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Murine immune response induced by *Leishmania major* during the implantation of paraffin tablets

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Abstract

We carried out a model of chronic inflammation using a subcutaneous paraffin tablet in mice experimentally infected with *Leishmania major*. It was previously reported that the parasite load following paraffin implantation occurred at a peak of 21 days in both BALB/c and C57BL/6 mice. At the present study, we have investigated what cytokines and chemokines are directly related to the parasite load in C57BL/6 mice. All mice were divided in four groups: mice implanted with paraffin tablets; mice experimentally infected with *L. major*; mice implanted with paraffin tablets and experimentally infected with *L. major*; and mice submitted only to the surgery were used for

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the Real-Time Polymerase Chain Reaction (RT-PCR) controls. Fragments of skin tissue and the tissue surrounding the paraffin tablets (inflammatory capsule) were collected for histopathology and RT-PCR studies. By 21 days, a diffuse chronic inflammatory reaction was mainly observed in the deep dermis where macrophages parasitized with *Leishmania* amastigotes were also found. RT-PCR analysis has shown that BALB/c mice showed strong IL-4 and IL-10 mRNA expression than controls with very little expression of IFN- γ . In contrast, both IFN- γ and IL-10 mRNA was found in higher levels in C57BL/6 animals. Moreover, in C57BL/6 mice the expression of chemokines mRNA of CCL3/MIP-1 α was more highly expressed than CCL2/MCP-1. We conclude that the Th1 immune response C57BL/6 did not change to a Th2 response, even though C57BL/6 animals presented higher parasitism than BALB/c mice 21 days after infection and paraffin implantation.

Keywords

Leishmania major; Paraffin tablets; Chronic inflammation; Cytokines; C57BL/6 and BALB/c mice

Introduction

Leishmania parasites are obligatory intracellular pathogens that replicate primarily within macrophages, and also in dendritic cells (DCs) and fibroblasts [1,2]. The initial step in protective host defense against *Leishmania* is confinement of the parasite to the site of infection. Infected macrophages are stimulated to produce antimicrobial molecules by interferons [3]. Twenty-four hours after infection, migrating NK cells at the site of the infection up-regulate IFN- γ , which amplifies the host immune defense [4,5]. Immature DCs in the skin (*Langerhans* cells) then take up *Leishmania* promastigotes and migrate to the regional lymph nodes where they differentiate into mature antigen-presenting DCs [6]. These DCs have two main functions: they present antigens to naïve T cells and they produce interleukin (IL)-12, the cytokine most crucial for the development of a Th1 adaptive immune response [7]. IFN- γ is produced in large amounts by activated Th1 cells, enhancing anti-*Leishmania* activity at the infection site [8]. In consequence, resolution of the infection depends on the early presence of macrophages and the activation of these cells by IFN- γ . Activated Th1 cells enhance parasite killing by macrophages, when they release IFN- γ [9]. However, these immunological processes differ in effectiveness among human individuals. In fact, *Leishmania* parasites can cause a wide spectrum of cutaneous diseases, including: (1) localized cutaneous leishmaniasis (LCL); (2) mucocutaneous leishmaniasis involving erosive lesions of the oral and nasal mucosa; (3) diffuse cutaneous leishmaniasis (DCL) characterized by multiple non-healing lesions; and (4) a distinct clinical form called disseminated cutaneous leishmaniasis [10–12].

In recent years, the regulation of cell migration to the site of inflammation has been intensively investigated. Chemokines, small chemotactic cytokines, are responsible for coordinating leukocyte trafficking with immune cell differentiation and effector functions [13,14]. LCL lesions show strong expression of Th1-associated chemokines such as CCL2/MCP-1, CXCL9/MIG, and CXCL10/IP10. In contrast, chronic DCL entails lower expression of Th1-associated chemokines and higher levels of CCL3/MIP-1 α [9].

