ORIGINAL PAPER

Eduardo A. Marques-da-Silva · Eduardo A. F. Coelho ·
Daniel C. O. Gomes · Márcia C. Vilela · Cássio Z. Masioli ·
Carlos A. P. Tavares · Ana Paula Fernandes ·
Luis Carlos C. Afonso · Simone A. Rezende

Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-γ production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge

Abstract Acute visceral leishmaniasis is a progressive disease caused by *Leishmania chagasi* in South America. The acquisition of immunity following infection suggests that vaccination is a feasible approach to protect against this disease. Since *Leishmania* homologue of receptors for activated C kinase (LACK) antigen is of particular interest as a vaccine candidate because of the prominent role it plays in the pathogenesis of experimental *Leishmania major* infection, we evaluated the potential of a p36(LACK) DNA vaccine in protecting BALB/c mice challenged with *L. chagasi*. In this study, mice received intramuscular (i.m.) or subcutaneous (s.c.) doses of LACK DNA vaccine. We evaluated the production of vaccine-induced cytokines and whether this immunization was able to reduce parasite load in liver and spleen. We detected a significant production of interferon gamma by splenocytes from i.m. vaccinated mice in response to *L. chagasi* antigen and to rLACK protein. However, we did not observe a reduction in parasite load neither in liver nor in the spleen of vaccinated animals. The lack of protection observed may be explained by a significant production of IL-10 induced by the vaccine.

Keywords *Leishmania chagasi* · Lack · Vaccine · IFN-γ · IL-4 · IL-10

E. A. Marques-da-Silva · D. C. O. Gomes · M. C. Vilela ·
C. Z. Masioli · L. C. C. Afonso · S. A. Rezende (✉)
Laboratório de Imunoparasitologia, DECBI/NUPEB-ICEB,
Campus Morro do Cruzeiro,
Universidade Federal de Ouro Preto,
CEP 35400-000,
Ouro Preto, MG, Brazil
e-mail: rezende@nupeb.ufop.br
Tel.: +55-31-3559-1702
Fax: +55-31-3559-1680

E. A. F. Coelho · C. A. P. Tavares
Departamento de Bioquímica e Imunologia, ICB,
Belo Horizonte, MG, Brazil

A. P. Fernandes
Departamento de Microbiologia, Escola de Farmácia, UFMG,
Belo Horizonte, MG, Brazil

E. A. F. Coelho
Setor de Patologia Clínica, COLTEC–UFMG,
Belo Horizonte, MG, Brazil

S. A. Rezende
Departamento de Análises Clínicas, Escola de Farmácia,
Universidade Federal de Ouro Preto,
CEP 35400-000,
Ouro Preto, MG, Brazil

Introduction

Human infection with *Leishmania chagasi*, the protozoan causing South American visceral leishmaniasis, causes diverse sequelae ranging from subclinical infection to progressive fatal disease (Wilson 1993). Subclinical infection results in the development of cellular immune response detected by a positive-delayed-type hypersensitivity skin test. This immune response often results in long-term protective immunity against reinfection (Pearson and Sousa 1996). The goal of antileishmanial 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 or disease exacerbation, respectively, has been demonstrated in some models of murine cutaneous leishmaniasis (Heinzel et al. 1989). In contrast, a similar pattern of T helper cell subsets has not been demonstrated in visceral leishmaniasis, where the production of interferon gamma (IFN-γ) is associated with resistance to the disease, but the expansion of a Th2-like pattern has not been observed during disease progression (Kaye et al. 1991). Furthermore, some studies in animal models have shown that protection in visceral leishmaniasis is associated with a mixed Th1/Th2 response (Ghosh et al. 2002; Ramiro et al. 2003).

The *Leishmania* homologue of receptors for activated C kinase (LACK) is a 36-kDa protein expressed in promastigote and amastigote forms of different *Leishmania*

species. This protein is of particular interest as a vaccine candidate because of the prominent role it plays in the immunopathogenesis of experimental BALB/c infection. During *L. major* infection of BALB/c mice, expression of the immunodominant LACK antigen drives the expansion of IL-4 secreting T cells responsible for the progressive disease observed in this model (Mougneau et al. 1995). Furthermore, immunization of susceptible BALB/c mice with a truncated (24 kDa) version of the 36-kDa protein delivered in either protein + IL-12 or DNA form confers protection against infection with *L. major* (Gurunathan et al. 1997, 2000).

In this study, we evaluated the potential of a LACK DNA vaccine to induce immune response and to protect BALB/c mice challenged with *L. chagasi*. Our findings demonstrated that, although the LACK DNA vaccine induced IFN-γ production by spleen cells, this vaccine was not able to reduce the parasite load in liver or spleen of vaccinated mice. Interestingly, the high level of IFN-γ was accompanied by the production of IL-10 that may be responsible for the inhibition of IFN-γ action.

