Germ-free mice produce high levels of interferon-gamma in response to infection with *Leishmania major* but fail to heal lesions

M. R. OLIVEIRA[1,2], W. L. TAFURI[3], L. C. C. AFONSO[4], M. A. P. OLIVEIRA[5,6], J. R. NICOLI[7], E. C. VIEIRA[5], P. SCOTT[8], M. N. MELO[1] and L. Q. VIEIRA[5]*

[1] *Departamento de Parasitologia, ICB, Universidade Federal de Minas Gerais, CP486, 30161-970, Belo Horizonte, MG, Brazil*
[2] *Departamento de Biologia Molecular, CCEN, Universidade Federal da Paraíba, Campus I. 58059-900, João Pessoa, PB, Brazil*
[3] *Departamento de Patologia Geral, Universidade Federal de Minas Gerais, CP486, 30161-970, Belo Horizonte, MG, Brazil*
[4] *Departamento de Ciências Biológicas/NUPEB, ICEB, Universidade Federal de Ouro Preto, Campus Universitário Morro do Cruzeiro, 35400-000, Ouro Preto, MG, Brazil*
[5] *Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais, CP486, 30161-970, Belo Horizonte, MG, Brazil*
[6] *current address: Instituto de Patologia Tropical e Saúde Pública/ DMIPP, Universidade Federal de Goiás, 74605-050, Goiânia, GO, Brazil*
[7] *Departamento de Microbiologia, Universidade Federal de Minas Gerais, CP486, 30161-970, Belo Horizonte, MG, Brazil*
[8] *Department of Pathobiology, School of Veterinary Medicine, 3800 Spruce Street, University of Pennsylvania, Philadelphia, PA, 19104, USA*

SUMMARY

In order to investigate the importance of the host microbiota on differentiation of T cell subsets in response to infection, Swiss/NIH germ-free mice and conventional (microbiota-bearing) mice were infected with *Leishmania major*, and lesion development, parasite loads, and cytokine production were assessed. Germ-free mice failed to heal lesions and presented a higher number of parasites at the site of infection than their conventional counterparts. In addition, histopathological analysis indicated a higher density of parasitized macrophages in lesions from germ-free mice than in conventional mice. The initial production of interleukin (IL)-12 and interferon-gamma (IFN-γ) in germ-free mice was comparable to the conventional controls. Also, germ-free mice produced elevated levels of IFN-γ and lower levels of IL-4 throughout the course of infection, suggesting the development of a Th1 response. Macrophages from germ-free mice exposed to IFN-γ and infected with amastigotes *in vitro* were not as efficient at killing parasites as macrophages from conventional animals. These observations indicate that the microbiota is not essential for the development of Th1 immune responses, but seems to be important for macrophage activation.

Key words: Leishmania major, leishmaniasis, microbiota, germ-free, Th1, gnotobiotic, gnotobiology.

INTRODUCTION

Experimental infections with the parasite *Leishmania major* have established the Th1/Th2 paradigm. Thus, C57BL/6 mice infected with *L. major* develop a Th1 response that results in IFN-γ production, macrophage activation, and control of parasite growth and lesions (Belosevic *et al.* 1989; Heinzel *et al.* 1989; Scott, 1991). In contrast, BALB/c mice respond to this parasite by developing a Th2 response, but no macrophage activation is achieved and parasite growth is poorly controlled (Heinzel *et al.* 1989; Sadick *et al.* 1990). Although resistance and susceptibility models with clear-cut Th1 or Th2 responses were not found for other *Leishmania* that cause cutaneous leishmaniasis (Afonso and Scott, 1993; Barral *et al.* 1993; Donnelly, Lima and Titus, 1998; Lima, Dekrey and Titus, 1999; Soong *et al.* 1996), the Th1/Th2 correlation with resistance or susceptibility has been found to be true for all *L. major* isolates or clones studied.

Many factors can influence the type of immune response mounted by the host, however, the effects of the intestinal microbiota on the outcome of infections caused by *Leishmania* parasites are little understood. It has been argued that development of the T cell

* Corresponding author: Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. C.P. 486, 30161-970, Belo Horizonte, MG, Brazil. Tel: +55 31 3499 2656. Fax: +55 41 3499 2614. E-mail: lqvieira@icb.ufmg.br

response (MacDonald and Carter, 1979) and macrophage activity (Nicaise *et al.* 1995, 1999) would be impaired in the absence of the normal microbiota.

We had previously shown that conventional Swiss/NIH mice are resistant to experimental *L. major* infection with the development of a Th1-type immune response against this infection. Moreover, we observed that germ-free animals infected with *L. major* which were conventionalized 3 weeks after infection presented larger lesions than conventional animals. However, similar to conventional mice, lesions in conventionalized mice peaked at 6 weeks and were progressively controlled (Oliveira *et al.* 1999).

In the present study, we investigated the outcome of *L. major* infection in germ-free Swiss/NIH mice for 13 weeks in the absence of microbiota, as compared to conventional mice. We demonstrate that germ-free Swiss mice developed non-healing lesions with high numbers of parasitized macrophages. However, in spite of the inability of germ-free mice to heal, cells from these mice produced high levels of IFN-γ. These observations indicate that the microbiota is not necessary for the development of a Th1 immune response, but is an adjuvant for macrophages to efficiently kill this intracellular parasite.

MATERIALS AND METHODS

Animals

Germ-free Swiss/NIH mice were derived from a germ-free nucleus (Taconic Farms, Germantown, USA) and maintained in flexible plastic isolators (Standard Safety Equipment Co., Pallatine, USA) using classical gnotobiology techniques (Pleasants, 1974). Conventional Swiss/NIH mice are derived from germ-free matrices, and considered conventional only after 2 generations in the conventional facility. All animals were 5 to 7-week-old females. All experimental procedures were carried out according to the standards set forth in the 'Guide for the Care and Use of Laboratory Animals' of the National Research Council (1996).

Parasites and antigen

The *Leishmania major* strain WHO MHOM/IL/80/Friedlin was used for these studies. Promastigotes were cultured in Grace's insect medium (Life Technologies, Grand Island, USA) supplemented with 20% fetal bovine serum (Nutricell, Campinas, SP, Brazil) 100 U/ml of penicillin and 100 g/ml of streptomycin. Metacyclic promastigotes were obtained from stationary phase of growth using *Arachis hypogae* agglutinin (Sigma Chemical Co., St Louis, MO) as described previously (Sacks, Hieny and Sher, 1985). Amastigotes were obtained by disruption of infected footpad tissue by homogenization

in a glass tissue grinder, and tissue debris was removed by centrifugation of the suspension at 200 $\boldsymbol{g}$. Antigen was prepared from *L. major* promastigote stationary phase cultures as earlier described (Hodes, 1995). In short, cells were washed 4 times in PBS, the concentration adjusted to 1×10^8 cells/ml, and frozen/ thawed 3 times. The antigen was homogenized, aliquoted, and maintained at $-20\,^{\circ}\text{C}$ until use.

Infection and monitoring of lesions

Mice were injected subcutaneously in the left hind footpad with 1×10^6 metacyclic promastigotes. For the infection of germ-free Swiss/NIH mice, metacyclic promastigotes in Grace's medium were aseptically transferred into sterile glass ampoules. The ampoules were sealed, chemically sterilized with 8% peracetic acid and introduced into the isolator. Another ampoule treated in the same manner was taken out of the isolator and the promastigotes were used to infect conventional Swiss/NIH mice. Measurements of the footpad thickness were taken using a caliper. The lesion size was calculated by subtracting the value of the uninfected contralateral footpad from that of the infected one.

