Available online at www.sciencedirect.com
ScienceDirect
Acta Tropica 104 (2007) 133–139
ACTA TROPICA
www.elsevier.com/locate/actatropica

Successful vaccination against *Leishmania chagasi* infection in BALB/c mice with freeze-thawed *Leishmania* antigen and *Corynebacterium parvum*

Márcia de Carvalho Vilela[a], Daniel Cláudio de Oliveira Gomes[b], Eduardo de Almeida Marques-da-Silva[a], Tiago Donatelli Serafim[a], Luis Carlos Crocco Afonso[a], Simone Aparecida Rezende[a,c,*]

[a] *Laboratório de Imunoparasitologia, DECBI/NUPEB, Campus Universitário, Morro do Cruzeiro, Universidade Federal de Ouro Preto, Cep: 35.400-000, Ouro Preto, MG, Brazil*
[b] *Instituto de Biofísica Carlos Chagas Filho/UFRJ, Brazil*
[c] *Depto. Análises Clínicas, Escola de Farmácia/UFOP, Brazil*

Received 16 April 2007; received in revised form 9 July 2007; accepted 28 August 2007
Available online 31 August 2007

Abstract

This study evaluated the potential of a *Leishmania* antigen vaccine in protecting BALB/c mice against *Leishmania chagasi*. Mice received two subcutaneous doses of *L. amazonensis* vaccine with *Corynebacterium parvum* and subsequent boost was done without adjuvant. One week later, mice were challenged with *L. chagasi*. We observed that this vaccine caused a significant reduction in parasite load in liver and spleen and induced a high production of IFN-γ and IL-4 by spleen cells from vaccinated mice in response to *Leishmania* antigen. Together, our data show that this vaccine is capable of inducing a Th1/Th2 response that is important to control parasite replication.

Keywords: Vaccine; *Leishmania chagasi*; Mice; IFN-γ; IL-4

1. Introduction

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan *Leishmania*. These diseases range from self-limiting cutaneous leishmaniasis to visceral leishmaniasis, also known as kalazar, which if a fatal infection if not treated successfully. Human infection with *Leishmania chagasi*, the protozoan causing South American visceral leishmaniasis, causes diverse sequel ranging from sub-clinical infection to progressive fatal disease (Wilson, 1993). Sub-clinical infection results in the development of a cellular immune response that often results in long-term protective immunity against re-infection (Pearson and Sousa, 1996). A goal of anti-leishmanial vaccine development is to replicate this naturally acquired protective immunity through immunization with parasite antigens.

The involvement of T helper 1 (Th1) and T helper 2 (Th2) subsets with protection and disease exacerba-

* Corresponding author at: Laboratório de Imunoparasitologia, DECBI/NUPEB, Campus Universitário, Morro do Cruzeiro, Universidade Federal de Ouro Preto, Cep: 35.400-000, Ouro Preto, MG, Brazil. Tel.: +55 31 3559 1702; fax: +55 31 3559 1680.
E-mail address: rezende@nupeb.ufop.br (S.A. Rezende).

0001-706X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2007.08.007

tion, respectively, has been demonstrated in the murine model of cutaneous leishmaniasis caused by *L. major* (Heinzel et al., 1989). In contrast, a similar pattern of T-helper cell subsets has not been demonstrated in visceral leishmaniasis. Wilson and Weinstock (1996) have shown that IFN-γ is critical for cure of visceral leishmaniasis in mice. Liver granuloma cells obtained from C3H.HeJ mice, that show a low parasite burden in liver, produce high levels of IFN-γ. Otherwise, cells obtained from BALB/c mice, that are susceptible to infection with *L. chagasi*, produce no IFN-γ. Afterwards, (Wilson et al., 1998) showed that liver granuloma cells produce TGF-β and this cytokine is responsible for the inhibition of IFN-γ production. Furthermore, the production of IL-4 is important for granuloma maturation (Stager et al., 2003), protection induced by vaccine (Ghosh et al., 2001) and for cure associated with chemotherapy (Alexander et al., 2000).