Materials and methods

Leishmania parasites and antigens

L. chagasi strain MHOM/BR/1974/M2682, kindly provided by Dra. Maria Norma de Melo, Departamento de Parasitologia, UFMG, Belo Horizonte, was used for the vaccine challenge experiments and preparation of *Leishmania* antigen. Promastigotes were grown in Dulbecco's Modified Eagle Medium (DMEM), pH 6.8, supplemented
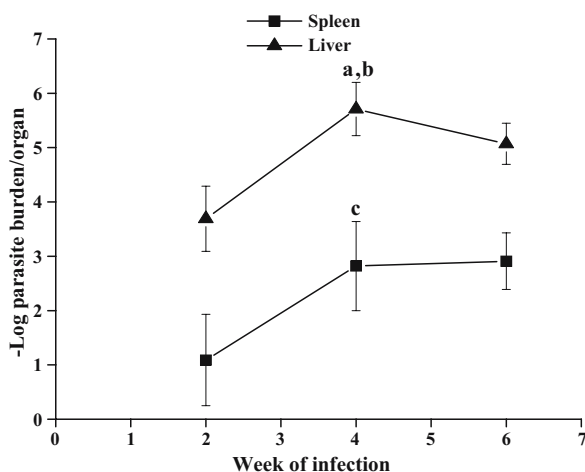

Fig. 1 Time course of *L. chagasi* infection in BALB/c mice. Mice were infected i.v. with $1 \times 10^7 L.$ *chagasi* promastigotes and sacrificed after 2, 4 and 6 weeks for parasite burden determination. The *letters* represent differences in parasite burden in the liver 4 weeks after infection compared with 2 weeks (**a**) ($P<0.01$) and with 6 weeks after infection (**b**) ($P<0.05$). In the spleen, the parasite burden 4 weeks after infection was compared with 2 weeks (**c**) ($P<0.01$). Three mice per group were used and the *points* represent the mean of two independent experiments±standard deviation. Statistical differences were determined by Student's *t* test
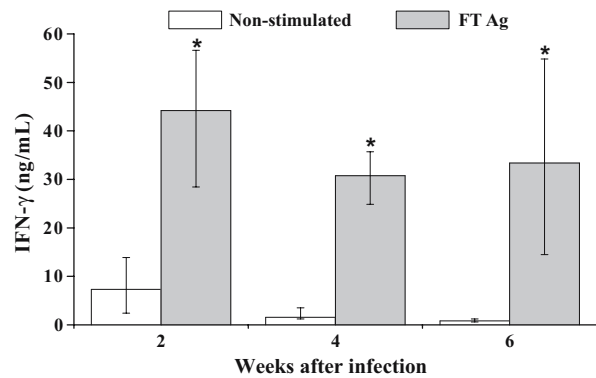

Fig. 2 IFN-γ production 2, 4 and 6 weeks after infection. BALB/c were inoculated with $1 \times 10^7 L.$ *chagasi* promastigotes in the lateral tail vein, and IFN-γ was determined by ELISA in supernatants from *L. chagasi* freeze–thawed antigen (FT Ag) (50 μg/mL) stimulated or non-stimulated spleen cells. Two or three mice per group were used and the *bars* represent the median of combined data from two independent experiments±25% of frequency distribution related to median values. Statistical differences between cytokine production by stimulated cells and their respective non-stimulated controls were determined by non-parametric Mann–Whitney's test (*$P<0.01$)

with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES) acid, 50 μM 2-mercaptoetanol and 20 μg/mL gentamicin (DMEM 20% FBS) at 25°C. Infectivity was maintained by serial passage in BALB/c
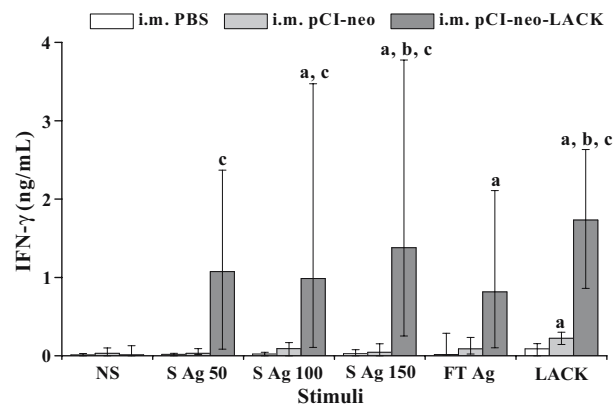

