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Cross-Protective Efficacy of a Prophylactic *Leishmania donovani* DNA Vaccine against Visceral and Cutaneous Murine Leishmaniasis

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The fucose-mannose ligand (FML) complex of *Leishmania donovani* is a promising vaccine candidate against murine and canine visceral leishmaniasis, and its main component is a 36-kDa nucleoside hydrolase (NH36). In this study, we tested the immune response and protection induced by the purified FML, the recombinant NH36 (rNH36), and NH36 DNA vaccines against the agents of visceral (*L. chagasi*) and cutaneous (*L. mexicana*) leishmaniasis in BALB/c mice. Mice developed weak humoral response to the vaccines alone, except for those immunized with FML. However, all three vaccine groups presented elevated immunoglobulin G (IgG), IgG1, and IgG2a levels after infection with *L. chagasi*, whereas no differences were observed between vaccine and control groups after infection with *L. mexicana*. A strong intradermal reaction to *L. donovani* and *L. mexicana* antigens was observed in mice immunized with rNH36 or FML, whereas mice immunized with NH36 DNA only reacted against *L. donovani* antigens. Experimental infection of immunized mice demonstrated that FML and rNH36 induced significant protection against *L. chagasi* infection with reductions in parasite loads of 79%. FML also conferred partial protection against *L. mexicana* infection. The best protection was observed in mice immunized with the VR1012-NH36 DNA vaccine, which induced an 88% reduction in *L. chagasi* parasite load and a 65% reduction in *L. mexicana* lesion size. Fluorescence-activated cell sorting analysis indicated the DNA vaccine induced a two- to fivefold increase in gamma interferon-producing $CD4^+$ T cells, indicating a Th1-type immune response. Our results showed that the NH36 DNA vaccine induced a strong immunoprotection against visceral and cutaneous leishmaniasis, suggesting that this DNA vaccine represents a very good candidate for use against several *Leishmania* species.

The leishmaniases are a group of diseases caused by protozoan parasites of the *Leishmania* genus. *Leishmania* spp. are obligate intracellular parasites of host macrophages and cause different forms of disease, depending on the *Leishmania* species. According to their clinical manifestations and affected tissues, leishmaniases are classified as either visceral leishmaniasis, which involves infection of the liver, spleen, and bone marrow and immunosuppression, causing severe damage and death if untreated, or cutaneous leishmaniasis, which is characterized by chronic or self-healing skin lesions (14).

Because of the lack of effective and low-cost treatments and the irreversibility of tissue damage during infection, intensive efforts have been devoted to vaccine development (15). Killed or live-attenuated parasites, as well as a large number of *Leishmania* antigens from different species, have been identified and tested as vaccines. Studies of recombinant protein vaccines in mice demonstrated that antigens such as GP63, p36/LACK, CPb, A-2, or PSA-2 proteins induced strong immune responses but weak and short-lived protection against *Leishmania* infection (19, 34, 44, 55, 56). Interestingly, these antigens used as DNA vaccines induced a stronger cellular immune response and a better protection than their recombinant counterparts (19, 20, 45, 55, 56, 58), suggesting that DNA vaccines may be more effective for controlling *Leishmania* infection. Indeed, DNA vaccines have been shown to induce a preferentially Th1 immune response, which is necessary for the elimination of intracellular parasites (9, 19, 52).

However, very little research has been done on the potential cross-protection induced by a vaccine derived from one *Leishmania* species against another. Initial studies using sequential infections with distinct species have suggested complex cross-protection relationships (1, 26, 27). For example, mice recovered from a *Leishmania major* infection are resistant to a subsequent *L. mexicana* infection, but a primary infection with *L. mexicana* does not protect against a secondary infection with *L. major* (2). Also, the immunization of mice with heat-killed *L. donovani* can induce protection against a subsequent infection with *L. major* (4). A few specific antigens have been tested for cross-protection with mixed success. For example, a DNA vaccine encoding the highly conserved LACK antigen cannot induce cross-species protection (16, 29), whereas *L. donovani* promastigote antigen dp72 can induce protection against both *L. donovani* and *L. major* in mice (43). A DNA vaccine encoding *L. amazonensis* P4 nuclease can also protect significantly

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against *L. amazonensis* and *L. major*, but different adjuvants (interleukin-12- or HSP70-encoding plasmids) are required to obtain such protection (8).

