Mimotope-Based Vaccines of *Leishmania infantum* Antigens and Their Protective Efficacy against Visceral Leishmaniasis

Louarena Emanuele Costa¹, Luiz Ricardo Goulart²,³,⁴, Nathália Cristina de Jesus Pereira¹, Mayara Ingrid Sousa Lima², Mariana Costa Duarte¹, Vivian Tamietti Martins⁴, Paula Sousa Lage¹, Daniel Menezes-Souza⁵, Tatiana Gomes Ribeiro⁶, Maria Norma Melo⁵, Ana Paula Fernandes⁷, Manuel Soto⁸, Carlos Alberto Pereira Tavares⁴, Miguel Angel Chávez-Fumagalli¹, Eduardo Antonio Ferraz Coelho¹,⁹*¹

¹Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, ²Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil, ³Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, United States of America, ⁴Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, ⁵Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, ⁶Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, ⁷Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, ⁸Centro de Biologia Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain, ⁹Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

**Abstract**

**Background:** The development of cost-effective prophylactic strategies to prevent leishmaniasis has become a high-priority. The present study has used the phage display technology to identify new immunogens, which were evaluated as vaccines in the murine model of visceral leishmaniasis (VL). Epitope-based immunogens, represented by phage-fused peptides that mimic *Leishmania infantum* antigens, were selected according to their affinity to antibodies from asymptomatic and symptomatic VL dogs’ sera.

**Methodology/Main Findings:** Twenty phage clones were selected after three selection cycles, and were evaluated by means of *in vitro* assays of the immune stimulation of spleen cells derived from naive and chronically infected with *L. infantum* BALB/c mice. Clones that were able to induce specific Th1 immune response, represented by high levels of IFN-γ and low levels of IL-4 were selected, and based on their selectivity and specificity, two clones, namely B10 and C01, were further employed in the vaccination protocols. BALB/c mice vaccinated with clones plus saponin showed both a high and specific production of IFN-γ, IL-12, and GM-CSF after *in vitro* stimulation with individual clones or *L. infantum* extracts. Additionally, these animals, when compared to control groups (saline, saponin, wild-type phage plus saponin, or non-relevant phage clone plus saponin), showed significant reductions in the parasite burden in the liver, spleen, bone marrow, and paws’ draining lymph nodes. Protection was associated with an IL-12-dependent production of IFN-γ, mainly by CD8⁺ T cells, against parasite proteins. These animals also presented decreased parasite-mediated IL-4 and IL-10 responses, and increased levels of parasite-specific IgG2a antibodies.

**Conclusions/Significance:** This study describes two phage clones that mimic *L. infantum* antigens, which were directly used as immunogens in vaccines and presented Th1-type immune responses, and that significantly reduced the parasite burden. This is the first study that describes phage-displayed peptides as successful immunogens in vaccine formulations against VL.

**Citation:** Costa LE, Goulart LR, Pereira NCdJ, Lima MIS, Duarte MC, et al. (2014) Mimotope-Based Vaccines of *Leishmania infantum* Antigens and Their Protective Efficacy against Visceral Leishmaniasis. PLoS ONE 9(10): e110014. doi:10.1371/journal.pone.0110014

**Editor:** Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil

**Received** May 27, 2014; **Accepted** September 5, 2014; **Published** October 15, 2014

**Copyright:** © 2014 Costa et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

**Funding:** This work was supported by grants from Pró-Reitoria de Pesquisa from UFMG (Edital 01/2014), Instituto Nacional de Ciência e Tecnologia em Nanobiofarmacêutica (INCT-Nanobiofar), FAPEMIG (PRONEX APQ-0101909, CBB-APQ-00496-11 and CBB-APQ-00819-12), CAPES (Rede Nanobiotec/Brasil) and CNPq (APQ-472090/2011-9 and APQ-482976/2012-8). MACF is a grant recipient of FAPEMIG/CAPES. EAFc and LRG are grant recipient of CNPq. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

⁹ Email: eduardoferrazcoelho@yahoo.com.br

* LRG and EAFc are co-senior authors on this work.
**Introduction**

Leishmaniasis is a disease with a wide spectrum of clinical manifestations caused by different species of protozoa belonging to the *Leishmania* genus [1]. The disease presents a high morbidity and mortality worldwide, with 350 million people in 98 countries at risk of contracting the infection. Moreover, approximately 1.0 to 1.5 million cases of cutaneous leishmaniasis (CL), and 200,000 to 500,000 cases of visceral leishmaniasis (VL), are registered annually [2,3]. Canine visceral leishmaniasis (CVL) due to *Leishmania infantum* is a major global zoonosis that is potentially fatal to dogs [4]. This disease can be found in Southern Europe, Africa, Asia, and Central and South America; and is endemic in approximately 70 countries worldwide [5]. CVL is expanding its geographic distribution throughout the Western hemisphere, where it now occurs from northern Argentina to the United States [6], even reaching as far as provinces of southern Canada [7]. This disease is also an important concern in non-endemic countries, where imported sick or infected dogs constitute a veterinary and public health problem [6,7].

The treatment of leishmaniasis is still based on the use of the parenteral administration of pentavalent antimonials; however, several side effects reported by patients, and increased parasite resistance have produced serious problems [8,9]. Therefore, the development of new strategies to prevent leishmaniasis has become a high priority [10].

The evidence of life-long immunity has inspired the development of vaccination protocols against the disease, but few have progressed beyond the experimental stage. Some studies have demonstrated that the Type-1 cells mediated immunity is important to a protective response against CL [11–15]. In addition, Th1 cells response has also been correlated with the protection against VL [16]. In this context, the protective immunity in murine VL primarily depends on an IL-12-driven Th1 cell response, leading to an IFN-γ and IL-2 production. Substantial up regulation of inducible NO synthase by IFN-γ generates NO from splenic and liver cells, thereby controlling parasite replication in these organs [17,18]. By contrast, TGF-β, IL-10, and IL-13 represent major disease promoting cytokines, in turn leading to the suppression of the Th1 response [18]. Low levels of IL-4 can enhance vaccine-induced protection by indirectly increasing IFN-γ production by Th1 cells [19]. Thus, antigens that are capable of stimulating the development of a Th1 immune response in murine models, primed by the production of cytokines, such as IFN-γ and IL-12, in *in vitro* spleen cell cultures, could be considered promising vaccine candidates for use against *Leishmania* infection [20,21].

One interesting approach toward the discovery of new antigens with biotechnological applications has been based on phage display technology [22]. This technique is based on DNA recombination, resulting in the expression of foreign peptide variants on the outer surface of phages. Using an *in vitro* selection process, based on binding affinity and so-called bio-panning cycles, peptides exposed in the selected phage clones are analyzed by DNA sequencing and identified [23–25]. Phage display has been used to identify mimotopes (peptides that mimic linear, discontinuous, and even non-peptide epitopes [26]) to be applied in the diagnosis of diseases, including malaria [27–29], leishmaniasis [30], toxoplasmosis [31,32], and Chagas disease [33]; as well as employed as vaccine candidates in the cysticercosis [34], trichinellosis [35], and Alzheimers disease [36].

