Phage-fused epitopes from *Leishmania infantum* used as immunogenic vaccines confer partial protection against *Leishmania amazonensis* infection

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SUMMARY

Two mimotopes of *Leishmania infantum* identified by phage display were evaluated as vaccine candidates in BALB/c mice against *Leishmania amazonensis* infection. The epitope-based immunogens, namely B10 and C01, presented as phage-fused peptides; were used without association of a Th1 adjuvant, and they were administered isolated or in combination into animals. Both clones showed a specific production of interferon-gamma (IFN-γ), interleukin-12 (IL-12) and granulocyte/macrophage colony-stimulating factor (GM-CSF) after *in vitro* spleen cells stimulation, and they were able to induce a partial protection against infection. Significant reductions of parasite load in the infected footpads, liver, spleen, bone marrow and paws' draining lymph nodes were observed in the immunized mice, in comparison with the control groups (saline, saponin, wild-type and non-relevant clones). Protection was associated with an IL-12-dependent production of IFN-γ, mediated mainly by $CD8^+$ T cells, against parasite proteins. Protected mice also presented low levels of IL-4 and IL-10, as well as increased levels of parasite-specific IgG2a antibodies. The association of both clones resulted in an improved protection in relation to their individual use. More importantly, the absence of adjuvant did not diminish the cross-protective efficacy against *Leishmania* spp. infection. This study describes for the first time two epitope-based immunogens selected by phage display technology against *L. infantum* infected dogs sera, which induced a partial protection in BALB/c mice infected with *L. amazonensis*.

Key words: Phage display, mimotopes, vaccine, leishmaniasis, Th1 immune response.

INTRODUCTION

Leishmaniasis presents a high morbidity and mortality throughout the world, where about 350 million people in 98 countries are at risk of contracting the infection (WHO, 2010). Moreover, approximately 1·0–1·5 million new cases of tegumentary leishmaniasis (TL) and 200 000–500 000 new cases of visceral leishmaniasis (VL) have been registered annually (Alvar *et al.* 2012). Several geographical regions are endemic for different *Leishmania* spp. species, being this the case of the South America, where the disease is caused by at least eight different species of the parasites (Grimaldi and Tesh, 1993; Coelho *et al.* 2003; Reithinger *et al.* 2007). Among them, *Leishmania amazonensis*, presents particular importance, since it is able to cause human disease (Garcez *et al.* 2002), as well as a broad spectrum of clinical manifestations, from cutaneous to VL (Barral *et al.* 1991). The treatment of the disease is hampered due to the side effects registered in the patients, by increased parasite resistance and the high cost of conventional drugs (Croft and Coombs, 2003; Minodier and Parola, 2007). Recently, Bacon *et al.* (2013) evaluated the potential economic value of a cutaneous leishmaniasis (CL) vaccine in endemic countries localized in the Americas, and the study predicted that if a vaccine could provide at least 5 years of protection, then this would be less costly. Therefore, the development of improved strategies to prevent

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leishmaniasis could be considered a priority (Costa *et al*. 2011; Coelho *et al*. 2012).

The evidence of life-long immunity against infection with some *Leishmania* spp. species has inspired the development of prophylactic vaccination models, but few have progressed beyond the experimental stage (Fernandes *et al*. 2008; Chávez-Fumagalli *et al*. 2010; Modabber, 2010; Das and Ali, 2012; Ramírez *et al*. 2013, 2014; Costa *et al*. 2014). Using murine models, it has been showed that an important challenge for the development of an effective vaccine is to find a formulation able to induce a Th1-type immunity, based on the production of cytokines, such as IFN-γ, IL-12 and GM-CSF, produced by $CD4^+$ and/or $CD8^+$ T cells; as well as by controlling the disease-associated, IL-4-mediated, humoral response (mainly in TL models) and IL-10 deactivating response (Afonso and Scott, 1993; Noben-Trauth *et al*. 2003). From these experimental models, vaccine candidates such as whole parasite (Mizbani *et al*. 2009; Dey *et al*. 2013), parasite fractions (Rosa *et al*. 2007; Iborra *et al*. 2008; Chávez-Fumagalli *et al*. 2010), recombinant proteins (Coelho *et al*. 2003; Fernandes *et al*. 2008; Martins *et al*. 2013), poly-proteins (Bertholet *et al*. 2009; Goto *et al*. 2011), DNA-based vaccine (Zanin *et al*. 2007; Carrión *et al*. 2008) and synthetic peptides (Spitzer *et al*. 1999; Basu *et al*. 2007), have been shown to be immune stimulatory, and induce variable degrees of protection against some *Leishmania* spp. species. There are two commercial vaccines against CVL available at Brazil, Leishmune® (Borja-Cabrera *et al*. 2008) and Leish-Tec® (Fernandes *et al*. 2008), which are formulated with the nucleoside hydrolase and A2 proteins, respectively, associated with saponin, as adjuvant. In Europe, there is the CaniLeish® (Moreno *et al*. 2012), which is based on the LiESP/QA-21 association. Protein-based vaccines, although offering considerable advantages in terms of safety and production' costs, usually necessity of association of adjuvants to be effective (Cerpa-Cruz *et al*. 2013). Notwithstanding, the adjuvants regulation for human use is far more rigorous than those applied to veterinary vaccines. In addition to preclinical studies on the adjuvant itself, the combined antigen–adjuvant formulation also requires toxicological evaluation for entering phase 1 clinical trials (Goldenthal *et al*. 1993). Amongst the biggest regulatory hurdles is the required population size that needs to be tested to prove efficacy and particularly safety of a new adjuvant or vaccine.

Therefore, effective vaccine formulations that do not have adjuvants will have one less safety and regulatory hurdle in comparison with vaccines that include adjuvants (Bazan *et al*. 2012). An interesting approach towards the discovery of new products to be evaluated in biotechnological applications has been based on phage display technology (Clark and March, 2004). This technology is focused on DNA recombination, resulting in the expression of foreign peptide variants, namely mimotopes, on the outer surface of phage clones (Smith and Petrenko, 1997). Using an *in vitro* selection process, based on binding affinity, so-called bio-panning cycles; these mimotopes (that correspond to peptides that mimic linear, discontinuous and even non-peptide epitopes) exposed on surface of phages are selected, analysed by DNA sequencing and identified (Barbas *et al*. 2001; Wang and Yu, 2004). Phage display has been used to select mimotopes to be applied as vaccine candidates against cysticercosis (Manoutcharian *et al*. 2004), trichinellosis (Gu *et al*. 2008), VL (Costa *et al*. 2014) and Alzheimer's disease (Frenkel *et al*. 2000); as well as therapeutic agents (Manoutcharian *et al*. 2001).

