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Splenectomy does not interfere with immune response to *Leishmania major* infection in mice

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Abstract

Spleen is one of the largest lymphoid organs in the body; it harbors immune cells including antigen presenting cells, B and T lymphocytes. It has an important role in humoral and cellular immune responses. Herein we investigated the role of spleen in the immune response to experimental *Leishmania major* infection. It is known that C57BL/6 mice are resistant to *L. major* infection whereas BALB/c mice are susceptible. Although splenectomy was associated with reduced serum levels of IFN-gamma, absence of the spleen did not change the profile of *L. major* infection in the resistant C57BL/6 and BALB/c susceptible mice. Both strains of mice maintained the same profile of cytokine production in regional lymph nodes after splenectomy and responded in the same way against the infection. Only splenectomized BALB/c mice had a reduction in IL-4 and IL-10 production by lymph node cells early in infection. Our data suggest that, in localized infections, regional lymph nodes may replace efficiently the immunological role of spleen in the cellular and humoral immune responses.

Keywords: Spleen; *Leishmania major*; Immune protection; IFN-gamma

1. Introduction

The spleen is a highly organized secondary lymphoid organ that provides an optimal structural framework for generation of efficient immune responses. It is a place that harbors antigen present cells (APC), T and B cells. Antigens that reach the circulation are captured and presented to T cells in the spleen [1–3]. The presence and integrity of the spleen are fundamental to immune responses against microorganisms [4]. Splenectomy in humans and animals has been associated with impairment in immune responses to microbial infections. Children with hepato-splenic form of schistosomiasis have a reduction in the production of reactive oxygen species (ROS) [5], and adults that have their spleens removed by surgery have a decrease in the number of naïve CD4+T cells [6]. In mice with streptococcal cell wall induced arthritis, splenectomy leads to a reduction in TNF-alpha, IL-6 and IL-1-alpha production in other lymphoid organs [7]. Moreover, splenectomy predisposes individuals to a risk of overwhelming infection, most often caused by encapsulated bacterium [8] and several explanations have been proposed to account for this increased risk of infection in asplenic individuals.

Moreover, spleen is an important organ for IFN-gamma production and Th1 responses and splenectomy has been associated with deficiency in the production of this cytokine in experimental models of infection by bacterias such as *Listeria monocytogenes* [9].

Although there is a close association between spleen and IFN-gamma production, there is no study on the consequences of splenectomy for immune responses to parasite infections where this cytokine plays an essential role such as *L. major* infection. In the case of experimental infection

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by *L. major*, the most studied *Leishmania*, infection resistance is correlated with Th1 type response characterized by IL-12 produced by dendritic cells and macrophages IFN-gamma and TNF-alfa production by T cells [2,10]. Participation of these cytokines in the control of lesion development is well documented in C57BL/6 mice [11]. On the other hand, susceptible BALB/c mice produce high amounts of IL-4 [12] and a vigorous humoral immune response [13] that is associated with high spleen parasitism and splenomegaly [12]. In all leishmaniasis models, a different immune response in each lymphoid organ can be detected, but even in the cutaneous leishmaniasis the participation of the spleen in cytokine production is clear [14].

Splenectomy during leishmania infection is still a common practice in the case of human visceral leishmaniasis, but little is known about the effects of the spleen absence in the immune response development during cutaneous leishmaniasis or another localized infection. Therefore, the aim of this study is to evaluate the effects of splenectomy in resistance and susceptibility models of the cutaneous leishmaniasis caused by *L. major* infection.

2. Materials and methods

2.1. Animals

Female C57BL/6 and BALB/c mice (4–6 weeks old) were obtained from our animal facility (CEBIO, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil). Animals were maintained in aseptic conditions and were given water and food *ad libidum*. All procedures with animals were approved by local ethical committee for animal research.