In the present study, phage display was used to identify mimotopes expressed on a foreign surface of phages, to be applied as vaccine candidates against *L. infantum*. Clones from a phage library were selected by antibodies present in sera samples of dogs with asymptomatic and symptomatic VL, and they were used for the *in vitro* immune stimulation of spleen cells obtained from naïve or chronically infected with *L. infantum* BALB/c mice; in an attempt to select those able to stimulate a higher production of INF-γ and lower levels of IL-4. Two phage clones presenting the best results of specificity and selectivity were selected and evaluated in vaccination experiments in BALB/c mice. Both clones, namely B10 and C01, were able to induce a Th1 immune response in the vaccinated mice, and were protective against *L. infantum* infection. In this context, these two antigens, identified by a non-described study using phage display and leishmaniasis; could be considered candidates to compose an effective vaccine against VL.

**Materials and Methods**

**Ethics Statement**

Experiments were performed in compliance with the National Guidelines, as set forth by the Institutional Animal Care (Law number 11.794, 2008), and the Committee on the Ethical Handling of Research Animals from the Federal University of Minas Gerais (UFMG), who approved this study under protocol number 043/2011. In addition, the owners of the domestic dogs (*Canis familiaris*) gave permission for their animals to be used in this study.

**Mice and parasites**

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), UFMG; and were maintained under specific pathogen-free conditions. Experiments were carried out using the *L. infantum* (MOM/BR/1970/BH46) strain. Parasites were grown at 24°C in Schneider’s medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.4. The soluble *Leishmania* antigenic (SLA) extract was prepared from stationary promastigotes of *L. infantum*, after few passages in liquid culture, as described [11]. Briefly, 2×10⁸ promastigotes per mL, in a volume of 5 mL, were washed 3 times in 5 mL of cold sterile phosphate-buffered saline (PBS). After five cycles of freezing and thawing, the suspension was centrifuged at 8,000 x g for 20 min at 4°C, and the supernatant containing SLA was collected in 500 μL aliquots and stored at −70°C, until use. The protein concentration was estimated by Bradford method [37].

**Sera samples**

The sample size used in this study consisted of 60 domestic dogs, made up of males and females of different breeds and ages, collected from an endemic area of Belo Horizonte, Minas Gerais, Brazil. Sera of CVL were selected on the basis of two serological tests (IFAT [IFAT-LVC Bio-Manguinhos kit] and ELISA [EIE-LVC Bio-Manguinhos kit], both from Bio-Manguinhos, Fiocruz, Brazil) for *Leishmania spp.* Dogs with an IFAT titre <1/40 or ELISA reactivity below the cut-off value indicated by the manufacturer were considered to be seronegative. Animals with an IFAT titre > 1/40 and an ELISA value over the cut-off were considered to be seropositive. Thus, symptomatic dogs (n = 20) were those positive by IFAT and ELISA, but also with positive parasitological results by PCR-RFLP (restriction fragment length polymorphism), as well as presenting more than three clinical symptoms (weight loss, alopecia, adenopathy, onychogryposis, etc.).
hepatomegaly, conjunctivitis, and exfoliative dermatitis on the nose, tail, and ear tips). Asymptomatic dogs (n = 20) presented positive serological (IFAT and ELISA) and parasitological (PCR-RFLP) results, but they did not present any clinical signals or symptoms of leishmaniasis. Healthy dogs (n = 20) were selected from an endemic area of Belo Horizonte, but they presented negative serological and parasitological results, as well as were free of any clinical signs or symptoms of leishmaniasis.

Purification of antibodies by coupling in protein G microspheres

In order to obtain microspheres incorporated with IgG molecules derived from different sera groups, a purification was performed using the coupling to magnetic microspheres (beads) conjugated to protein G (Dynabeads, Invitrogen). For this, 2 × 10^9 microspheres were prepared by washing 3 times with 1 mL of 0.1 M MES buffer pH 5.0, been this procedure repeated for three times. After this, the recovered phage clones were transferred to a new tube containing the IgGs purified from asymptomatic CVL, and the process was repeated for three times. After this, the recovered phage clones were transferred to a new tube containing the IgGs that had been purified from asymptomatic CVL, and the process was also repeated for three times, when the selected clones reactive to IgGs from asymptomatic and symptomatic sera of CVL were then recovered and titrated.

Titration of phage clones

Phage clones were diluted 10^{-1} to 10^{-11} in 500 μL of sterile TBS buffer, mixed with an *Escherichia coli* culture (OD<sub>600nm</sub> ~ 0.5), and plated on LB agar plates containing 1 mL of a IPTG/N-gal solution (composed by 200 mg/mL isopropyl β-D-L-thiogalactopyranoside and 20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside, diluted in dimethyl sulfoxide PA). Colonies were individually quantified, and the titration was performed for each bio-panning cycle. After the 3rd round of positive selection using IgGs from CVL sera, 96 colonies selected from the plate were added in 200 μL of LB (Luria Bertani) medium in a sterile culture plate (BD Falcon TM clear, 96-well microtest TM plate), then the plate was sealed and incubated for 5 h, under constant stirring, at 37°C. After incubation, the plate was centrifuged for 20 min by 2,250xg, and the supernatant was transferred to a new plate, where a PEG/NaCl solution (20% PEG 8,000 and 2.5 M NaCl) was added (1/6 of the total volume of supernatant), and the plate was incubated for 16 h at 4°C. After the plate was centrifuged for 1 h, the supernatant was removed, and the pellet was resuspended in 500 μL of a solution composed by 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 4 M NaCl. The plate was shaken vigorously for 5 min, when 250 μL of a 70% ethanol solution was added. After having been incubated for 10 min, the plate was centrifuged (2,250xg at 4°C for 10 min), and the supernatant was discarded. The pellet containing the DNA of interest of each clone was washed with 500 μL of 70% ethanol, and again centrifuged. Finally, the DNA was diluted in 20 μL of ultra-pure water, and its quality was evaluated in a 1% agarose gel, which was stained with an etidium bromide solution (10 μg mL⁻¹). The individual DNA of clones was used for the sequencing and identification of target peptides.

DNA sequencing and bioinformatics

The sequencing reaction was performed using 500 ng of DNA for each selected phage clone, 5 pmol primer 96 gII (5’-OH CC TCA TAG TTA GCG TAA CG-3’, Biolabs), plus a pre-mix (Dye Terminator Cycle ET Journal Kit, Amersham Biosciences). Thirty-five cycles were performed in a thermocycler, under the following conditions: denaturation at 95°C for 20 sec, ringing at 58°C for 15 sec, and extension at 60°C for 60 sec. Ten microliters of the generated amplicons were precipitated with 1 μL of ammonium acetate (1:10 ratio), added with 27.5 μL of ethanol PA. The plate was centrifuged for 45 min to 2,432xg, when the supernatant was discarded and 150 μL of 70% ethanol was added to the pellet. The resuspended DNA was centrifuged for 10 min at 2,432xg, and the supernatant was discarded again. The plate was inverted on a paper towel and, in this position, was centrifuged at 486xg for 1 min. Next, the plate was covered for 5 min, until the complete evaporation of the remaining ethanol had been achieved. The pellet was resuspended in dilution buffer, and the sequencing was performed in a MegaBace 1000 automatic sequencer (Amersham Biosciences). Peptide sequences of 20 valid
phage clones were deduced using the Expasy server (www.expasy.org), and analyzed with the Peptide [38], MimoDB [39] and SAROTUP [40] programs to identify possible false-positive sequences.

