

INFLUENCE OF MICROBIOTA IN EXPERIMENTAL CUTANEOUS LEISHMANIASIS IN SWISS MICE

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SUMMARY

Infection of Swiss/NIH mice with *Leishmania major* was compared with infection in isogenic resistant C57BL/6 and susceptible BALB/c mice. Swiss/NIH mice showed self-controlled lesions in the injected foot pad. The production of high levels of interferon- γ (IFN- γ) and low levels of interleukin-4 (IL-4) by cells from these animals suggests that they mount a Th1-type immune response. The importance of the indigenous microbiota on the development of murine leishmaniasis was investigated by infecting germfree Swiss/NIH in the hind footpad with *L. major* and conventionalizing after 3 weeks of infection. Lesions from conventionalized Swiss/NIH mice were significantly larger than conventional mice. Histopathological analysis of lesions from conventionalized animals showed abscesses of variable shapes and sizes and high numbers of parasitized macrophages. In the lesions from conventional mice, besides the absence of abscess formation, parasites were rarely observed. On the other hand, cells from conventional and conventionalized mice produced similar Th1-type response characterized by high levels of IFN- γ and low levels of IL-4. In this study, we demonstrated that Swiss/NIH mice are resistant to *L. major* infection and that the absence of the normal microbiota at the beginning of infection significantly influenced the lesion size and the inflammatory response at the site of infection.

KEYWORDS: *Leishmania*; Swiss mice; Microbiota ; Germfree; Th1; Leishmaniasis.

INTRODUCTION

Leishmaniasis is a disease caused by parasites of the genus *Leishmania* that produce diversified clinical manifestation depending upon the characteristics of the parasite and on the immune response of the host^{2,5}. The present understanding of the factors that lead to this diversity of clinical symptoms was obtained, in large part, through studies using isogenic mice experimentally infected with *L. major*. In these studies, the resistance to *L. major* is correlated with the development of Th1 CD4+ lymphocytes that produce high levels of IFN- γ , a cytokine capable of activating macrophages to kill the intracellular forms of the parasite. On the other hand, susceptibility has been associated with the lack of IFN- γ production and the presence of Th2 CD4+ lymphocytes that produce IL-4 which acts inhibiting the activation of macrophages^{12,18,23}. There is, however, no report on infection of Swiss/NIH mice with *L. major*.

Many factors can influence the type of immune response mounted by the host. The effects of the intestinal microbiota on the outcome of

infections are not well investigated. There are reports including ours showing that the indigenous microbiota can play an important role in the outcome of parasitic infections, making germfree animals either more susceptible or more resistant to infection when compared with the corresponding animals that are associated to the normal microbiota (conventional animals)^{9,26,28,29,30}. Several investigators have reported that the indigenous microbiota affects the development of the host's immune response⁴. However, the mechanisms by which the microbiota exerts its effect on the host immune response are not well understood. Both the development of T cell responses (delayed-type hypersensitivity)¹³ and macrophage activity^{4,16} have been found impaired in the absence of the indigenous microbiota, whereas antibody responses seemingly is not affected¹³. Hence, it would be reasonable to suppose that the Th1 arm of the immune response could be deficient in the absence of the normal microbiota, while the Th2 arm could be intact.

In the present work, Swiss/NIH mice were characterized as to their response to infection with *L. major* by measuring the development

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of the primary lesion and cytokine production after subcutaneous injection with the parasite. We then investigated whether the absence of the microbiota at the onset of infection, and during the time period in which the development of a susceptible or resistant T cell response occurs^{3,12,23}, would alter sizes of lesions and the cytokine profile when compared with animals bearing the microbiota from birth. We found that the absence of the normal microbiota early in infection renders Swiss/NIH mice more susceptible to *L. major*, and that this increased susceptibility is not due to an impaired Th1 response.