Fig. 3 IFN-γ production by spleen cells after intramuscular vaccination with pCI-neo-p36(LACK). BALB/c mice were vaccinated by intramuscular route with 100 μg of DNA (pCI-neo or pCI-neo-LACK) in a 25% sucrose solution (50 μL) or inoculated with PBS (50 μL). Three weeks after, a booster was given with the same quantities and concentrations. Two weeks after booster, the mice were killed, and IFN-γ was determined by ELISA in spleen cells culture supernatants submitted to stimuli with soluble *L. chagasi* antigen (S Ag 50, 100 or 150 μg/mL), freeze–thawed *L. chagasi* antigen (FT Ag 50 μg/mL), p36(LACK) recombinant protein (LACK 5 μg/mL) or without stimulus. *Letters over the bars* represent significant IFN-γ production ($P<0.05$) in the following comparisons: (*a*) stimulated cultures × non-stimulated cultures, (*b*) pCI-neo-p36(LACK) × pCI-neo and (*c*) pCI-neo-p36(LACK) × PBS. Three mice per group were utilized, and the *bars* represent the median of combined data from two independent experiments±25% of frequency distribution related to median values. Statistical differences were determined by non-parametric Mann–Whitney's test

mice. Promastigotes of *L. chagasi* were harvested from late-log-phase cultures by centrifugation, washed three times in phosphate-buffered saline (PBS) and disrupted by three rounds of freezing and thawing (freeze–thawed antigen). To obtain soluble *L. chagasi* antigen, the previously washed parasites were lysed in a sonifier and centrifuged at 8,500×*g*/4°C/30 min. The supernatant was centrifuged at 100,000×*g*/4°C/1 h 30 min and sterilized by filtration. The protein content was estimated in both preparations by the Lowry method (Lowry et al. 1995), and the antigens were frozen at −20°C until use. The rLACK was obtained as described elsewhere (Coelho et al. 2003).

Mice and infection

Female BALB/c mice (5–8 weeks old) were obtained from CEBIO, UFMG, Belo Horizonte and were maintained at Biotério Central, UFOP. To determine the course of infection, the BALB/c mice were infected in the tail vein with 1.0×10^7 late-log-phase promastigotes of *L. chagasi*. Two, 4 and 6 weeks later, mice were killed, the parasite load was determined in liver and spleen, and cytokine response was studied in spleen cells stimulated with *L. chagasi* antigen.

Plasmid extraction and purification

L. chagasi p36-LACK gene was cloned into the pCI-neo plasmid. *Escherichia coli* DH-5α™ was transformed with pCI-neo plasmid or pCI-neo-LACK at Laboratório de Bioquímica e Imunologia de Parasitos, Departamento de Bioquímica e Imunologia/UFMG. Plasmid extraction and purification was done by using the Wizard *Plus* Maxipreps DNA Purification System (A7270, Promega). After purification, plasmid concentration was determined by spectophotometry at λ=260 and 280 nm. The 260:280 UV absorption ratio was higher than 1.8.