2.2. Splenectomy protocol

Mice received 0.1 ml of an injection containing 1.7 mg ketamine and 0.33 mg xylazine in physiological buffer. After anesthesia, animals had their hair removed in the section area, a small incision was performed and the spleen was removed. Incision was sutured and animals were kept until wakeup at 37 °C. Control group underwent a sham surgery and they were maintained in the same conditions. All experiments were performed one month afterwards when there was no sign of inflammation found in the abdominal cavity.

2.3. Parasites and antigen preparation

Leishmania major (MHOM/IL/8/Friedlin) promastigotes were cultured in Gracés insect medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal calf serum (FSC) (CUTLAB, Campinas, SP, Brazil) 2 mM of L-glutamine and 20 μg/ml de gentamicyn sulfate at 26 °C. Mice were inoculated in the hind footpad with 1×10^6 *L. major* promastigotes in stationary phase. Lesion development was accompanied weekly with a caliper (Dial Lens Meter – Mitugoyo Mfg. Ltda, Japan) and expressed as the difference in size between the infected footpad and the contra lateral uninfected footpad. *L. major* antigen was obtained from logarithmic phase promastigotes by centrifugation and submitted to cycles of freezing and thawing [15]. Parasite number in the footpad was estimated by limiting dilution assay [15].

2.4. Analysis of cytokine production

Single-cell suspensions were prepared from popliteal lymph nodes of the infected footpad harvested at 2, 4 or 8 weeks after infection. Cells were placed in tissue culture plates and adjusted to a concentration of 5×10^6 cells/ml in RPMI Medium 1640 (GIBCO BRL, Grand Island, N.Y., USA) containing 10% of FCS, 2 mM L-glutamine, 50 μM 2-mercapto-ethanol, 100 U/ml penicillin, 100 μg/ml fungizone, 1 mM sodium piruvate, 0.1 mM essential amino acids and 25 mM Hepes. They were stimulated with 50 μg/ml *L. major* antigen. After 72 h, supernatants were harvested and IFN-gamma, IL-4 and IL-10 were measured by ELISA using antibodies to capture and detection in accordance with the manufacturer's protocol (PharMingen, San Diego, CA, USA) in appropriated plates (NUNC, Naperville, IL). To measure serum IFN-gamma, blood was collected and sera were separated by centrifugation, and ELISA was preceded in the same way for supernatants.

3. Results

3.1. IFN-gamma production after splenectomy

Spleen is reported as an important organ for IFN-gamma production and Th1-type immune responses against bacterial infections. To access the effect of spleen removal in the basal levels of IFN-gamma production, concentration of this cytokine was measured in serum. Indeed, reduced levels of IFN-gamma were observed in splenectomized C57BL/6 mice as compared to control mice (Table 1).

3.2. Lesion development and local parasitism

To evaluate the importance of the spleen in the development of immune response against protozoa parasites, we used *L. major* infection as a model. BALB/c and C57BL/6 mice, splenectomized or not, were used to show the effects

Table 1
INF-gamma levels in serum one month after surgery

Groups	Surgery	IFN-gamma in serum (ng/ml)
C57BL/6 with spleen	Sham	7.0 ± 8.0
C57BL/6 without spleen	Splenectomy	$0.8 \pm 0.5^*$
BALB/c with spleen	Sham	1.01 ± 0.13
BALB/c without spleen	Splenectomy	1.0 ± 0.17

* $p < 0.05$ between same strain of mice with and without spleen.

of splenectomy on susceptibility and resistance to this parasite, respectively. One month after surgery, animals were infected in the hind footpad with 1×10^6 stationary phase promastigotes and lesion development was evaluated at different time points. Fig. 1A shows that animals without spleen had the same pattern of infection observed in control mice; this result was observed in BALB/c and C57BL/6 mice. Usually, control of infection is associated with reduction in parasitism and in lesion development. Local parasitism was determined by limiting dilution and no difference was found between control and splenectomized mice (Fig. 1B).