The mouse is generally considered a valuable model for human disease. For cutaneous leishmaniasis, it provides an excellent model for identifying the factors involved in developing T helper subsets, since Th1 cells confer protection in resistant strains of mice (C57BL/6), whereas Th2 cells are associated with a fatal outcome in susceptible mice (BALB/c) [15]. Despite technological advances in characterizing the influx of different

monocyte–macrophage subpopulations at the molecular level [16,17], the role of inflammatory macrophages in leishmaniasis remains relatively poorly defined. In 2000, Tafuri et al. [18] described a model of monocytic inflammation using a subcutaneous paraffin tablet. This inert irritant induces the recruitment of monocytes to the site of inflammation and the localized immobilization of macrophages around the tablet. Monocyte infiltration and macrophage accumulation following paraffin implantation was similar in BALB/c and C57BL/6 mice. When paraffin implantation was coupled with *Leishmania major* infection, both strains of mice had large numbers of parasites associated with these inflammatory macrophages at 21 days post-infection. Thus, the early accumulation of parasites in macrophages occurs in both genetically susceptible (BALB/c) and resistant (C57BL/6) strains of mice. Despite these heavy early parasite burdens, the C57BL/6 mouse is able to resolve infection, whereas the BALB/c mouse does not. In that time, based on Murray et al. [19], we discussed that the heavy parasitism that we observe is dependent on the type of macrophages recruited into lesions, and that paraffin implantation can recruit a population of permissive macrophages even in the genetically resistant C57BL/6 mouse. However, in the previous histological examination, the nature of cytokines and chemokines that were produced by these inflammatory cells was not determined [18].

Considering that C57BL/6 mice presented a parasite load similar to BALB/c mice 21 days after infection during the implantation of paraffin tablets, the aim of this study was to determine the chemokines that are produced in this tissue. We hypothesized that the chemokines could be directly related to the parasite load increasing in C57BL/6 mice after paraffin implantation associated to the *Leishmania* infection. We show that over the first 3 weeks post-implantation, there was a remarkable similarity in the histological features and the degree of parasitism in these two mouse strains. However, unlike the BALB/c mouse that is unable to clear infection, the parasite infection was transient in C57BL/6 mice, which had begun to clear the infection by 30 days. Real-time PCR analysis has shown that both IFN- γ and IL-10 mRNA was found in higher levels in the skin and inflammatory capsule of C57BL/6 animals that were infected with *L. major* when compared to paraffin implant alone. In addition, in C57BL/6 mice the expression of chemokines mRNA of CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES, CXCL9/MIG, and CXCL10/IP-10 were found in the infected tissues. Particularly, CCL3/MIP-1 α was more highly expressed than CCL2/MCP-1, suggesting a differential and important role for them in the recruitment and activation of different immune cells that can define a permissive profile at the site of infection with *L. major*.

Materials and methods

Subcutaneous implantation of paraffin tablets

Paraffin tablets (220.0 mg; Queel-Indústrias Químicas Ltda, São Paulo, Brazil) with the dimensions - 1.0×0.5 cm (HXL) were prepared as described previously. These tablets have a smooth surface and are maintained under aseptic conditions. To implant them, mice were anesthetized and shaved, and then a skin incision (1.5 cm) was made on the dorsum using blunt point scissors. Tablets were inserted into a small subcutaneous cavity, and the wound was stitched [18].

Mice

Section “Mice”—One hundred mice, 4–6 weeks old, obtained from our animal facility at ICB/UFGM, were divided into two groups: 50 C57BL/6 and 50 BALB/C mice where they were subdivided in four groups.

Group 1 (P) This group comprised mice implanted with paraffin tablets ($n=30$; 15 of each mice strain);

Group 2 (L) This group comprised mice experimentally infected with *L. major* promastigotes ($n=30$; 15 of each mice strain)

Group 3 (P+L) This group comprised mice implanted with paraffin tablets and infected with promastigotes immediately thereafter ($n=30$; 15 of each mice strain);

Group 4 (Group sham–“Wound Healing Surgery Group”) This group comprised of ten mice that were submitted only to the surgery (skin incision) where the wound was stitched (wound healing). We collected the skin samples from dorsal area. ($n=10$; 5 of each mice strain)

These animals were sacrificed at 7, 21 and 30 days.