Evaluation of the in vitro immune stimulation

BALB/c mice (n = 8) were subcutaneously infected with 1×10^7 stationary phase promastigotes of *L. infantum*, and they were monitored by 10 weeks. Liver, spleen, paws draining lymph nodes (dLN), and bone marrow (BM) of the animals were collected for parasite quantification, following a limiting-dilution technique [11]. The parasite load was performed to confirm that the animals were chronically infected (data not shown). After this, spleen cells were collected, pooled and in vitro cultured in 24-well plates (Nunc, Nunclon, Roskilde, Denmark), at 5×10^6 cells per mL, in duplicate. Cells were incubated in RPMI 1640 medium (Sigma-Aldrich; non-stimulated control), or separately stimulated with SLA (20 μg/mL-1), or individual phage clones (1×10^10 phages per mL), at 37°C in 5% CO₂ for 48 h. The same experimental conditions were performed using spleen cells of naive mice. Wild-type and random non-specific phage clones were used like controls. The IFN-γ and IL-4 levels were determined in the culture supernatants, using commercial kits (Pharmingen, according to manufacturer instructions. In order to block IL-12, CD4, and GM-CSF levels were determined in the culture supernats, using commercial kits (Pharmingen), according to manufacturer instructions. Experiments were repeated twice and presented similar results.

Immunization and challenge infection

After the in vitro immunogenicity experiments, two from 20 phage clones were selected, namely B10 and C01, based on their polarized Th1 immune response, and were used in the in vivo experiments. For this, BALB/c mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with two selected clones or wild-type phage (1×10^11 phages, per each one), associated with 25 μg of saponin (Quillaja saponaria bark saponin; Sigma Aldrich), only adjuvant or received the diluent (saline). Three doses were administered at 2-week intervals. Four weeks after the last immunization, animals (n = 4, per group) were euthanized and/or infected mice was performed by one-way D’Agostino & Pearson test. Statistical analysis of the results of the immunized and/or infected mice was performed by one-way analysis of variance (ANOVA), using Tukey's post-test for multiple comparisons between the groups. Differences were considered significant with P < 0.05. Data showed in this study are representative of two independent experiments, which presented similar results.

**Cytokine production**

Splenocytes cultures and cytokine assays were performed before infection and at 10th week after challenge, as described [11]. Briefly, single-cell preparations from spleen tissue were plated in duplicate in 24-well plates (Nunc), at 5×10^6 cells per mL. Cells were incubated in RPMI 1640 medium (background control), or separately stimulated with SLA (20 μg/mL-1), or individual phage clones (1×10^10 phages, per each one), at 37°C in 5% CO₂ for 48 h. The 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 instructions. In order to block IL-12, CD4, and CD8 mediated T cells cytokine release, spleen cells of mice vaccinated and infected were in vitro stimulated with SLA (20 μg/mL-1), and incubated in the presence of 5 μg/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).

**Analysis of the humoral response**

SLA-specific IgG1 and IgG2a antibodies were measured by ELISA, as described elsewhere [11]. Briefly, previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Falcon flexible microrot immunosay plates (Becton Dickinson) were coated with SLA (1.0 μg per well) in 100 μL coating buffer (50 mM carbonate buffer) pH 9.6 for 18 h, at 4°C. After this, free binding sites were blocked using 200 μL of TBS-T buffer containing 5% casein for 1 h, at 37°C. After washing the plates 7 times using TBS-T, they were incubated with 100 μL of individual sera for 1 h at 37°C. Samples were diluted 1:100 in TBS-T containing 0.5% casein solution. Plates were washed 7 times using TBS-T, and incubated with the peroxidase-labeled antibodies specific to mouse IgG1 or IgG2a isotypes (Sigma Aldrich) diluted at 1:5,000, and incubated for 1 h at 37°C. After washing 7 times with TBS-T, the reaction was developed through incubation with H₂O₂, o-toluidine, and citrate-phosphate buffer pH 5.0, for 30 min in the dark. The reaction was stopped by adding 25 μL H₂SO₄ 2 N, and optical density was read in an ELISA microplate spectrophotometer, at 492 nm.

**Statistical analysis**

The statistical analysis was made using the GraphPad Prism software (version 6.0 for Windows). Results were expressed by mean ± standard deviation (SD) of the groups. Outliers were evaluated using ROUT test, and excluded from statistical analyses. The normality analysis of the data was performed using the D’Agostino & Pearson test. Statistical analysis of the results of the immunized and/or infected mice was performed by one-way analysis of variance (ANOVA), using Tukey’s post-test for multiple comparisons between the groups. Differences were considered significant with P < 0.05. Data showed in this study are representative of two independent experiments, which presented similar results.

**Results**

**Selection of the phage clones**

First, the phage clones selection was performed by excluding those that were recognized by antibodies present in sera samples of healthy dogs. After, a positive selection process was performed, by recovering the phage clones that were recognized by antibodies present in the sera samples of dogs with asymptomatic and
symptomatic VL. The technical protocol is summarized in the Figure 1. Approximately 96 clones were randomly selected from individual colonies, and their DNA sequences were amplified by PCR and sequenced. Twenty clones had their sequences clearly identified (Table 1), and an alignment showed that no identical consensus motif could be detected between them (data not shown). In addition, none of the 20 sequences selected were identified as previously described sequences, or as non-specific binders to the commons reagents used in the bio-panning cycles (data not shown).

Spleen cells derived from naive or chronically infected with *L. infantum* BALB/c mice were collected, pooled and separately stimulated with each one of the 20 phage clones, at which time the IFN-γ and IL-4 levels were determined in the cultures supernatants. A wild-type phage clone, identical to the phage clones present in the library, but that did not express foreign exogenous mimotopes; a random non-specific to *Leishmania* phage clone (NMSDFLRQLQRS), and non-stimulated cells (medium, background) were used as controls (Supplementary Figure). To select the phage clones that presented the best results of immune stimulation, their specificity and selectivity were calculated, comparing the results obtained after the stimulation of spleen cells with the values obtained from wild-type and non-relevant phages. The specificity was defined as the ability of a clone to bind to its target, based on the presence of mimotopes displayed on its surface; whereas the selectivity was defined as the ability of a clone to identify its cognate target in a mixture of different targets [41]. Initially, the specificity was calculated by dividing the IFN-γ and IL-4 values obtained of each evaluated individual clone by respective values of these cytokines obtained after the wild-type phage stimulus, using spleen cells derived from naive mice. Then, with the corrected values, a ratio between IFN-γ and IL-4 using these new values was calculated, and the generated number was defined as the specificity of clone. In the same way, the selectivity was calculated by dividing the IFN-γ and IL-4 values obtained of each evaluated individual clone by respective values of these cytokines obtained after the non-relevant phage stimulus, using spleen cells derived from infected mice. Then, with the corrected values, a ratio between IFN-γ and IL-4 using these new values was calculated, and the generated number was defined as the selectivity of clone (Fig. 2). The phage clones that presented a ratio $\geq 1.0$ were selected, being this case observed with the B10 and C01 phages; in this context, these molecules were evaluated as vaccine candidates against *L. infantum* infection.