Fig. 4 Spleen (**a**, **b**) and liver (**c**, **d**) parasite burden of vaccinated BALB/c mice challenged 4 or 12 weeks after booster. BALB/c mice were vaccinated, as described in "Materials and methods", and challenged by intravenous route with 1×10^7 *L. chagasi* late-log-phase promastigotes 4 weeks (**a**, **c**) or 12 weeks (**b**, **d**) after booster. Four weeks after challenge, mice were killed, and their spleens and livers were harvested for parasite quantification by quantitative limiting dilution culture. Statistical differences between groups from **a** and their respective groups from **b** (and **c** × **d**) are represented by *asterisks* (*P<0.05). Two to four mice per group were used, and the *bars* represent the medians of combined data from three independent experiments±25% of frequency distribution related to median values. Statistical differences were determined by non-parametric Mann–Whitney's test
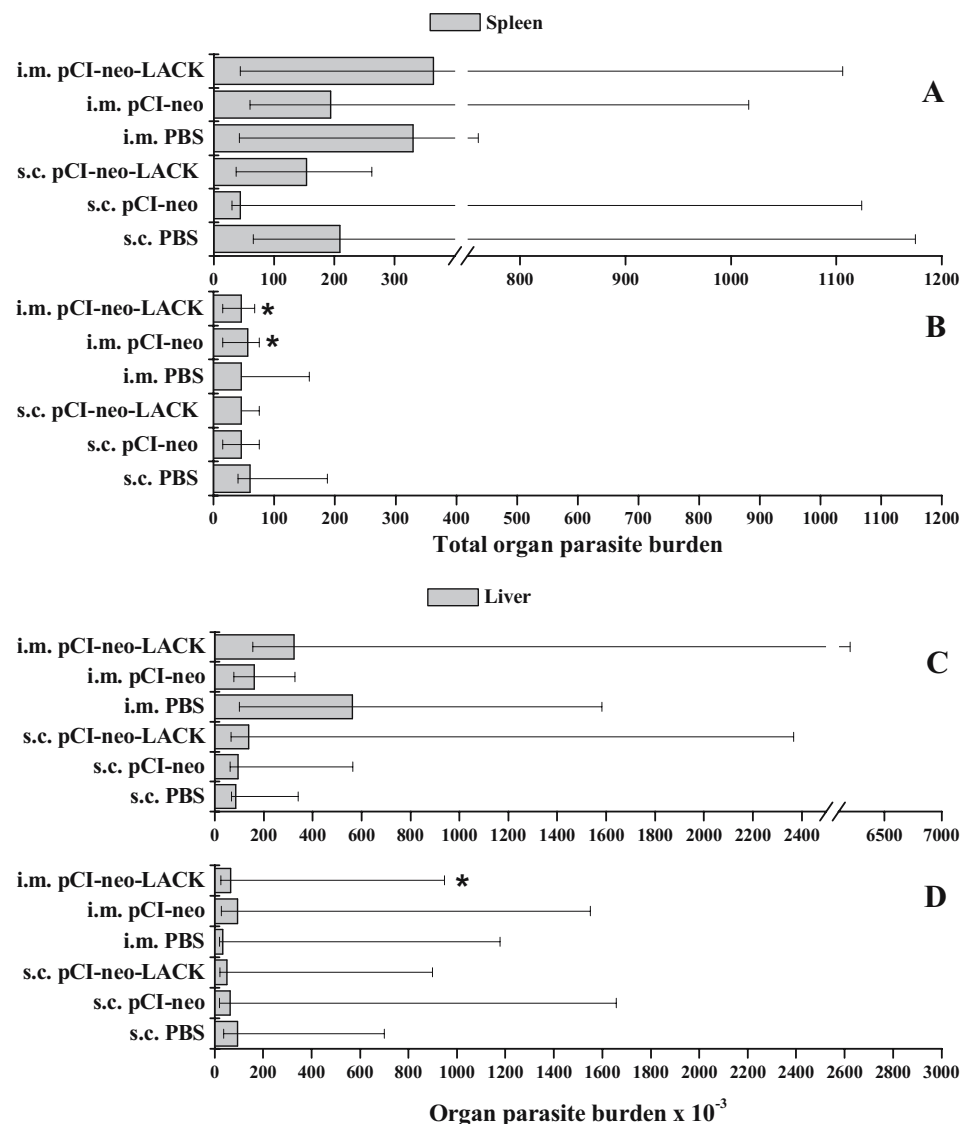
Vaccination and challenge of mice

Five- to eight-week-old female mice were vaccinated by intramuscular (i.m.; hind leg thigh) or subcutaneous (s.c.; hind footpad) route. Mice were given two injections of 100 µg of DNA (pCI-neo or pCI-neo-LACK) in 25% of sucrose (i.m. route) or PBS (s.c. route), 3 weeks apart. Two weeks later, mice were killed, and cytokine production by splenocytes stimulated with *L. chagasi* antigen was determined. Alternatively, mice were challenged 4 or 12 weeks after the second dose with 1×10^7 promastigotes of *L. chagasi* given intravenously in the lateral tail vein. Four weeks after the challenge, mice were killed, and spleen and liver parasite load was determined by quantitative limiting dilution culture. Cytokine production was also determined.

Determination of vaccine-induced cytokine production

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 FBS, 2 mM L-glutamine, 25 mM HEPES, 50 µM 2-mercaptoetanol and 20 µg/mL gentamicin (DMEM 10% FBS) at 5×10^6 cells/mL. These cells were cultured in 48-well flat-bottom microtiter plates in medium alone (non-stimulated) or stimulated with *L. chagasi* soluble antigen (50, 100 or 150 µg/mL), freeze–thawed antigen (50 µg /mL) or the purified rLACK protein (5 µg/mL) for 72 h. The production of IFN-γ and IL-4 was determined in cell culture supernatant by ELISA (Afonso and Scott 1993). The production of IL-10 was assayed by ELISA (Duo Set®, R&D Systems).