Parasite quantification

Parasites were quantified by limiting dilution, as previously described (Vieira *et al.* 1996). Briefly, single-cell suspensions from individual excised lesions were plated in log-fold serial dilutions in Grace's insect tissue culture medium starting with a 1:10 dilution. Each sample was plated in quadruplicates and read 5 days after the beginning of the culture. Results are expressed as the mean of the negative log of the titre, i.e., the dilution corresponding to the last positive well.

Histopathology

Four animals from each group were sacrificed at 2, 6 and 13 weeks of infection and footpad tissues were collected and fixed in 10% neutral buffered formalin. Later the material was dehydrated, cleared, embedded in paraffin, cut (3–5 μm thick), and stained with haematoxylin and eosin for microscopical examination.

Preparation and culture of cells

Spleen cells and a pool of popliteal lymph node cell suspensions were prepared as described previously (Oliveira *et al.* 2000). Pools of popliteal lymph nodes were used since our Swiss/NIH mice did not show a positive mixed lymphocyte reaction in several experiments performed (our unpublished observations;

Ribeiro-Sobrinho *et al*. 2002). This result 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), the concentration of cells was adjusted to 5×10^6 cells/ml RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, 25 mM HEPES, and 0·05 mM-mercaptoethanol (Gibco Laboratories), and 1 ml was plated in 24-well tissue culture plates. Some cultures were stimulated *in vitro* with concanavalin A (ConA, 5 g/ml, Sigma Chemical Co., St Louis, MO, USA) or 50 μl of *L. major* antigen. Supernatants were harvested at 72 h for IFN-gamma and IL-4 analysis and at 24 h for tumor necrosis factor (TNF) and IL-12 assays.

Treatment of macrophages with IFN-gamma

Peritoneal exudate cells were harvested 5 days after injection of 1 ml of thioglycollate. The concentration of cells was adjusted to 1×10^6 cells/ml in RPMI 1640 (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, 25 mM HEPES, and 0·05 mM β-mercaptoethanol) and 1 ml plated onto glass cover-slips in 24-well tissue culture plates in the presence or absence of recombinant IFN-gamma (100 U/ml, Genzyme, Cambridge, MA, USA). Cultures were incubated for 2 h at 37 °C, 5% CO_2 and humidified atmosphere, after which the macrophages were exposed to 2 amastigotes per macrophage for 2 h. After this incubation, the supernatants containing non-adherent cells were removed and adherent cells were washed 3 times with warm RPMI 1640 medium to remove non-ingested amastigotes and fresh medium with or without IFN-gamma (100 U/ml) was added again. The cells were cultured for 72 h (37 °C, 5% CO_2, humidified atmosphere). Supernatants were collected for NO assays. The percentage of macrophages containing intracellular amastigotes was determined by optical microscopy examination of stained cover-slips (Panótico Rápido Laborclin, Pinhais, PR, Brazil).

Cytokine and nitric oxide measurement

IFN-gamma, IL-4 and IL-12 were measured using specific 2-site ELISA as previously described (Oliveira *et al*. 2000). IFN-gamma was measured using R46A2 rat monoclonal antibody as a capture antibody and a rabbit polyclonal anti-IFN-gamma antiserum (kind gift from Dr J. P. Farrell, University of Pennsylvania, Philadelphia, PA, USA) was used as a secondary antibody followed by donkey horse-radish peroxidase-conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). IL-4 was measured using 11B11 rat monoclonal antibody as a capture

antibody and biotinylated BVD6 rat monoclonal antibody as a secondary antibody followed by streptavidin-peroxidase (Sigma Chemical Co. St Louis, MO, USA). In the IL-12 p40 ELISA, the mAb C17.15 and biotinylated C15.6 were used (kind gifts from Dr Giorgio Trinchieri, The Wistar Institute, Philadelphia, PA, USA). The levels of IFN-gamma, IL-4 and IL-12 p40 were calculated by comparison with a standard curve using recombinant IFN-gamma, IL-4 and IL-12 (Genzyme, Cambridge, MA, USA). The sensitivity for the assays were as follows: IFN-gamma: 30 pg/ml, IL-4: 0·2 U/ml and IL-12: 30 pg/ml. TNF-alpha was measured according to the manufacturer's instructions using a kit (Mouse TNF- DUOSET Genzyme Corporation, USA). Nitric oxide production was assessed using the Griess reagent (Green *et al*. 1982).

Statistical analysis

The data obtained in each experiment were analysed using the Student's *t*-test or the paired Mann Whitney test (non-parametric). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Germ-free mice fail to resolve lesions caused by infection with Leishmania major

The outcome of the experimental infection by *L. major* in conventional and germ-free Swiss/NIH mice was compared by measuring the development of primary lesions after infection with *L. major* in the hind footpad (Fig. 1). As previously described, Swiss/NIH conventional mice infected with *L. major* developed a lesion at the site of infection, which was comparable to that of other resistant models (Oliveira *et al*. 1999), and eventually healed these lesions. In contrast, Swiss/NIH germ-free mice developed larger lesions than their conventional counterparts and were unable to heal over a period of 13 weeks after the infection (Fig. 1A–C). Accordingly, parasite numbers in footpads harvested from germ-free animals were higher than in footpads from conventional mice (Fig. 2). Furthermore, 13 weeks after infection, parasites were cultured from the spleens and liver in 4 out of 5 germ-free animals in 1 experiment, while parasites were not detected in the viscera of conventional mice at this time-point.

Presence of associated microbiota modulates the inflammatory process

In order to determine if the presence of the microbiota would interfere with the inflammatory process induced by infection with *L. major*, fragments of lesions from conventional and germ-free mice were
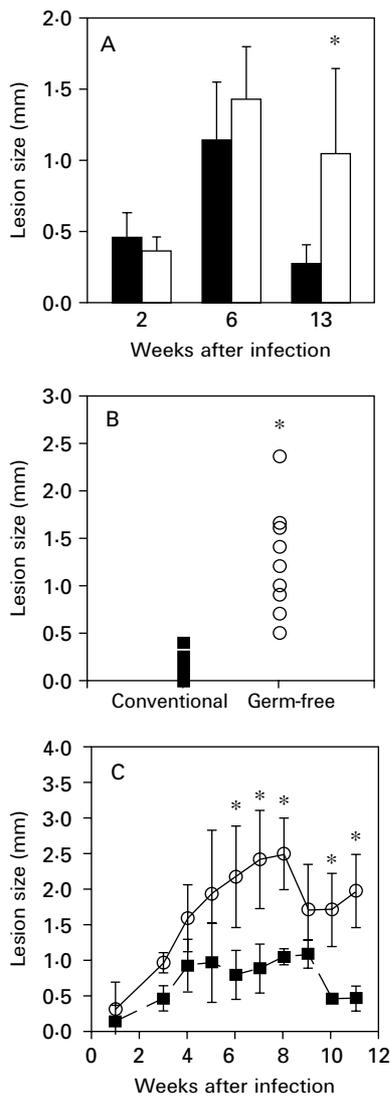
Fig. 2. Parasite numbers in lesions from conventional (black bars) and germ-free (white bars) mice infected with 1×10^6 metacyclic promastigotes of *Leishmania major*. Parasite numbers were evaluated by limiting dilution, as described in the Materials and Methods section. Represented are the results of 1 of 2 experiments performed, with similar results. *Indicates statistical difference, $P < 0.05$, # indicates $P = 0.073$.