As mentioned above, these observations have important implications for the diagnosis of *Leishmania* infections and vaccine development (26). Indeed, it would be of key importance to develop vaccines that are effective against more than one species of *Leishmania*. For example, in Mexico, localized cutaneous leishmaniasis caused by *L. mexicana* and, to a lesser extent by *Leishmania* (*Viannia*) *braziliensis* are the most widespread forms of the disease (10, 23, 47), but cases of visceral leishmaniasis caused by species of the *L. donovani* complex and *L. mexicana* have been reported (32, 46). On the other hand, in Brazil, infection by *L. chagasi* (the American agent of visceral leishmaniasis) is more frequent, even though cases of visceral leishmaniasis due to *L. mexicana* (22) or *L. amazonensis* (3) have also been reported, and cutaneous leishmaniasis is caused principally by *Leishmania* (*Viannia*) *braziliensis* (41).

The fucose-mannose ligand (FML) complex of *L. donovani* has been characterized as a major antigenic complex of this parasite species (35, 36), and its most immunogenic fraction, a glycoprotein of 36 kDa (40), has a proteic moiety identified as nucleoside hydrolase 36 (NH36) (48). NHs participate in parasite DNA metabolism, hydrolyzing the N-glycosidic bond of purine and pyrimidine ribosides to yield the ribose and base, and are present in a wide variety of *Trypanosomatidae* parasites (12, 18, 31, 42). Both the purified FML complex and NH36 can induce strong immune responses and significant protection against *L. donovani* infection in mouse models (50, 51, 40). FML in saponin formulation has also been shown to protect dogs in field trials as a prophylactic (5, 13) or therapeutic vaccine (6). In the present study, we thus evaluated the protective immune response induced by purified *L. donovani* FML, recombinant NH36 (rNH36), and NH36 DNA vaccines against both *L. chagasi* and *L. mexicana* in mice.

MATERIALS AND METHODS

Purification of the FML complex. Isolation of the FML glycoproteic complex from stationary-phase *L. donovani* promastigotes (LD1S/MHOM/SD/00-strain 1S) was carried out as previously described (13, 50). Briefly, promastigotes were subjected to an aqueous extraction, followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilized and fractionated by gel filtration on a Bio-Gel P-10 column, yielding the FML complex (13, 50). The FML vaccine is registered as a patent under INPI number PI1100173-9 (18.3.97; Federal University of Rio de Janeiro, Rio de Janeiro, Brazil).

Recombinant NH36. *Escherichia coli* transformed with the pMAL-c2-NH36 plasmid, containing the NH36 coding sequence, was grown in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) to induce rNH36 biosynthesis as previously described (48). The recombinant protein was purified from the culture by passage on an amylose-resin column eluted with 10 mM maltose according to the manufacturer's instructions (New England Biolabs, Inc.).

DNA vaccine construction. NH36 coding sequence was PCR amplified from the pMAL-c2-NH36 plasmid (48) to create new XbaI and BamHI restriction sites at the 5′ and 3′ ends, respectively, by using the following oligonucleotides: forward, 5′-ATTCTAGAATGCCGCGCAAGATTATTCTC-3′; and reverse, 5′-AGGATCCTTTTATTGAGGATCGCCGATGCG-3′. After restriction digestions, the NH36 cDNA was ligated into the VR1012 DNA vaccine vector (Vical, Inc.) by using standard molecular biology techniques to form the VR1012-NH36 plasmid. Insert size and orientation were checked by extensive restriction digestions, and the final plasmid was fully sequenced. This plasmid vector drives the expression of the inserted gene through a cytomegalovirus promoter with the intron A enhancer and the bovine growth hormone terminator, and it provides an immunogenicity comparable to that of the pcDNA3 vector.

Purification of plasmids. VR1012 and VR1012-NH36 plasmids were purified from transformed *E. coli* DH5α cultures by using a Qiagen Endofree Plasmid Maxi kit (Qiagen) according to the manufacturer's instructions. Plasmid concentration and quality were evaluated by spectrophotometric analysis at 230, 260, 280, and 325 nm and by agarose gel electrophoresis. The ratio of the optical density at 260 nm to that at 280 nm ranged from 1.8 to 2.0.

Immunization and challenge of mice. Female BALB/c mice (6 to 8 weeks old) were used in all experiments. For the evaluation of vaccine efficacy against visceral leishmaniasis, mice were immunized with either three subcutaneous (s.c.) doses of 100 μg of purified FML or 100 μg of rNH36 with 100 μg of saponin as adjuvant, one week apart as previously reported (40, 50, 51) or via intramuscular with 100 μg of VR1012-NH36 plasmid DNA in 100 μl of saline solution and boosted 2 weeks later by a second injection (15). Control groups included the empty VR1012 vector and saline solution. At 2 weeks after immunization, animals were challenged by intravenous injection of 2×10^8 amastigotes of *L. chagasi* (MHOM/BR/72/BH46), obtained from the spleens of infected hamsters, as previously described (50). Sera were collected 7 days after the last vaccine injection and 30 days after infection when animals were sacrificed. Livers and spleens were removed for assessment of parasite loads on Giemsa-stained imprints, expressed as Leishman-Donovan units (i.e., [the number of amastigotes/1,000 cell nuclei] × the organ weight in milligrams). For the evaluation of vaccine efficacy against cutaneous leishmaniasis, the same immunization protocol was used injecting three s.c. doses of 30 μg of FML or 16 μg of rNH36 with 100 μg of saponin as adjuvant or two intramuscular doses of 100 μg of VR1012-NH36 plasmid DNA, with saline and empty VR1012 plasmid as controls. Mice were challenged 2 weeks after the last immunization with 10^6 stationary-phase promastigotes of *L. mexicana* (MNYC/BZ/62/379) by s.c. injection in the hind footpad. The time course of infection was monitored weekly by measuring footpad swelling with a vernier caliper for up to 12 weeks. Mice were sacrificed at 12 weeks or at earlier time points if lesion development was excessive. Serum was then collected. After sacrifice, the relative spleen-to-body weight ratio was assessed in all animals and compared to that of untreated controls.