MATERIAL AND METHODS

Animals

C57BL/6 and BALB/c mice were obtained from CEBIO (UFMG, Belo Horizonte, Brazil). Germfree Swiss/NIH mice were derived from a germfree nucleus (Taconic Farms, Germantown, USA) and maintained in flexible plastic isolators (Standard Safety Equipment Co., Pallatine, USA) using classical gnotobiology techniques²⁰. Conventionalized Swiss/NIH mice were obtained by transferring germfree animals from a sterile isolator environment to the experimental animal facility. Conventional Swiss/NIH mice were derived from germfree matrices, and considered conventional only after two generations in the conventional facility. All animals were females and 5-7 weeks old.

Parasite and antigen

A clone of *Leishmania major* (WHO MHOM/IL/80/Friedlin) was used for these studies. Parasites were cultured in Grace's insect medium (Life Technologies, Grand Island, USA) supplemented with 20% fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Metacyclic promastigotes were obtained from stationary phase of growth using *Arachis hypogae* agglutinin (Sigma Chemical Co., St. Louis, MO) as described previously²¹. Antigen was prepared from *L. major* stationary phase cultures as described previously¹. In short, cells were washed four times in PBS (0.9% NaCl buffered with 0.01M sodium phosphate, pH 7.2-7.4, the concentration adjusted to 1×10^8 cells/ml, freeze/thawed three times, the antigen was homogenized, aliquoted and maintained at -20 C until use.

Infection and monitoring of lesions

All mice were injected subcutaneously on the left hind footpad with 1×10^6 metacyclic promastigotes of *L. major*. For the infection of germfree Swiss/NIH mice, metacyclic promastigotes in Grace's medium (Life Technologies, Grand Island, USA) were aseptically transferred into sterile glass ampoules. The ampoules were sealed, the outer surface was chemically sterilized with 8% peracetic acid and introduced into the isolator. Another ampoule containing promastigotes and treated in the same way was used to infect conventional Swiss/NIH mice. Measurements of the footpad thickness were taken using a calliper (Starret S. A., Itu, SP, Brazil). The lesion size was calculated by subtracting the value of the uninfected contralateral footpad from that

of the infected one. The lesions from conventionalized Swiss/NIH mice were monitored starting from the third week of infection when they were taken out of the isolator.

Conventionalization

Germfree Swiss/NIH mice were infected with *L. major* as described above. Three weeks after infection, these animals were placed in bedding which contained fresh faeces from conventional Swiss/NIH animals, in order to induce the colonization by the different species of microorganisms normally found in conventional mice. The conventionalized animals were kept in the experimental animal facility under the same conditions as the conventional mice.

Histopathology

Histopathological analysis of lesions were performed in conventional and conventionalized Swiss/NIH mice. Four animals from each group were sacrificed after seven or fifteen weeks of infection and footpad tissues were collected and fixed in 10% formalin in PBS. Later the material was dehydrated, cleared, embedded in paraffin, cut (3 to 5 µm thick) and stained with hematoxylin and eosin for microscopical examination.

Cell cultures and cytokine measurement

Spleen cells and a pool of popliteal lymph node cells were prepared as described previously²⁴. Pools of popliteal lymph nodes were used since our Swiss/NIH mice did not give, in several experiments, a positive mixed lymphocyte reaction (our unpublished observations), which indicates that these animals are not genetically different as to their MHC antigens. The organs were homogenized in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) and the concentration of cells adjusted to 5×10^6 cells/ml RPMI 1640 supplemented with 10% fetal bovine serum (Nutricell, Campinas, SP, Brazil), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES and 0.05 mM β-mercaptoethanol (Gibco Laboratories), and 1 ml/well plated in 24-well tissue culture plates either with or without ConA (5µg/ml, Sigma Chemical Co., St. Louis, MO, USA) or 50µl of *L. major* antigen. Supernatants were harvested at 72 h for IFN-γ and IL-4 analyses. IFN-γ and IL-4 were measured by capture ELISA as previously described¹. IFN-γ was measured using R46A2 rat monoclonal antibody as capture antibody (kind gift from Dr. P. Scott, University of Pennsylvania, Philadelphia, PA, USA) and a rabbit polyclonal anti-IFN-γ anti serum (kind gift from Dr. J. P. Farrell, University of Pennsylvania, Philadelphia, PA, USA) was used as a secondary antibody followed by donkey peroxidase conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, INC, West Grove, PA, USA). IL-4 was measured using 11B11 rat monoclonal antibody as capture antibody and biotinilated BVD6 rat monoclonal antibody as a secondary antibody (both kind gifts from Dr. J. P. Farrell, University of Pennsylvania, Philadelphia, PA, USA) followed by streptoavidin-