Fig. 1. Lesion sizes in conventional (black symbols and bars) and germ-free (white symbols and bars) mice infected with 1×10^6 metacyclic promastigotes of *Leishmania major* in the hind footpad. Results from 3 experiments are shown. (A) Lesion size in conventional and germ-free mice at 2, 6 and 13 weeks of infection taken from separate groups of mice, as they were sacrificed for experiments. Bars for second and sixth week represent the average of the footpad thickness of 8 animals, the results of the thirteenth week post-infection is the average of the lesions of 20 animals per group. Lines stand for the standard deviation of the means. (B) Lesion size from individual conventional ($n = 8$) and germ-free ($n = 10$) mice 13 weeks after infection. (C) Course of infection in conventional and germ-free mice, measurements were taken weekly inside isolators or in the conventional animal house using a caliper. Each point represents the mean of 5 mice per group; lines stand for the standard deviation of the means. *Indicates statistical difference, $P < 0.05$.

histologically examined. At the sixth week after infection, cutaneous lesions from conventional mice presented a diffuse and moderate chronic inflammator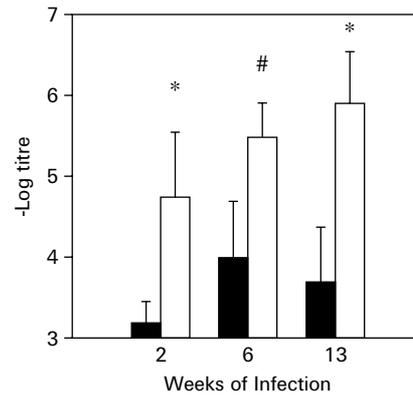y process (Fig. 3A) with the presence of macrophages parasitized with *L. major* amastigotes (Fig. 3B). In germ-free mice an intense and diffuse chronic inflammatory reaction was observed, associated with necrotic areas (Fig. 3C). The cellular exudate was characterized by several macrophages loaded with amastigotes associated with the presence of necrotic cells (Fig. 3D).

At the thirteenth week of infection sections of cutaneous lesions in footpads from conventional animals showed a moderate and diffuse chronic inflammatory reaction (Fig. 4A). The cellular exudate was comprised of macrophages, lymphocytes and rare neutrophils. Higher magnification showed no signs of parasites (Fig. 4B). In contrast, germ-free mice showed an intense and diffuse chronic inflammatory reaction (Fig. 4C), and the cellular exudate contained macrophages, lymphocytes and some plasma cells. Moreover, a severe chronic granulomatous inflammatory reaction was observed. The granuloma contained mainly vacuolated macrophages heavily parasitized with amastigotes (Fig. 4D).

Germ-free mice develop a type 1 immune response when infected with Leishmania major

Early production of IL-12 and IFN-gamma is essential for resistance to infection with *L. major* (Heinzel *et al.* 1993; Sypek *et al.* 1993). Hence, we investigated the production of these cytokines by lymph node cells from Swiss/NIH conventional and germ-free mice 2 days after infection. As seen in Fig. 5, lymph node cells from both groups of animals secreted similar levels of IL-12 and IFN-gamma. Moreover, no differences in *in vitro* IFN-gamma production was found between the two groups at 2, 6 and 13 weeks after infection, both in lymph node
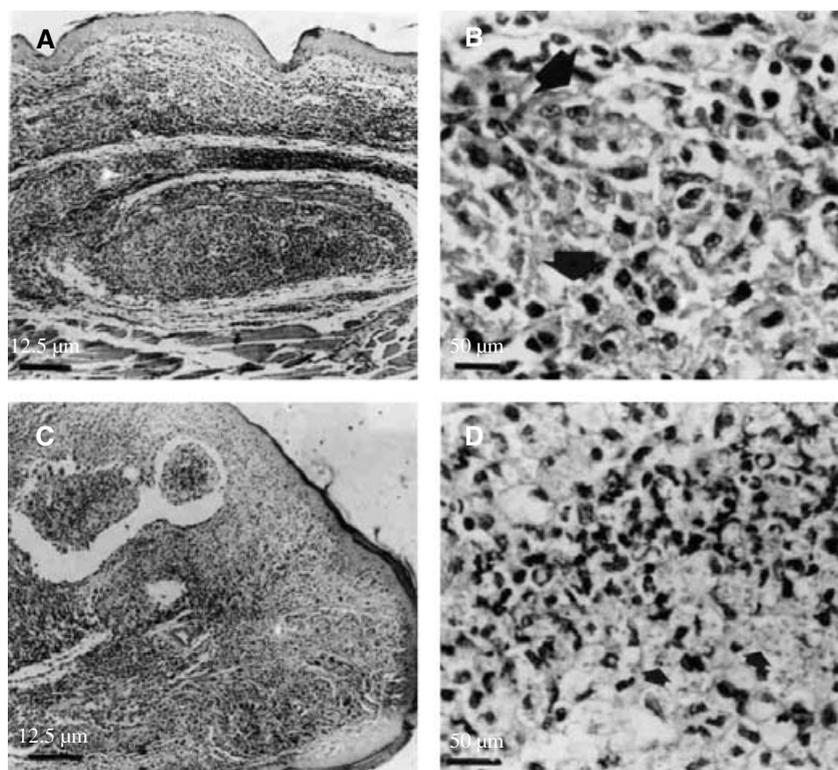
Fig. 3. Skin sections of hind footpads lesions of conventional and germ-free Swiss/NIH mice infected with 1×10^6 metacyclic promastigotes of *Leishmania major* after 6 weeks post-infection. (A) Lesions from conventional mice showing a diffuse and moderate chronic inflammatory process (H.&E. 40×). (B) Higher magnification showing the presence of macrophages parasitized with *L. major* amastigotes (arrow) (H.&E. 400×). (C) Lesions from germ-free mice showing an intense and diffuse chronic inflammatory reaction (H.&E. 40×). (D) Higher magnification indicating several macrophages loaded with *L. major* amastigotes.

and spleen cell cultures stimulated with *L. major* antigens (Fig. 6A and B). Importantly, IL-4 production was detected only in lymph node cell cultures, at the second week of infection and in similarly low levels in both groups of animal (Fig. 6C). At 2 weeks post-infection, similar levels of TNF-alpha were also found in cultures of lymph node cells from conventional and germ-free mice, and the addition of *L. major* antigen *in vitro* increased the production of TNF-alpha by lymph node cells (Fig. 6D).

Macrophages from germ-free mice kill fewer parasites than macrophages from conventional animals after activation with IFN-gamma

Peritoneal macrophages from conventional and germ-free mice were infected *in vitro* with *L. major* amastigotes in the presence or absence of IFN-gamma and incubated for 72 h. It was found consistently that macrophages from germ-free mice were less effective in killing parasites than macrophages from conventional mice. Both macrophages were equally permissive to infection with parasites (percentage infection of $39 \cdot 7 \pm 19 \cdot 9$ for cells from germ-free mice and $30 \cdot 2 \pm 24 \cdot 3$ for conventional mice). Similar results were found when promastigotes were used to infect macrophages (data not shown).