Recently, our group reported the use of this technology to identify mimotopes that were highly reactive with sera from 100% of dogs infected by *L. infantum*. Two clones, B10 and C01, were selected and evaluated in vaccination experiments in BALB/c mice. Both immunogens, when associated with saponin, were able to induce a Th1 immune response before infection, which was based on the production of high levels of IFN-γ, IL-12 and GM-CSF; as well as low levels of IL-4 and IL-10 and, after challenge, they were protective against infection (Costa *et al*. 2014).

In the present study, these two phage clones were further tested as cross-protective immunogens against *L. amazonensis* infection, with and without added adjuvants, in order to evaluate the inherent immune-enhancing potential of the phage capsid, as has been previously described (García *et al*. 2005; Manoutcharian, 2005). The partial cross-protection induced by both clones against infection was demonstrated with and without saponin, suggesting that elicitation of the protective Th1 immune response was phage-dependent. Thus, we present evidence that a simple vaccine formulation composed of the combination of two epitope-based immunogens can induce partial protection against *L. amazonensis* infection.

MATERIALS AND METHODS

Mice

Female BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG); and were maintained under specific pathogen-free conditions. Experiments were performed in compliance with the Brazilian Guidelines for Animal Care (Law number 11 794, 2008), and under the approval of the Ethics Committee on the Handling of Research Animals from the UFMG, protocol number 043/2011.

Parasites

Experiments were carried out using the *L. amazonensis* (IFLA/BR/1967/PH-8) strain. Parasites were grown at 24 °C in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 20 mM L-glutamine, 200 U mL^{-1} penicillin and 100 $\mu g\ mL^{-1}$ streptomycin, at pH 7·4. The soluble *Leishmania* antigenic (SLA) extract was prepared from stationary-phase promastigotes, as described (Coelho *et al.* 2003). Briefly, 1×10^9 promastigotes of *L. amazonensis* per mL, in a volume of 5 mL, were washed three times in 5 mL of cold sterile phosphate-buffered saline (PBS). After seven cycles of freezing (−196 °C) and thawing (+37 °C), the suspension was centrifuged at 8000 ***g*** for 20 min at 4 °C, and the supernatant containing SLA was collected in 500 μL aliquots and stored at −80 °C, until use. The protein concentration was estimated by the Bradford method (Bradford, 1976).

Selection of the phage clones

The technical procedures used to select the B10 and C01 phage clones were performed as previously described (Costa *et al.* 2014).

Immunization and challenge infection

Seven groups of 8 BALB/c mice were inoculated into the left hind footpad with diluent (PBS); or immunized with 1×10^{11} non-adjuvanted bacteriophages of the parent wild-type clone (WTP), a non-relevant phage (NRP), the B10 clone, the C01 clone or a combination of the B10 and C01 clones (5×10^{10} phages, each) in equal proportions; or with 25 μg of saponin adjuvant (*Quillaja saponaria* bark saponin; Sigma 178 Aldrich) mixed with the combined B10 and C01 clones. Three doses were administered at 2-week intervals. Four weeks after the last immunization, animals ($n = 4$, per group) were euthanized to analyse the immune response elicited by vaccination. At the same time, the remaining animals were infected subcutaneously in their right hind footpad with 1×10^6 stationary-phase promastigotes of *L. amazonensis*. The course of the disease was monitored at weekly intervals by measuring footpad thickness with a metric calliper, and expressed as the increase in thickness of the infected footpad compared with the uninfected footpad. Ten weeks post-challenge, animals were euthanized and their sera samples, infected footpads and some organs were harvested for immunological analysis and parasite quantification. Experiments were repeated twice and presented similar results.

Estimation of parasite load

The infected footpad, liver, spleen, bone marrow (BM) and infected paws' draining lymph nodes (dLN) were collected for parasite quantification, using a limiting-dilution technique (Coelho *et al.* 2003). Briefly, tissue and organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris were removed by centrifugation at 150 ***g***, and cells were concentrated by centrifugation at 2000 ***g***. Pellets were resuspended in 1 mL of Schneider's insect medium supplemented with 20% FBS. Two hundred and twenty microlitres were plated onto 96-well flat-bottom microtitre plates (Nunc), and diluted in log-fold serial dilutions using the supplemented Schneider's medium, to a 10^{-1}–10^{-12} dilution. Each sample was plated in triplicate and read 7 days after the beginning of the cultures, at 24 °C. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Results are expressed as the negative log of the titre (i.e. the dilution corresponding to the last positive well) adjusted per microgram of tissue.

Cytokine response and nitric oxide (NO) production

Spleen cells cultures and cytokine assays were performed before infection and at 10th week after challenge, as described (Coelho *et al.* 2003). Briefly, single-cell suspensions from spleen tissue were plated in duplicate in 24-well plates (Nunc), at 5×10^6 cells mL^{-1}. Cells were incubated in RPMI 1640 medium (negative control), which was supplemented with 10% FBS, 20 mM L-glutamine, 200 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin, at pH 7·4; or separately stimulated with individual B10, C01, WTP or NRP clones (1×10^{11} phages, each one) or with SLA *L. amazonensis* (25 $\mu g\ mL^{-1}$); for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12 and GM-CSF levels were determined in the culture supernatants, using commercial kits (Pharmingen), according to manufacturer's instructions. In order to block IL-12, $CD4^+$ and $CD8^+$ mediated T cells cytokine release, spleen cells of mice vaccinated with B10/C01, added or not with saponin, and lately infected with *L. amazonensis*, were *in vitro* stimulated with SLA *L. amazonensis* (25 $\mu g\ mL^{-1}$), and incubated in the presence of 5 $\mu g\ mL^{-1}$ of monoclonal antibodies (mAb) against mouse IL-12 (C017·8), CD4 (GK 1·5) or mouse CD8 (53–6·7). Appropriate isotype-matched controls – rat IgG2a (R35-95) and rat IgG2b (95-1) – were employed in the assays. Antibodies (no azide/low endotoxin) were purchased from BD (Pharmingen).