peroxidase (Sigma Chemical Co. St. Louis, MO, USA). The levels of IFN- γ and IL-4 were calculated by comparison with a standard curve using recombinant IFN- γ and IL-4 (Genzyme, Cambridge, MA, USA). The sensitivity for the assays were as follows: IL-4, 0.2 U/ml and IFN- γ , 30 pg/ml.

Statistical analysis

The data obtained in each experiment were analysed by the Student's *t* test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Development of the lesion and cytokine profile in Swiss/NIH mice after *L. major* infection

The outcome of infection with *L. major* in Swiss/NIH mice was compared with that in resistant C57BL/6 and susceptible BALB/c mice by measuring the development of primary lesions after subcutaneous infection with *L. major* metacyclic promastigotes into the left hind footpad. Footpad thickness in Swiss/NIH mice, like in C57BL/6 mice, peaked at 4-5 weeks and resolved to pre-infection size, whereas footpads of BALB/c mice continued to swell without any evidence of resolution (Figure 1A).

IFN- γ and IL-4 production by lymph node cells from Swiss/NIH mice after 2 weeks of infection was measured and compared with that

of lymph node cells taken from C57BL/6 and BALB/c mice. Cells from both Swiss/NIH and C57BL/6 mice secrete similar amounts of IFN- γ (Figure 1B). Clearly, these cells secreted substantially more IFN- γ upon antigenic stimulation “*in vitro*” than cells from BALB/c mice. The IL-4 production occurred in lower levels in Swiss/NIH mice when compared with BALB/c mice (Figure 1C).