However, a higher percentage of macrophages were infected when amastigotes were used, therefore results were more clear-cut in these conditions. Variability among experiments was very large. Still, when the data from 4 experiments were pooled and the results were expressed as the percentage reduction of infected macrophages upon activation with IFN-gamma (Fig. 7), a statistical difference was found, which indicated that, indeed, macrophages from germ-free mice were less effective in killing *L. major* when activated with IFN-gamma. Slightly less nitric oxide was produced by macrophages from germ-free mice in these cultures ($88 \cdot 3$ mM $\pm 34 \cdot 0$ for germ-free and $121 \cdot 5$ mM $\pm 50 \cdot 9$ for conventional mice, $P = 0 \cdot 062$ by the Mann Whitney paired test).

DISCUSSION

Animals are associated with a variety of microorganisms, which are called the normal or indigenous microbiota (Berg, 1996). These associated microbiota influence the host homeostasis, and several studies have indicated that exposure to microorganisms may influence the host immune response and susceptibility to disease (Rodrigues *et al.* 1996; Vieira *et al.* 1998; Maia *et al.* 2001). Germ-free animals have been major tools in investigating
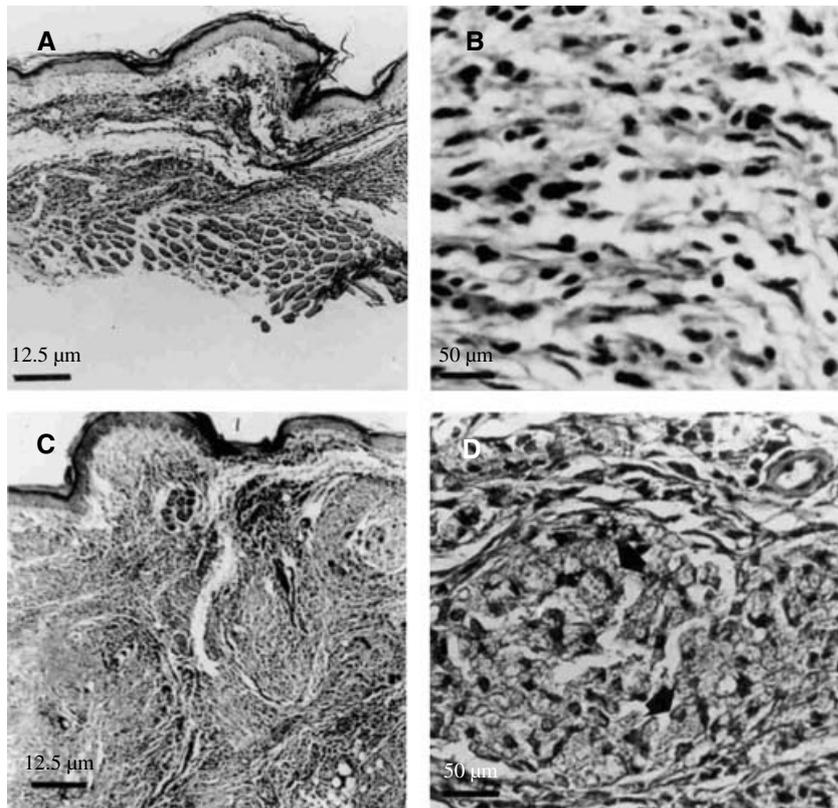
Fig. 4. Skin sections of hind footpads lesions of conventional and germ-free Swiss/NIH mice infected with 1×10^6 metacyclic promastigotes of *Leishmania major* after 13 weeks post-infection. (A) Healed lesion from a conventional mouse (H.&E. 40×). (B) Higher magnification showing no signs of parasites, but showing a discreet inflammatory process (H.&E. 400×). (C) Lesion from germ-free mouse showing an intense and diffuse granulomatous inflammatory reaction (H.&E. 40×). (D) Higher magnification showing nodules of macrophages loaded with *L. major* amastigotes (arrow).

host-microbiota relationships (Rodrigues *et al*. 1996; Vieira *et al*. 1998; Filho-Lima, Vieira and Nicoli, 2000; Maia *et al*. 2001). When infected with pathogens in the absence of the normal microbiota, animals may be either more resistant or more susceptible to parasites. Hence, intestinal microbiota is essential for the establishment of some gut infections (Phillips and Wolfe, 1959; Torres *et al*. 1992). On the other hand, infection with the intracellular parasite *Trypanosoma cruzi* is more severe in germ-free mice, as shown by a higher mortality, more intense parasitism, and more aggressive inflammatory response when compared to the infection in conventional mice (Silva *et al*. 1987; Furarah *et al*. 1991). Infection with *Leishmania amazonensis* was less severe in germ-free mice if compared to conventional controls when we used Swiss/CFW mice (Vieira *et al*. 1987). However, Swiss/NIH germ-free and conventional mice behaved similarly when infected with *L. amazonensis*, regardless of the association with the normal microbiota (our unpublished observation). We had previously shown that conventional Swiss/NIH mice develop an ultimately self-healing lesion around the inoculation site. This pattern of resistance to experimental *L. major* infection is similar to that observed in C57BL/6 mice, characterized as resistant to this infection (Oliveira *et al*. 1999). In addition, resistance to *L. major* in Swiss/NIH mice is associated with the development of a Th1-type immune response against *L. major* (Oliveira *et al*. 1999; and data presented herein), since similar levels of IFN-gamma are produced by both strains at 2 weeks of infection. We also found that germ-free mice infected with *L. major* while in the germ-free state and conventionalized 3 weeks after infection (i.e. associated with the normal mouse microbiota) had significantly larger lesions than the conventional controls. However, similar to conventional mice, these conventionalized animals resolved their lesions (Oliveira *et al*. 1999). Here, we show that germ-free mice infected with *L. major* and kept in the gnotobiotic state (associated only with *L. major*) for 13 weeks after infection failed to resolve lesions. Accordingly, these mice did not control parasite growth for the duration of the experiments, as determined both by limiting dilution and histopathological analysis. At the thirteenth week of infection, while conventional mice showed a discrete inflammatory reaction indicating regression of the inflammatory process, germ-free mice showed an intense chronic inflammatory reaction with many vacuolated macrophages, loaded with *Leishmania* amastigotes. Taken together, these
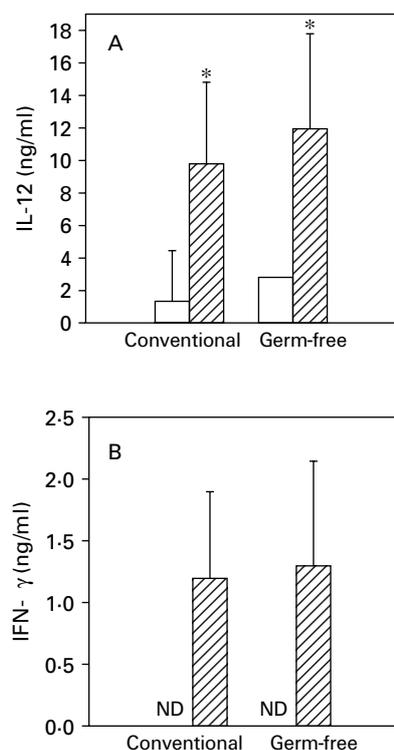
Fig. 5. IL-12 (A) and IFN-gamma (B) production by lymph nodes cells from conventional and germ-free Swiss/NIH mice in the beginning of infection. Cells from non-infected animals (white bars) and from animals infected with 1×10^6 metacyclic promastigotes of *Leishmania major* for 2 days (hatched bars) were cultured *in vitro* for 24 h in the absence of antigen, as described in the Materials and Methods section. The bars represent the average of 4 animals per group, vertical lines stand for the standard deviations of the means. Data are from 1 of 2 experiments performed with similar results. ND, not detected.

results show that the presence of the indigenous microbiota is important for effective resistance to infection with *L. major*.