**Immunogenicity of the selected phage clones**

The immunogenicity of B10 and C01 clones was evaluated in BALB/c mice, four weeks after the last vaccine dose (Fig. 3). After having undergone *in vitro* immune stimulation, spleen cells from vaccinated mice significantly produced high levels of IFN-γ and IL-12 than did those secreted by spleen cells from all control groups. No significant production of IL-4 and IL-10 could be

**Figure 1. Schedule of technical protocol used in the bio-panning cycles of phage display.**
doi:10.1371/journal.pone.0110014.g001
observed in any experimental groups after stimulation with individual clones (Fig. 3A). The ratios between IL-12/IL-4 and IL-12/IL-10 (Fig. 3B), and between IFN-\(\gamma\)/IL-4 and IFN-\(\gamma\)/IL-10 (Fig. 3C) showed that vaccinated animals with B10/saponin or C01/saponin presented an elevated Th1 immune response after receiving a specific phage-stimulus. In addition, mice vaccinated presented a humoral response with a higher predominance of SLA-specific IgG2a isotype, in comparison to the IgG1 levels (Fig. 3D).

**Protective efficacy of the phage clones against *L. infantum***

Next, this study analyzed whether the immunization with the B10 or C01 clones plus saponin was in fact able to induce protection against *L. infantum*. After the three vaccine doses, the infection was performed and the animals were followed-up over a 10-weeks period, at which time the parasite burden in the liver, spleen, dLN, and BM was determined. Significant reductions in the number of parasites were observed in all of the evaluated organs of the vaccinated mice, as compared to those that received saline, or that were immunized with the wild-type phage clone plus saponin (Fig. 4). Vaccinated mice with B10 or C01 clones plus saponin, as compared to the saline group, as an infection control; presented significant reductions in the parasite load in the liver (2.5- and 5.3-log reductions, respectively; Fig. 4A), spleen (2.8- and 4.4-log reductions, respectively; Fig. 4B), dLN (2.5- and 4.0-log reductions; respectively, Fig. 4C), and BM (2.5- and 5.3-log reductions, respectively; Fig. 4D). In the same way, immunized mice with B10 or C01 clones plus saponin, as compared to the wild-type phage clone plus saponin group, as a phage control; showed significant reductions in the parasite load in the liver (2.0- and 4.2-log reductions, respectively), spleen (2.3- and 3.6-log reductions, respectively), dLN (1.9- and 3.0-log reductions; respectively), and BM (1.9- and 4.0-log reductions, respectively). In both cases, the results showed an effective protection induced by B10 and C01 phage clones against the *Leishmania* infection, which could be represented by exposed mimotopes in their foreign surface, mainly leading in count by their absence in the wild-type phage clone.

**Cellular response elicited after *L. infantum* infection**

The production of cytokines in the supernatants of spleen cell cultures stimulated with SLA, 10 weeks after infection, was analyzed to evaluate the immunological correlates of protection induced by previous immunization. The spleen cells derived from mice vaccinated with B10 or C01 clones plus saponin produced Table 1. Amino acid sequences from target peptides present in the selected phage clones.

| Clone | Sequence |
|-------|----------|
| A10   | LLSSKTL  |
| B10   | LSFPFPG  |
| C01   | FTFSFSPY |
| C02   | QATHFS   |
| D09   | LASLPFR  |
| E03   | TMFSWI   |
| E10   | ACDPSPN  |
| E11   | TPSLRHS  |
| F01   | TAMARSA  |
| F03   | VALLPHH  |
| F04   | QSPPALL  |
| F08   | FSLGGL   |
| F09   | VLLPFFP  |
| F11   | YFPLSLH  |
| G02   | SLGPQIK  |
| G05   | MSPTLYL  |
| G09   | DRAALSL  |
| G11   | ALTPQLL  |
| G12   | QTPSSPLA |
| H05   | FPFLGLS  |

doi:10.1371/journal.pone.0110014.t001

Figure 2. Evaluation of the selectivity and specificity of the selected phage clones. Spleen cells suspensions were obtained from naive or chronically infected with *L. infantum* BALB/c mice. Cells were non-stimulated (medium; background control), or separately stimulated with each phage clone, as well as the wild-type and non-relevant clones (1x10^10 phages, each one) for 48 h at 37°C, 5% CO_2. IFN-\(\gamma\) and IL-4 levels were measured in culture supernatants by capture ELISA. The black circles indicate the specificity of the phage clones, which was calculated by dividing the IFN-\(\gamma\) and IL-4 values obtained of each evaluated individual clone by respective values of these cytokines obtained after the wild-type phage stimulus, using spleen cells derived from naive mice. With the corrected values, the ratio between IFN-\(\gamma\) and IL-4 with these new values was calculated, and the specificity of the each clone was defined. The white circles indicate the selectivity, which was calculated by dividing the IFN-\(\gamma\) and IL-4 values obtained of each evaluated individual clone by respective values of these cytokines obtained after the non-relevant phage stimulus, using spleen cells derived from infected mice. With the corrected values, a ratio between IFN-\(\gamma\) and IL-4 using these new values was calculated, and the selectivity of each clone was defined.

doi:10.1371/journal.pone.0110014.g002
higher levels of SLA-specific IFN-γ, IL-12, and GM-CSF, than did those secreted by spleen cells from control groups (Fig. 5A). In contrast, the SLA-driven production of IL-4 and IL-10 showed that vaccination with both phage clones induced no production of these cytokines in vaccinated and infected animals. In this context, the ratios between IL-12/IL-4 and IL-12/IL-10 (Fig. 5B), and between IFN-γ/IL-12 and IFN-γ/IL-4 levels (Fig. 5C), indicated that vaccinated mice developed a Th1 response, which was maintained after infection. It was also possible to observe that vaccinated and infected mice presented a predominance of SLA-specific IgG2a antibodies, which was significantly higher than the IgG1 levels (Fig. 5D). The contribution of CD4+ and CD8+ T cells, as well as the dependence of IL-12 production for the SLA-specific IFN-γ response from the spleen cells of mice immunized with B10 or C01 phage clones plus saponin and infected with *L. infantum*, was also evaluated (Fig. 6). The IFN-γ production was significantly suppressed using an anti-CD8+ monoclonal antibody in the spleen cell cultures. The addition of anti-CD4+ or anti-IL-12 antibodies to the cultures also decreased the production of this cytokine, as compared to the control cells culture without treatment; however, the production of this cytokine proved to be greater than that produced by the use of the anti-CD8+ monoclonal antibody.