The NO production was evaluated in all groups, at 10th week after challenge, for which 100 μL of SLA-stimulated culture supernatants were mixed with an equal volume of Griess reagent (Sigma). After 30-min incubation at room temperature, nitrite concentration was calculated using a standard curve of known concentrations. Data were expressed as μM per 5×10^6 cells.

Analysis of the humoral response

The B10- and C01-specific IgG1 and IgG2a antibodies, as well as SLA *L. amazonensis*-specific IgG1 and IgG2a antibodies were measured by an enzyme-linked immunosorbent assay (ELISA) technique. Briefly, previous titration curves were performed to determine the appropriate antigens concentration and antibodies dilution. Microtitre immunoassay plates (Biofil®, Belo Horizonte, Minas Gerais, Brazil) were coated with either B10 or C01 clone (1×10^{10} phages per well) or with SLA *L. amazonensis* (1·0 μg per well) diluted in 100 μL coating buffer (50 mM carbonate buffer, pH 9·6) for 18 h at 4 °C. After incubation, free binding sites were blocked using 200 μL of TBS-T (50 mM Tris–HCl pH 7·5, 150 mM NaCl and 0·1% Tween 20) buffer containing 5% casein for 1 h at 37 °C. After having been washing three times using PBS-T, the plates were incubated with sera samples diluted 1:100 in TBS-T containing 0·5% casein solution for 1 h at 37 °C. Then, plates were washed seven times using TBS-T, and incubated with the peroxidase-labelled antibodies specific to mouse IgG1 or IgG2a isotypes (Sigma-Aldrich) diluted at 1:5000 and 1:10 000, respectively; and incubated for 1 h at 37 °C. Plates were again washed seven more times with TBS-T, and the reaction was developed through incubation with 2 mg orto-phenylenediamine, 2 μL H_2O_2 (30 vol.) and 10 mL citrate-phosphate buffer (pH 5·0), for 30 min and in the dark. The reaction was stopped by adding 25 μL H_2SO_4 2 N, and optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada), at 492 nm.

Statistical analysis

Results were entered into Microsoft Excel (version 10.0), and analysed using GraphPad Prism™ (version 6.0 for Windows). Results were expressed by mean ± standard deviation (S.D.) of the groups. Statistical analysis with the data from vaccinated and/or infected mice was performed by one-way analysis of variance (ANOVA), using Tukey's post-test for comparisons among groups. Differences were considered significant with $P < 0{\cdot}05$. Data shown in this study are representative of two independent vaccination experiments, which presented similar results.

RESULTS

Immunogenicity of selected phage clones in BALB/c mice

Two phage clones (B10 and C01) isolated by their antigenicity in CVL, and tested as protective against *L. infantum* (Costa *et al.* 2014), were analysed for their protective role against *L. amazonensis* infection. Clones were administered independently in the absence of any adjuvant and in combination, in this case, associated or not with saponin. The immunogenicity of the clones was evaluated in the immunized mice 4 weeks after the last vaccine dose (Fig. 1). Spleen cells' cultures derived from vaccinated mice with the B10 and/or C01 clones significantly secreted higher levels of IFN-γ and IL-12 than cells from control groups (WTP, NRP and saline) after specific stimuli with each phage clone. In addition, very low levels of IL-4 and IL-10 were observed in all experimental groups, after stimulation with specific clones employed in the vaccination regimens (Fig. 1A). Also, spleen cells cultured from the saline group were separately stimulated with the individual clones; however, no significant cytokines production was observed (data not shown). The ratios between the IL-12/IL-4 and IL-12/IL-10 levels (Fig. 1B), as well as between the IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig. 1C), were calculated and the results showed that vaccinated animals with B10, C01, B10/C01 and B10/C01/saponin were able to induce a specific Th1 response before infection, when the specific phage-stimulus was employed in culture cells. The association between B10 and C01 clones was able to increase these cytokines' ratios. In addition, when the humoral response was evaluated, mice vaccinated with B10 and/or C01 clones presented higher predominance of the phage-specific IgG2a isotype in comparison with the phage-specific IgG1 levels (Fig. 1D), corroborating with the higher production of IFN-γ and lower levels of IL-4 in these vaccinate animals, representing a typical and specific Th1 immune response mounted in these vaccinated animals.

Protective efficacy of the phage clones against L. amazonensis

The protective effect of immunization of BALB/c mice with the B10 and C01 clones against *L. amazonensis* infection was evaluated by measuring lesion development in infected footpads, as well as by determination of the parasite burden in infected tissues and some organs of the animals (Fig. 2). Animals vaccinated with B10, C01, B10/C01 and B10/C01/saponin presented partial protection and significant reductions in oedema in the infected footpads when compared with control groups (saline, WTP and NRP). The reduction in the lesion development observed in these groups correlated with a 3 mm decrease in the infected footpad swellings, when compared with control groups (Fig. 2A). No significant differences were observed in lesion development among immunized groups with B10, C01, B10/C01 or B10/C01/saponin, corroborated by the diminished footpad swellings and parasite burden in the infected tissue in comparison with the