Histological analysis was performed to search for differences in lesion features such as cellular infiltrate and tissue damage. Fig. 2A and D shows infected footpad tissue of control C57BL/6 mice and C57BL/6 mice without spleen 2 weeks post infection. There was no difference in the inflammatory infiltrated or in parasitism between the two groups of mice, and the same was observed for BALB/c mice with or without spleen (Fig. 2G and J, respectively).

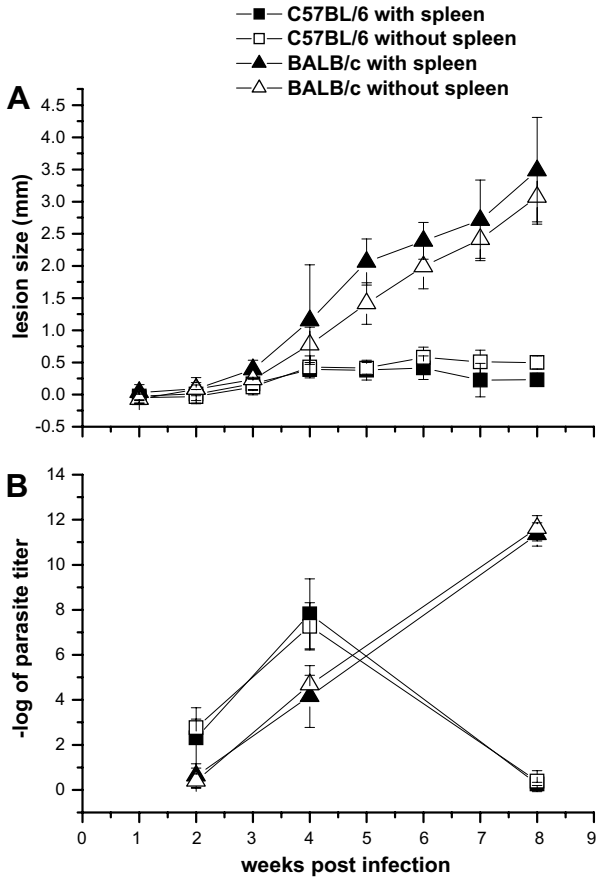

Fig. 1. Course of infection and parasite quantification Mice were inoculated with 1×10^6 promastigotes of *L. major* in the hind footpad 30 days after splenectomy. (A) Lesion measurements were carried out weekly with a metric caliper. Each point represents mean ± SD of three independent experiments with four or six animals per group. (B) Parasite load in lesions was performed two, four or eight weeks post infection. Animals were sacrificed and parasite load determined by limiting dilution assay in the infected footpad.

The pattern of infection and tissue damage were similar between both groups. Comparing C57BL/6 mice with and without spleen 4 weeks post infection (Fig. 2B and E) and 8 weeks post infection (Fig. 2C and F), there were no detectable differences either between groups. At the eighth week post infection, tissue damage was almost resolved.

Comparing control and splenectomized BALB/c mice, there were also similarities between the groups after 4 weeks (Fig. 2H and K) and 8 weeks post infection (Fig. 2I and L). Differences in the progression of infection in this susceptible strain were observed in both groups when compared to resistant C57BL/6 mice. At eighth weeks post infection, footpad tissue was severely damaged and some necrotic foci could be detected in lesions of BALB/c.

3.3. Cytokine production and induction of Th1 and Th2 immune responses

In *L. major* infection, susceptibility is associated with Th2-type response that is characterized by IL-4 production and isotype switch to IgG1. On the other hand, C57BL/6 mice show a resistance profile with IFN-gamma production and less involvement of humoral immune response. IL-10 plays regulatory functions in both strains. To verify whether the spleen is an important organ for the development of both Th1 and Th2 immune responses as well as for anti-inflammatory responses during infection with *L. major*, levels of IFN-gamma, IL-4, and IL-10 were measured in tissue culture supernatants of spleen (of control mice only) and popliteal lymph node cells after 72 h of stimulation with *L. major* antigen. Fig. 3A shows that spleen cells from control C57BL/6 mice produce higher levels of IFN-gamma at 4 weeks post-infection. Fig. 3B shows that IL-4 production is higher in BALB/c than in C57BL/6 mice at week 2 post-infection. There was no difference in IL-10 production by spleen cells between control C57BL/6 and BALB/c control mice at any time point examined after infection (Fig. 3C).