Fig. 5 IFN-γ and IL-4 production by spleen cells after subcutaneous or intramuscular vaccination with pCI-neo-p36 (LACK) and *L. chagasi* challenge. BALB/c mice were vaccinated by subcutaneous or intramuscular route. After 4 (**a**, **c**) or 12 weeks (**b**, **d**), mice were challenged by lateral tail vein with 1×10^7 *L. chagasi* late-log-phase promastigotes and sacrificed 4 weeks after challenge in order to determine IFN-γ (**a**, **b**) and IL-4 (**c**, **d**) production by spleen cells stimulated with freeze–thawed *L. chagasi* antigen (FT Ag) (50 µg/mL). The *letters* represent statistical differences (*P*<0.05) in the following comparisons: (**a**) stimulated cultures × non-stimulated cultures; (**b**) pCI-neo-p36(LACK) (stimulated); × pCI-neo (stimulated); (**c**) pCI-neo-p36(LACK) (stimulated) × PBS (stimulated) and (**d**) stimulated cultures (**a**) × stimulated cultures (**b**). 2 to 4 mice per roup were utilized and the *bars* represent the medians of combined data from three independent experiments±25% of frequency distribution related to median values. Statistical differences were determined by non-parametric Mann–Whitney's test
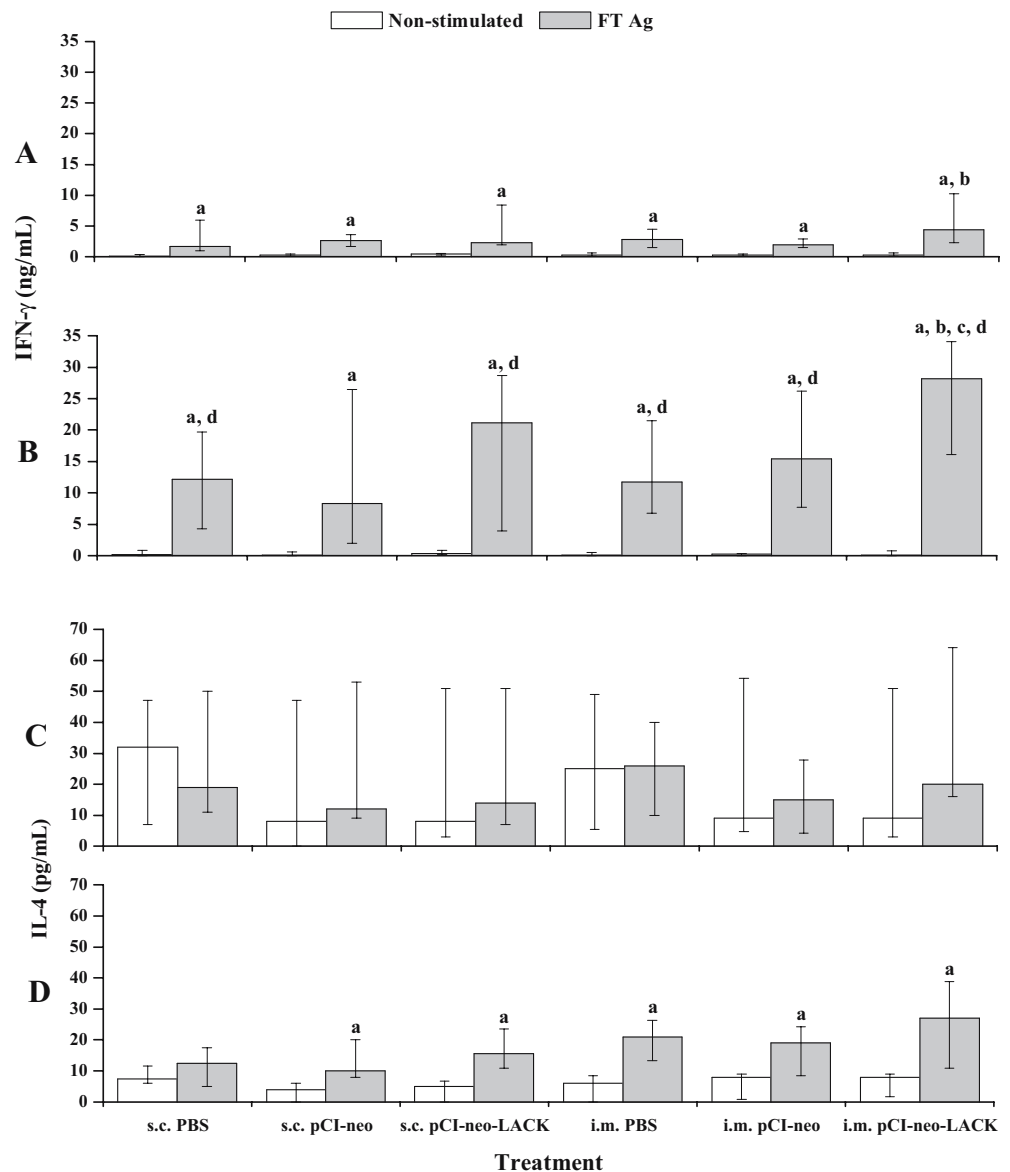
Determination of the tissue parasite burden

