltrate of inflammatory cells at the inoculation site forming organized granulomas mainly made up of eosinophils, unlike the C57BL/6 mice, which had infiltrated more expressive, but no organized granulomas were observed.

The eosinophils constitutively express few TLRs on their surface and the direct activation of eosinophils through TLR-2 is controversial (Sabroe *et al.* 2002; Nagase *et al.* 2003; D'Avila *et al.* 2007; Driss *et al.* 2009). Although a role for TLR-2 in regulating eosinophils recruitment and activation through direct and indirect mechanisms has been described in bacterial and parasitic infections (D'Avila *et al.* 2007; Driss *et al.* 2009), similar to our observations, a lack of impairment or increased recruitment of eosinophils in TLR-2 deficient animals has been reported following ocular filarial infection and also in a ear model of contact dermatitis (Daehnel *et al.* 2007; Jin *et al.* 2009). The tissue eosinophilia presented in TLR-2$^{-/-}$ mice is characteristic of a Th2 response, as observed with asthma and helminthic infections (Del Prete. 1992; Mehlotra *et al.* 1998). However, a Th2 response would be favourable for parasite proliferation, which was not observed in TLR-2 deficient mice. Of note, eosinophils may participate in the process of killing parasites through their ability to phagocytize, mount a respiratory burst and mobilize cytotoxic proteins from specific granules after infection, suggesting an immunomodulatory and in some conditions protective role of eosinophils in infections (Akuthota *et al.* 2008; Blanchard & Rothenberg 2009).

On the other hand, TLR-2$^{-/-}$ mice showed a significant reduction in the number of neutrophils during the second week of infection when compared with C57BL/6 WT mice. This reduction can be related to the involvement of TLR-2 in the recruitment, activation and apoptosis of these cells (Sabroe *et al.* 2005; Jablonska *et al.* 2006).

Furthermore, we observed that these mice showed a low parasite load from the first day of infection when compared with C57BL/6 WT mice. This fact indicates that these receptors have an effective participation in the adhesion and internalization of the parasite in the host cells present in the initial stages of infection. The difference in the parasite load observed in the second week of infection in TLR-2$^{-/-}$ and C57BL/6 WT mice may be related to the association of macrophages with eosinophils present at the inoculation site. This association was described by Grimaldi *et al.* (1984) who found that the eosinophils could serve as donors of peroxidase for mature macrophages. Moreover, these eosinophils may be acting in direct control of amastigotes free through the release of extracellular peroxidase, can then be adsorbed at plasma membrane of *Leishmania* by making it more susceptible to death after phagocytosis by macrophages as suggested by Grimaldi *et al.* (1984) and described by Pimenta *et al.* (1987).

The presence of mast cells in TLR-2$^{-/-}$ and C57BL/6 WT mice from the first day of infection is supported by data from the literature that show the presence of these cells in the dermis during infections caused by protozoa of the genus *Leishmania* and their direct participation in the initial immune response through the production of several inflammatory mediators (Bidri *et al.* 1997; Saha *et al.* 2004). So, we can suggest that mast cell degranulation, mainly seen in TLR-2$^{-/-}$ mice infected with *L. (L.) amazonensis*, is helping to reduce the number of free amastigotes in the extracellular matrix.

With these studies, we have demonstrated the importance of TLR-2 in the initial response to *L. (L.) amazonensis* infection, where the absence of these receptors in initial stages of infection favours the control of the parasite load. Thus, we suggest the study of TLR pathways as an alternative for the development of new medicines for the treatment of infections caused by *L. (L.) amazonensis*.

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