**Discussion**

In a recent study, our group developed a subtractive phage display strategy that led to the identification of novel phage antigens, represented by specific mimotopes, which were applied to a sensitive and specific serodiagnosis of CVL [30]. In the present study, phage display was applied to the identification of antigens based on phage clones that could be evaluated in murine models, such as vaccines against VL. The selected phages expressing the target mimotopes were selected after bio-panning cycles, which had been performed using a negative selection process, employing sera samples from healthy dogs, as well as by a positive selection process, based on the use of sera samples from dogs with asymptomatic and symptomatic VL.

Figure 3. Cellular and humoral response induced in BALB/c mice by immunization with B10 or C01 phage clones plus saponin. Single cells suspensions were obtained from the spleen of mice, four weeks after the last immunization. Cells were non-stimulated (medium; background control), or separately stimulated with individual clones (1 × 10^11 phages per mL, each one) for 48 h at 37°C, 5% CO2. 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 ± standard deviation (SD) of the groups. Statistically significant differences in the IFN-γ and IL-12 levels between the B10 or C01 clone plus saponin groups and control mice (saline, saponin and wild-type phage plus saponin groups) were observed (***P < 0.0001). The ratio between IL-12/IL-10 and IL-12/IL-4 levels (B), and between IFN-γ/IL-10 and IFN-γ/IL-4 levels (C) are also showed. Statistically significant differences in the ratios between the B10 or C01 clone plus saponin groups and control groups were also observed (***P < 0.0001). The ratio between the *Leishmania*-specific IgG1 and IgG2a antibodies was obtained from the different groups, and statistically significant differences between the B10 or C01 clone plus saponin and control groups were observed (***P < 0.0001) (D).

doi:10.1371/journal.pone.0110014.g003
It has been proposed that parasite antigens that are recognizable by antibodies present in sera of dogs with asymptomatic VL may be well-associated with a protective response, and could represent potential vaccine candidates against leishmaniasis [42]. Here, the selected phage clones were used for in vitro immune stimulation experiments using spleen cells derived from naive or chronically infected with L. infantum BALB/c mice. Two clones were selected based on their ability of inducing a specific and selective higher production of IFN-γ, which was associated with low levels of IL-4. These clones were then evaluated in BALB/c mice, according to their ability to induce protection against L. infantum infection.

In the present study, spleen cells derived from naive or chronically infected BALB/c mice were used in the selection of the phage clones. Murine models have been used widely for the development of vaccines against VL [20,43]. Studies employing the mouse model have led to the characterization of the immune mechanisms needed to develop organ specific immune responses, which would clear the parasites from the liver, but not from the spleen [43]. BALB/c mice infected with L. donovani or L. chagasi/ L. infantum are the most widely studied models of VL, but they are also considered to be rather vulnerable, given that the infection progresses during the first two weeks and become chronic. In the murine VL, C57BL/6 mice mount early Th2 response, which results in a non-healing lesion and an exaggeration of the disease [45]. In addition, the respective resistance and susceptibility of C57BL/6 and BALB/c strains depend not only on the Th1 and Th2 types of immune responses from CD4⁺ T cells, but also on the genetic background of the host [46]. In this manner, the immune response, following the infection of inbred mouse strains with viscerotropic Leishmania species, such as L. donovani and L. infantum; can be considered to be similar to those observed in the L. major mouse model. In this context, in the present study, spleen cells derived from naive and chronically infected with a high inoculum of L. infantum BALB/c mice were used to the in vitro immune stimulation experiments, in order to select only the candidate antigens able to subvert the undesirable Th2 profile in a mouse strain highly susceptible to L. infantum infection.

Additional studies may well be carried out in order to extend the observations present herein of the protective efficacy of the B10 and C01 phage clones plus saponin vaccination to other mouse models and/or experimental conditions.

Phage display, since its first description [47] and introduction into laboratory practice [48], has proven to be useful in selecting antigens derived from phages, either using the phage clone itself or the synthetic mimotopes, for vaccine development against diseases, such as Burkitt’s lymphoma [49], melanoma [50], colorectal cancer [51], hepatitis B virus [52], and rotavirus [53]; or against
pathogens, such as Vibrio cholera [54], Candida albicans [55], and Brugia malayi [56].

One could speculate that a major requirement for a successful vaccine will be based not only on the most effective way of inducing immunity, but also on the technical feasibility of its production [57]. Phage-displayed peptides as candidates possess two main advantages: first, peptides exposed in filamentous phages are antigenic and immunogenic. As very potent carriers, phages can be taken up and processed efficiently by mechanism of major histocompatibility complex class I and II pathways [58,59]. Second, the amplification of phage is much simpler and inexpensive than the conventional route of peptide synthesis or production of recombinant proteins, besides of the final product is composed by a high number of virion copies, reflecting in a high exposition of the mimotopes to the immune system of the immunized hosts. Also, phages can replicate inside the phagocyte cells, and taken more time to be eliminated of the organism, in comparison to use of synthetic peptides or recombinant proteins, as well as they are not pathogenic to humans [60,61]. Other important difference is based on that the immune stimulatory non-methylated CpG motifs, present in the phage genome, can contribute to the activation of the immune system of the mammal hosts, through Toll-like receptors [62,63], reducing the necessity of employ of immune adjuvants that are necessary to be incorporated together to synthetic peptides and recombinant proteins.

On the other hand, some factors could be considered to improve the protection observed in the present study using the B10 and C01 phage clones. In this context, these phages were screened from a Ph.D.-C7C Phage Display Peptide Library Kit, which displayed peptides fused to the pIII protein of them. Minor coat protein pIII is presented at five copies per virion, of which all five can be fused to short peptides without interfering in the phage infectivity. In contrast, major coat protein pVIII is presented at 2,700 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 [61]. Therefore, protection observed in this study could be improved performing a construction of hybrid phage fusions of B10 and C01 epitopes to the pVIII of the filamentous phages. In addition, the incorporation of other protective epitopes could be considered an efficient strategy to develop a more protective