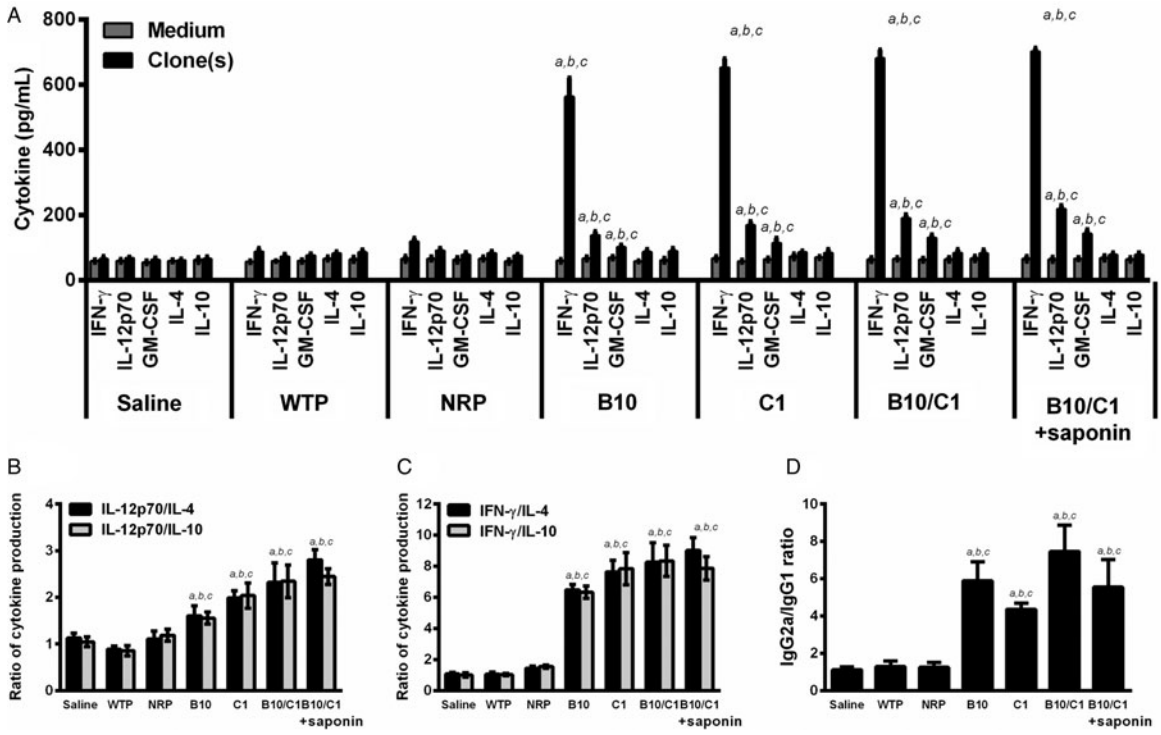

Fig. 1. Immune response induced in BALB/c mice by immunization with B10 and/or C01 phage clones. Single cells suspensions were obtained from the spleen of mice, 4 weeks after the last immunization. Cells were non-stimulated (negative control), or separately stimulated with the B10, C01, wild-type (WTP) or NRP clones (1×10^{11} phages, each one), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured in culture supernatants by capture ELISA (A). Each bar represents the mean ± S.D. of the different groups. The ratios between the IL-12/IL-10 and IL-12/IL-4 levels (B), as well as between the IFN-γ/IL-10 and IFN-γ/IL-4 levels (C); are showed. Also, the ratio between the levels of specific phage-IgG2a and IgG1 antibodies were calculated, and are shown (D). (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0{\cdot}001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0{\cdot}001$).

control groups (Fig. 2B). Also, vaccinated mice with B10 and/or C01 phage clones presented significant reductions in the number of parasites in the liver, spleen, dLN and BM in comparison with control groups (saline, WTP or NRP) (Fig. 3). On the other hand, the combination of B10 and C01 clones was able to induce a better protection against infection, when compared with the use of individual clones. The employ of saponin as adjuvant to the B10/C01 vaccine did not induce any significant alteration in the vaccine efficacy, when compared with the group of animals immunized with both clones, but without the association of this adjuvant.

Cellular response elicited after L. amazonensis *infection*

The production of cytokines in the supernatants of spleen cell cultures stimulated with specific phages or SLA *L. amazonensis*, 10 weeks after infection, was analysed in order to evaluate the immunological correlates of protection induced by previous immunization (Fig. 4). The spleen cells derived from mice vaccinated with B10, C01, B10/C01 or B10/C01/saponin produced higher levels of phages- and SLA-specific IFN-γ, IL-12 and GM-CSF than did those secreted by spleen cells from control groups (saline, WTP and NRP). In contrast, the phages- and SLA-driven production of IL-4 and IL-10 showed that vaccination with both clones induced no significant production of these cytokines in the vaccinated and infected animals (Fig. 4A). The ratios between the IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig. 4B), as well as between the IL-12/IL-4 and IL-12/IL-10 levels (Fig. 4C) obtained after the SLA *L. amazonensis* stimulus were calculated, and showed that vaccinated and infected animals mounted a typical Th1 immune response against parasites, which has possibly contributed to the partial protection observed against the challenge.

The involvement of $CD4^+$ and $CD8^+$ T cells, as well as the dependence of IL-12 production for the SLA *L. amazonensis*-specific IFN-γ response from the spleen cells of mice immunized with B10/C01 or B10/C01/saponin, and lately infected with *L. amazonensis*, was evaluated (Fig. 5). The IFN-γ production was significantly suppressed using the

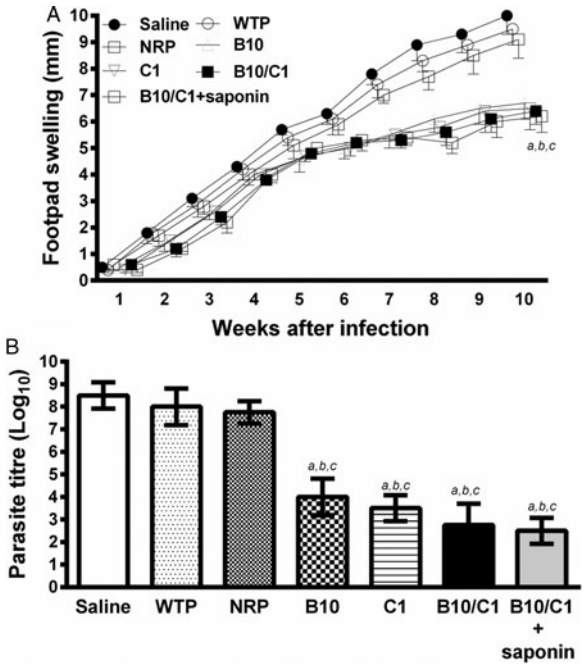

Fig. 2. Protection of BALB/c mice vaccinated with B10 and C01 phage clones against *Leishmania amazonensis* infection. Mice were inoculated with saline or immunized with wild-type (WTP), non-relevant (NRP), B10 or C01 clones (1×10^{11} phages, each one), or with the association between B10 and C01 clones (5×10^{10} phages, each), with or without 25 μg of saponin (*Quillaja saponaria* bark saponin; Sigma-Aldrich). Three doses were administered at 2-week intervals, and 4 weeks after the last immunization; animals were subcutaneously infected with 1×10^{6} stationary-phase promastigotes of *L. amazonensis*. The course of the disease was monitored at weekly intervals and expressed as the increase in thickness of the infected footpad compared with the uninfected footpad (A). The number of parasites in the infected footpads was also measured (B), 10 weeks after challenge, by a limiting-dilution technique. Each bar represents the mean ± S.D. of the different groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0{\cdot}001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0{\cdot}001$).