Day 7–20 mice were distributed in groups 1, 2, 3 and 4 (five mice in each group)

Day 21–15 mice were distributed in groups 1, 2 and 3 (five mice in each group)

Day 30–15 mice were distributed in groups 1, 2 and 3 (five mice in each group)

Experimental infection

C57BL/6 mice were injected with 1×10^6 *L. major* promastigotes in 100 μ l (subcutaneous) in the dorsum nearest the paraffin tablet, but not inside of the paraffin tablets and immediately after implantation. Control mice (without paraffin) were infected at the same time. BALB/c mouse experiments followed the same protocol.

Parasites

The Friendlin strain of *L. major* clone IV (MHOM/IL/80/Friedlin) was used in all experiments. Promastigotes were grown at 25°C in Grace's insect cell culture medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin G (100 U/ml) and streptomycin (100 μ g/ml). Stationary-phase promastigotes, obtained from 7- to 9-day cultures, were used for all experimental infections.

Disease progression and collection of tissue samples for histopathology

Five animals from each group were sacrificed at 7, 21 and 30 days after the experimental infection. At each of the designated days, fragments of skin tissue and the tissue surrounding the paraffin tablets (inflammatory capsule) were collected for histopathology [18]. Tissues were fixed in 10% buffered formalin, dehydrated, cleared, embedded in paraffin, cut (3–4 μ m thick) and stained with Hematoxylin and Eosin (HE).

Microtitration

Parasites were quantified by limiting dilution, as previously [20]. The inflammatory capsule formed by the paraffin implantation was homogenized using a glass tissue grinder in sterile PBS. Tissue debris was removed by centrifugation at $150 \times g$ and the cells were concentrated by centrifugation at $2,000 \times g$. Pellets were resuspended in 400 μ l supplemented Grace's culture medium (see above). Samples were plated on to culture plates and serially diluted 1:10 in supplemented Grace's insect tissue culture medium. Each sample was plated in duplicate and read 5 days after the start of culture. Pipette tips were discarded after each

dilution to avoid carrying adhering parasites from one well to another. Results are expressed as the negative log of the titer (i.e., the dilution corresponding to the last positive well).

Tissue extraction and determination of *N*-acetyl- β -D-glucosaminidase activity

Fragments of the inflammatory capsule were collected, weighed and homogenized (vortex) in 1.9 ml of 0.1 M NaCl, 0.02 M NaH₂PO₄ 0.015 M sodium EDTA, pH 4.7, 4°C, per 100 mg tissue. Infiltration of mononuclear cells was quantified by measuring the levels of the lysosomal enzyme *N*-acetylglucosaminidase (NAG), which is present at high levels in activated macrophages [21]. Part of the pellet remaining after hemoglobin measurement was retained for this assay. These pellets were weighed, homogenized in 0.9% w/v NaCl containing 0.1% v/v Triton X-100 (Promega, Madison, WI, USA) and centrifuged (10,000×g, 10 min, 4°C). Samples of the resulting supernatant (100 µl) were incubated for 10 min with 100 µl of 2.24 mM *p*-nitrophenyl-*N*-acetyl-h-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA), prepared in 0.1 M citrate/phosphate buffer (pH 4.5). The reaction was terminated by adding 100 µl 0.2 M glycine buffer, pH 10.6. Hydrolysis of the substrate was determined by measuring the absorption at 405 nm. NAG activity was expressed as the change in OD per gram wet tissue.

Quantitative real-time polymerase chain reaction

Fragments of the inflammatory capsule were collected and Total RNA was extracted using a single-step phenol/chloroform procedure (TRIZOL[®]) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, the tissue was soaked with TRIZOL solution and homogenized using a PowerGen Generator (Fisher Scientifics, Franklin, MA, USA). Chloroform (0.25 ml) was added to 1 ml of TRIZOL homogenate and mixed thoroughly. After centrifugation, the upper aqueous phase was transferred to a fresh tube and 0.7 ml of isopropyl alcohol was added to precipitate the RNA. The RNA pellet was washed once with 70% ethanol and air-dried for no more than 10 min at room temperature. Contaminating genomic DNA was eliminated by dissolving the RNA pellet in 10 mM Tris-HCl, pH 7.5, containing RNase-free DNase and 1 mM MgCl₂ and incubating for 30 min at room temperature. TRIZOL[®] solution was then added to stop the reaction and the total RNA was further purified as described above. The final RNA pellet was dissolved in RNase-free water and the A260/280 ratio was measured to evaluate quality [22].