Figure 5. Analysis of the cellular and humoral response in BALB/c mice after L. infantum challenge infection. BALB/c mice (n = 8, per group) received saline or were immunized subcutaneously in their left hind footpad with 25 µg of saponin, and with B10, C01 or wild-type phage clones (1 × 10^{11} phages, each one), associated with saponin. Three doses were administrated at 2-week intervals, and four weeks after the last immunization, the animals (n = 4) were infected subcutaneously in their right hind footpad with 1 × 10^7 stationary promastigotes of L. infantum, and were followed for 10 weeks. Single-cells suspensions were obtained from the spleens of mice in this time, and were non-stimulated (medium; background control), or stimulated with SLA (20 µg mL^{-1}) for 48 h at 37 C, 5% CO_2. Levels of IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 were measured in culture supernatants by capture ELISA. Each bar represents the mean ± standard deviation (SD) of the cytokines levels of the different groups (A). Statistically significant differences between the B10 and C01 clones plus saponin and control groups (salmie, saponin and wild-type phage plus saponin groups) were observed (**P < 0.0001). The ratio between IL-12/IL-10 and IFN-γ/IL-4 levels (B), and between IFN-γ/IL-10 and IFN-γ/IL-4 levels (C), are also showed. Statistically significant differences between the B10 and C01 clones plus saponin and control groups were observed (**P < 0.0001). The ratio between SLA-specific IgG1 and IgG2a antibodies levels were calculated and statistically significant differences between the B10 and C01 clones groups and the saline and saponin were also observed (P < 0.005) (D). doi:10.1371/journal.pone.0110014.g005
Although the induction of a life-long protection against reinfection, after recovering from a primary challenge, demonstrates that a vaccine can be achieved, an effective candidate did not exist. Some antigens, such as GP63, GP46, LACK, CPB, CPA, A2, PSA-2, HASPB1, LeIF, and LCR1, have been tested like recombinant proteins, synthetic peptides, and/or DNA vaccines against leishmaniasis, but they have induced only partial protection [69–71]. Recently, to solve this problem, the use of remodeled live vaccines has become attractive, presenting notable arguments concerning immunogenicity and efficacy [69,72,73]. The main disadvantage of such vaccines may well be based on the difficulty of their large-scale production, as well as due to their difficult standardization [70].

The present study described, for the first time, the employ of the phage display technology to select antigens to be used as vaccines against *L. infantum*, in a known murine model. The immunization using B10 or C01 phage clones associated with saponin was able to induce a specific Th1 response in vaccinated BALB/c mice, which was characterized by an *in vitro* phages-specific production of IFN-γ, IL-12, and GM-CSF, combined with the presence of low levels of IL-4 and IL-10; as well as a predominance of SLA-specific IgG2a antibodies. After infection, immunized mice, as compared to the controls, including phage (wild-type phage) and mimotope (non-relevant phage) controls, displayed significant reductions in the parasite burden in all evaluated organs, which was correlated with a higher production of IFN-γ by their spleen cells; one of the main cytokines implicated in the protective immunity against *Leishmania* [28,29,31].

As regards the profile of T cells involved on this production, CD8<sup>+</sup> T cells proved to be the major source of IFN-γ in the protected mice, since the depletion of these cells in the cultures of spleen cells stimulated with SLA, significantly nullified this response. Although previous reports have shown that the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> cells subsets may be important for the killing of parasites in vaccinated mice using several parasite recombinant antigens [15,32,33], the present study’s data suggest that CD4<sup>+</sup> cells may contribute to a lesser extent to the induction of an IFN-γ mediated response elicited by immunization using B10 and C01 clones; being that the CD8<sup>+</sup> T cells were the main resource of IFN-γ, and these cells could directly contribute to infection control through their direct cytotoxic effect.

Figure 6. Involvement of IL-12, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the IFN-γ production after *L. infantum* infection. Single-cells suspensions were obtained from the spleens of mice that were immunized with B10 clone plus saponin (A) or C01 clone plus saponin (B), 10 weeks after *L. infantum* infection. Levels of IFN-γ were measured in culture supernatants by capture ELISA of spleen cells cultures stimulated with SLA (20 μg mL<sup>−1</sup>) for 48 h at 37°C, 5% CO<sub>2</sub>. Cultures were incubated in the absence (positive control) or in the presence of 5 μg mL<sup>−1</sup> of monoclonal antibodies (mAb) against mouse IL-12 (C017.8), CD4 (GK 1.5), or mouse CD8 (53-6.7). Statistically significant differences between non-treated control cells and cultures incubated with anti-CD4, anti-CD-8 and anti-IL-12 monoclonal antibodies were observed (**P <0.0001). Each bar represents the mean ± standard deviation (SD) of the IFN-γ levels of the different groups.

doi:10.1371/journal.pone.0110014.g006

Phage Display against Visceral Leishmaniasis

Usually, in studies using the phage display technology applied to select vaccine [64–66], or diagnosis [30,67] candidates to diseases; the employed controls in the *in vivo* experiments are the own phage molecules that not express the foreign peptides, namely as wild-type phage. Normally, this control is derived of phage display library used in the selection process. In the present study, the wild-type phage used as a control was a filamentous phage derived from Ph.D.-C7C Phage Display Peptide Library Kit, which did not displayed exogenous peptides fused to the pIII protein, unlike to the other clones employed in the selection. This fact could be considered relevant, once the own phage particles present proteins that can interact with the immune system of the hosts, leading to the development of an immune response specific to them, and interfering in the immune system response specific to the target, in this case, to the selected phage-displayed mimotopes.

In consequence, one could speculate that the higher or lower obtained protection in the case, to the selected phage-displayed mimotopes. In consequence, the immunogens are usually administered in mice associated with difficulties of their large-scale production, as well as due to their difficulty of their large-scale production, as well as due to their difficulty of their large-scale production. The main disadvantage of such vaccines may well be based on the difficulty of their large-scale production, as well as due to their difficulty of their large-scale production. The main disadvantage of such vaccines may well be based on the difficulty of their large-scale production, as well as due to their difficulty of their large-scale production.
The present study also demonstrates that the protection of BALB/c mice was associated with a significant decrease in the production of IL-4 and IL-10. Very low levels of parasite-specific IL-10 were detected after the stimulation of spleen cells derived from vaccinated mice, 10 weeks after infection. By contrast, spleen cells from both control mice showed a significantly higher production of this cytokine. Indeed, control of the parasite-mediated IL-10 response may be critical for protection, since this cytokine is considered to be the most important factor for VL progression after infection with visceralotropic Leishmania species in IL-10 deficient mice [74,75], or in mice treated with an anti-IL-10 receptor antibody [76]. In BALB/c mice, the IL-4-dependent production of IgG1 antibodies is associated with disease progression into some Leishmania species, including L. amazonensis [77], and L. infantum [78]. Here, immunized mice with the phage clones presented higher levels of SLA-specific IgG2a antibodies, as compared to IgG1 levels, also demonstrating the development of a Th1 immune response in these animals.

Like observed to the IFN-γ and IL-12 levels, spleen cells from vaccinated mice, as compared to the control groups, produced higher levels of clones-specific GM-CSF, a cytokine related to macrophage activation and resistance in murine models against intracellular pathogens, such as L. infantum [77], L. major [79], and L. donovani [80]. It has also been reported that the administration of a therapeutic vaccine containing Leishmania antigens plus GM-CSF could be correlated with the curing of cutaneous leishmaniasis patients [81].

In conclusion, the present study describes a subtractive phage display strategy that led to the identification of antigens to be evaluated as vaccines against Leishmania infection. The results indicated that two phage clones expressing target mimotopes conferred protection in BALB/c mice against L. infantum. Protection was correlated with CD4+ and, mainly, CD8+ T cells response, which was characterized by high levels of IFN-γ, IL-12, and GM-CSF, associated with low levels of IL-4 and IL-10. The data leads to the conclusion that the B10 and C01 phage clones may well be effective vaccine candidates against VL. Studies are in progress to identify and clone the native proteins that express these mimotopes, in an attempt to compare their efficacy with those found for mimotopes in the present study.