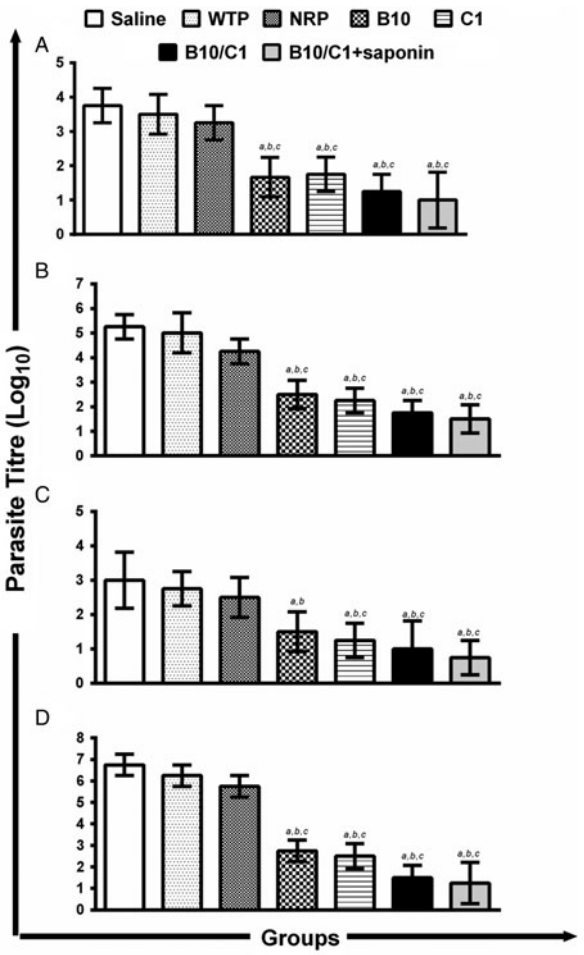

Fig. 3. Parasite burden in different organs of the immunized animals with B10 and C01 phage clones, and challenged with *Leishmania amazonensis*. Mice were inoculated with saline or immunized with wild-type (WTP), non-relevant (NRP), B10 or C01 clones (1×10^{11} phages, each one) or with the association between B10 and C01 clones (5×10^{10} phages, each), with or without 25 μg of saponin (*Quillaja saponaria* bark saponin; Sigma-Aldrich). After the immunization schedules, animals were infected with 1×10^{6} stationary-phase promastigotes of *L. amazonensis*, and 10 weeks after challenge, the parasite burden was determinate in the liver (A), spleen (B), bone marrow (C) and infected paws' draining lymph nodes (D), by a limiting-dilution technique. Each bar represents the mean ± standard deviation (S.D.) of the different groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0{\cdot}001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0{\cdot}001$).

anti-CD8 monoclonal antibody in the spleen cell cultures in both B10/C01 (Fig. 5A) and B10/C01/saponin (Fig. 5B) groups. On the other hand, the addition of anti-CD4 or anti-IL-12 antibodies to the cultures also decreased the production of this cytokine when compared with the control cells culture without treatment; however, this production proved to be greater than that occurred by use of anti-CD8^{+} monoclonal antibody (Fig. 5).

Humoral response and nitrite production after infection

Evaluating the humoral response induced after challenge, it was also possible to observe that mice vaccinated with B10, C01, B10/C01 or B10/C01/saponin and infected presented a significant predominance of SLA *L. amazonensis*-specific IgG2a antibodies, which was significantly higher than the observed SLA-specific IgG1 levels. The ratio between IgG2a and IgG1 levels (Fig. 6A) corroborated with the Th1 profile found in the vaccinated and protected animals.

In an attempt to evaluate the influence of the phage clones on the *L. amazonensis* specific killing