Indirect ELISA. In mice vaccinated against visceral leishmaniasis, the humoral response was monitored by the FML–enzyme-linked immunosorbent assay (ELISA) as previously described (50), 1 week after the last immunization and 30 days after infection with *L. chagasi*, by using 2 μg of antigen per well and goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Sigma) at 1:4,000 or goat anti-mouse IgG1- or IgG2a-horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates, Birmingham, Ala.) in a 1:4,000 dilution in blocking buffer. The reaction was developed with *o*-phenylenediamine (Sigma), interrupted with 1 N sulfuric acid, and read at 492 nm. Sera were analyzed at a 1/100 dilution in triplicate. Positive and negative control sera were included in each test. The humoral immune response induced by the vaccines was also evaluated against *L. mexicana* by measuring total specific IgG, IgG1, and IgG2a levels by indirect ELISA from serum samples collected from the tail of mice 2 weeks after the last immunization and 12 weeks after infection with *L. mexicana*. Briefly, 96-well microplates were coated with 0.2 mg of *L. mexicana* (MNYC/BZ/62/M379) soluble antigen/ml, and a 200-fold dilution of sera from immunized and infected mice was placed in each well. Anti-IgG, -IgG1, and -IgG2a secondary antibodies labeled with alkaline phosphatase (Gibco-BRL) were added, and the phosphatase activity was detected with *p*-nitrophenyl phosphate (Sigma) as substrate. Plates were read at 405 nm.

DTH. A delayed type hypersensitivity (DTH) test against visceral leishmaniasis was performed by injecting 37.4 μg of *L. donovani* (LD1S/MHOM/SD/00-strain 1S) freeze-thawed stationary-phase promastigote antigen in 50 μl of saline solution, measuring the footpad thickness with a Mitutoyo apparatus both before and 0, 24, and 48 h after injection. Previous experiments carried out in CB hamsters and mice demonstrated that, 24 h after inoculation, saline-treated footpads returned to basal levels (37). Similarly, 30 μg of *L. mexicana* (MNYC/BZ/62/379) soluble antigen in 50 μl of saline solution was administered in the right footpad, whereas the left footpad received 50 μl of saline solution, to evaluate the DTH reaction against this species. Footpad thickness was measured with a vernier caliper at 24 and 48 h after injection. In both experiments, at each time values of the saline control injected on the left footpad were subtracted from the reaction due to *Leishmania* antigen.

Spleen cell phenotyping. $CD4^+$ and $CD8^+$ spleen cell populations were identified and quantified by immunostaining and flow cytometry analysis. Spleen cells were collected 2 weeks after immunization (saline, VR1012, and VR1012-NH36 groups only). Pooled cells from six mice per group were stimulated for 6 h in vitro with anti-CD28 monoclonal antibody or concanavalin A in the presence of monensin, stained with PerCP-labeled anti-CD3 and fluorescein isothiocyanate-labeled anti-CD4 (GK1.5 clone) or fluorescein isothiocyanate-labeled anti-CD8 (53–6.7 clone), permeabilized, and then stained with phycoerythrin-labeled anti-