Fragments of spleen and liver were obtained and weighed separately for parasite quantification. Quantitative limiting dilution culture was performed as described previously (Titus et al. 1985), with some modifications. A weighed fragment of each organ (liver and spleen) was homogenized in tissue grinder and suspended in 500 μL of DMEM 20% FBS in 96-well flat-bottom microtiter plates (160 μL per well). Fivefold serial dilutions were done, and, after 12 days, 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 12 days of incubation at 25°C.

Statistical analyses

Data deriving from parasite burden determination were logarithmically transformed to homogenization of variance. All data were analysed by Kolmogorov–Smirnov normality test. Data with normal distribution were analysed by Student's t test. Data whose distributions were not considered normal were submitted to non-parametric Mann–Whitney's test.

Results

Parasite load in liver and spleen

In this work, we initially determined the parasite load in the liver and spleen after intravenous infection with $1 \times 10^7 L.$ *chagasi* promastigotes. We observed a distinct rate of parasite growth in the spleen and the liver (Fig. 1). Although parasites were detected in both organs 2 weeks after infection, the parasite load was higher in the liver in all time points studied. We also observed a peak of parasitism in the liver 4 weeks after infection, time chosen to evaluate parasite load after vaccination. A high production of IFN-γ in response to *L. chagasi* freeze–thawed antigen by spleen cells from these mice as compared with their respective controls (non-stimulated) (Fig. 2) was observed in all time points tested.

Determination of vaccine-induced cytokine production

Mice were vaccinated by s.c. or i.m. injection with *L. chagasi* p36(LACK) DNA cloned into the pCI-neo vector. Initially, we compared DNA vaccine immunogenicity by evaluating IFN-γ and IL-4 production by spleen cells prior to challenge
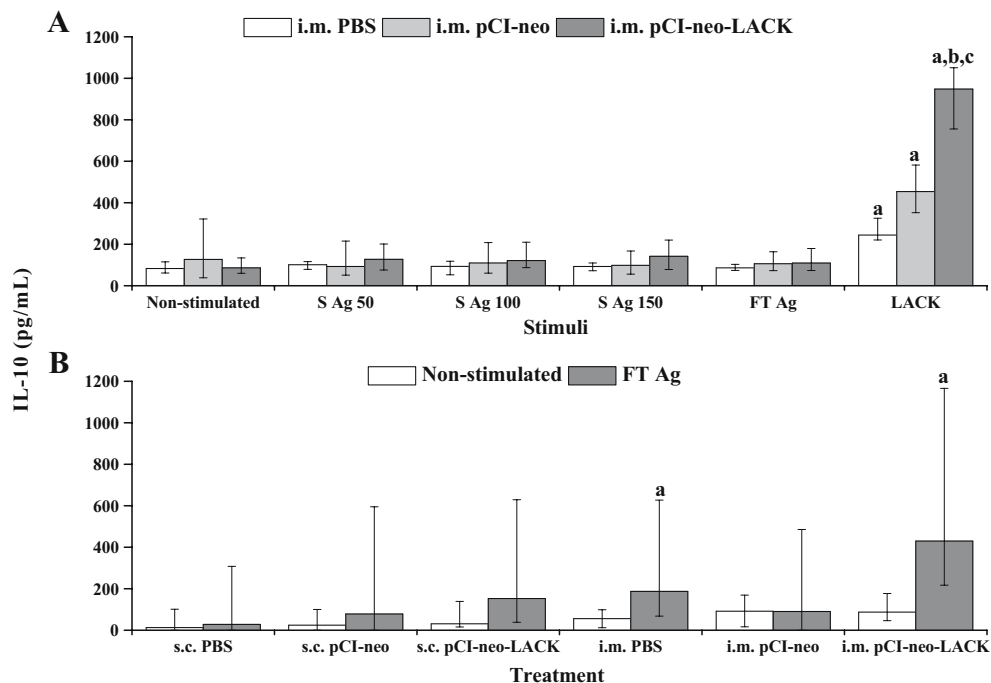