One mechanism that could explain the differences in behaviour between germ-free and conventional animals is that the microbiota would influence the kind of response that is made when animals are faced with infection. The ideal model to address this issue would the infection with *L. major*. IFN-gamma secreted by CD4^{+} T (Th1) lymphocytes is known to be essential for effective killing of parasites by macrophages (Titus, Kelso and Louis, 1984; Belosevic *et al.* 1989;). It has also been demonstrated that IFN-gamma secreted early during infection with *L. major* plays an important role in the development of Th1 phenotype (Titus *et al* 1984; Scharton and Scott, 1993); development of this phenotype was shown to be dependent on IL-12 (Scharton-Kersten *et al.* 1995). On the other hand, susceptibility to *L. major* infection in mice is characterized by the differentiation of CD4^{+} T lymphocytes into the Th2 subtype, which secretes high amounts of IL-4 (Locksley

et al. 1991). IL-4 inhibits macrophage activation leading to high intracellular parasitism and a non-healing lesion at the inoculation site with *L. major* (Liew *et al.* 1989; Locksley *et al.* 1991). In addition, the development of the Th2 subtype of CD4^{+} T cells inhibits the development of a Th1 response and the activation of macrophages by IFN-gamma. This archetypal response correlated well with the initial findings by Parish (1972), Parish and Liew (1972) and Lagrange, Mackaness and Miller (1974) who had suggested that there was an inhibition of the production of antibodies to a given antigen when the cell-mediated immune response prevailed, and vice-versa. Additionally, McDonald and Carter (1979) established that the development of a delayed-type hypersensitivity to sheep red blood cells (a putative Th1 response) was dependent on the presence of the normal microbiota, while germ-free mice could produce antibodies to this antigen (a putative Th2 response) similar to conventional animals. Subsequently, it was shown that the normal microbiota enhanced IL-12 production in spleen-derived macrophages (Nicaise *et al.* 1999). Hence, it was tempting to predict that germ-free mice, which are more susceptible than conventional mice to infection with *L. major*, would tend to respond to this parasite in a Th2 fashion. Indeed, preliminary data using a different source of Swiss mice than the one used herein suggested that infection with *L. major* would trigger a higher level of IL-4 production by germ-free mice (Vieira and Scott, 1996). However, in this paper we show that the enhanced susceptibility of germ-free animals to *L. major* was not due to a higher production of IL-4. IFN-γ was produced at similar levels by germ-free and conventional animals in response to *L. major* at the time points investigated. Also, early production of IL-12 and IFN-γ in the lymph nodes, which are clearly shown to be essential for the development of a Th1 response during infection with *L. major* (Scharton and Scott, 1993; Scharton-Kersten *et al.* 1995) are similar in both groups of mice. Peritoneal cells from germ-free and conventional mice injected intraperitoneally with *L. major* produce equivalent levels of IL-12 (data not shown). Thus, the higher susceptibility of germ-free mice to *L. major* could not be explained by their bias toward a Th2 response.

Although the increased susceptibility of BALB/c mice to *L. major* seems to be related to the presence of the cytokine IL-4 (Scharton-Kersten *et al.* 1995; Launois *et al.* 1995, 1997), the reason for this bias towards IL-4 production is unknown. An early IL-4 burst in both susceptible and resistant strains occurs (Scharton-Kersten *et al.* 1995). It has been demonstrated that there is expansion of a memory IL-4 producing CD4^{+} T cell population a few hours after infection of BALB/c mice with *L. major*, but the same population is present and expands in B10D2 (resistant) mice (Julia *et al.* 2000; Malherbe *et al.*
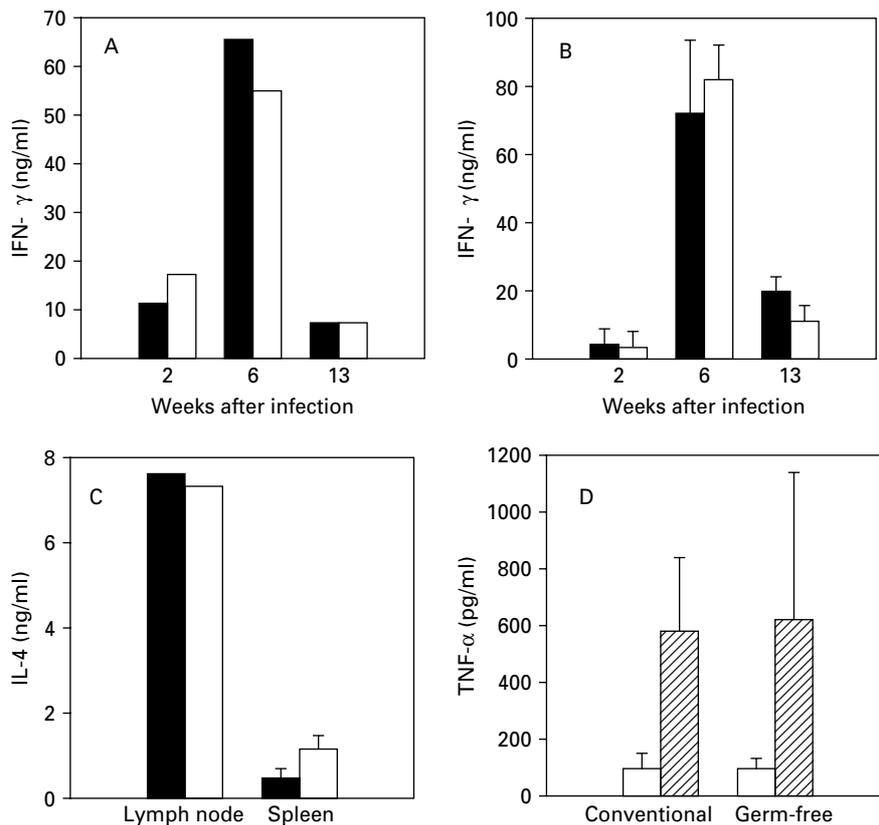
Fig. 6. Cytokine production by conventional and germ-free Swiss/NIH mice infected with *Leishmania major*.
(A) IFN-gamma production by antigen-stimulated lymph node cells from conventional (black bars) or germ-free (white bars) 2, 6 and 13 weeks after infection with *L. major*. (B) IFN-gamma production by antigen-stimulated spleen cells from conventional (black bars) or germ-free (white bars) 2, 6 and 13 weeks after infection with *L. major*. (C) IL-4 production by antigen-stimulated lymph node or spleen cells from conventional (black bars) or germ-free (white bars) mice 2 weeks after infection with *L. major*. (D) TNF-alpha production by lymph node cells from conventional and germ-free mice 2 weeks after infection in the presence (white bars) or absence (hatched bars) of *Leishmania major* antigens. Lymph node cells were pooled from 5 mice in each group. Spleen cell results are the mean production of individual cultures from 5 mice in each group. All experiments were repeated once and similar results were obtained.