First-strand cDNA synthesis by reverse-transcription reaction—First strand cDNA was generated using Thermo-Script[®] Reverse Transcriptase (Invitrogen,). A 5 µg of genomic DNA-free total RNA and oligo(dT)₂₀ primer were used; the detailed procedure is described in the manufacturer's instructions (Invitrogen).

Quantitative-PCR—This was performed using a Light-Cycler[®]480 Real-time PCR System (Roche Applied Science, Indianapolis, IN, USA) with SYBR Green PCR reagents purchased from BioRad. Melting curve analyses were carried out to ensure that a single product with the expected melting curve characteristics was obtained. The relative differences among samples were analyzed using the $\Delta\Delta C_t$ method described previously. A ΔC_t value was determined for each sample using the C_t value for GAPDH to normalize loading differences. A $\Delta\Delta C_t$ value was then obtained by subtracting the ΔC_t value for the mice submitted only to the surgery (group sham-without paraffin and Leishmania infection) from the corresponding experimental ΔC_t . The fold difference over the value for the sham mice was calculated as $2^{\Delta\Delta C_t}$.

Statistical analysis—GraphPad Prism software was used. Data were analyzed statistically by the Student *t* test. Data are expressed as mean±SD. The level of significance was set at $p<0.05$.

Results

Histology

C57BL/6 and BALB/c mice implanted with paraffin alone (Group P)—Sterile paraffin tablets were implanted subcutaneously in the back of C57BL/6 and BALB/c mice. According to Tafuri et al. (2000) [18], both strains of mice had an inflammatory capsule of tissue formed around the paraffin tablets as a result of granulomatous tissue formation.

C57BL/6 and BALB/c mice experimentally infected with *Leishmania* alone (Group L)—By 21 days, the inflammatory reaction in the BALB/c mice was intense and large numbers of parasitized macrophages were present throughout the lesion. In C57BL/6 mice, a discrete chronic inflammatory process in the deep dermis was observed. Only a few macrophages parasitized with *L. major* were observed (Fig. 1a, b).

C57BL/6 and BALB/c mice experimentally infected with *Leishmania* and implanted with paraffin (Group P+L)—By 21 days, a diffuse chronic inflammatory reaction was mainly observed in the deep dermis and hypodermis (Fig. 1c, d, and e). Macrophages parasitized with *Leishmania* amastigotes were also found in the dermis (Fig. 1e). Moreover, a new formation of blood vessels and fibroblast proliferation with mature collagen deposition were observed mainly in the capsules (Fig. 1f). However, collagen deposition was observed in both strains of mice, and the degree of deposition was not substantially different between the two strains. Some formation of giant cells was observed, and some of these cells had parasites within their cytoplasm (Fig. 1g, h).

C57BL/6 and BALB/c mice submitted only to the surgery without *Leishmania* and paraffin implanted (sham group)—Macroscopical lesions were not observed in the dorsum of all mice as an extension of the wound healing. In fact, microscopically skin sections of all mice did not show any histological alteration in the epidermis or dermis of all the samples at 21 days post-surgery assay.

Tissue parasite load—According to Tafuri et al. [18], higher parasite load was observed at 21 days post-infection in BALB/c mice and C57BL/6 when compared to the other groups, at 7 and 21 days. Interestingly, C57BL/6 mice showed higher parasitism than BALB/c mice in the tissue capsules ($p \leq 0.01$). By 30 days, the parasite load had decreased in C57BL/6 but not in BALB/c (Fig. 2).