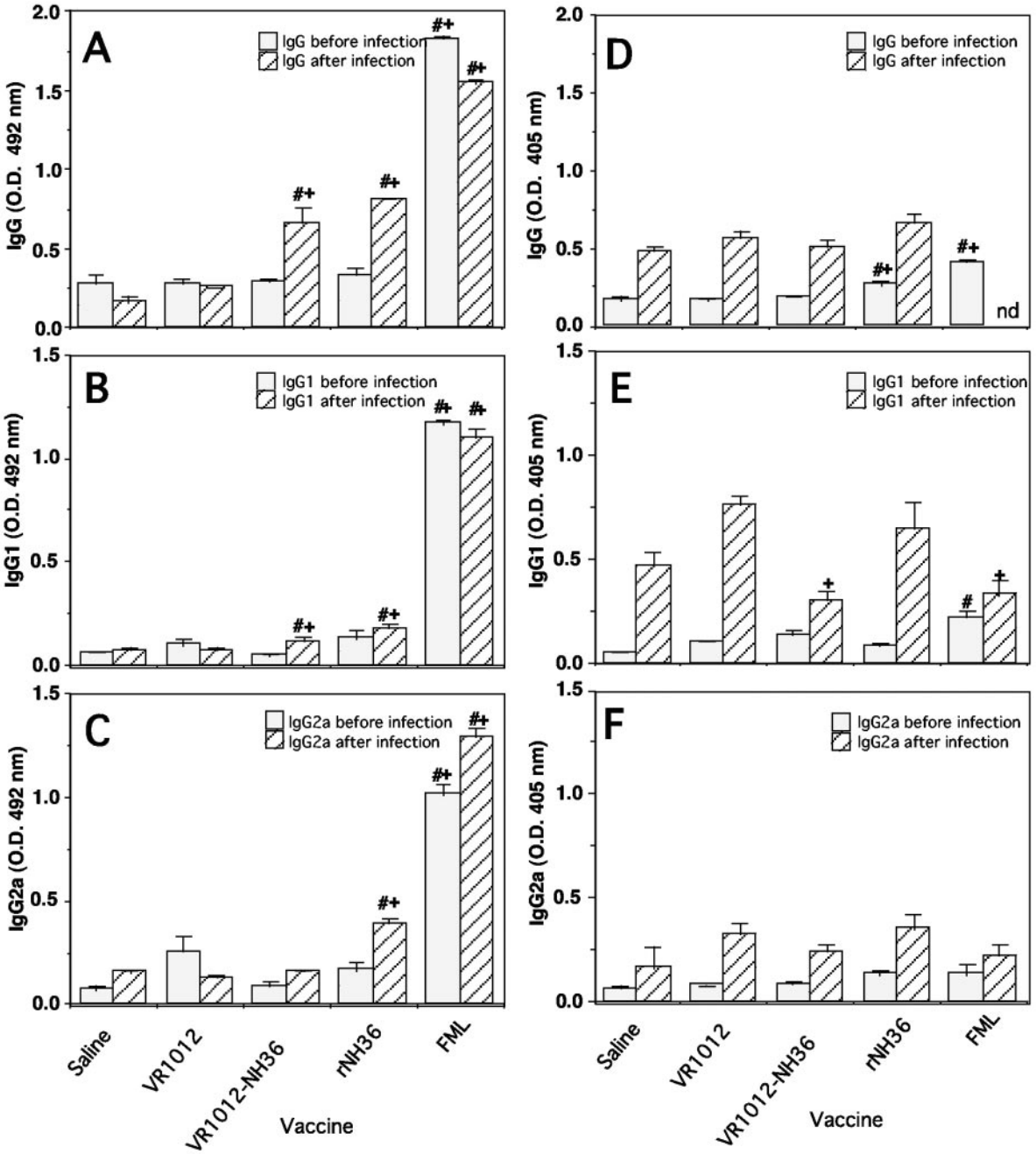

FIG. 1. Humoral responses of immunized mice before and after infection. Levels of *L. donovani*-FML (A, B, and C) and *L. mexicana*-specific (D, E, and F) total IgG (A and D), IgG1 (B and E), and IgG2a (C and F) levels induced by the indicated vaccines were determined by ELISA 2 weeks after the last immunization (dotted bars) and after infection (hatched bars) of the immunized mice with the respective *Leishmania* species. The data are presented as means ± the standard errors of the mean (SEM) of six mice per group. The "#" and "+" symbols indicate a significant difference (as determined by Dunnett's or Newman-Keuls post hoc tests, $P < 0.05$) from the saline and VR1012 control groups, respectively. nd, not done; O.D., optical density.

gamma interferon (IFN-γ) monoclonal antibodies (Pharmingen). Then, 100,000 cells were analyzed by flow cytometry on a Becton Dickinson FACScalibur apparatus and further analyzed by using WinMDI software.

Statistical analysis. Differences between time and treatments were assessed by one-way analysis of variance (ANOVA) using SPSS for Windows, followed by Tukey's or Dunnett's or Newman-Keuls post hoc tests for comparison between groups or with controls, respectively. Differences in footpad swelling after infection were assessed by Welch ANOVA for unequal variances.

RESULTS

Antibody responses to vaccines and infection. We first evaluated both the homologous and the heterologous humoral responses induced by the distinct vaccines by measuring the anti-*L. donovani* FML and anti-*L. mexicana* total IgG, IgG1, and IgG2 levels at 2 weeks after the last immunization (Fig. 1).