Analysis of cytokine production by cells from the draining lymph nodes showed no difference between control and splenectomized mice in the development of Th1 immune response against the parasite (Fig. 3D). There was an increased production of IFN-gamma in the fourth week post infection and this response was maintained up to the eighth week of infection when control of parasite growth was observed. Splenectomized BALB/c mice showed a decrease in IL-4 production in the second week post infection (Fig. 3E), but this result was not maintained during the course of infection. In the fourth week after infection, control as well as splenectomized BALB/c but not C57BL/6 mice showed very high levels of IL-4 production by popliteal lymph node cells (Fig. 3E). In accordance to that, it was possible to identify increase in the levels of specific serum IgG1 in BALB/c but not C57BL/6 mice regardless of the surgical procedure performed (BALB/c with

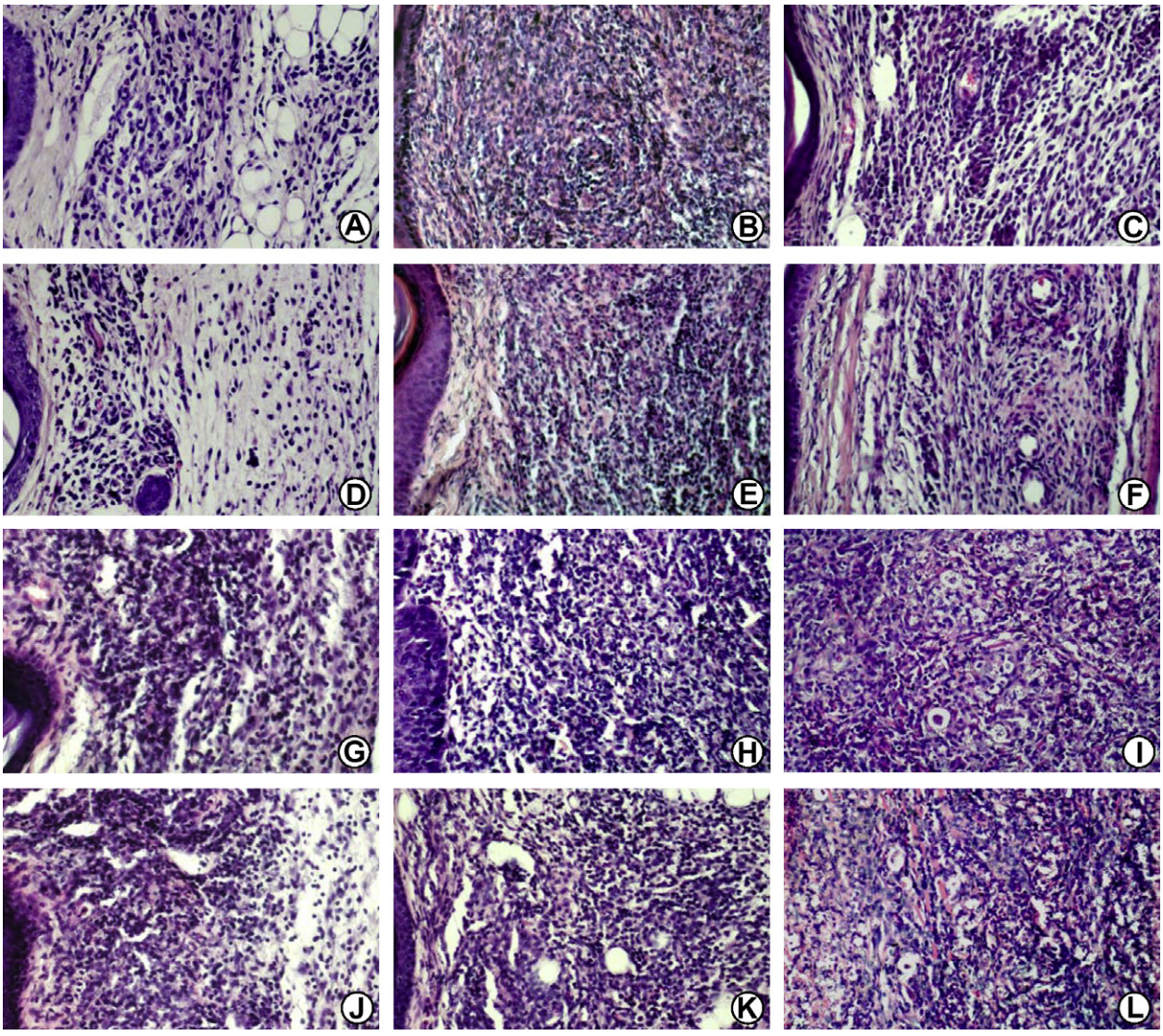

Fig. 2. Histological analysis of the infected footpad 2, 4 and 8 weeks post infection showing the presence of parasites and inflammatory infiltrate. Mice were inoculated with 1×10^6 promastigotes of *L. major* in the hind footpad 30 days after splenectomy. The infected footpad was collected 2, 4 and 8 weeks post infection and histological analysis was performed after haematoxilyn and eosin staining. (A–C) Tissue from C57BL/6 mice with spleen 2, 4 and 8 weeks post infection, respectively; (D–G) tissues from C57BL/6 mice without spleen 2, 4 and 8 weeks post infection, respectively; (H–J) tissues from BALB/c mice with spleen 2, 4 and 8 weeks post infection, respectively and (K–L) tissues from BALB/c mice without spleen 2, 4 and 8 weeks post infection, respectively.