Supporting Information

Figure S1 Analysis of the cellular immune response. The cellular immune response induced in the in vitro cultured spleen cells obtained from naive and Leishmania infantum-infected BALB/c mice was evaluated. For this, single cells suspensions of spleen cells were collected, pooled and in vitro cultured in 24-well plates (Nunc), at 5 × 10⁶ cells per mL, in duplicate. Cells were incubated in RPMI 1640 medium (Sigma; non-stimulated control), or separately stimulated with each phage clone (20 individual clones, with 1 × 10⁶ phages per well) for 48 h at 37°C, 5% CO2. The same experimental conditions were performed using spleen cells of naive mice. Wild-type and random non-specific phage clones were used as controls. The IFN-γ and IL-4 levels were determined in the culture supernatants, using commercial kits (BD OptEIA, Pharmingen), according to manufacturer instructions. In A, the results obtained using spleen cells of naive mice are showed. In B, the results obtained using spleen cells of L. infantum-infected mice are showed. Each bar represents the mean ± standard deviation (SD) of the cytokines levels. Experiments were repeated twice and presented similar results.

(TIF)

Author Contributions

Conceived and designed the experiments: EAFC CAPT MACF LRG APF. Performed the experiments: LEC MPN MCD VTM PS TGR DMS. Analyzed the data: EAFC MACF MS LRG APF. Contributed reagents/materials/analysis tools: MNM. Wrote the paper: EAFC MACF MS LRG CAPT.

References

1. Desjeux P (2004) Leishmaniasis: current situation and new perspectives. Comparative Immunology, Comp ImmunoL Microbiol Infect Dis 27: 305–318.
2. World Health Organization (2010) Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases. Available: http://www.who.int/tdr/docs/WHO_TRS_949_eng.pdf. Accessed 2014 September 28.
3. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7: 1–12.
4. Baneeth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L (2008) Canine leishmaniasis: new concepts and insights on an expanding zoonosis: part I. Trends Parasitol 24: 324–330.
5. Gramatica M, Gradoni L (2005) The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol 35: 1169–1180.
6. Cruz I, Cuestas MN, Niero J, Caraveo C (2010) A canine leishmaniasis pilot survey in an emerging focus of visceral leishmaniasis: Posadas (Misiones, Argentina). BMC Infect Dis 10: 342.
7. Duprey ZH, Steuber EJ, Rooney JA, Kirkhoff L-V, Jackson JE, et al. (2006) Canine Visceral Leishmaniasis in the United States and Canada, 2000–2003. Emerg Infect Dis 12: 440–446.
8. Córfio SL, Coimbra GH (2003) Leishmaniasis-current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 19: 502–508.
9. Mündler P, Parola P (2010) Cutaneous leishmaniasis treatment. Travel Med Infect Dis 5: 150–156.
10. Costa CHN, Peters NG, Maruyama SR, Brito Jr EC, Santos IKFM (2011) Identification of proteins in promastigote and amastigote-like Leishmania using an immunoproteomic approach. PLoS Negl Trop Dis 6: e1430.
11. Coelho VT, Oliveira JS, Valadares DG, Chávez-Fumagalli MA, Duarte MC, et al. (2012) Identification of proteins in promastigote and amastigote-like Leishmania using an immunoproteomic approach. PLoS Negl Trop Dis 6: e1430.
12. Vieira LQ, Goldschmidt M, Nashleman M, Pfeffer K, Mak T, et al. (1996) Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with Leishmania major, but control parasite replication. J Immunol 157: 827–835.
13. Lima MM, Sarquis O, Oliveira TG, Coutinho C, et al. (2012) Investigation of Chagas disease in four periurban areas in northeastern Brazil: epidemiologic survey in man, vectors, non-human hosts and reservoirs. Trans R Soc Trop Med Hyg 106: 143–149.
14. Roque AL, Xavier SC, Gerhardt M, Silva MF, Lima VS, et al. (2012) Trypanosoma cruzi among wild and domestic mammals in different areas of the Alhezetteba municipality (Pará State, Brazil), an endemic Chagas disease transmission area. Vet Parasitol 193: 71–77.
15. Zanin F, Coelho EA, Tavares CA, Marques-da-Silva EA, Costa MMP, et al. (2007) Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against Leishmania chagasi and Leishmania amazonensis experimental infections. Microbes Infect 9: 1070–1077.
16. Fernandes AP, Costa MMP, Coelho EAF, Michalik MSM, Freitas E, et al. (2008) Protective immumity against challenge with Leishmania (Leishmania)
chagasi in dogs vaccinated with recombinant A2 protein. Vaccine 26: 5808–5895.

22. Clark JR, March JB (2004) Bacteriophage-mediated nucleic acid immunisation. FEMS Immunol Med Microbiol 40: 21–26.

23. Smith PG, Petrenko YA (1997) Phage display. Chern Rev 97: 391–410.

24. Barbas CF, Burton DR, Scott JK, Silverman GJ (2001) Phage display: a laboratory manual. New York: Cold Spring Harbor Laboratory Press. 738 p.

25. Wang LF, Yu M (2004) Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr Drug Targets 5: 1–15.

26. Gemschweizer V, Murray K (1995) Screening a monoclonal antibody with a fusion-phage display library shows a discontinuity in a linear epitope within sporozoite protein as antigens. J Mol Biol 220: 821–827.

27. Mouette M, Opella S, Greenwood J, Willis A, Perham R (2003) Structure of a mammalian parasite antigenic determinant displayed on filamentous bacteriophage determined by NMR spectroscopy: implications for the structure of continuous pep-tide epitopes of proteins. Protein Sci 10: 1150–1159.

28. Demangel C, Lafaye P, Mazie J (1996) Reproducing the immune response against the Plasmodium vivax merozoite surface protein 1 with monomers selected from a phage-displayed peptide library. Mol Immunol 33: 909–916.

29. Costa LE, Lima MGS, Chávez-Fumagalli MA, Menezes-Souza D, Martins VT, et al. (2014) Subtractive phage display selection from canine visceral leishmaniasis identifies novel epitopes that mimic Leishmania infantum antigens with potential serodiagnosis applications. Clin Vaccine Immunol 21: 96–106.

30. Bergotto E, Spadoni A, Buffolano W, Pezzò M, Minenkova O, et al. (2003) Molecular dissection of the human B-cell response against Toxoplasma gondii infected targets by lambda phage display of cDNA libraries. Int J Parasitol 33: 163–175.

31. Cunha-Junior J, Silva D, Silva N, Souza M, Souza G, et al. (2010) A4D12 monoclonal antibody recognizes a new linear epitope from SAG2A Toxoplasma gondii tachyzoites, identified by phage display bioselection. Immunoinununol 215: 26–32.