Significant differences in IgG antibodies were observed between vaccine groups ($F = 486.0$ and $P < 0.0001$ in Fig. 1A and $F = 7.37$ and $P = 0.0005$ in Fig. 1D [ANOVA]). As expected, before infection, control mice that received saline solution or the empty plasmid vector had very low total specific IgG levels in response to both species of *Leishmania* (Fig. 1A and D). Similarly, mice immunized with VR1012-NH36 DNA did not present any significant antibody response against either species, suggesting a low immunogenicity of the DNA vaccine. Immunization with rNH36 in saponin induced significant IgG levels in response to *L. mexicana* (Fig. 1D) but not in response to *L. donovani*-FML, whereas immunization with FML in saponin induced the highest humoral response against both species (Fig. 1A and D), indicating that the rNH36 and FML-saponin vaccines were capable of inducing a specific humoral immune response against different species of *Leishmania*.

Humoral response was also assessed after *L. chagasi* and *L. mexicana* infection. At this stage, most groups of mice presented increased in total IgG levels ($P < 0.001$ for Fig. 1A and $P < 0.005$ for Fig. 1D [ANOVA]), compared to their respective levels before infection. In mice infected with *L. chagasi* (Fig. 1A), elevated secondary IgG responses were observed in all three vaccine groups compared to the saline control ($P < 0.001$), being again FML the most immunogenic formulation, followed by NH36 saponin ($P < 0.001$) and VR1012-NH36 ($P < 0.0001$). The immunogenicity of the VR1012-NH36 vaccine was specifically due to the NH36 gene, since the empty plasmid control group presented low IgG levels (Fig. 1A). On the other hand, no vaccine-specific humoral response was detected after infection with *L. mexicana*, with all vaccine and control groups presenting similar increases in IgG levels compared to preinfection levels, and this was likely due to the infection (Fig. 1D). Although a direct comparison of the humoral response against *L. chagasi* and *L. mexicana* is not possible due to differences in the ELISA methodologies, there seemed to be a greater immunogenic response to *L. chagasi* than to *L. mexicana*.

The levels of IgG isotypes IgG1 and IgG2a induced by the distinct *L. donovani* vaccines and infection were also evaluated. Before infection only the FML vaccine induced elevated IgG1 and IgG2a against *L. chagasi* compared to saline (Fig. 1B, $P = 0.005$ [ANOVA]), and only IgG1 levels were elevated in response to *L. mexicana* (Fig. 1E, $P = 0.0005$ [ANOVA]). Thus, all groups of mice appeared to develop mixed Th1/Th2 responses after immunization with the different vaccines. After infection with either *Leishmania* species, IgG1 and IgG2a antibody levels were increased in most experimental groups (Fig. 1B to E), but most groups maintained an antibody isotype profile indicative of a mixed Th1/Th2 response. Only mice immunized with rNH36 or FML and infected with *L. chagasi* presented higher IgG2a levels compared to IgG1, suggesting some Th1 bias.

Cell-mediated response to the vaccines. The cellular response after immunization was evaluated by measuring the DTH reaction after footpad injection of *L. donovani* or *L. mexicana* promastigote antigens. As shown in Fig. 2, control mice that received saline or VR1012 DNA did not present any significant DTH reaction, whereas mice immunized with rNH36 and FML both reacted significantly to *L. donovani* and *L. mexicana* antigens 24 h after injection ($P < 0.001$ and $P < 0.05$, respectively). Mice immunized with VR1012-NH36 presented

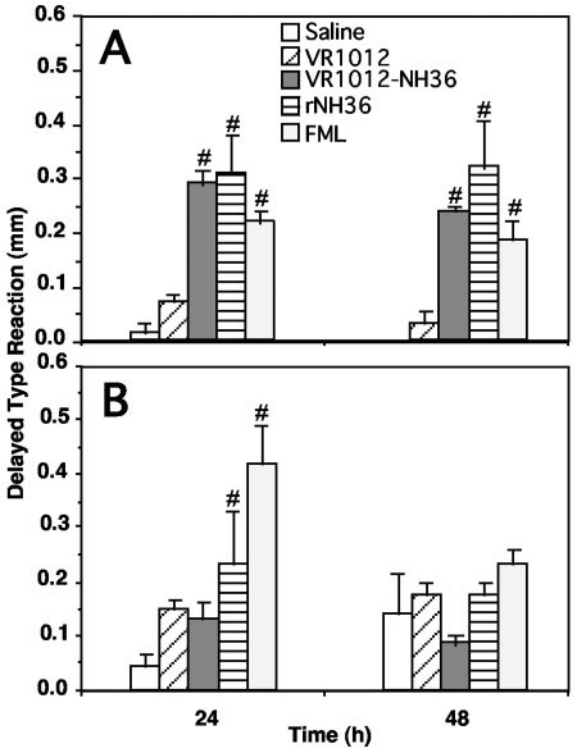

FIG. 2. DTH reaction of immunized mice. The cellular immune response induced by the indicated vaccines was evaluated by a DTH reaction at 24 and 48 h after the administration of *L. donovani* (A) or *L. mexicana* (B) antigen. The data are presented as means ± the SEM of six mice per group. The "#" symbol indicates a significant difference with the saline control group (as determined by Tukey's post hoc test, $P < 0.05$).