N-acetyl- β -D-glucosaminidase activity—We had shown by histological examination that the capsule tissue obtained from C57BL/6 presented an intense chronic inflammatory reaction by 21 days. We therefore investigated the accumulation of activated inflammatory macrophages by an indirect method, NAG activity. At the site of the lesion (capsule tissue) induced by the paraffin implantation associated with *L. major*, NAG activity was significantly increased ($p \leq 0.05$) at 21 days after experimental infection, compared to paraffin-implantation group (Fig. 3).

Cytokines and chemokines at the site of infection—mRNA expression of the cytokines IL-12, TNF- α , and IFN- γ , which are associated with the resistant phenotype to *L. major* infection (C57BL/6 mice), and IL-4 and IL-10, which are associated with the susceptible phenotype (BALB/c), were determined by real-time polymerase chain reaction (RT-PCR). Expression of chemokines related to monocyte-macrophage (CCL2/MCP-1, CCL3/MIP-1 α and CCL5/RANTES) and lymphocyte (CXCL9/MIG and CXCL10/IP-10) recruitment was also investigated. For these studies, all capsule samples from BALB/c and C57BL/6 mice were obtained 21 days after paraffin implantation concurrent with

Leishmania infection. This time point was chosen because the kinetics of the inflammatory response peaked and the parasite load was prominent in both BALB/c and C57BL/6 mice. Skin tissue samples of sham group were used as controls to compare the cytokine levels. Figure 4a shows that C57BL/6 mice have a resistant phenotype marked by the higher expression of IFN- γ and IL-12 mRNA. Both of these expressions were increased in mice receiving parasites plus paraffin (gray bars), relative to mice receiving paraffin implantation alone (black bars). TNF- α and IL-10 mRNA were detected in lower levels in these mice, and there was no increase relative to paraffin alone. BALB/c mice, on the other hand, expressed higher levels of IL-10 and IL-4 mRNA than C57BL/6 mice, and these levels were higher in mice receiving both parasites and paraffin implantation (Fig. 4b).

C57BL/6 mice from the P+L group showed higher levels of CCL3/MIP-1 α , CCL5/RANTES, CXCL9/MIG and CXCL10/IP-10 mRNA when compared to the paraffin alone group (Fig. 5a). In particular, CXCL9/MIG level in the P+L group was dramatically increased. BALB/c mice, in contrast, expressed higher levels of CCL2/MCP-1 and CCL3/MIP-1 α than C57BL/6 (Fig. 5b).

Discussion

In these paraffin implantation model, both C57BL/6 and BALB/c mouse strains showed an early increase in the numbers of parasites in the lesions accompanies the inflammation. This increase in parasite numbers was observed over the first 3 weeks of infection with a peak at 21 days. In fact, these results were the same observed by Tafuri et al. (2000) [18] following the same experimental protocol. The increase in parasite burden due to paraffin implantation was associated with an intense and diffuse chronic inflammatory reaction, with macrophages heavily loaded with parasites. Moreover, NAG activity was higher at 21 days in the inflammatory capsule of C57BL/6 mice than BALB/c. However, this increase in parasites was transient and these mice subsequently began to resolve their infections. In fact, 30 days after infection, the number of parasites in their lesions begun to be reduced relative to those in the BALB/c mice.