In this study, we evaluated the potential of a freeze-thawed *Leishmania* antigen vaccine (composed of PH8 strain of *L. amazonensis*) as a vaccine candidate against *L. chagasi* infection. This vaccine was developed in 1979 (Mayrink et al., 1979) and has been used not only in the prevention of the disease (Nascimento et al., 1990) but also as an immunotherapeutic agent in cases where antimonium salts cannot be used (Hermeto et al., 1994). Cross-species protection has been documented in other studies in leishmaniasis (Gicheru et al., 1997, Misra et al., 2001). These findings tempted us to investigate if subcutaneous immunization with a *Leishmania amazonensis* vaccine in conjunction with *C. parvum* was able to protect BALB/c mice against *L. chagasi* infection.

2. Materials and methods

2.1. Leishmania parasites and antigen

The strain of *L. chagasi* used in this study was kindly provided by Dr. Maria Norma de Melo, Dep. de Parasitologia, UFMG, Belo Horizonte. Promastigotes were grown in DMEM pH 6,8 supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoetanol and 20 μg/mL garamicin (DMEM 20% FBS) at 25 °C. Parasites used in this study were maintained up to 15 passages in culture. Infectivity was maintained by serial passage through mice. *L. amazonensis* was grown in Grace's Medium (Grace's medium supplemented with 20% FBS, 2 mM L-glutamine and 20 μg/mL garamicin). For mice inoculation and vaccine preparation promastigotes from *L. chagasi* or *L. amazonensis* were harvested from late-log-phase cultures by centrifugation, washed three times with PBS and disrupted by three rounds of freezing and thawing. The protein content was estimated by the Lowry method (Lowry et al., 1951) and the antigen was frozen at −70 °C until use.

2.2. Mice and vaccine

Female BALB/c mice (4–6 weeks old) were obtained from CEBIO, UFMG, Belo Horizonte and were maintained at Biotério Central/UFOP. The vaccine used in this study was produced with one strain of *Leishmania amazonensis* (IFLA/BR/67/PH8). Promastigotes of *L. amazonensis* were harvested as described previously. Vaccine was prepared with a concentration of 800 μg of protein/mL and 0.01% merthiolate. This vaccine was kindly provided by Dr Wilson Mayrink, Departamento de Parasitologia, UFMG, Belo Horizonte.

BALB/c mice were immunized with two subcutaneous doses (2 weeks apart) of 100 μg of *L. amazonensis* vaccine along with 250 μg of *Corynebacterium parvum* and subsequent boost was done 4 weeks later with 10 μg of *L. amazonensis* vaccine without adjuvant. Injections were given at the tail base. Control mice were inoculated with PBS without adjuvant. One week later, mice were challenged with 1×10^7 promastigotes of *L. chagasi* given intravenously by lateral tail vein. Two, 4 and 6 weeks later, mice were sacrificed and spleen and liver parasite load was determined by quantitative limiting-dilution culture with some modifications (Titus et al., 1985).

2.3. Determination of vaccine-induced cytokine production

Spleen and liver from PBS-inoculated and vaccinated mice were harvested and their weight was determined. One fragment of each organ was obtained and weighed separately for parasite quantification. Single cell suspensions of spleen were obtained by tissue grinder homogenization. The erythrocytes were lysed with ammonium chloride lysis buffer and the cells were washed and cultured in DMEM pH 7,2 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoetanol and 20 μg/mL garamicin (DMEM 10% SFB) at 5×10^6 cells/mL. These cells were cultured in 96-well flat-bottom microtiter plates in medium alone (control) or stimulated with 50 μg of *L. chagasi* or *L. amazonensis* Ag/mL of culture for 72 h. The production of IFN-γ and IL-4 was determined in cell culture supernant by ELISA (Afonso and Scott, 1993).

2.4. Determination of the tissue parasite burden

Quantitative limiting-dilution culture was performed as described previously with some modifications (Titus et al., 1985). A weighed piece of each organ (liver and spleen) was homogenized in tissue grinder and re-suspended in 500 μL of DMEM 20% FBS in 48-well flat-bottom microtiter plates. Fivefold serial dilution was done and after 2 weeks the plates were scored microscopically for parasite growth. The number of parasites was determined from the reciprocal of the highest dilution at which promastigotes could be detected at 2 weeks of incubation at 25 °C and was expressed as parasites per organ.

2.5. Statistical analysis

All data were analyzed by Kolmogorov-Smirnov normality test. All data were not considered normal and were submitted to non-parametric Mann–Whitney's test.