a significant DTH reaction against *L. donovani* (Fig. 2A, $P < 0.001$) but not against *L. mexicana* antigen (Fig. 2B). The DTH reaction against *L. donovani* was maintained for up to 48 h after injection of the antigen, whereas the response against *L. mexicana* disappeared at 48 h. No significant differences were found in the intensity of the DTH response at 24 or 48 h in mice injected with either *L donovani* or *L. mexicana* antigens ($P > 0.05$ [ANOVA]). These results clearly indicate the induction of an homologous cellular immune response by the three vaccines, as well as a heterologous response to *L. mexicana* in the case of rNH36 and FML vaccines.

Protection against infection. We then evaluated whether the *L. donovani* vaccines were able to induce protection against infection with either *L. chagasi* or *L. mexicana*. Immunized mice were challenged by intravenous injection of 2×10^8 *L. chagasi* amastigotes, and the parasite burden in the liver was evaluated 1 month after infection. Figure 3A shows that control mice presented a high parasite burden, whereas mice immunized with any of the vaccines had significantly lower parasite loads ($P < 0.0007$ [ANOVA]). A 79% reduction in liver parasitic load was achieved in animals immunized with FML or NH36 saponin vaccines (Fig. 3A, $P < 0.01$). The highest reduction in parasite burden (88%, $P < 0.01$) was observed in mice immunized with the VR1012-NH36 DNA vaccine. The differences between the DNA and FML vaccines were significant ($P < 0.025$). No protection was induced by the saline or empty plasmid control ($P > 0.05$) (Fig. 3A).

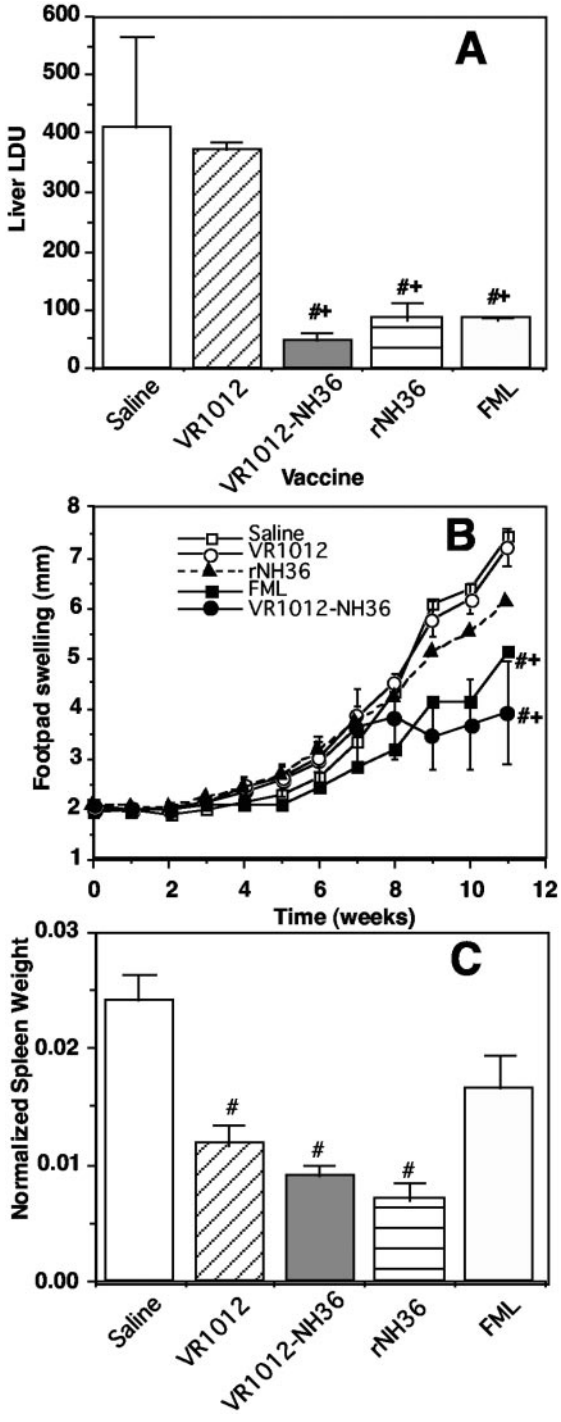

FIG. 3. Protection of immunized mice against infection. (A) Protection induced by the different vaccines against infection by *L. chagasi* was evaluated by measuring the parasite burden in the liver (in Leishman-Donovan units [LDU]). (B and C) Protection against *L. mexicana* infection was assessed by measuring footpad swelling (B) and normalized spleen weight (C). The data are presented as means ± the SEM of six mice per group, except in panel B where error bars have been omitted from the saline, rNH36, and FML vaccine groups for clarity. The "#" and "+" symbols indicate a significant difference ($P < 0.05$) from the saline and VR1012 control groups, respectively.