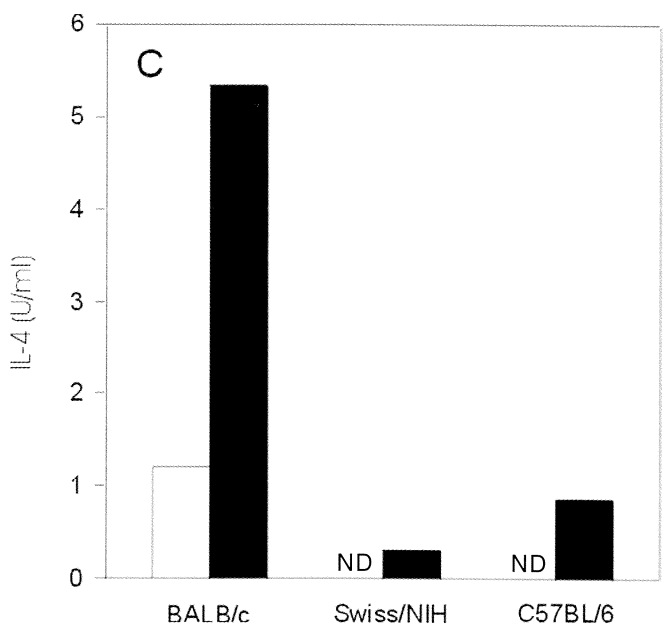
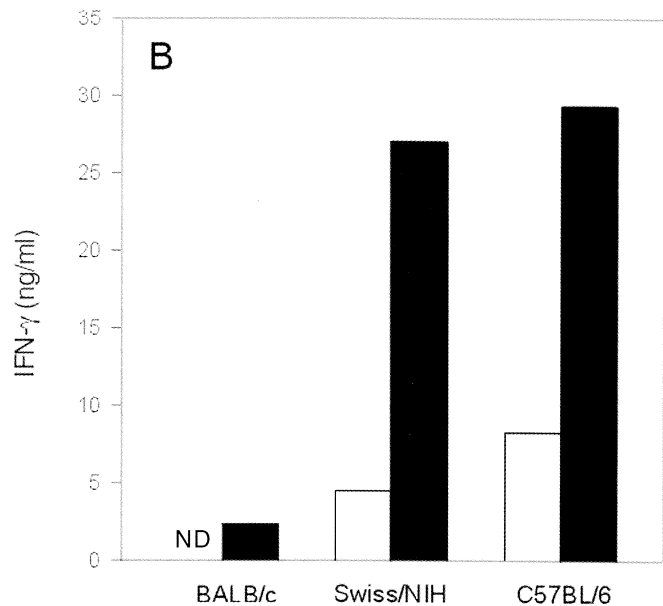
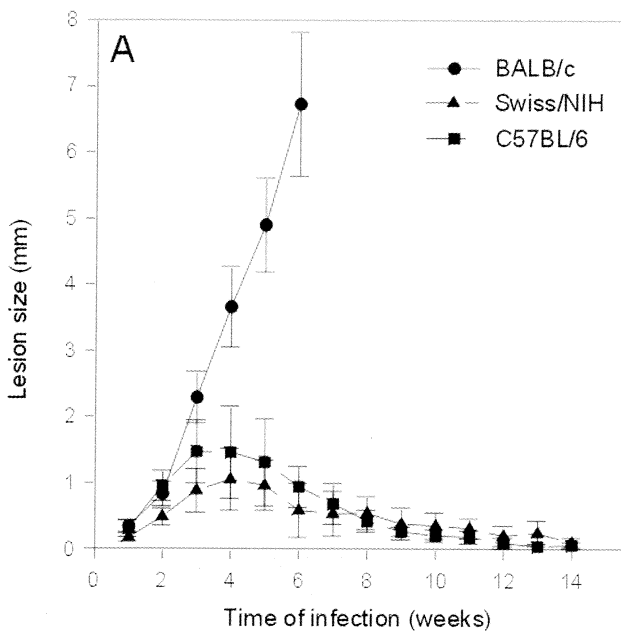


Fig. 1 - Course of infection and cytokine production in conventional Swiss/NIH, BALB/c and C57BL/6 mice infected in the hind footpad with *L. major*. (A) Each point represents the mean of lesion size of at least five mice per group, subtracted of the contralateral control foot. Vertical lines represent SD of means. IFN- γ (B) and IL-4 (C) levels in supernatant of lymph node cell culture from mice at 2 weeks of infection. Cells were unstimulated (white bars) or stimulated (black bars) “*in vitro*” with *L. major* antigen for 72 hours, supernatants were harvested and the presence of cytokines was assayed by capture ELISA as described in Materials and Methods. ND: not detectable. Data are from one representative experiment of three performed.

Infection of conventionalized Swiss/NIH mice with *L. major*

We had previously shown that germfree mice do not heal lesions when infected with *L. major* and that their higher susceptibility was not due to a Th2 type response³⁰. In order to investigate whether we could revert this picture by associating germfree mice with the conventional microbiota after the T cell response was already established, we infected germfree mice with *L. major* and associated them with the normal microbiota three weeks after infection. The severity of infection in conventionalized and conventional Swiss/NIH mice with *L. major* was compared by measuring lesion were weekly after conventionalization (Figure 2). Like conventional mice, conventionalized animals showed cutaneous lesions restricted to the site of infection, presenting no evidence of metastasis. However, conventionalized mice had lesions significantly larger than those observed in conventional control mice (Figure 2A).