32. Piccosky TA, Mucci J, Alvarez P, Leguizamon MS, Burrone O, et al. (2001) Epitope mapping of trans-sialidase from Trypanosoma cruzi reveals the presence of several cross-reactive determinants. Infect Immun 69: 1809–1875.

33. Manoutcharian K, Diaz-Arce A, Gevorkian G, Arceo G, Arceo G, et al. (2004) Reombinant bacteriophage-based multipeptide vaccine against Taenia solium pig cysticercosis. Vet Immunol Immunopathol 99: 11–24.

34. Gu Y, Li J, Zhu X, Yang J, Li Q, et al. (2008) Trichinella spiralis: characterization of phage-displayed specific epitopes and their protective immunity in BALB/c mice. Exp Parasitol 118: 66–74.

35. Frenkel D, Katz O, Solomon B (2000) Immunization against Alzheimer’s B-amyloid plaques via FFRH phage administration. Proc Natl Acad Sci USA 97: 11455–11459.

36. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

37. Shiffland T, Gurtler D, Kossooo M, Povcarov M, Weissler D (2007) Phage display of peptides based on sequence text mining and public peptide data sources. BMC Bioinformatics 8: 280.

38. Ru B, Huang J, Dai P, Li S, Xia Z, et al. (2010) MimoDB—a database of peptides based on sequence text mining and public peptide data sources. BMC Bioinformatics 8: 280.

39. Wu W, Huang L, Mendez S (2010) A live Leishmania major vaccine containing C57BL/6 mice. Vaccine 23: 4088–4096.

40. Gnanasekar M, Rao KV, He Y, Mishra PK, Nutman TB, et al. (2004) Novel phage display-based subtractive screening to identify vaccine candidates of Brugia malaya. Infect Immun 72: 4707–4715.

41. Wu Y, Kurta XM, Wang Y, Zeng JF, et al. (2006) Protective immunity induced by phage displayed mitochondrial related peptides of Schistosoma japonicum. Acta Tropica 99: 200–207.

42. Manoutcharian K, Terrazas LI, Gevorkian G, Arceo G, Periasamy P, et al. (1999) Phage-displayed T-cell epitope from the circumsporozoite protein of Plasmodium falciparum shows a discontinuity in a linear epitope within a filamentous bacteriophage. Peptides 20: 163–175.

43. Wang LF, Yu M (2004) Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr Drug Targets 5: 1–15.

44. Leishman C, Dunn B, Niraj K, Cheng A, Hou X, et al. (2010) Influenza A virus-based vaccine protects against systemic candidiasis in C57BL/6 mice. Vaccine 28: 5808–5895.

45. Shtatland T, Guettler D, Kossodo M, Pivovarov M, Weissleder R (2007) MimoDB: a new repository for mimotope data derived from phage display technology. Molecules 15: 8279–8292.

46. Afonso LC, Scott P (1993) Immune responses associated with susceptibility of mice to Leishmania amazonensis. Infect Immun 61: 2952–2959.

47. Ploegh HL, Foy C, Radhakrishnan J (2005) Phage display of peptides specific to alpha-1-proteinase inhibitor enhances tumor response to fractionated radiotherapy targeting toll-like receptor 9 in mice. Clin Cancer Res 11: 361–369.

48. Hashiguchi S, Yamauchi Y, Takeuchi O, Akira S, Sugimura K (2010) Immunological basis of M13 phage vaccine: regulation under MyD88 and TLR9 signaling. Biochem Biophys Res Commun 402: 19–22.

49. Cui L, Ren H, Liu Z (2012) Phage display-specific peptide epitopes induce significant protective immunity against Trichinella spiralis infection in BALB/c mice. Vaccine 6: 1171–1177.

50. Pouyband S, Baudard T, Hashemi Y, Bandeloup M, Kaenni B (2012) Induction of protective anti-CTL epitope responses against HER2-positive breast cancer bearing on multivalent T7 phage nanoparticles. PLoS One 7: e49359.

51. Prudencio CR, Rodrigues AAR, Cardoso R, Souza GR, Szabol MP (2011) Cytotoxic hypersensitivity test to evaluate phage display anti-tick vaccine borne virus antigen candidates. Exp Parasitol 129: 388–392.

52. Ribeiro VS, Araujo TG, Gonçalves HT, Nascimento R, Goulart LR (2013) Development of specific scFv antibodies to detect neurocysticercosis antigens and potential applications in immunodiagnosis. Immunol Lett 136: 59–67.

53. Gumpy A, Louis J, Launois P (2004) The murine model of infection with Leishmania major and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic backgrounds. Int J Parasitol 34: 433–444.

54. Mihani A, Teheri T, Zahedifard F, Taslimi Y, Azizi H, et al. (2009) Reombinant Leishmania tarentolae expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis. Vaccine 27: 53–62.

55. Handman E (2001) Leishmaniasis: current status of vaccine development. Clin Microbiol Rev 14: 229–245.

56. Spitzer N, Ardoin J, Lippert D, Olfason RW (1999) Long-term protection of mice against Leishmania major with a synthetic peptide vaccine. Vaccine 17: 1290–1296.

57. Wu W, Huang L, Mendez S (2010) A live Leishmania major vaccine containing CpG motifs induces the de novo generation of Th17 cells in C57BL/6 mice. Eur J Immunol 40: 2517–2527.

58. Bruhn KW, Birnbaum R, Haskell J, Vanchinathan V, Garey S, et al. (2012) Killed but metabolically active Leishmania infantum as a novel whole cell vaccine for visceral leishmaniasis. Clin Vaccine Immunol 19: 490–498.

59. Avashali A, Mathur RK, Saha B (2004) Immune response to Leishmania infection. Indian J Med Res 119: 238–258.
75. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP (2001) IL-10 mediates susceptibility to Leishmania donovani infection. Eur J Immunol 31: 2848–2856.
76. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, et al. (2002) Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect Immun 70: 6284–6293.
77. Chávez-Fumagalli MA, Costa MA, Oliveira DM, Ramírez L, Costa LE, et al (2010) Vaccination with the Leishmania infantum ribosomal proteins induces protection in BALB/c mice against Leishmania chagasi and Leishmania amazonensis challenge. Microbes Infect 12: 967–977.
78. Garg R, Dube A (2006) Animal models for vaccine studies for visceral leishmaniasis. Indian J Med Res 123: 439–454.
79. Dumas G, Mayombwe A, Roy G, Matte C, Ouellette M, et al. (2003) Recombinant Leishmania major secreting biologically active granulocyte-macrophage colony-stimulating factor survives poorly in macrophages in vitro and delays disease development in mice. Infect Immun 71: 6499–6509.
80. Murray HW, Cervia JS, Hariprashad J, Taylor AP, Stoeckle MY, et al. (1995) Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. J Clin Invest 95: 1183–1192.
81. Badaro R, Lobo I, Nakatani M, Muinos A, Netto EM, et al. (2001) Successful use of a defined antigen/GM-CSF adjuvant vaccine to treat mucosal leishmaniasis refractory to antimony: a case report. Braz J Infect Dis 5: 223–232.