2000). Interestingly, these memory cells bind to a peptide from the LACK antigen of *Leishmania* which sequence is homologous to peptides found in some subdominant components of the normal microbiota (*Escherichia coli* and *Enterococcus faecalis*) but not in other residual (*Proteus mirabilis*) or residual and potentially pathogenic (*Clostridium perfringens*) components of the microbiota. Hence, when conventional BALB/c mice were decontaminated with antibiotics the early IL-4 burst was abrogated. In addition, germ-free BALB/c mice did not present the early IL-4 production found in conventional mice. Germ-free BALB/c mice were, however, as susceptible as conventional mice to *L. major*, ruling out the importance of the microbiota-primed memory cell-mediated early IL-4 production in susceptibility. Moreover, contrary to conventional BALB/c mice, germ-free BALB/c mice were still somewhat susceptible to *L. major* after anti-IL-4 treatment (Julia *et al*. 2000). Here we show that in Swiss/NIH mice (a resistant mouse strain) the germ-free state confers greater susceptibility to infection with *L. major* and

that this susceptibility does not correlate with higher IL-4 production. We had previously shown that conventional Swiss mice produce lower levels of IL-4 than BALB/c mice, and that the IL-4 production by Swiss mice at 4 weeks of infection was similar to that of C57BL/6 mice (Oliveira *et al*. 1999). Hence, the levels of IL-4 found at 2 weeks of infection are not typical of a Th2 response. More importantly, mice remain susceptible even whilst producing similar levels of IFN-gamma to conventional mice. It is important to note that lesions found in germ-free Swiss mice are in no way similar to the ones found in BALB/c mice: BALB/c mice present large uncontrolled lesions that can reach 7 mm (Oliveira *et al*. 1999) while germ-free Swiss mice displayed nonhealing lesions that were always smaller than 3 mm. Furthermore, the histological aspect of the lesions was also very diverse: Swiss/NHI germ-free mice had a large lymphocytic infiltrate that is mostly absent in BALB/c mice (Santiago *et al*. 1999). One possible explanation for the higher susceptibility would be that germ-free mice produce higher
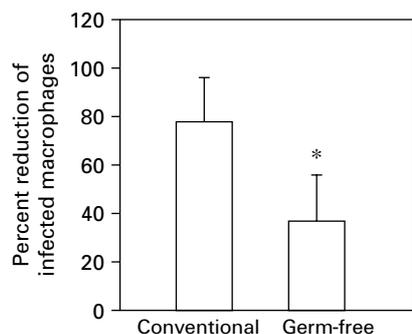
Fig. 7. Killing of *Leishmania major* by elicited peritoneal macrophages from conventional or germ-free mice. The percentage reduction of the number of macrophages infected with *L. major* in response to treatment with 100 U/ml of IFN-gamma is presented. The percentages of infected macrophages in control (no IFN-gamma added) wells were $39\cdot7\pm19\cdot9$ for cells from germ-free mice and $30\cdot2\pm24\cdot3$ for conventional mice. The percentage of infected macrophages was determined after 72 h of culture, as described in the Materials and Methods section. A total of 400 macrophages were counted per group. Bars stand for the average of the 4 experiments performed, lines represent the standard deviations of the means. *Indicates statistically significant difference by the paired Mann Whitney test ($P=0\cdot0286$).

levels of IL-10, since this cytokine has also been shown to be important for susceptibility to *L. major* (Noben-Trauth *et al*. 2003). Indeed, during acute inflammation, germ-free mice produce higher levels of IL-10 (Souza *et al*. 2004). Moreover, spleen cells from germ-free mice, when cultured with *Trypanosoma cruzi* homogenate, produce higher levels of IL-10 than cells from conventional mice, albeit there is no difference in the IL-10 levels during infection (Duarte *et al*. 2004). This possibility deserves further investigation and would explain the deficient activation of macrophages to kill parasites.

Peritoneal and bone-marrow macrophages from germ-free mice that were exposed to the normal microbiota as adults produce higher levels of IL-1, IL-6 and TNF-alpha than macrophages from germ-free mice (Nicaise *et al*. 1993). Subsequently, it was shown that the normal microbiota enhanced IL-12 production in spleen-derived macrophages (Nicaise *et al*. 1999). Pro-inflammatory cytokine basal levels were also higher in sera from microbiota-associated mice (Dahlgren, Midtvedt and Tarkowski, 1995; Nicaise *et al*. 1999). Germ-free mice show impaired clearance of bacteria injected intravenously (Neumann *et al*. 1998; Rodrigues *et al*. 2000) and respond to this stimulus with secretion of TNF-*α*, IL-12 and IFN-*γ* more slowly than mice associated with one gut microorganism (Rodrigues *et al*. 2000). Hence, macrophages from mice that are exposed to the normal microbiota would seem to be in a more

activated state than macrophages from an animal that has not been exposed to living bacteria in their organism. Although germ-free animals are not antigen-free and are not free of exposure to dead bacteria present in commercial diets, the presence of the conventional microbiota seems to keep macrophages secreting cytokines at higher basal levels. Thus, it is not surprising that macrophages from germ-free mice could not be activated to kill *L. major* as efficiently as macrophages from conventional mice. In a different experimental model we showed that germ-free mice produce more IL-10 than conventionals (data not shown). Since IL-10 is a potent inhibitor of macrophage activation (Gazzinelli *et al*. 1992; Oswald *et al*. 1992), we could hypothesize that, in spite of the addition of IFN-gamma *in vitro* and of the presence of IFN-gamma and TNF-alpha *in vivo*, macrophages from germ-free mice were not as activated as macrophages from conventional mice, which allowed growth of parasites.

The hygiene hypothesis states that in the absence of a certain level of exposure to microorganisms, an exacerbated immune response may occur, either of the Th1 or of the Th2 type (Wills-Karp, Santeliz and Karp, 2001; Yazdanbakhsh, Kremsner and Van Ree, 2002). On the other hand, recent data have pointed to IL-6 as a putative regulator of the inflammatory response, even before it is triggered (Hori, Nomura and Sakaguchi, 2003; Pasare and Medzhitov, 2003; Powrie and Maloy, 2003). Our data show that the lack of antigenic stimulus in the adult mouse does not induce an exacerbated immune response against a single invasive microorganism. On the contrary, it seems that the effector mechanisms in germ-free mice are kept on hold due to the lack of constant stimulation by the normal microbiota.

In conclusion, we have shown that the inability of germ-free mice to heal lesions during *L. major* infection could not be explained by low levels of IFN-gamma or higher production of IL-4. Instead, higher susceptibility to this parasite correlated with a less efficient killing of parasites *in vitro* by macrophages exposed to IFN-gamma. Hence, our results indicate that the normal microbiota does not bias towards a type 1 or type 2 immune response, but seems to be essential for the maintenance of macrophages in a more activated state that allows them to be responsive to activating stimuli.

REFERENCES

Afonso, L. C. and Scott, P. (1993). Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infection and Immunity* **61**, 2952–2959.

Barral, A., Barral-Netto, M., Yong, E. C., Brownell, C. E., Twardzik, D. R. and Reed, S. G. (1993). Transforming growth factor beta as a virulence mechanism for *Leishmania braziliensis*. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 3442–3446.