Assays to analyze IFN- γ and IL-4 production were carried out after seven and fifteen weeks of infection. Spleen cell cultures from conventional and conventionalized mice produced similar levels of IFN- γ when stimulated “*in vitro*” with *L. major* antigen (Figure 2B). IL-4 production occurred at levels lower than 2 U/ml, and were similar in both groups (data not shown).

Histopathological analysis

To determine if the absence of the microbiota at three weeks of

infection would interfere with the inflammatory process associated with *L. major* infection, cutaneous lesions from conventional and conventionalized mice were histologically examined. At the seventh week after infection, histological sections from both groups showed a diffuse chronic inflammatory cellular exudate (Figure 3A and 3B). However, the footpad skin fragments of all the conventionalized animals showed a chronic focal inflammation with abscesses of variable sizes constituted of collagen fibers associated with the cellular exudate (pyogenic membrane), vacuolated macrophages with the presence of several amastigotes (sometimes found outside the cells); and finally, in the central region, the presence of cellular acidophilic residue (necrotic cells) and a large quantity of neutrophils in fatty degeneration (Figure 3C) was noted. In conventional animals, besides the absence of abscess formation, amastigotes inside the macrophages or even in the interstitial space were rarely observed.

At the fifteenth week of infection the footpad skin fragments from both groups showed the presence of a chronic productive diffuse inflammation, mainly observed in the deep dermis. However, in all of the conventionalized animals, the reactions were more intense (Figure 4 A and B).

DISCUSSION

There are well established isogenic mouse models for leishmaniasis, specially concerning infections with *L. major*. Upon infection with

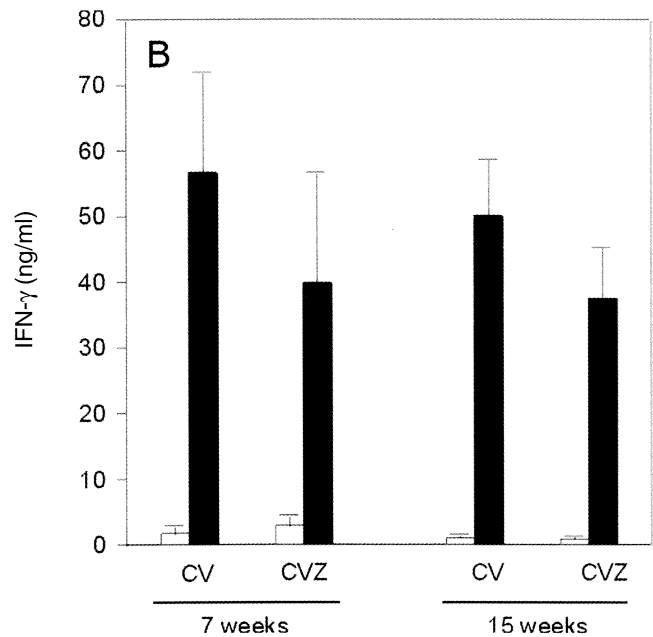
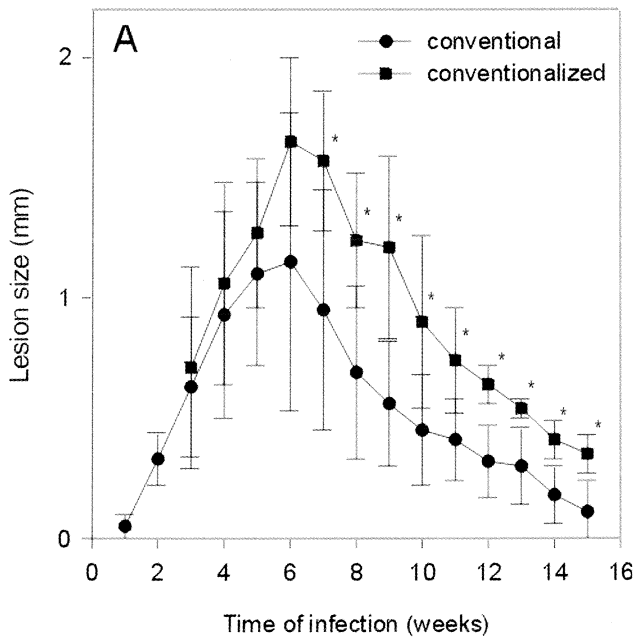