3. Results

3.1. IFN-γ and IL-4 production by splenocytes 3 days after challenge

Initially, we determined whether immunization of mice with a *L. amazonensis* vaccine was able to induce cytokines that were protective against infection with *L. chagasi*. Mice were immunized with this vaccine and, 1 week later, they were challenged with 1×10^7 *L. chagasi* promastigotes. Three days after infection, spleen cells from PBS-inoculated and vaccinated mice were obtained and stimulated with *L. chagasi* or *L. amazonensis* antigen. Higher levels of IFN-γ were produced 3 days after challenge by PBS-inoculated and vaccinated mice cells in response to *L. chagasi* and *L. amazonensis* antigen when compared to non-stimulated cells (Fig. 1A). However, there was no difference in the level of IFN-γ between spleen cells obtained from PBS-inoculated and vaccinated mice stimulated with *Leishmania* antigen. Spleen cells from vaccinated mice produced a higher level of IL-4 when stimulated with both antigens when compared non-stimulated cells. Furthermore, spleen cells obtained from vaccinated mice produced a higher level of IL-4 in response to both antigens when compared with cells obtained from PBS-inoculated mice (Fig. 1B).

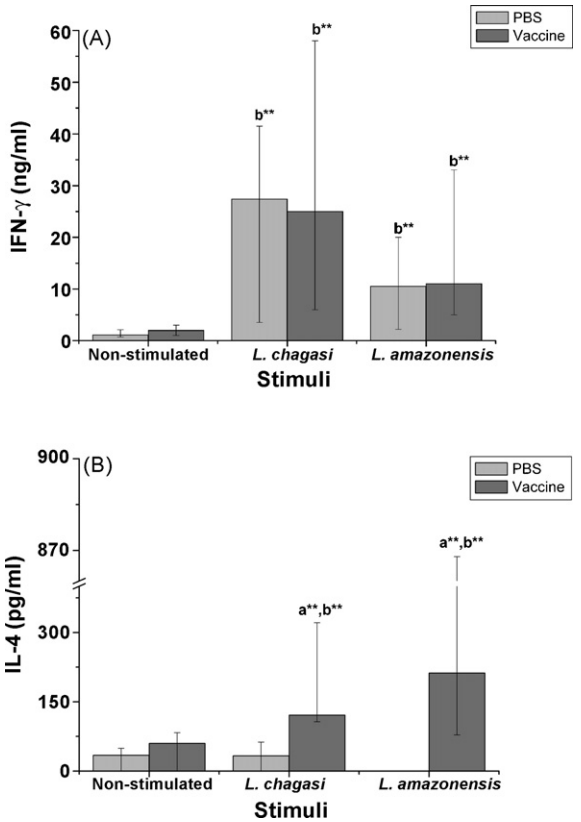

Fig. 1. IFN-γ (A) and IL-4 (B) production by splenocytes 3 days after challenge. BALB/c mice were immunized with *Leishmania amazonensis* + *C. parvum* or with PBS as a control. One week later, mice were challenged with *L. chagasi*. Three days later, mice were killed and spleen was collected. Splenocytes were stimulated with *L. chagasi* or *L. amazonensis* Ag and 3 days later supernatants were collected and cytokine level was determined by ELISA. These results are represented as the median ±25% of data distribution from two to four experiments with four to six mice. Letter over the bars represent significant IFN-γ production by spleen cells in the following comparisons: (a): vaccinated × PBS-inoculated, (b): Ag stimulated × non-stimulated. Statistical differences between cytokine production by stimulated cells and their respective non-stimulated control were determined by non-parametric Mann–Whitney's test (**$p < 0.01$).