Fig. 6 IL-10 production by spleen cells after intramuscular or subcutaneous vaccination with pCI-neo-p36(LACK) before and after challenge. BALB/c mice were vaccinated by intramuscular or subcutaneous route with 100 μg of DNA (pCI-neo or pCI-neo-LACK) in a 25% sucrose solution (50 μL) or inoculated with PBS (50 μL). Three weeks after, a booster was given with the same quantities and concentrations. Two weeks after booster, mice were sacrificed and IL-10 was determined by ELISA in spleen cells culture supernatants submitted to stimuli with soluble *L. chagasi* antigen (S Ag 50, 100 or 150 μg/mL), freeze–thawed *L. chagasi* antigen (FT Ag-50 μg/mL), p36(LACK) recombinant protein (LACK 5 μg/mL) or without stimulus (**a**). The IL-10 production was also evaluated in supernatant from spleen cells obtained from mice challenged 4 weeks after booster that were sacrificed 4 weeks after infection (**b**). The *letters over the bars* represent significant IL-10 production (*P*<0.05) in the following comparisons: (**a**) stimulated cultures × non-stimulated cultures, (**b**) pCI-neo-p36(LACK) × pCI-neo and (**c**) pCI-neo-p36(LACK) × PBS. Three mice per group were utilized and the *bars* represent the median of combined data from two independent experiments±25% of frequency distribution related to median values. Statistical differences were determined by non-parametric Mann–Whitney's test

infection. Mice were killed 2 weeks after booster, and spleen cells were incubated with soluble (50, 100 and 150 μg/mL) or freeze–thawed (50 μg/mL) *L. chagasi* antigen or with the purified rLACK protein (5 μg/mL). We observed that vaccination by the i.m. route induced a significant production of IFN-γ by splenocytes from pCI-neo-LACK-vaccinated mice in response to rLACK and to soluble or freeze–thawed *L. chagasi* antigen when compared to cytokine production by spleen cells of mice inoculated with PBS or pCI-neo (Fig. 3). Furthermore, we did not detect a significant production of IL-4 by these cells (data not shown). Vaccination by s.c. route was not able to induce significant production of IFN-γ or IL-4 (data not shown).

Parasite load in liver and spleen and cytokine production after challenge

In spite of the ability of i.m. inoculation of p36(LACK)DNA vaccine to induce an antigen-specific response with IFN-γ production, we observed that this vaccine, given either by i.m. or s.c. route, was not able to reduce parasite load in liver and spleen when BALB/c mice were challenged by intravenous route with *L. chagasi* 4 or 12 weeks after vaccination (Fig. 4). The 12 week challenge was chosen initially to evaluate if the vaccine was able to induce a long-term protection. However, we observed a higher IFN-γ production by splenocytes from vaccinated mice that were challenged 4 or 12 weeks after booster (Fig. 5a,b). This production was even higher by splenocytes from mice that were challenged after 12 weeks from booster. We only detected a significant IL-4 production in response to *Leishmania* antigen in mice challenged 12 weeks after booster (Fig. 5c,d). This response was associated with a smaller parasite load in both liver and spleen in mice that were challenged after 12 weeks from i.m. vaccination with pCI-neo-LACK when compared to that in mice challenged after 4 weeks (Fig. 4). A smaller parasite load was also observed in the spleen of mice vaccinated by the i.m. route with pCI-neo.

Determination of vaccine-induced IL-10 production

To better understand the lack of protection observed, we also studied the IL-10 production by spleen cells prior and after infection. We did not observe a reduced production of IL-10 by splenocytes from pCI-neo-LACK-vaccinated mice in response to rLACK and to soluble or freeze–thawed *L. chagasi* antigen when compared to the cytokine production by spleen cells from mice inoculated with PBS or pCI-neo (Fig. 6a). In fact, there was an increase in IL-10 production by spleen cells from pCI-neo-LACK-vaccinated mice in response to rLACK protein when compared to that by spleen cells from mice inoculated with pCI-neo or PBS. We also observed that IL-10 production by spleen cells from i.m. vaccinated and PBS-inoculated mice that were challenged 4 weeks after booster was higher in response to particulate *Leishmania* antigen if compared to

non-stimulated cells. However, the vaccine was not able to reduce IL-10 production in response to *Leishmania* antigen if compared to PBS-inoculated mice. There was no difference in IL-10 production by spleen cells from s.c. inoculated mice (Fig. 6b).

Discussion

In this study, we demonstrated that vaccination of BALB/c mice with *L. chagasi* p36(LACK) DNA vaccine induced a strong IFN-γ production, but it did not confer protection against an intravenous challenge with *L. chagasi* promastigotes. These data support the findings of a previous study that shows that, although the generation of a type 1 response is important for protection against visceral infection caused by *L. donovani*, it may not be sufficient (Melby et al. 2001).