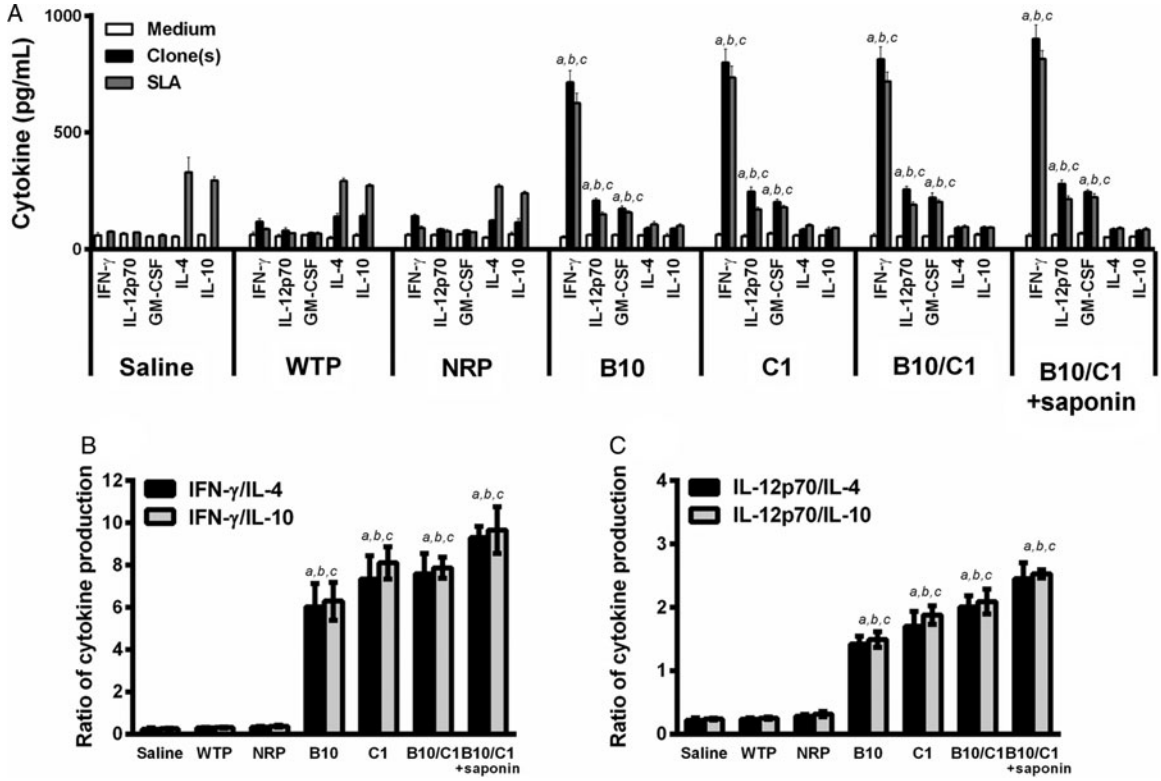

Fig. 4. Analysis of the cellular response in BALB/c mice after *Leishmania amazonensis* infection. Mice were inoculated with saline or immunized with wild-type (WTP), non-relevant (NRP), B10 or C01 clones (1×10^{11} phages, each one), or with the association between B10 and C01 clones (5×10^{10} phages, each), with or without 25 μg of saponin (*Quillaja saponaria* bark saponin; Sigma-Aldrich). Four weeks after the last immunization, animals were subcutaneously infected with 1×10^6 stationary-phase promastigotes of *L. amazonensis*, and they were monitored until 10 weeks after challenge. Then single-cell suspensions were obtained from the spleens of animals, and they were non-stimulated (negative control), or separately stimulated with SLA (25 μg mL^{-1}), or with the B10, C01, WTP or NRP clones (1×10^{11} phages, each one); for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured in the culture supernatants by capture ELISA. Each bar represents the mean ± S.D. of the different groups (A). The ratios between the IFN-γ/IL-10 and IFN-γ/IL-4 levels (B), as well as between the IL-12/IL-10 and IL-12/IL-4 levels (C); are shown. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0{\cdot}001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0{\cdot}001$).

effectors functions in the spleen of vaccinated and infected mice; the nitrite concentration was evaluated as an indicator of NO production in the spleen cell cultures, using either specific phage- or SLA-stimulus (Fig. 6B). The nitrite production was significantly higher in mice vaccinated with the B10, C01, B10/C01 and B10/C01/saponin after stimulation with phages or SLA, when compared with the control groups (saline, WTP and NRP).

DISCUSSION

We have recently identified novel antigens based on a subtractive phage display strategy, represented by *L. infantum* mimotopes fused to the phage capsid, which were strongly reactive against positive sera from VL-affected dogs and their immunoprotective role was investigated. The subtraction against sera from non-infected animals, followed by a positive selection against asymptomatic and symptomatic VL dogs, led to the selection of two immunodominant antigens, represented by the phage clones B10 and C01, which were successfully employed in the partial protection of BALB/c mice against *L. infantum* infection (Costa *et al.* 2014). Due to their immunodominance and protective activity, we hypothesized that such immunogens could be cross-protective against other *Leishmania* spp. species, including those causing American TL. Interestingly, besides proving that such mimotopes could protect against *L. amazonensis*, we also showed that a simple vaccine formulation is possible just by using the bacteriophage as immunogenic carrier, which was able to generate antibodies against the two recombinant peptides displayed at the amino terminus of phage coat proteins without the need of additional adjuvants.

Two experimental controls were used in this study: the wild-type phage (WTP) and a non-relevant peptide (NRP) fused to the phage capsid. The WTP is the same filamentous bacteriophage derived from the Ph.D.-C7C Phage Display Peptide Library Kit, without exogenous peptides fused to the pIII

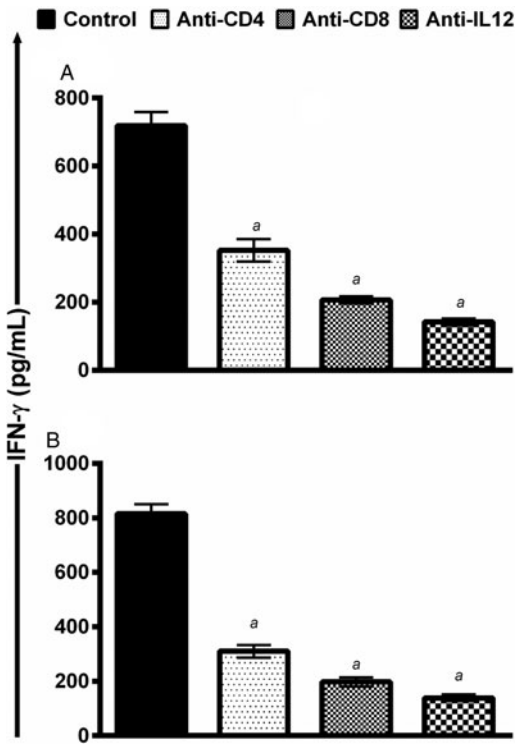

Fig. 5. Involvement of $CD4^+$ and $CD8^+$ T cells, as well as IL-12, in the IFN-γ production after challenge infection. Single-cells suspensions were obtained from the spleens of mice that were immunized with B10/C01 (A) or B10/C01/saponin (B), at 10 weeks after infection. Levels of IFN-γ were measured in the culture supernatants by capture ELISA of spleen cells cultures stimulated with SLA (25 $\mu g\ mL^{-1}$), for 48 h at 37 °C in 5% CO_2. Cultures were incubated in the absence (positive control) or in the presence of 5 $\mu g\ mL^{-1}$ of mAb against mouse IL-12 (C017·8), CD4 (GK 1·5) or mouse CD8 (53–6·7). Each bar represents the mean ± S.D. of the different groups. (*a*) indicate statistically significant differences between non-treated control cells and cultures incubated with anti-CD4, anti-CD8 or anti-IL-12 mAb ($P < 0{\cdot}001$).