Belosevic, M., Finbloom, D. S., van der Meide, P. H., Slayter, M. V. and Nacy, C. A. (1989). Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. *Journal of Immunology* **143**, 266–274.

Berg, R. D. (1996). The indigenous gastrointestinal microflora. *Trends in Microbiology* **4**, 430–435.

Dahlgren, U. I., Midtvedt, T. and Tarkowski, A. (1995). Transient appearance of circulating interleukin-6 and tumor necrosis factor in germ-free C3H/HeJ and C3H/HeN mice upon intestinal exposure to *E. coli*. *Advances in Experimental Medicine and Biology* **371A**, 459–462.

Donnelly, K. B., Lima, H. C. and Titus, R. G. (1998). Histologic characterization of experimental cutaneous leishmaniasis in mice infected with *Leishmania braziliensis* in the presence or absence of sand fly vector salivary gland lysate. *Journal of Parasitology* **84**, 97–103.

Duarte, R., Silva, A. M., Vieira, L. Q., Afonso, L. C. and Nicoli, J. R. (2004). Influence of normal microbiota on some aspects of the immune response during experimental infection with Trypanosoma cruzi in mice. *Journal of Medical Microbioogy* **53**, 741–748.

Filho-Lima, J. V., Vieira, E. C. and Nicoli, J. R. (2000). Antagonistic effect of *Lactobacillus acidophilus*, *Saccharomyces boulardii* and *Escherichia coli* combinations against experimental infections with *Shigella flexneri* and *Salmonella enteritidis* subsp. *typhimurium* in gnotobiotic mice. *Journal of Applied Microbiology* **88**, 365–370.

Furarah, A. M., Crocco-Afonso, L. C., Silva, M. E. C., Silva, M. E., Bambirra, E. A., Vieira, E. C. and Nicoli, J. R. (1991). Immune responses of germ-free mice to experimental infection with *Trypanosoma cruzi*. *Brazilian Journal of Medical and Biological Research* **24**, 1223–1231.

Gazzinelli, R. T., Oswald, I. P., James, S. L. and Sher, A. (1992). IL-10 inhibits parasite killing and nitrogen oxide production by IFN-g-activated-macrophages. *Journal of Immunology* **148**, 1792–1796.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry* **126**, 131–138.

Heinzel, F. P., Rerko, R. M., Hatam, R. and Locksley, R. M. (1993). IL-2 is necessary for the progression of leishmaniasis in susceptible murine hosts. *Journal of Immunology* **150**, 3924–3931.

Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L. and Locksley, R. M. (1989). Reciprocal expression of interferon gamma or IL4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *Journal of Experimental Medicine* **169**, 59–72.

Hodes, R. J. (1995). Molecular alterations in the aging immune system. *Journal of Experimental Medicine* **182**, 1–3.

Hori, S., Nomura, T. and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.

Julia, V., McSorley, S. J., Malherbe, L., Breittmayer, J. P., Girard-Pipau, F., Beck, A. and Glaichenhaus, N. (2000). Priming by microbial antigens from the intestinal flora determines the ability of CD4+ T cells to rapidly secrete IL-4 in BALB/c mice infected with *Leishmania major*. *Journal of Immunology* **165**, 5637–5645.

Lagrange, P. H., Mackaness, G. B. and Miller, T. E. (1974). Influence of dose and route of antigen injection on the immunological induction of T cells. *Journal of Experimental Medicine* **139**, 528–542.

Launois, P., Maillard, I., Pingel, S., Swihart, K. G., Xénarios, I., Acha-Orbea, H., Diggelmann, H., Locksley, R. M., MacDonald, H. R. and Louis, J. A. (1997). IL-4 rapidly produced by Vb4Va8 CD4+ cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. *Immunity* **6**, 541–549.

Launois, P., Ohteki, T., Swihart, K., MacDonald, H. R. and Louis, J. A. (1995). In susceptible mice, *Leishmania major* induce very rapid interleukin-4 production by CD4+ T cells which are NK1.1. *European Journal of Immunology* **25**, 3298–3307.

Liew, F. Y., Millott, S., Li, Y., Lelchuk, R., Chan, W. L. and Ziltener, H. (1989). Macrophage activation by interferon-gamma from host-protective T cells is inhibited by interleukin (IL)3 and IL4 produced by disease-promoting T cells in leishmaniasis. *European Journal of Immunology* **19**, 1227–1232.

Lima, H. C., Dekrey, G. K. and Titus, R. G. (1999). Resolution of an infection with *Leishmania braziliensis* confers complete protection to a subsequent challenge with *Leishmania major* in BALB/c mice. *Memorias do Instituto Oswaldo Cruz* **94**, 71–76.

Locksley, R. M., Heinzel, F. P., Holaday, B. J., Mutha, S. S., Reiner, S. L. and Sadick, M. D. (1991). Induction of Th1 and Th2 CD4+ subsets during murine *Leishmania major* infection. *Research in Immunology* **142**, 28–32.

MacDonald, T. T. and Carter, P. B. (1979). Requirement for a bacterial flora before mice generate cells capable of mediating the delayed hypersensitivity reaction to sheep red blood cells. *Journal of Immunology* **122**, 2624–2629.

Maia, O. B., Duarte, R., Silva, A. M., Cara, D. C. and Nicoli, J. R. (2001). Evaluation of the components of a commercial probiotic in gnotobiotic mice experimentally challenged with *Salmonella enterica* subsp. *enterica* ser. Typhimurium. *Veterinary Microbiology* **79**, 183–189.

Malherbe, L., Filippi, C., Julia, V., Foucras, G., Moro, M., Appel, H., Wucherpfennig, K., Guery, J. C. and Glaichenhaus, N. (2000). Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with *Leishmania major*. *Immunity* **13**, 771–782.

Neumann, E., Oliveira, M. A., Cabral, C. M., Moura, L. N., Nicoli, J. R., Vieira, E. C., Cara, D. C., Podoprigora, G. I. and Vieira, L. Q. (1998). Monoassociation with Lactobacillus acidophilus UFV-H2b20 stimulates the immune defense mechanisms of germ-free mice. *Brazilian Journal of Medical and Biological Research* **31**, 1565–1573.

Nicaise, P., Gleizes, A., Forestier, F., Quéro, A. M. and Labarre, C. (1993). Influence of intestinal bacterial flora on cytokine (IL-1, IL-6 and TNF-alpha) production by mouse peritoneal macrophages. *European Cytokine Network* **4**, 133–138.

Nicaise, P., Gleizes, A., Forestier, F., Sandre, C., Quero, A. M. and Labarre, C. (1995). The influence of *E. coli* implantation in axenic mice on cytokine production by peritoneal and bone marrow-derived macrophages. *Cytokine* **7**, 713–719.

Nicaise, P., Gleizes, A., Sandre, C., Kergot, R., Lebrec, H., Forestier, F. and Labarre, C. (1999). The intestinal microflora regulates cytokine production positively in spleen-derived macrophages but negatively in bone marrow-derived macrophages. *European Cytokine Network* **10**, 365–372.

National Research Council (1996). *Guide for the Care and Use of Laboratory Animals.* Institute of Laboratory Animal Resources, Washington, D.C., National Academy Press.

Noben-Trauth, N., Lira, R., Nagase, H., Paul, W. E. and Sacks, D. L. (2003). The relative contribution of IL-4 receptor signaling and IL-10 to susceptibility to Leishmania major. *Journal of Immunoogy* **170**, 5152–5158.