spleen: 0.877 ± 0.269; BALB/c without spleen: 0.839 ± 0.214; C57BL/6 with spleen: 0.204 ± 0.06; C57BL/6 without spleen: 0.139 ± 0.05). IL-10 is known to be produced by regulatory T cells in chronic infections caused by *L. major* [16], but in our model of infection we did not see differences among groups in the production of IL-10 by draining lymph node cells (Fig. 3F).

4. Discussion

Very few studies have addressed the effects of splenectomy in the immune response. In this study, we accessed the effects of spleen removal in *L. major* infection in mice. Our results suggest that the spleen is not fundamental for Th1 or Th2 immune response development against localized leishmaniasis. Most of the studies on the consequences of splenectomy have been performed for bacterial infections. In human cases of visceral leishmaniasis splenectomy is a common practice to eliminate amastigote foci and ameliorate leucopenia, but this procedure may increase the susceptibility to opportunistic infections [17].

In rats, splenectomy causes decrease in mediators of inflammation such as IL-6 and TNF-alfa during streptococcally induced arthritis [7]. During *Listeria monocytogenes* infection in C57BL/6 mice, there is a reduction in the serum levels of IFN-gamma and IL-12 and consequent impairment of Th1 immune response [9]. In humans, splenectomy has been associated with increased susceptibility to bacterial and viral infections [4]. In children with hepatosplenic schistosomiasis mansoni, for instance, splenec-