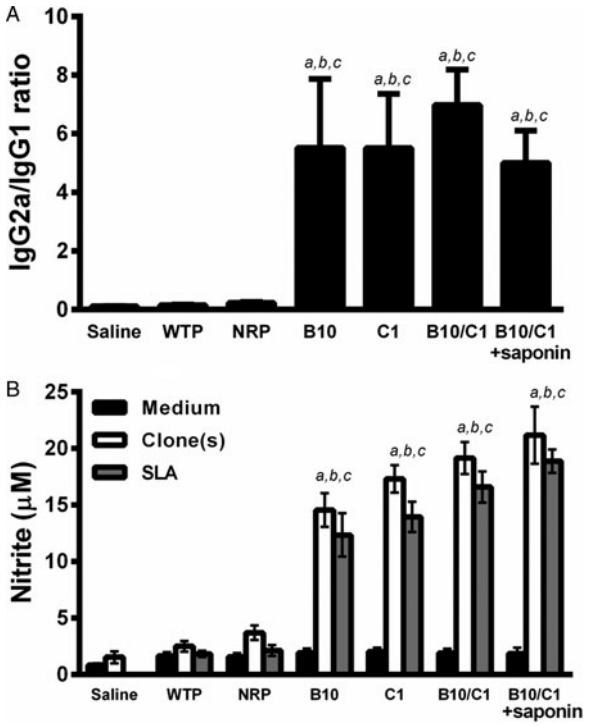

Fig. 6. Humoral response and nitrite production in BALB/c mice after *Leishmania amazonensis* infection. Sera samples of the immunized and infected animals were collected, at 10 weeks after infection. The levels of SLA *L. amazonensis*-specific IgG1 and IgG2a antibodies levels were determined, and a ratio between the values (IgG2a/IgG1) was calculated (A). The NO production was also evaluated in all groups, at 10 weeks after challenge; when single-cell suspensions were obtained from the spleens of animals, and they were non-stimulated (negative control), or separately stimulated with SLA (25 $\mu g\ mL^{-1}$), or with the B10, C01, WTP or NRP clones (1×10^{11} phages, each one); for 48 h at 37 °C in 5% CO_2. Then, 100 μL of the individual culture supernatants were mixed with an equal volume of Griess reagent. After an incubation of 30 min at room temperature, nitrite concentration was calculated using a standard curve of known concentrations (B). Each bar represents the mean ± S.D. of the different groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the WTP group ($P < 0{\cdot}001$). (*c*) indicates statistically significant difference in relation to the NRP group ($P < 0{\cdot}001$).

protein. The WTP was used to demonstrate the specific immune activation in the host, and how this could interfere in the target response. The NRP was used as a mimotope control, in order to verify if the partial protection induced by clones was due to the specific mimotopes. In the results, no protection was observed in both controls, confirming that the foreign antigens exposed in the selected phage particles were the main responsible to partial protection observed in this study.

Despite the fact that the science behind the treatment of infectious diseases through vaccination was first shown by Edward Jenner more than two centuries ago (Riedel, 2005), and that use of vaccine in treatment of diseases is widespread now, there are still problems and limitations in preparing vaccines by traditional means. The approach used in the present study can be considered as a new recombinant technology employed to select vaccine candidates against *Leishmania* spp. species, and it could be well considered as a tool to perform other studies aiming to produce better vaccines against diseases (O'Hagan and Rappuoli, 2004). In this context, for instance, the phage display technology has proven to be useful in selecting antigens based on exposed foreign peptides on phages, either using their own clones or their synthetic mimotopes, to be experimentally evaluated to protect against Burkitt's lymphoma (Hardy and Raiter, 2005), melanoma (Wagner *et al.* 2005), colorectal cancer (Coomber and Ward, 2001), hepatitis B virus (Wan *et al.* 2001), VL (Costa *et al.* 2014) and rotavirus (Van der Vaart *et al.* 2006).

Whole phage particles possess many intrinsic characteristics that make them ideal as vaccine delivery vehicles. Phage vaccines are cheap and can be easily produced on a large scale. Phages are highly stable and are even stable within the pH range 3·0–11·0, over a 24 h period. Because the displayed protein is fused with a coat protein, it is not susceptible to nuclease degradation under the protective protein matrix (Gao *et al*. 2010). The safety issue of phage vaccine is an important matter and should be outlined. Phage vaccines have been shown to be safe in mice (Hashemi *et al*. 2010), pigs (Gamage *et al*. 2009) and humans (Bruttin *et al*. 2005). Hence, phages themselves may act as strong adjuvants and thus enhance excellent immune responses against any antigens present along with phage (Frenkel *et al*. 2000; Manoutcharian *et al*. 2004). Additionally, vaccination with phage particles also induces a highly immunogenic signal against phage coat protein, which provides an easily detectable marker to confirm the vaccination effect in animals (Margonari *et al*. 2006).

The B10 and C01 phages evaluated in the present study are expressed fused to pIII proteins of the phage structure. The minor coat protein pIII is presented in five copies per virion, of which all five can be fused to short peptides, without interfering in its infectivity. In contrast, the major coat protein pVIII is presented as 2700 copies per virion, of which 10% can be reliably fused to peptides. As a result, peptides expressed as pIII fusions are present at lower valency, whereas pVIII fusions are present at higher valency (Gu *et al*. 2008). Therefore, one could speculate that the partial protection found here could be improved by using a construction based on the hybrid phage fusion of B10 and C01 epitopes to the pVIII molecules from filamentous phages. In addition, the incorporation of other immunogenic mimotopes could be considered also as a strategy to develop a phage-displayed multi-epitope-based vaccine protective to a wider range of *Leishmania* spp. species.

There is an emerging trend in experimental vaccinology that focuses more on multi-strain or multi-species rather than strain-specific vaccines (Hamad, 2011). The concept of universal vaccines is chiefly based on the presence of common antigens among pathogens and on the ability of properly formulated vaccines to elicit cross-protective adaptive immunity. Support for antigenic commonality among pathogens derives from both indirect and direct evidence (Barbour and Restrepo, 2000; Kyes *et al*. 2007). In this sense, genomics is fast expanding the list of common proteins among organisms (Pizza *et al*. 2000; Kanduc *et al*. 2008; Real *et al*. 2013), and has proven to be useful in selecting common antigens for vaccine development against diseases, such as brucellosis (Cherwonogrodzky *et al*. 2014), salmonellosis (Heithoff *et al*. 2015) and encephalitis (Lobigs and Diamond, 2012). Since multiple *Leishmania* spp. species are distributed in the same or adjacent geographical regions (Duthie *et al*. 2012), it would be desirable to develop vaccines containing candidates capable of inducing protection against the infection caused by different parasite species. Thus, to be effective as a human vaccine against leishmaniasis its components should be shared by different parasite species and, prior to its use in humans, the protective efficacy of these candidates should be analysed in different models of experimental leishmaniasis (Coelho *et al*. 2003; Chávez-Fumagalli *et al*. 2010; Nico *et al*. 2014).