3.2. IFN-γ and IL-4 production by spleen cells at 2, 4 and 6 weeks after challenge

We also evaluated IFN-γ and IL-4 production by spleen cells from PBS-inoculated and vaccinated mice 2, 4 and 6 weeks after challenge. In all weeks evaluated, it was observed a higher IFN-γ production by spleen cells from vaccinated mice in response to both antigens when compared to IFN-γ production by cells from PBS-inoculated mice, with one exception – spleen cells obtained from mice with 6 weeks of infection stimulated with *L. chagasi* Ag (Fig. 2A). We also observed that this vaccine was able to induce a higher production of IL-4 in all weeks studied when spleen cells were stimulated with *L. chagasi* or *L. amazonensis* Ag. This increase was much more pronounced than the one observed with IFN-γ, mainly because spleen cells obtained from PBS-

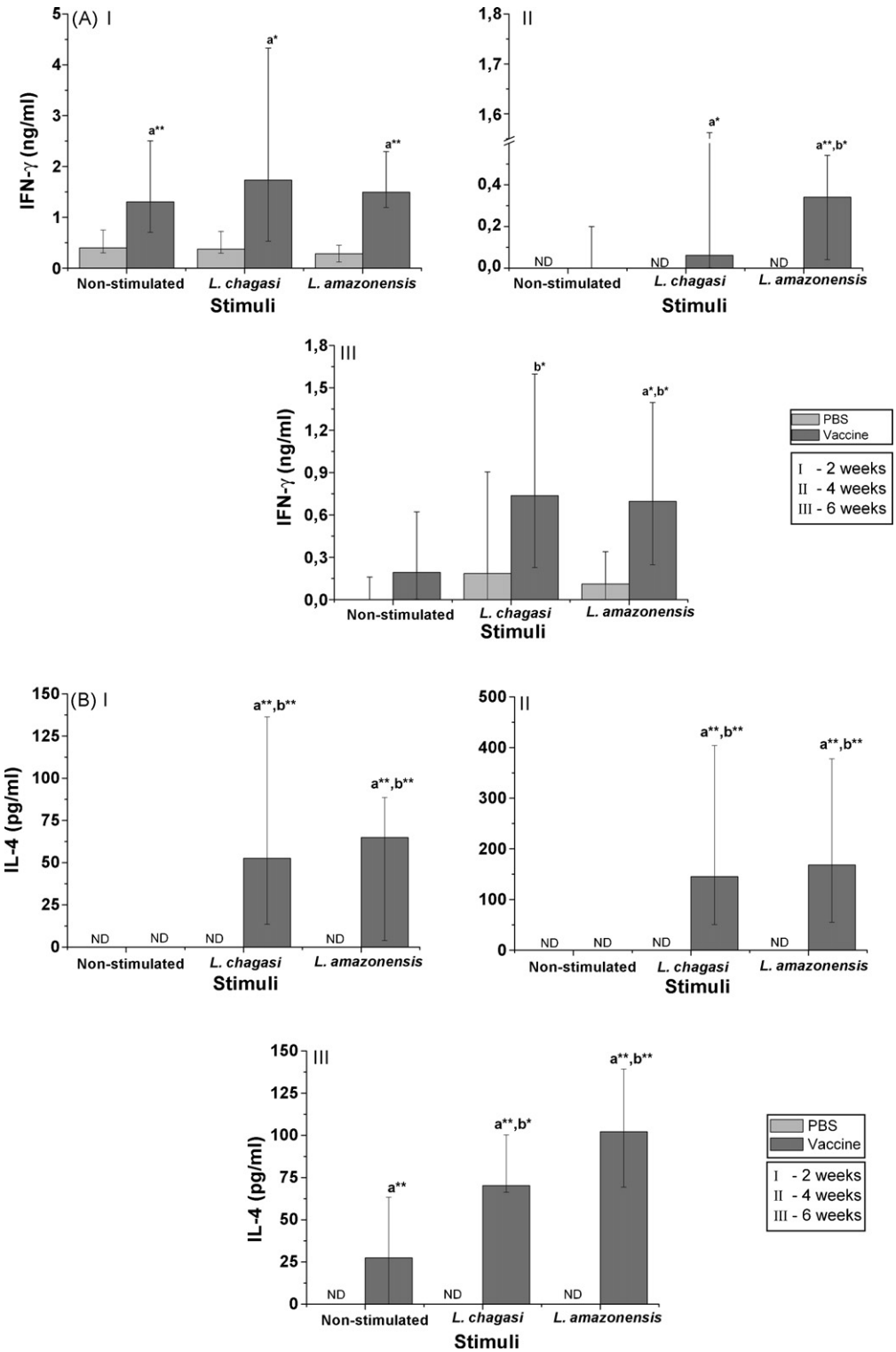

Fig. 2. IFN-γ (A) and IL-4 (B) production by splenocytes in different weeks after challenge in PBS-inoculated and vaccinated mice. BALB/c mice were immunized with *Leishmania amazonensis* + *C. parvum* or with PBS as a control. One week later, mice were challenged with *L. chagasi*. 2, 4 or 6 weeks later, mice were killed and spleen was collected. Splenocytes were stimulated with *L. chagasi* or *L. amazonensis* Ag and 3 days later supernatants were collected and cytokine level was determined by ELISA. These results are represented as the median and 25% of data

inoculated mice did not produce IL-4 in response to *Leishmania* antigen.