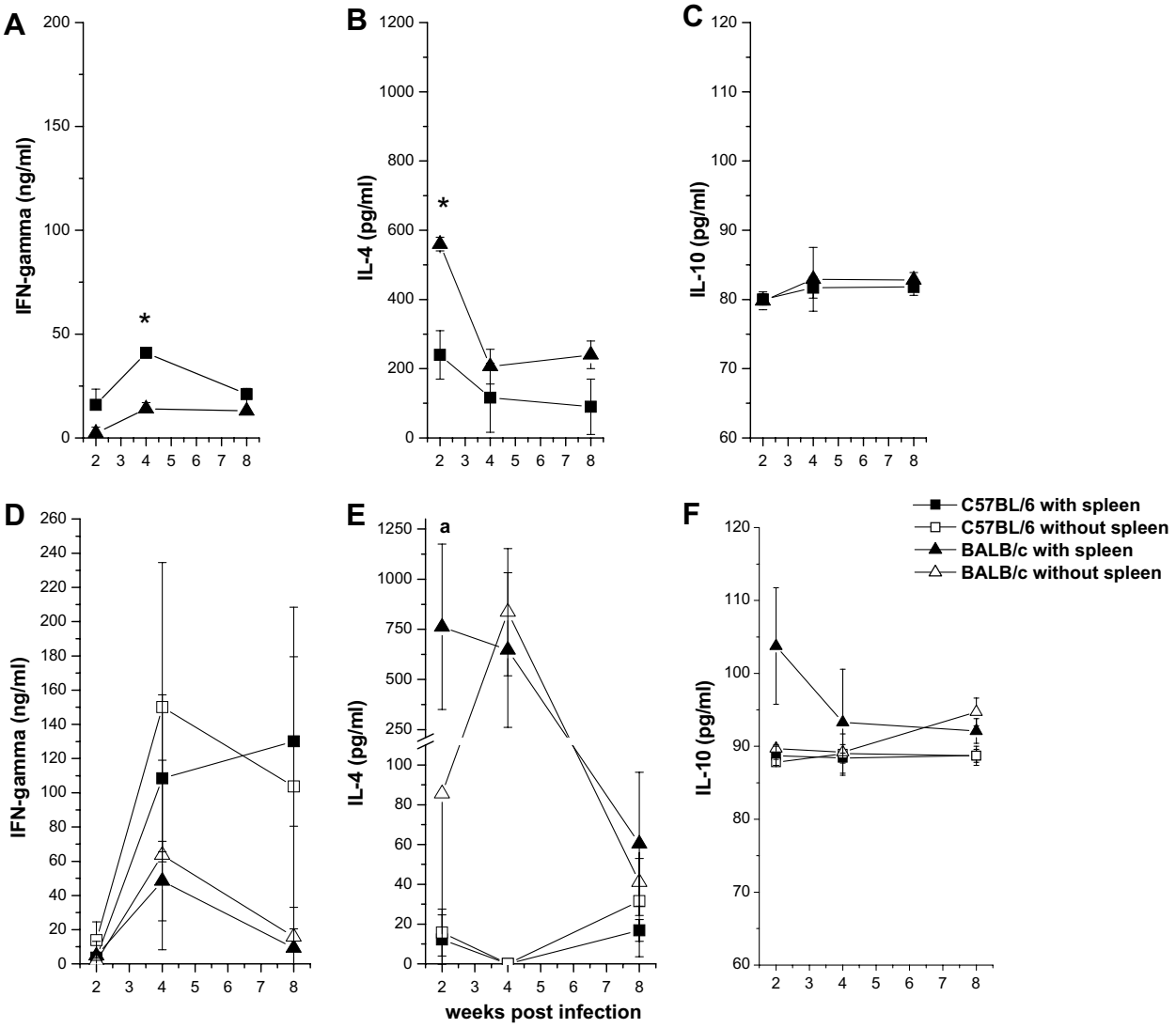

Fig. 3. Cytokine production by spleen and popliteal lymph node cells of *L. major* infected mice Cytokine concentrations of IFN-gamma, IL-4 and IL-10 were determined by capture ELISA with specific antibodies in supernatants of cells from spleen or popliteal lymph nodes after 72 h of stimulation with 50 μg/ml *L. major* antigen. (A–C) IFN-gamma, IL-4 and IL-10 production, respectively, by spleen cells isolated from *L. major* infected mice. (D–F) IFN-gamma, IL-4 and IL-10 production, respectively, by popliteal lymph node cells isolated from *L. major* infected mice. Each point represents mean ± SD of three independent experiments with four or six animals per group. $^{*}p < 0.05$ between control (with spleen) BALB/c and C57BL/6 mice. $^{a}p < 0.05$ between BALB/c mice with and without spleen.