Fig. 2- Course of infection and IFN- γ production in conventional or conventionalized Swiss/NIH mice infected in the hind footpad with *L. major*. (A) Each point represents the mean of lesion size of at least four mice per group, subtracted from the value obtained for the contralateral control foot. Vertical lines represent SD of means. Asterisks denote statistical difference between conventional and conventionalized mice at each time point, ($p \leq 0.05$). (B) IFN- γ production by spleen cells at 7 and 15 weeks of infection. Cells were unstimulated (white bars) or stimulated (black bars) “*in vitro*” with *L. major* antigen for 72 hours, supernatants were harvested and the presence of IFN- γ was assayed by capture ELISA as described in Materials and Methods. CV: conventional mice, CVZ: conventionalized mice. Data are from one representative experiment of three performed.

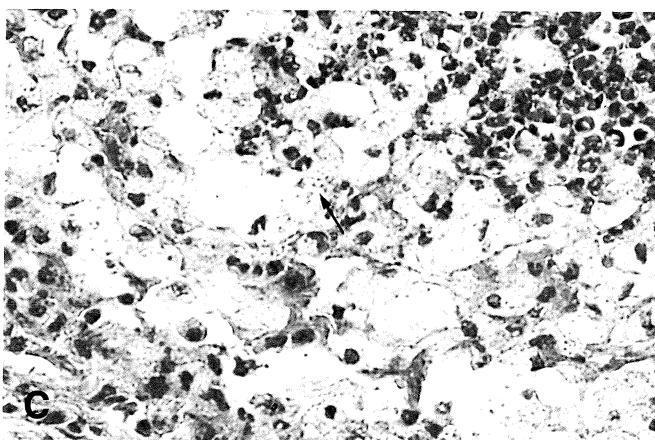
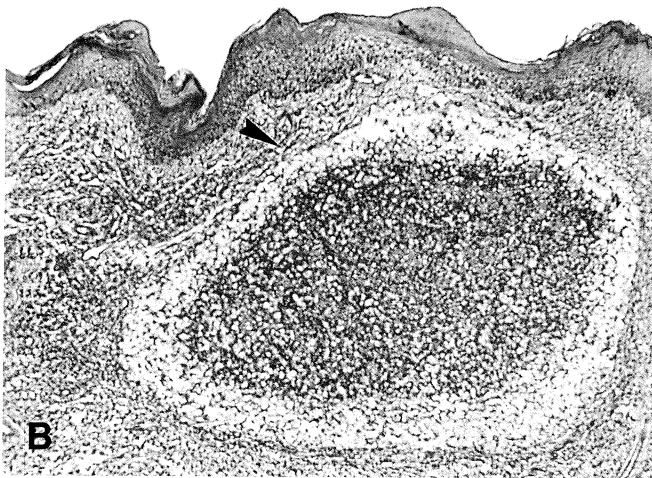


Fig. 3 - Histologic sections of lesions from conventional (A) and conventionalized (B, C) mice after 7 weeks of infection with *L. major*. (A) Typical lesion from a conventional mouse, showing chronic and diffused inflammation reaching the superficial and deep dermis (H.E. 4x). (B) Lesion from conventionalized mouse, showing chronic diffuse and focal inflammation with abscess formation (arrow head) (H.E. 4x). (C) Detail of structure of abscess. From right to left: pyogenic membrane (fibrosis and leukocytic exudate), vacuolated macrophages with several intracellular amastigotes (arrow), central area with neutrophils in fatty granule degeneration and acidophilic residue (H.E. 40x).

this intracellular parasite, most isogenic mouse strains, such as C57BL/6 and C3H, will develop an ultimately self-healing lesion around the inoculation site. Thereafter, these mouse strains will remain refractory to other infections by the same parasite. On the other hand, BALB/c mice are extremely susceptible to this infection and develop a non-healing lesion and subsequent visceral infection^{7,8}.