3.3. Parasite load in liver and spleen

In order to determine if *L. amazonensis* Ag vaccine was able to protect BALB/c mice from *L. chagasi* infection, we evaluated parasite load in liver and spleen by limiting-dilution analysis. We evaluated the magnitude of parasite load in vaccinated and PBS-inoculated mice in different times after intravenous challenge with 1×10^7 parasites. A significant reduction in parasite load was observed both in liver at 2, 4 and 6 weeks of infection (Fig. 3A) and in spleen, at 2 weeks of infection (Fig. 3B) from immunized mice when compared to PBS-inoculated mice. Thus, this vaccine (composed of PH8 strain of *L. amazonensis*) was able to provide a partial protection in BALB/c mice infected with *Leishmania chagasi* associated with a mixed Th1/Th2 response.

4. Discussion

Leishmaniasis has always been one of the promising diseases for vaccine development, since it has been known that exposure and cure from infection usually results in long lasting immunity (Pearson and Sousa, 1996). Although some works have tried vaccination with live parasites (Streit et al., 2001), there are ethical considerations against live parasite vaccine and attention has shifted to killed parasite vaccines.

A vaccine against cutaneous leishmaniais was developed by Mayrink et al. (1979) and was prepared from 5 stocks of parasites isolated from patients with different forms of leishmaniasis. Subsequently, it was developed another vaccine that used only PH8 strain of *Leishmania amazonensis*. This vaccine was licenced in 1999 by Brazil Healthy Minister for use in therapy and it was commercialized as Leishvacin. Since then, the vaccine has been used as an immunotherapeutic agent in cases where antimonium salts cannot be used. In this manner, it was shown that combining therapy was able to reduce the time necessary for complete healing of the lesions when compared to conventional therapy (Toledo et al., 2001).

During this study, we evaluated if this vaccine was able to provide heterologous protection against *L. chagasi* infection in BALB/c mice. Cross-species protection

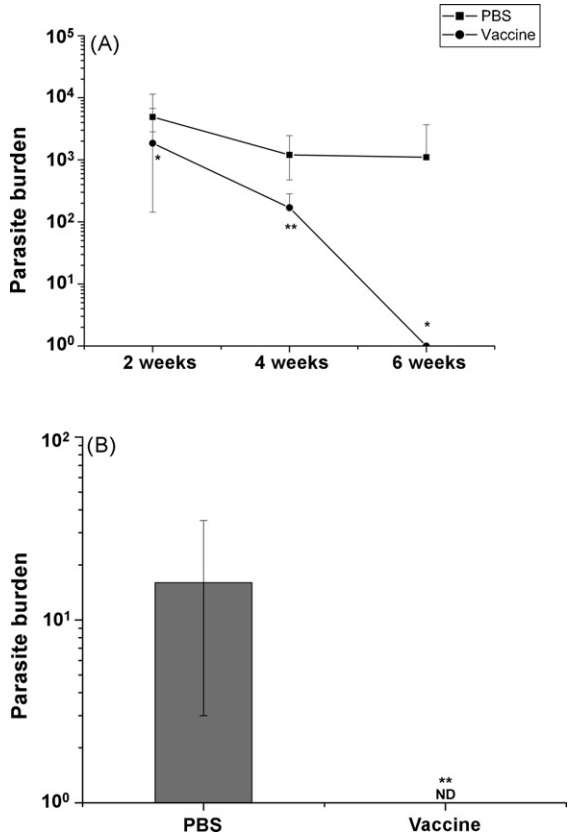

Fig. 3. Liver (A) and spleen (B) parasite burden from vaccinated BALB/c mice challenged 1 week after booster. BALB/c mice were vaccinated, as described in Section 2, and challenged by intravenous route with 1×10^7 *L. chagasi* late-log-phase promastigotes 1 week after booster. Two, four and six weeks after challenge, mice were sacrificed and their liver and spleen were harvested for parasite quantification by limiting-dilution culture. Results are represented as the median and 25% of data distribution from two to four experiments with four to six mice. Statistical differences in parasite load between vaccinated and PBS-inoculated mice were determined by non-parametric Mann–Whitney's test (*$p < 0.05$; **$p < 0.01$).