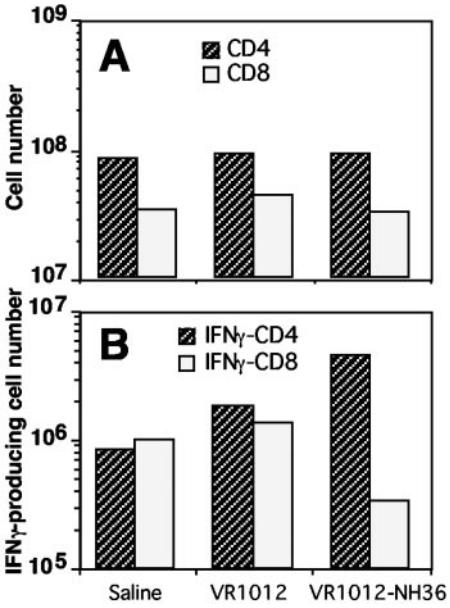

FIG. 4. Analysis of spleen T-cell populations. At 2 weeks after immunization, spleens were removed and single cell suspensions of six mice were pooled for staining with monoclonal antibodies and analyzed on a FACScalibur flow cytometer. The $CD4^+$ and $CD8^+$ total cell number (A) and IFN-γ-producing $CD4^+$ and $CD8^+$ cell numbers (B) are shown for the indicated vaccine groups.

Other groups of immunized mice were infected with 10^6 *L. mexicana* promastigotes in the footpad, and the time course of footpad swelling was determined. We observed significant differences between the different vaccine groups ($P < 0.05$ [Welch ANOVA], Fig. 3B). As expected, control mice that received saline solution or the empty plasmid developed large lesions. Mice immunized with rNH36 tended to show a small but not significant reduction in lesion size. On the other hand, mice immunized with FML-saponin were partially protected, as evidenced by a significant reduction in footpad swelling compared to control mice (42% reduction, $P < 0.05$, Fig. 3B). The best protection was achieved in mice immunized with VR1012-NH36 DNA, which presented a reduction in lesion size of 65% ($P < 0.05$, Fig. 3B), with three of six mice completely protected (not shown).

Spleen weight (normalized to body weight) was also measured after infection with *Leishmania* as an indicator of disease progression. Control mice infected with *L. chagasi* or *L. mexicana* showed important splenomegaly. No significant reduction of splenomegaly was observed after any vaccine treatment and infection with *L. chagasi* (not shown). In contrast, *L. mexicana*-infected mice previously immunized with VR1012-NH36 DNA or rNH36-saponin had a smaller spleen weights, suggesting milder inflammatory responses ($P < 0.0001$ [ANOVA], Fig. 3C) and confirming the protection induced by NH36 DNA against infection by *L. mexicana*. Surprisingly, mice immunized with the empty plasmid vector also presented a significantly minor spleen enlargement, suggesting a strong effect of the vector itself, and mice immunized with FML only had a small but not significant reduction of the splenomegaly (Fig. 3C).

T-cell phenotypes induced by the DNA vaccine. Because NH36 DNA vaccine induced the highest protection against both

L. chagasi and *L. mexicana*, we further analyzed, by immunostaining and flow cytometry analysis, the spleen T-cell phenotypes after immunization with this vaccine. Although immunization with VR1012-NH36 did not affect the total $CD4^+$- and $CD8^+$-T-cell populations (Fig. 4A), it induced a two- to fivefold increase in IFN-γ-producing $CD4^+$ T cells (Fig. 4B), characteristic of the induction of a Th1 type immune response.