In this work, our interest was to study the effect of the association of Swiss/NIH non-isogenic germfree mice with the normal microbiota on the outcome of infection with *L. major*. Thus, we found it necessary to characterize the course of infection and cytokine production in conventional Swiss/NIH mice. In order to achieve this goal, we compared infection with *L. major* in Swiss/NIH mice with the infection in the isogenic resistant C57BL/6 and susceptible BALB/c mice. We



Fig. 4 - Histologic sections of lesions from conventional and conventionalized mice after 15 weeks of *L. major* infection. (A) Lesion from a conventional mouse showing chronic productive and diffused moderate inflammatory process, preferentially localized in the deep dermis (H.E. 4x). (B) Lesion from a conventionalized mouse, showing greater intensity of the inflammatory reactions qualitatively similar to the conventional shown in (A) (H.E. 10x).

observed that conventional Swiss/NIH mice presented a pattern of resistance to experimental *L. major* infection, showing a self-controlled lesion in the injected footpad. This pattern was similar to that observed in C57BL/6 mice, characterized as resistant to this infection.

Several evidences support the idea that the ability to control infection with *L. major* is due to the development of T CD4+ helper 1 (Th1) lymphocytes, which secrete high levels of the potent macrophage activator IFN- γ , thus stimulating effective killing of the parasite. Mice genetically resistant to infection by *L. major* become susceptible after the neutralization of IFN- γ ³, moreover, the deletion of the gene that codes for this cytokine also renders resistant mice susceptible³¹. On the other hand, the susceptibility to this infection is correlated to the development of T CD4+ helper 2 (Th2) lymphocytes, which inhibit macrophage activation by producing mainly IL-4 and IL-10^{12,14}. Despite conflicting results about the exact role of IL-4 *in vivo*^{9,17}, there are significant correlations between Th2 type response and disease progression^{14,22}. In order to determine the cytokine profile induced by *L. major* in Swiss/NIH mice, IFN- γ and IL-4 production were analyzed as markers of Th1- and Th2-type responses, respectively. We observed that conventional Swiss/NIH mice produced high levels of IFN- γ and low levels of IL-4, comparable to the cytokine production by C57BL/6 mice. Our results show that resistance in Swiss/NIH mice is associated with the development of a Th1-type immune response against *L. major*.

To determine if the absence of the microbiota at the early weeks of infection could influence the response to *L. major*, germfree animals were infected with the parasite and later conventionalized. We observed that the conventionalized Swiss/NIH mice presented lesions significantly larger than those observed in conventional animals, suggesting that the absence of the microbiota in the first three weeks of infection induces an impaired resistance to *L. major*. However, the higher susceptibility was not due to a lower production of IFN- γ or the presence of IL-4, since cells from conventional and conventionalized mice produced similar levels of these cytokines when cultured *in vitro*. Besides the lesion sizes, the histopathological picture observed in the footpad lesions from conventionalized Swiss/NIH mice was qualitative and quantitatively different from conventional animals. At the seventh week of infection, when lesions were at their peak, multiple abscesses constituted by neutrophils and macrophages heavily loaded with amastigotes were observed only in conventionalized mice. At the fifteenth week of infection, when both conventional and conventionalized mice were healing, there was regression of the inflammatory process in both experimental groups, in agreement with the lesion size at this time. In conventionalized mice the abscesses present at seven weeks of infection were replaced by a chronic productive diffuse inflammation. Besides, this inflammatory reaction was more intense than in the conventional animals.