C57BL/6 mice produce IFN- γ , TNF- α and IL-12 in response to *Leishmania* infection, and are able to resolve their infection and develop long-lasting immunity. BALB/c mice, in contrast, produce IL-4 and IL-10 rather than IFN- γ and fail to resolve their lesions, ultimately succumbing to fulminating infection [15,23]. In this work, C57BL/6 implanted with paraffin concurrently with *L. major* infection showed a marked expression of IFN- γ mRNA by 25 times greater than controls. Small amounts of IL10 (10 ng/ml) inhibit IL-12, TNF- α , and IFN- γ production by macrophages and also render the macrophages refractory to the activating effect of IFN- γ have shown by Kane and Mosser [24]. In consequence, IL-10 prevents macrophages from eliminating intracellular parasites. IL-10 mRNA was also expressed concomitantly with IFN- γ expression. In addition to macrophages, one subset of CD4+CD25-Foxp3-Th1 cells with IFN- γ production produces high amounts of IL-10. IL-10 derived from these Th1 cells and macrophages play a pathologic role in the pathogenesis of *Leishmania* infection, as reported by Anderson [25]. Thus, our data are consistent with these earlier studies and could explain the increased tissue parasite load at 21 days (transient susceptibility), related to higher IL-10 mRNA expression at the site of infection, probably without changing the systemic Th1 profile in C57BL/6. In contrast, BALB/c mice showed strong IL-10 mRNA expression by 51 times higher than controls with very little expression of IFN- γ ; IL-4 message was also mainly detected in BALB/c mice. IL-4 production induces Th2 differentiation and progressive leishmaniasis in susceptible mice, but the persistence of infection in these mice could be related to an overproduction of IL-10, which inhibits Th1 cells, and not by exacerbation of the Th2 response [26]. In addition to the Th2 cell-polarizing conditions that underlie the susceptibility of BALB/c mice to cutaneous

Leishmania infection, overproduction of endogenous IL-10 by the host has been well recognized as a central factor that compromises Th1 response to efficiently clean parasites, not only in a variety of mouse models but also in human beings.

On the other hand, IL-12 is essential for an effective polarization of naïve Th cells to the Th1 phenotype characterized by the expression of IFN- γ . IL-12 is composed of two subunit, p35 and p40. IL-12p40 expression is readily detectable in C57BL/6 strain with higher expression in paraffin-*Leishmania* group than paraffin one. However, IL-12p40 expression is hardly detected in BALB/c strain. Scharton-Kersten and Scott found that BALB/c mice lose the capacity to produce IL-12 in the initial phases of *L. major* infection owing to genetic defect [27]. Furthermore, IL-12p40 reduction can also be due to the inhibitory effect of Fc γ -mediated signaling induced by interaction of opsonized parasites on the macrophages [22].

Matte and Olivier found that *L. major* is a strong inducer of an early inflammatory response, and this immunological scenario could potentially restrain the parasite to the inoculation site, favoring the development of local swelling and cutaneous lesions [28]. In our paraffin model, we found increased expression of all chemokines investigated in the skin inflammatory capsule tissue (CCL2, CCL3, CCL5, CXCL9 and CXCL10), especially those induced by IFN- γ and directly involved in the Th1 response, such as CCL3/MIP1- α , CXCL9/MIG and CXCL10/IP-10. These last two chemokines are chemoattractants for CXCR3 T lymphocytes [13]. The onset of *Leishmania donovani*-induced hepatic inflammation and granuloma formation was found to be delayed in CXCR3 $^{-/-}$ C57BL/6 mice [29]. The increased expressions of CXCL9/MIG and CXCL10/IP-10 may attract regulatory T cell populations with subsequent IL-10 production at the site of inflammation [30,31].

CCL5/RANTES up-regulates IL-12, IFN- γ and migration of Th1 cells, particularly memory T cells to the site of infection [32,33]. Here, we observed significantly greater production of the chemokine CCL5/RANTES in the skin capsules of C57BL/6 mice implanted with paraffin concurrently with experimental infection with *L. major* promastigotes compared to mice that had received only paraffin implantation. ELISA assays (data not shown) revealed a notably greater production of CCL5/RANTES in the initial phases of experimental infection (7 days), especially in mice with both paraffin implantation and *L. major* infection. Consistently with the kinetics of this experimental protocol (LP) in C57BL/6 mice, the CCL5/RANTES mRNA levels decreased by 21 days and fell to the levels found in non-infected mice by 30 days. Thus, the decrease in CCL5/RANTES might be directly correlated with the inflammatory response at the site of experimental *Leishmania* infection in C57BL/6 mice. Santiago and co-authors have demonstrated that treatment with Met-RANTES or anti-CCL5/RANTES rendered C57BL/6 animals more susceptible to *L. major* and skewed the immune response from type 1 to type 2 by diminishing IFN- γ production by draining lymph node cells and increasing IL-4 mRNA expression in the lesions [33].