Oliveira, M. A., Santiago, H. C., Lisboa, C. R., Ceravollo, I. P., Trinchieri, G., Gazzinelli, R. T. and Vieira, L. Q. (2000). *Leishmania* sp: comparative study with *Toxoplasma gondii* and *Trypanosoma cruzi* in their ability to initialize IL-12 and IFN-gamma synthesis. *Experimental Parasitology* **95**, 96–105.

Oliveira, M. R., Tafuri, W. L., Nicoli, J. R., Vieira, E. C., Melo, M. N. and Vieira, L. Q. (1999). Influence of microbiota in experimental cutaneous leishmaniasis in Swiss mice. *Revista do Instituto de Medicina Tropical de Sao Paulo* **41**, 87–94.

Oswald, I. P., Gazzinelli, R. T., Sher, A. and James, S. J. (1992). IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. *Journal of Immunology* **148**, 3578–3582.

Parish, C. R. (1972). The relationship between humoral and cell-mediated immunity. *Transplantation Reviews* **13**, 35–66.

Parish, C. R. and Liew, F. Y. (1972). Immune response to chemically modified flagellin. 3. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. *Journal of Experimental Medicine* **135**, 298–311.

Pasare, C. and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* **299**, 1033–1036.

Phillips, B. P. and Wolfe, P. A. (1959). The use of germ-free guinea pigs in studies on the microbial interrelationships in amoebiasis. *Annals of the New York Academy of Sciences* **78**, 308–314.

Pleasants, J. R. (1974). Gnotobiotics. In *Handbook of Laboratory Animal Science.* (ed. Melby Jr., E. C. & Altman, N. H.), pp. 119–174. CRC Press, Cleveland.

Powrie, F. and Maloy, K. J. (2003). Immunology. Regulating the regulators. *Science* **299**, 1030–1031.

Ribeiro-Sobrinho, A. P., Maltos, S. M. M., Faria, L. M., Carvalho, M. A., Nicoli, J. R., Uzeda, M. and Vieira, L. Q. (2002). Cytokine production in response to endodontic infection in germ-free animals. *Oral Microbiology and Immunology* **17**, 344–353.

Rodrigues, A. C., Cara, D. C., Fretez, S. H., Cunha, F. Q., Vieira, E. C., Nicoli, J. R. and Vieira, L. Q. (2000). *Saccharomyces boulardii* stimulates sIgA production and the phagocytic system of gnotobiotic mice. *Journal of Applied Microbiology* **89**, 404–414.

Rodrigues, A. C., Nardi, R. M., Bambirra, E. A., Vieira, E. C. and Nicoli, J. R. (1996). Effect of *Saccharomyces boulardii* against experimental oral infection with *Salmonella typhimurium* and *Shigella flexneri* in conventional and gnotobiotic mice. *Journal Applied Bacteriology* **81**, 251–256.

Sacks, D. L., Hieny, S. and Sher, A. (1985). Identification of cell surface carbohydrate and antigenic changes between noninfective developmental stages of *Leishmania major* promastigotes. *Journal of Immunology* **135**, 564–569.

Sadick, M. D., Heinzel, F. P., Holaday, B. J., Pu, R. T., Dawkins, R. S. and Locksley, R. M. (1990). Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *Journal of Experimental Medicine* **171**, 115–127.

Santiago, H. C., Oliveira, M. A., Bambirra, E. A., Faria, A. M., Afonso, L. C., Vieira, L. Q. and Gazzinelli, R. T. (1999). Coinfection with *Toxoplasma gondii* inhibits antigen-specific Th2 immune responses, tissue inflammation, and parasitism in BALB/c mice infected with *Leishmania major*. *Infection and Immunity* **67**, 4939–4944.

Scharton, T. M. and Scott, P. (1993). Natural killer cells are a source of IFN-γ that drives differentiation of CD4+ T cell subsets and induces early resistance to *Leishmania major* in mice. *Journal of Experimental Medicine* **178**, 567–577.

Scharton-Kersten, T., Afonso, L. C. C., Wysocka, M., Trinchieri, G. and Scott, P. (1995). IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *Journal of Immunology* **154**, 5320–5330.

Scott, P. (1991). IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *Journal of Immunology* **147**, 3149–3155.

Silva, M. E., Evangelista, E. A., Nicoli, J. R., Bambirra, E. A. and Vieira, E. C. (1987). American trypanosomiasis (Chagas'disease) in conventional and germ-free rats and mice. *Revista do Instituto de Medicina Tropical de São Paulo* **29**, 284–288.

Soong, L., Xu, J. C., Grewal, I. S., Kima, P., Sun, J., Longley, B. J. Jr., **Ruddle, N. H, McMahon-Pratt, D. and Flavell, R. A.** (1996). Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. *Immunity* **4**, 263–273.

Souza, D. G., Vieira, A. T., Soares, A. C., Pinho, V., Nicoli, J. R., Vieira, L. Q. and Teixeira, M. M. (2004). The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *Journal of Immunology* **173**, 4137–4146.

Sypek, J. P., Chung, C. L., Mayor, S. E. H., Subramanyam, J. M., Goldman, S. J., Sieburth, D. S., Wolf, S. F. and Schaub, R. G. (1993). Resolution of cutaneous leishmaniasis: Interleukin 12 initiates a protective Th1 immune response. *Journal of Experimental Medicine* **177**, 1797–1802.

Titus, R. G., Kelso, A. and Louis, J. A. (1984). Intracellular destruction of *Leishmania tropica* by macrophages activated with macrophage activating factor/interferon. *Clinical and Experimental Immunology* **55**, 157–165.

Torres, M. R. F., Silva, M. E. C., Vieira, E. C., Bambirra, E. A., Sogayar, M. I. T., Pena, F. J. and Nicoli, J. R. (1992). Intragasgric infection of conventional and germ-free mice with *Giardia lamblia*. *Brazilian Journal of Medical and Biological Research* **25**, 349–353.

Vieira, L. Q., Goldschmidt, M., Nashleanas, M., Pfeffer, K., Mak, T. and Scott, P. (1996). Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with *Leishmania major*, but control parasite replication. *Journal of Immunology* **157**, 827–835.

Vieira, E. C., Nicoli, J. R., Moraes-Santos, T., Silva, M. E., Costa, C. A., Mayrink, W. and Bambirra, E. A. (1987). Cutaneous leishmaniasis in germ-free, gnotobiotic, and conventional mice. *Revista do Instituto de Medicina Tropical de São Paulo* **29**, 385–387.

Vieira, L. Q., Oliveira, M. R., Neumann, E., Nicoli, J. R. and Vieira, E. C. (1998). Parasitic infections in germ-free animals. *Brazilian Journal of Medical and Biological Research* **31**, 105–110.

Vieira, L. Q. and Scott, P. (1996). Germ-free mice exhibit higher levels of IL-4 than conventional mice when infected with *Leishmania major*. *Microecology and Therapy* **24**, 231–236.

Wills-Karp, M., Santeliz, J. and Karp, C. L. (2001). The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nature Reviews Immunology* **1**, 69–75.

Yazdanbakhsh, M., Kremsner, P. G. and van Ree, R. (2002). Allergy, parasites, and the hygiene hypothesis. *Science* **296**, 490–494.