DISCUSSION

In this study, we evaluated the immune response and the protection induced by *L. donovani* FML and NH36 antigens against *L. chagasi* and *L. mexicana* infection. The FML and recombinant NH36 antigens have been shown to induce significant protection against the agents of visceral leishmaniasis (*L. donovani* and *L. chagasi*) in a variety of animal models (5, 13, 40, 50, 51). A major finding of the present study is that these *L. donovani* antigens are also immunogenic against *L. mexicana* and can induce significant heterologous protection against this species, particularly when administered as a DNA vaccine. This is a relevant finding since, epidemiologically, *L. mexicana* and *L. chagasi* coexist in wide areas of the world, causing very different forms of disease, i.e., visceral and cutaneous forms. In previous studies, cross-protection between *Leishmania* species has been mostly investigated by sequential infections with distinct species (1, 17, 26, 27) or by infecting animal models with homologous or heterologous species after immunization with killed parasites (4). However, little is known about the specific antigens that may be involved. Sera from infected patients seem to react to a 112- to 116-kDa antigen common to *L. major*, *L. tropica*, and *L. donovani* (33), but the nature of this antigen remains unknown. Purified *L. donovani* promastigote antigen dp72 was one of the first purified antigens to induce protection against both *L. donovani* and *L. major* (43). We found here that a single *L. donovani* antigen can similarly induce heterologous protection against both *L. chagasi* and *L. mexicana*.

All three vaccine formulations tested induced a significant immune response against both *L. donovani* FML and *L. mexicana* antigens. Overall, the immunogenicity of the vaccines seemed somewhat higher against *L. chagasi* than against *L. mexicana*, and the FML antigen always induced the strongest immune response. The higher immunogenicity of FML versus NH36 may be due to additional epitopes present in this complex. Also, the FML and its GP36/NH36 glycoprotein antigen were purified (35, 40) and cloned from *L. donovani* (48). NH36 sequences present 96.2% similarity between *L. donovani* and *L. major* (48), although a very species-specific response to both FML and NH36 antigen was observed in murine macrophage-*Leishmania* in vitro interactions (36) and serology (39). Three species of *Leishmania*, however, compose the donovani complex and are responsible for visceral leishmaniasis. Although *L. donovani* is the agent of human kala-azar in India and Africa, the disease is caused by *L. chagasi* in Brazil and by *L. infantum* in Europe and the Mediterranean region. The two last species are considered very closely related (28). *L. donovani* FML is recognized by sera from kala-azar human and canine patients infected with *L. chagasi* with 100% sensitivity and 96 to 100% specificity (7, 38). Sera from human and canine patients infected with *L. infantum* also react with FML in ELISAs (C. B. Palatnik de Sousa et al., unpublished results), indicating that this antigen can give a strong cross-reactivity with all of the species of the donovani complex. On the other hand, although *L. mexicana* belongs to the same parasitic genus and subgenus, it is the agent of a very different pathology, and it is related to a different complex within the genus *Leishmania*, which would make cross-reactivity against this species more difficult to obtain. However, Santos (49) demonstrated that mice vaccinated with FML antigen obtained from either *L. donovani* or *L. amazonensis* developed better humoral responses against *L. donovani* FML, suggesting that FML isolated from this species is the best immunogen.

The NH36 DNA vaccine appeared to be the least immunogenic of the three tested vaccines, as assessed by ELISA and DTH assays. It induced low antibody levels associated with a good DTH response to *L. donovani* but no detectable response to *L. mexicana*. Such a low or even absent humoral response is in fact common for DNA vaccines, which are biased toward cellular responses (21, 16, 30, 53–57). Also, it is likely that the DTH assay may not have been sensitive enough for the detection of a cellular response induced by the DNA vaccine. Indeed, very weak DTH responses have also been obtained after DNA immunization against papillomavirus in mice, even though a strong protection was subsequently observed (24). In our study, the analysis of T cells from mice immunized with NH36 DNA clearly indicated a strong increase in IFN-γ-producing $CD4^+$ cells compared to controls, confirming the induction of a Th1-type immune response with this vaccine.

Experimental infection of immunized mice confirmed that FML and NH36 protein vaccines could protect significantly against an infection with *L. chagasi*, but only the FML vaccine could induce some protection against infection with *L. mexicana*. This FML-saponin vaccine has been shown to induce very high levels of cross-protection in dogs infected with *L. chagasi* (5, 6, 13). On the other hand, we found in the present study that the NH36 DNA vaccine induced the highest protection against both species. This result provides strong evidence that a vaccine against several *Leishmania* species is feasible, in spite of the wide species diversity of this parasite. These observations also confirm that DNA vaccines may have a greater potential than protein vaccines to protect against *Leishmania*, an observation that has been repeatedly made in vaccines studies comparing various recombinant and DNA vaccines (16, 21, 30, 53–57). Furthermore, even in the case of successfully protective recombinant vaccines against cutaneous leishmaniasis, their plasmid DNA counterparts have the additional advantage of being more stable and easier to prepare (9).

In conclusion, we demonstrated here that *L. donovani* antigen NH36 as a DNA vaccine can provide homologous and heterologous protection against *L. chagasi* and *L. mexicana*, respectively. These results suggest that, in spite of major differences in the mechanisms of pathogenesis between these different *Leishmania* species (11), the development of a vaccine with broad species specificity may be feasible, and thus *L. donovani* NH36 may be a leading antigen for further development.

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