In murine leishmaniasis, the development of an IL-4 or IL-10-mediated immune response inhibits the protective effects from the IFN-γ response, which may be well related to the deactivation of macrophages and the onset of the disease in infected animals (Gumy *et al*. 2004). In studies evaluating vaccine candidates against leishmaniasis, immunogens are usually administered in mice and, after only a few weeks, they are infected and followed up for a couple of months. In this time, spleen cells are collected and cultured *in vitro* with the antigens used in the immunization process, and/or with *Leishmania* spp. extracts, in order to evaluate their immunogenicity. In this point, cytokines, such as IFN-γ and IL-12, markers of a Th1 response, and IL-4 and IL-10, indicators of a Th2 response, have their levels determined and, together with the results of the parasite burden, the efficacy of immunogens is evaluated (Martins *et al*. 2013). Thus, antigens capable of stimulating the development of a Th1 response, based on the production of high levels of IFN-γ and IL-12, could be considered a promising candidate for use against *Leishmania* spp. In the present study, the immunization using the B10 and/or C01 clones was able to induce a robust and phage-specific Th1 immune response in the immunized animals, which was primed by high levels of IFN-γ, IL-12, combined with the presence of low levels of IL-4 and IL-10. After infection, these animals, when compared with the controls, including the wild-type and non-relevant clones; displayed significant reductions in the parasite burden in the infected footpad, liver, spleen, BM and dLN, which was correlated with a higher production of *Leishmania*-specific IFN-γ by spleen cells. Additionally, spleen cells also produced higher levels of IL-12 and GM-CSF when compared with the control groups, and GM-CSF is the last cytokine related to macrophage activation and resistance in murine models against infection with some *Leishmania* spp. species, such as *L. infantum* (Chávez-Fumagalli *et al*. 2010; Costa *et al*. 2014), *Leishmania major* (Dumas *et al*. 2003) and *Leishmania donovani* (Murray *et al*. 1995). The NO production was also evaluated in the spleen cell cultures, and showed that the protected animals

produced higher levels of NO when compared with the control groups, demonstrating the possible activation of these cells by a NO-dependent mechanism.

Evaluating the profile of T cells involved on the IFN-γ production in the protected animals, the $CD8^+$ T cells proved to be an important source of IFN-γ in the vaccinated and infected animals. Depletion of these cells in cultures of spleen cells stimulated with SLA has significantly reduced this production. Although previous reports have shown that the activation of both $CD4^+$ and $CD8^+$ T cells subsets may be important for the killing of parasites in mice vaccinated with different parasite recombinant antigens (Pitcovsky *et al*. 2001; Cunha-Júnior *et al*. 2010; Martins *et al*. 2013), the data of the present study suggested that $CD4^+$ T cells presented a lower influence in the induction of IFN-γ-mediated response elicited by vaccination with B10 and C01 phage clones. The immunization using isolated B10 and C01 phages induced partial protection against infection, but the association between clones was more effective in cross-protect the infected animals, inducing a more polarized Th1 response and followed by significant reductions in the parasite load in the infected animals.

This study also demonstrates that the partial protection of mice against *L. amazonensis* was associated with a significant reduction of IL-4 and IL-10 levels. Very low levels of IL-4 and IL-10 were detected after the stimulation of spleen cells derived from vaccinated and infected mice. On the other hand, spleen cells from control mice showed a significantly higher production of these cytokines. In the evaluation of the humoral response, usually, in *L. amazonensis*-infected BALB/c mice, the IL-4-dependent production of IgG1 antibodies is associated with the progression and severity of disease; whereas IFN-γ is related to the production of IgG2a isotype, and with protection against infection (Coelho *et al*. 2003; Chávez-Fumagalli *et al*. 2010). Here, immunized mice with phage clones that were protected against infection presented higher levels of SLA-specific IgG2a antibodies, as compared with IgG1 levels; correlating with the development of a Th1 immune response observed in these animals. On the other hand, animals of the control groups; which were not protect against infection, showed high levels of *L. amazonensis*-specific IgG1 antibodies.

A limiting condition of the present study could be considered the fact that immunized animals were not infected lately of 4 weeks after the last immunization. However, others have also showed a partial protection in their studies evaluating different vaccine candidates against VL or TL, using the same period of time evaluated in the present study (Iborra *et al*. 2008; Chávez-Fumagalli *et al*. 2010; Costa *et al*. 2014; Nico *et al*. 2014). Clearly, a vaccine able to induce a long protection would be desirable, but the main purpose of the present study was to present mimotopes that were selected in a viscerotropic *Leishmania* spp. species as being able to confer a partial cross-protective efficacy against a *Leishmania* spp. species able to causes American TL. Also, the present study could be considered a proof-of-concept about the efficacy of these mimotopes to be employed in a vaccine composition to protect against VL, once the number of animals evaluated in each experimental group could be considered low. However, since the level of cytokines, the antibody reactivities and the evolution of the disease have been homogeneous in all the components from each analysed group, it could be postulated that the vaccine can be also effective against a higher evaluated population.

A critical aspect for *Leishmania* vaccine development refers to the pre-clinical model chosen for the initial screening of vaccine candidates. Although sand fly transmitted infection in hamsters more closely resemble the natural transmission and the human disease, this infection model requires specific laboratory conditions and trained personnel staff, which are not widely available, hindering its general use as a first step for testing vaccine efficacy against VL (Gomes *et al*. 2008). In contrast, BALB/c mice infected with *L. donovani* or *L. infantum* is one the most widely studied murine model of VL, and is therefore naturally selected over other models for this purpose (Afrin *et al*. 2000; Coelho *et al*. 2003; Carrión *et al*. 2006). Murine models have also allowed the characterization of the immune mechanisms required to develop organ-specific immune response against different *Leishmania* spp. species (Requena *et al*. 2004). Therefore, the evaluation of the parasite burden in different organs is an important marker of vaccine efficacy against VL in these models. Nevertheless, additional studies may well be carried out in order to extend the observations present herein of the protective effect of the phage clones vaccination to other infection models and experimental conditions.

In conclusion, we have shown that a simple vaccine formulation based on phage-fused peptides could be well used to induce partial protection in BALB/c mice against *L. amazonensis* infection. This effect was correlated by a $CD8^+$ T cells response, which was based on the production of high levels of IFN-γ, IL-12, GM-CSF and NO; as well as low levels of IL-4, IL-10 and antileishmanial IgG1-isotype antibodies. The combination of these phage clones was able to improve the vaccine efficacy against *L. amazonensis*, and this result has led us to the conclusion that these antigens may be well used as vaccine candidates to, alone or in combination to other immunogens, be evaluated against *Leishmania* spp. infections. Additional studies are under way in order to identify the native proteins that express these target mimotopes.

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