Some studies have shown that LACK protein or its gene is able to protect BALB/c against *L. major* infection (Gurunathan et al. 1997, 2000). Moreover, Ramiro et al. (2003) demonstrated that LACK is able to protect dogs against visceral infection caused by *L. infantum*. The differences in protection observed between our study and those on cutaneous leishmaniasis may relate to differences in the role of IL-4 in the pathogenesis of these two infections. Many studies have shown that protection in the visceral model is associated with a mixed Th1/Th2 pattern. Ghosh et al. (2002) 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. These studies suggest that, although protection in experimental visceral infection demands IFN-γ production response, IL-4 is also necessary in this model. This may be related to the role of IL-4 in visceral infection (Stager et al. 2003) or in the development of a T CD8 response (Carvalho et al. 2002). Stager et al. (2003) showed that IL-4 may be beneficial against *L. donovani* infection, and that mice knockout for the α chain of IL-4 receptor had a retardation of granuloma maturation and an increase in parasite load both in the liver and spleen. Also, Carvalho et al. (2002) showed that IL-4 is essential to the development of a T CD8 response against malaria liver stages.

Another important point is the challenge route used in our study. Natural infection is characterized by a low-dose infection with 100–1,000 metacyclic promastigotes of *Leishmania* and intradermal inoculation (Belkaid et al. 1998). The present study was done using the intravenous route, where the parasite gains direct access to the blood stream surpassing many immunological barriers present in the skin. Furthermore, studies with the murine model of visceral leishmaniasis have shown that the level of protection in vaccine approaches using the intravenous route is lower than the one observed in cutaneous leishmaniasis (Ahmed et al. 2003).

Another interesting point is the difference between parasite loads in vaccinated mice that were challenged 12 weeks after vaccination when compared to that in mice challenged after 4 weeks. We observed that mice challenged 12 weeks after i.m. vaccination with pCI-neo-LACK had a smaller parasite load in liver and spleen than mice challenged after 4 weeks. This decrease was also observed in the spleen of mice inoculated by i.m. route with pCI-neo. This finding was associated with a higher IFN-γ production by spleen cells in response to *L. chagasi* antigen in mice challenged at 12 weeks when compared to that in mice challenged at 4 weeks after vaccination, and a production of IL-4 in response to *Leishmania* antigen when compared to non-stimulated cells in mice challenged at 12 weeks. This phenomenon may be caused by the response induced by the p36(LACK) DNA vaccine associated with a change in cytokine production that happens in older mice. Humphreys and Grencis (2002) have shown that older mice (between 19 and 28 months old) are more susceptible to *Trichuris muris* infection than younger mice (3 months old), and this is associated with a higher IFN-γ and IL-12 production and a lower IL-4 and IL-5 production by lymph node cells. It is possible that the age difference due to the waiting period before the challenge infection may have influenced the cytokine pattern in response to *L. chagasi*, leading to a stronger type 1 response in older mice. Moreover, these data confirm the long-term immune response induced by the LACK DNA vaccine.

Recent data obtained by Uzonna et al. (2004) have shown that vaccination with *L. major* lpg²⁻ mutants is able to induce a long-term protection in BALB/c mice challenged with virulent *L. major*. This protection was not associated with a strong Th1 response but with a decreased IL-4 and IL-10 production in response to *Leishmania* antigen. IL-10 is induced in both experimental and human infection, and it is associated to a decrease in the secretion and macrophage responsiveness to activating Th1-cell-associated cytokines (Bogdan et al. 1991). These authors hypothesize that low levels of IFN-γ induced by a vaccine against *Leishmania* may be sufficient for protection in the absence of a strong IL-10 (or IL-4) response. In the present study, LACK DNA vaccine was able to induce an increase in IFN-γ production, but it is possible that this level of IFN-γ was not able to induce a high-macrophage activity against *L. chagasi* in the presence of high levels of IL-10.

Thus, this study demonstrates that i.m. vaccination with p36(LACK) DNA vaccine in a 25% sucrose solution is able to induce IFN-γ production by spleen cells from vaccinated mice, but it is not able to reduce parasite load in liver or spleen. This lack of protection might be related to the IL-10 induced by the vaccine. Furthermore, it is possible that the IFN-γ production induced by the vaccine could contribute to the response induced by a multicomponent vaccine. To this point, we are studying a vaccine protocol in which we associated p36(LACK) DNA vaccine with the *Leishmania amazonensis* promastigote extract, which is able to induce a high level of IL-4 in response to *L. chagasi* antigen (Vilela et al. 2002), to see if this combination protects against *L. chagasi* infection. Furthermore, since

LACK protein contains a single epitope (amino acids 156–173) that is able to induce IL-10 synthesis, another possibility is trying to vaccinate mice with a protein in which this epitope was removed.

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