CCL2/MCP-1 is known to attract monocytes, DCs, natural killer cells, and memory T lymphocytes [32]. It has been suggested that CC chemokine receptor 2 (CCR2), the receptor that binds CCL2/MCP-1, increases monocyte recruitment, whereas CCL2/MCP-1 plays a variety of roles in host defense against *Leishmania* [34–36]. It has been reported that high doses of CCL2/MCP-1 activate anti-*Leishmania* macrophage killing mechanisms, either directly by inducing reactive oxygen intermediates [6], or indirectly via nitric oxide production [37]. Recently, a subset of CCR2-positive monocytes has been identified and shown to migrate into inflammatory sites. This population of cells may play a role in pathogen clearance [16]. Here, C57BL/6 mice showed low levels of CCL2/MCP-1 at 21 days after infection. Thus, the lower levels of CCL2/MCP-1 detected in C57BL/6 mice may be consistent with the intense tissue parasite load in the skin capsules. In fact, Conrad and

others, working with CCL2/MCP-1 transgenic *L. major* promastigotes, considered that CCL2/MCP-1 is the main chemoattractant recruiting a restrictive CCR2-positive macrophage subset to the site of infection in resistant mouse strains [38]. These macrophages may assist in resolving the lesions when coactivated with CCL2/MCP-1, whereas the lack of CCL2/MCP-1 production may preclude any such recruitment of CCR2-positive monocytes/macrophages in susceptible BALB/c mice. In our work, BALB/c mice showed no statistical difference from controls in CCL2/MCP-1 production. It has been reported in humans that CCL2/MCP-1 is present in self-healing lesions but absent in non-healing diffuse cutaneous leishmaniasis [9].

Ritter and Heinrich-Korner described divergent expression of inflammatory dermal chemokines in self-healing localized cutaneous leishmaniasis and progressive diffuse cutaneous leishmaniasis [9]. LCL is characterized by strong expression of Th1-associated chemokines such as CCL2/MCP-1, CXCL9/MIG and CXCL10/IP-10, and small amounts of CCL3/MIP-1 α . Chronic DCL is associated with the opposite expression pattern: CCL3/MIP-1 α is dominant and CCL2/MCP-1, CXCL9/MIG and CXCL10/IP-10 are expressed at low levels. In our model, C57BL/6 mice concurrently implanted with paraffin and infected with *Leishmania* dominantly expressed CXCL9/MIG whereas in BALB/c mice, CCL3/MIP-1 is the highest expressed chemokines. In parallel with Th1-phenotype in C57BL/6 and Th2-phenotype in BALB/c as demonstrated by the expression pattern of cytokines and chemokines, the lesion in C57BL/6 is transient and healed after 30 days whereas BALB/c mice have a persistent and non-healing lesion. It has been suggested that immigrating monocytes–macrophages act more like host cells that are permissive for *Leishmania* growth than as effector cells that restrict parasite replication [17]. This permissiveness exists even in the face of the intense inflammatory reaction that occurs in response to paraffin. In fact, Tafuri et al. (2000) [18], in a morphological study, showed that *L. major* lesions were more intense when induced along with the inert inflammatory agent. In both the BALB/c and C57BL/6 strains of mice, an early increase in the numbers of parasites in lesions accompanied this inflammation. This increase in parasite numbers in the lesion was observed over the first 3 weeks of infection. In this present study, we have attempted to determine the immunological response in C57BL/6 during this transiently susceptibility picture related to the highest tissue parasite load. We conclude that the Th1 immune response C57BL/6 did not change to a Th2 response, even though C57BL/6 animals presented higher parasitism than BALB/c mice 21 days after infection and paraffin implantation. Importantly, our models of C57BL/6 mice implanted with paraffin and infected with *Leishmania* vs. BALB/c strain with same manipulation will be useful models to mimic self-healing localized cutaneous leishmaniasis (LCL) vs. progressive diffuse cutaneous leishmaniasis (DCL) in human beings.