The reason for the delay in healing of lesions from conventionalized mice is unknown, but certainly is independent of IFN- γ and IL-4 production since the levels of these cytokines were similar in both

groups. However, several evidences suggest that macrophage function is impaired in germfree animals³⁰, which could explain the slower healing. One candidate cytokine to play a role in resistance in conventional animals is TNF- α , which production is impaired in the absence of the normal microbiota¹⁶. TNF- α , endogenously produced by activated infected macrophages, was shown by several authors to be essential for NO production and consequent killing of parasites^{6,11}. Anti-TNF- α treatment during infection with *L. major* resulted in larger lesions that healed, similarly to our results^{10,27}. It is possible that a lower capacity of TNF- α production in conventionalized mice is responsible for an impaired capacity to kill parasites. This issue is currently under investigation.

The association with the microbiota has diverse effects on virulence and pathogenicity of parasites. For example, the pathogenicity of both *Giardia lamblia*²⁸ and *Entamoeba histolytica*¹⁹ in mice is dependent on the presence of the microbiota. In contrast, infection with the intracellular parasite *Trypanosoma cruzi* is more severe in germfree mice, resulting in a higher mortality of these animals, when compared to their conventional counterparts²⁶. Moreover, in contrast to the results described here, germfree mice infected with another species of *Leishmania*, *L. amazonensis*, were more resistant than conventional controls, showing no lesions although parasites could be seen at the site of infection²⁹. Nevertheless, we should point out that the species of *Leishmania*, the lineage of the mice and the site of infection were distinct in the two experiments and these are factors that have been described to influence the outcome of infection^{15,25}.

In summary, Swiss/NIH mice were resistant to infection with *L. major* and this resistance was accompanied by an increase in IFN- γ production by antigen-specific cells. The absence of the microbiota during the first few weeks of infection significantly influenced the lesion size and the inflammatory process in Swiss/NIH mice infected with *L. major*. Conventional mice were more resistant to this infection when compared with conventionalized animals, as revealed by significantly smaller lesions and by histopathological analysis showing scarce parasitized macrophages. Differences in production of other cytokines between conventional and conventionalized Swiss/NIH mice may explain our results.

RESUMO

Influência da microbiota normal na leishmaniose cutânea experimental em camundongos suíços

Neste trabalho, estudamos a infecção experimental de camundongos Suíços/NIH com *Leishmania major* em comparação com camundongos isogênicos C57BL/6 e BALB/c que são resistentes e susceptíveis a esta infecção, respectivamente. Camundongos Suíços mostraram lesões que se curaram espontaneamente e se restringiram ao local de inoculação. Células linfóides derivadas destes animais desenvolveram uma resposta do tipo Th1, caracterizada pela produção de níveis altos de IFN- γ e níveis baixos de IL-4. Com o objetivo de estudar a impor-

tância da microbiota no desenvolvimento da leishmaniose cutânea neste modelo, camundongos Suíços sem germes foram infectados na pata com promastigotas de *L. major*, convencionalizados após 3 semanas de infecção e as lesões comparadas com as observadas em camundongos convencionais. Os camundongos convencionalizados apresentaram lesões significativamente maiores do que as observadas nos camundongos convencionais. A análise histopatológica das lesões de todos os animais convencionalizados mostrou abscessos de tamanhos e formas variáveis e a presença de numerosos macrófagos parasitados. Nas lesões dos camundongos convencionais não houve a formação de abscessos e foram observados poucos macrófagos parasitados. Entretanto, células de ambos os grupos experimentais desenvolveram uma resposta similar do tipo Th1, caracterizada pela produção de altos níveis de IFN- γ e baixos níveis de IL-4. Neste trabalho, demonstramos que os camundongos Suíços/NIH são resistentes à infecção por *L. major*, com o desenvolvimento de um fenótipo do tipo Th1 e que a ausência da microbiota no início da infecção influencia significativamente o tamanho da lesão e no processo inflamatório nestes animais.

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