has been documented in other studies in leishmaniasis. Gicheru et al. (1997) demonstrated that Vervet monkeys that had been previously infected with *Leishmania donovani* were protected against *L. major* infection. Furthermore, Misra et al. (2001) showed that alum-precipitated autoclaved *Leishmania major* along with BCG protected monkeys against *Leishmania donovani* infection. In this study, mice were vaccinated *L. amazonensis* vaccine and we observed that this vaccine was able to reduce parasite load in the liver (2, 4 and 6 weeks

distribution from two to four experiments with four to six mice. Letter over the bars represent significant IFN-γ production by spleen cells in the following comparisons: (a): vaccinated × PBS-inoculated, (b): Ag stimulated × non-stimulated. Statistical differences between cytokine production by stimulated cells and their respective non-stimulated control were determined by non-parametric Mann–Whitney's test (*$p < 0.05$; **$p < 0.01$). IL-4 production was not detected by spleen cells obtained from PBS-inoculated mice. ND: not detectable.

of infection) and spleen (2 weeks of infection). These data suggest that different mechanisms are involved in the protection in these organs, since at 4 and 6 weeks of infection, protection was observed only in the liver.

Furthermore, we evaluated the production of IFN-γ and IL-4 by spleen cells stimulated with *Leishmania* Ag and the parasite load in both liver and spleen. Cytokine evaluation was done initially 3 days after challenge and it was observed a higher production of IFN-γ by spleen cells in response to both antigens by PBS-inoculated and vaccinated mice in comparison to non-stimulated cells. We believe that this rapid production of IFN-γ is due the cross-reaction among *Leishmania* and microbial Ag found in the flora. One of these antigens, *Leishmania* homologue of mammalian RACK (LACK) is able to induce a rapid production of IL-4 by Vβ4Vα8 $CD4^+$ T cells in the draining lymph nodes within 16 h after the subcutaneous infection of *L. major* promastigotes (Mougneau et al., 1995). Furthermore, there was no increase in the production of IFN-γ by spleen cells obtained from vaccinated mice as compared to PBS-inoculated mice. Otherwise, the level of IL-4 was higher by spleen cells from vaccinated mice as compared to PBS-inoculated mice when these cells were stimulated with *Leishmania* Ag.

Cytokine evaluation was done also 2, 4 and 6 weeks after infection and we observed that *L. amazonensis* vaccine was able to induce a significant production of IFN-γ by spleen cells from vaccinated mice when compared to spleen cells from mice inoculated with PBS. Furthermore, a much more pronounced increase was observed in the level of IL-4. Many studies have shown that this cytokine is important in visceral model of leishmaniasis. Stager et al. (2003) have shown that IL-4 (and IL-13) is important for granuloma maturation and anti-leishmanial activity in the murine model of *L. donovani* infection. Furthermore, some studies have shown that protection induced by vaccines against *L. donovani* and *L. infantum/chagasi* is associated with a mixed Th1/Th2 pattern. Ghosh et al. (2001) showed that immunization with A2 protein protects mice against *L. donovani* infection and this protein induces a mixed Th1/Th2 and a humoral response. Ramiro et al. (2003) showed that LACK immunization in a heterologous prime-boost regime (plasmid DNA and recombinant vector) was able to protect dogs against *L. infantum* infection, and this protection was associated with an increase in the mRNA level of IL-4 and IFN-γ in peripheral blood mononuclear cells.

Thus, this study shows that a vaccine composed of *L. amazonensis* is able to protect BALB/c mice against an endovenous challenge with *L. chagasi* promastigotes and this protection is associated with the induction of both IFN-γ and IL-4. Furthermore, it shows that cross-protection between *Leishmania* species presents a major practical implication, and that this vaccine can be part of a vaccination scheme that offers protection against *L. chagasi* in the mouse model of visceral leishmaniasis.