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Fig. 1.

a–c: **a, b** Skin fragment of C57BL/6 mice infected with *L. major* (21 days). **a** Note the presence of a discrete chronic inflammatory exudate in the deep dermis (DD; white arrows). Hematoxylin and eosin (HE) stain. Bar=32 μ m. E Epidermis; UD Upper dermis; DD Deep dermis; ML Muscular layer. **b** High magnification showing mononuclear cells and some macrophages with amastigotes forms of *Leishmania* (white arrow). HE stain. Bar= 16 μ m. **c–e** Skin fragment of C57BL/6 implanted with paraffin tablets and infected with *L. major* (21 days). **c** There is an intense chronic inflammatory exudate dissecting the muscular layer (ML) in the deep dermis. HE stain. Bar=62 μ m. **d, e** Higher magnification showing inflammatory mononuclear cells also provoking tissue damage (black triangle). Amastigotes

forms of *Leishmania* can be observed inside macrophages (*white arrows*). HE stain. *Bar*=32 and 16 μm , respectively. **f–h** Inflammatory capsule of C57BL/6 implanted with paraffin tablets and infected with *L. major* (21 days). **f** Granulomatous tissue formation with blood vessels (*arrowheads*), collagen and mononuclear exudate can be noted. HE stain. *Bar*=16 μm . **g** Giant cell formation (*white arrowheads*) and macrophages parasitized with *Leishmania* amastigotes were readily observed (*white arrows*). HE stain. *Bar*=16 μm . **h** Granuloma formation with giant cells (*white arrowhead*) and epithelioid cells (*white arrows*). HE stain. *Bar*=16 μm

Fig. 2.
Parasite load of inflammatory capsule of C57BL/6 and BALB/c implanted with paraffin tablets and infected with *L. major* (21 days). Statistical difference was noted by 21 days (Bars, mean $\pm$ SD; asterisk, $p < 0.01$ compared to BALB/c group)

Fig. 3. *N*-acetyl- β -D-glucosaminidase activity in C57BL/6 mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 7, 21, and 30 days after the infection and implantation. The NAG activity was determined individually and each *symbol* represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only.). Statistical difference was noted by 21 days (*Bars*, mean $\pm$ SD; *asterisk*, $p < 0.05$ compared to paraffin group)

Fig. 4.

a mRNA expression by RT-PCR of cytokines in C57BL/6 mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each bar represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and infection with *L. major*; *P* represents mice with paraffin-tablets implantation only.). Statistical difference was noted by 21 days (Bars, mean±SD; asterisk $p < 0.05$ compared to *L+P* group). **b** mRNA expression by RT-PCR of cytokines in BALB/c mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each bar represents the average and standard deviation of five animals for each group. *L+P* represents mice with the paraffin-tablet implantation and

infection with *L. major*; *P* represents mice with paraffin-tablets implantation only. Statistical difference was noted by 21 days (*Bars*, mean $\pm$ SD, *asterisk*, $p<0.05$ compared to L+P group)

Fig. 5.

a mRNA expression by RT-PCR of chemokines in C57BL/6 mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each bar represents the average and standard deviation of five animals for each group. L+P represents mice with the paraffin-tablet implantation and infection with *L. major*; P represents mice with paraffin-tablets implantation only. Statistical difference was noted by 21 days (Bars, mean±SD; asterisk, $p < 0.05$ compared to L+P group).

b mRNA expression by RT-PCR of chemokines in BALB/c mice: Fragments of a granulomatous tissue formation (inflammatory capsule) were obtained from 21 days after the infection and implantation. The results are expressed of increasing numbers in relation to skin tissue samples from animals that were submitted only to the surgery, group sham (considered zero-axis graph). Each bar represents the average and standard deviation of five animals for each group. L+P represents mice with the paraffin-tablet implantation and infection with *L. major*; P represents mice with paraffin-tablets implantation only. Statistical difference was noted by 21 days (Bars, mean±SD; asterisk, $p < 0.05$ compared to L+P group).