tomy is followed by a reduction in ROS production [5]. The absence of the spleen is also associated with a decrease in humoral responses. To our knowledge, no study on the effects of splenectomy in mice protozoa parasite infection is available.

Spleen is an important organ for IFN-gamma production and we observed that splenectomy was correlated with reduction in the systemic levels of this cytokine. Thus, it would be plausible to surmise that splenectomy would interfere with resistance to *L. major* infection in C57BL/6 mice that show a Th1 response development with IFN-gamma production and low levels of immunoglobulin [18].

Spleen has been shown to have a role in protective immune responses to visceral leishmaniasis when the infective parasite is *L. donovani*. In this infection, there is clear change in the cytokine production by spleen T cells and macrophages during infection. Early production of the inhibitory cytokines IL-10 and TGF-beta is followed by IFN-gamma and IL-12 at 28 days of infection in the mouse model. At this time, T cells and dendritic cells had moved out of the lymphoid follicle and marginal zone into the red pulp of the spleen where the parasites were located. The Th1-related cytokines that these cells secrete are strongly correlated with protective immune responses [19].

Although the cutaneous leishmaniasis is a localized disease, the immune response against the parasites is systemic. In experimental models of the disease, spleen parasitism and proliferation of immune cells in this organ are observed [14]. In our study, we also observed higher levels of IFN-gamma production by spleen cells of resistant

C57BL/6 mice whereas spleen cells of susceptible BALB/c had increased IL-4 production. Nevertheless, the absence of the spleen did not change the profile of *L. major* infection in C57BL/6 mice. Splenectomized infected animals kept their ability to develop an efficient local Th1 immune response, adequate control of parasite growth and lesion healing.

Spleen is also an important organ for immunoglobulin production and B cell activity [20] with a high frequency of B cells [3,21]. There are reports showing that cross-linking of FcR in macrophages of BALB/c mice by anti-parasite antibodies seems to be important for IL-10 secretion and immunomodulation of the lesion caused by *L. major* infection [22]. In spite of that, no difference was observed in IgG1 specific antibody production against *L. major* antigens, in any of the two strains tested, although BALB/c mice showed higher levels of these antibodies (data not shown). In concert with this result, BALB/c mice without spleen also maintained a normal development of Th2 immune response with production of high amounts of IL-4 by popliteal lymph node cells. Therefore, spleen was not fundamental to Th2 immune response development in adult mice.

Thus, *Leishmania major* infection in mice seems to have clear systemic immunological effects, even if it is a regional infection that affects the epidermis. Indeed, in susceptible BALB/c but not in resistant C57BL/6 mice after 8 weeks of infection, it was possible to see visceral growth of parasites, mostly in the popliteal lymph nodes and spleen (data not shown).

In spite of the fact that spleen seems to mount an immune response to the parasite, the immune responses in the regional lymph nodes were unchanged in both strains of splenectomized mice and it was probably enough to control parasite growth in resistant C57BL/6 mice as well as to stimulate Th2 immune response in susceptible BALB/c mice. Our data suggest that, in localized infections, regional lymph nodes may replace efficiently the immunological role of spleen in the cellular immune responses.

Splenectomy is still a common clinical procedure used to ameliorate the hepato-splenic form of human schistosomiasis and also the visceral form of leishmaniasis in humans. Our study brings important clinical information on the possible effects of this procedure in individuals living in endemic areas where these diseases usually cohabit. It also suggests that regional lymph nodes may take over the role of spleen when local stimulation is delivered to the immune system.

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