References

Afonso, L.C., Scott, P., 1993. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. Infect. Immun. 61, 2952–2959.

Alexander, J., Carter, K.C., Al-Fasi, N., Satoskar, A., Brombacher, F., 2000. Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur. J. Immunol. 30, 2935–2943.

Ghosh, A., Zhang, W.W., Matlashewski, G., 2001. Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections. Vaccine 20, 59–66.

Gicheru, M.M., Olobo, J.O., Anjili, C.O., 1997. Heterologous protection by *Leishmania donovani* for *Leishmania major* infections in the vervet monkey model of the disease. Exp. Parasitol. 85, 109–116.

Heinzel, F.P., Sadick, M.D., Holaday, B.J., Coffman, R.L., Locksley, R.M., 1989. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J. Exp. Med. 169, 59–72.

Hermeto, M.V., Vieira-Dias, D., Genaro, O., Rotondo-Silva, A., Da Costa, C.A., Toledo, V.P., Michalick, M.S., Williams, P., Mayrink, W., 1994. Outbreak of cutaneous leishmaniasis in the Rio Doce Valley, Minas Gerais, Brazil. Mem. Inst. Oswaldo Cruz 89, 519–521.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mayrink, W., Da Costa, C.A., Magalhaes, P.A., Melo, M.N., Dias, M., Lima, A.O., Michalick, M.S., Williams, P., 1979. A field trial of a vaccine against American dermal leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 73, 385–387.

Misra, A., Dube, A., Srivastava, B., Sharma, P., Srivastava, J.K., Katiyar, J.C., Naik, S., 2001. Successful vaccination against *Leishmania donovani* infection in Indian langur using alum-precipitated autoclaved *Leishmania major* with BCG. Vaccine 19, 3485–3492.

Mougneau, E., Altare, F., Wakil, A.E., Zheng, S., Coppola, T., Wang, Z.E., Waldmann, R., Locksley, R.M., Glaichenhaus, N., 1995. Expression cloning of a protective *Leishmania* antigen. Science 268, 563–566.

Nascimento, E., Mayrink, W., Da Costa, C.A., Michalick, M.S., Melo, M.N., Barros, G.C., Dias, M., Antunes, C.M., Lima, M.S., Taboada, D.C., 1990. Vaccination of humans against cutaneous leishmaniasis: cellular and humoral immune responses. Infect. Immun. 58, 2198–2203.

Pearson, R.D., Sousa, A.Q., 1996. Clinical spectrum of Leishmaniasis. Clin. Infect. Dis. 22, 1–13.

Ramiro, M.J., Zarate, J.J., Hanke, T., Rodriguez, D., Rodriguez, J.R., Esteban, M., Lucientes, J., Castillo, J.A., Larraga, V., 2003. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. Vaccine 21, 2474–2484.

Stager, S., Alexander, J., Carter, K.C., Brombacher, F., Kaye, P.M., 2003. Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect. Immun. 71, 4804–4807.

Streit, J.A., Recker, T.J., Filho, F.G., Beverley, S.M., Wilson, M.E., 2001. Protective immunity against the protozoan *Leishmania chagasi* is induced by subclinical cutaneous infection with virulent but not avirulent organisms. J. Immunol. 166, 1921–1929.

Titus, R.G., Marchand, M., Boon, T., Louis, J.A., 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunol. 7, 545–555.

Toledo, V.P., Mayrink, W., Gollob, K.J., Oliveira, M.A., Costa, C.A., Genaro, O., Pinto, J.A., Afonso, L.C., 2001. Immunochemotherapy in American cutaneous leishmaniasis: immunological aspects before and after treatment. Mem. Inst. Oswaldo Cruz 96, 89–98.

Wilson, M.E., 1993. Leishmaniasis. Curr. Opin. Infect. Dis. 6, 331–340.

Wilson, M.E., Weinstock, J.V., 1996. Hepatic granulomas in murine visceral leishmaniasis caused by *Leishmania chagasi*. Methods 9, 248–254.

Wilson, M.E., Young, B.M., Davidson, B.L., Mente, K.A., McGowan, S.E., 1998. The importance of TGF-beta in murine visceral leishmaniasis. J. Immunol. 161, 6148–6155.