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(54) Title: LEISHMANIA ANTIGENS AND RELATED COMPOSITIONS AND USES

(57) Abstract: The disclosure provides methods and formulations for eliciting an immune response to a Leishmania protein in a mammal, particularly a human or a canine.



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LEISHMANIA ANTIGENS AND RELATED COMPOSITIONS AND USES

RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. Provisional
5 Patent Application No. 60/630,986, filed November 24, 2004, entitled "Vaccines,
Diagnostics, Treatments and Delivery Systems for Leishmaniasis." The provisional
application is incorporated by reference herein.

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BACKGROUND

15 The leishmanias are a group of vector-borne parasitic diseases caused by
protozoa of the genus *Leishmania*. The disease is endemic in the tropics, subtropics
and southern Europe. The clinical forms range from the self-healing cutaneous
leishmaniasis (CL) to visceral leishmaniasis (VL) in which the parasite spreads into
the reticuloendothelial system with fatal outcome.

20 The causative parasite is transmitted by sandflies. The sandfly vector
becomes infected when feeding on the blood of an infected individual or an animal
reservoir host. The leishmania parasites live in the macrophages as round, non-
motile amastigotes (3-7 micrometers in diameter). The macrophages are ingested by
the fly during the blood-meal and the amastigotes are released into the stomach of
25 the insect. Almost immediately, the amastigotes transform into the motile, elongated
(10-20 micrometers), flagellate promastigote form. The promastigotes then migrate
to the alimentary tract of the fly, where they multiply in the extracellular space.
Four to five days after feeding, the promastigotes move to the esophagus and the
salivary glands of the insect. When the sandfly next feeds on a mammalian host, the

proboscis pierces the skin and saliva containing anti-coagulant is injected into the wound along with the leishmania promastigotes. Once in the host, the promastigotes are taken up by macrophages where they rapidly revert to the amastigote form. The leishmania are resistant to the microbicidal action of the macrophage acid
5 hydrolases, allowing the parasites to survive and multiply inside the macrophages, eventually lysing the host cells. The released amastigotes are taken up by additional macrophages, thus continuing the infective cycle. Ultimately all organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow.

10 The World Health Organization (WHO) has estimated that some 12 million people worldwide are infected and some 350 million people are at risk of infection. In Mediterranean countries the infection is zoonotic and the domestic dog is the main reservoir. The endemic strain of Leishmania in the Mediterranean area is *Leishmania infantum*, which infects both humans and dogs, producing cutaneous
15 and visceral leishmaniasis.

Although drug treatment exists for VL, alternative approaches for the control of this disease, including improved vaccine technology, are still needed.

SUMMARY OF THE INVENTION

20 In part, the disclosure provides single antigens and combinations of antigens that may be used to elicit immune responses to leishmania, and to generate vaccines for leishmaniasis, particularly for VL. The disclosure further demonstrates that NKT-active agents may be used as effective adjuvants to enhance the immune response to a leishmania antigen in both CL and VL.

25 In certain aspects, the disclosure provides formulations for eliciting an immune response to a leishmania antigen in a mammal, and, in a preferred embodiment, such formulation is a vaccine. A formulation may comprise a single antigen, or at least two antigens. Antigens may be selected from the group consisting of: GP46, P4, GP63, P36-LACK and P8. A formulation may also
30 comprise at least two nucleic acids encoding at least two antigens selected from the

group consisting of: GP46, P4, GP63, P36-LACK. The antigen-encoding nucleic acids may be joined into a single construct, such as a vector, and may even be joined so as to express two or more antigens as a single fusion protein. A formulation may also comprise one or more antigens selected from the group consisting of: GP46, P4, GP63, P36-LACK and P8, and one or more nucleic acids encoding an antigen selected from the group consisting of: GP46, P4, GP63, P36-LACK. GP46, P4, GP63 and P36-LACK are polypeptides and may therefore be used as complete proteins, fusion proteins, or antigenic portions thereof. Variants and fragments of the antigens, and the encoding nucleic acids, are also acceptable for use insofar as such variants and fragments elicit an immune response. Certain P4 polypeptides disclosed herein are novel P4 polypeptides from *L. infantum*, and accordingly, the disclosure provides isolated or purified polypeptides comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, as well as formulations comprising such protein or an immunogenic portion thereof. The antigens may be derived from any *Leishmania spp.* For example, antigens may be derived from a causative agent for visceral leishmaniasis. Preferred antigens may be derived from an organism selected from the group consisting of: *Leishmania donovani*, *Leishmania pifanoi*, *Leishmania chagasi*, *Leishmania amazonensis*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania infantum*. A formulation may further comprise an adjuvant, such as an NKT-active adjuvant. An adjuvant may be selected from the group consisting of: a glycosyl ceramide, a P-8 antigen and *P. acnes/C. parvum*.

In certain aspects, the disclosure provides formulations for eliciting an immune response to a leishmania protein in a mammal, particularly vaccines, wherein such formulations may comprise an NKT-active adjuvant and an antigen from a causative agent for leishmaniasis (or a nucleic acid encoding such antigen). The adjuvant may be, for example, glycosyl ceramide. The glycosyl ceramide may be selected from the group consisting of: a c-GalCer and an alpha-GalCer. Another example of an adjuvant is the P-8 antigen.

In certain aspects, the disclosure provides a formulation for eliciting an immune response to a leishmania protein in an outbred canine, particularly a vaccine, the formulation comprising an antigen selected from the group consisting of: D-13, D-2, D-6/D-15, and D-14. The outbred canine may be a domestic dog. D-

13, D-2, D-6/D-15, and D-14 may be used as complete antigens or as antigenic portions thereof. The antigens may be derived from any *Leishmania spp.* For example, antigens may be derived from a causative agent for visceral leishmaniasis. Preferred antigens may be derived from an organism selected from the group consisting of: *Leishmania donovani*, *Leishmania pifanoi*, *Leishmania chagasi*,
5 *Leishmania amazonensis*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania infantum*. A formulation may further comprise an adjuvant, such as an NKT-active adjuvant. An adjuvant may be selected from the group consisting of: a glycosyl ceramide, a P-8 antigen and *P.acnes/C. parvum*.

10 In certain aspects, the disclosure provides methods for eliciting an immune response to a leishmania protein in a mammal by administering, to the mammal, any of the antigens, nucleic acids or formulations disclosed herein. The antigen and/or nucleic acid may be administered as a single formulation or may be administered separately. In certain aspects, the disclosure provides methods for eliciting an
15 immune response to a leishmania protein in a mammal, the method comprising administering to a subject an NKT-active adjuvant and an antigen from a causative agent for leishmaniasis.

In certain aspects, the disclosure provides methods for reducing the likelihood that a mammal will contract leishmaniasis, particularly a visceral
20 leishmaniasis, by administering, to the mammal, any of the vaccines disclosed herein. The vaccine may be administered as a single formulation or may include a plurality of components that are administered together or separately.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1. Wild type (C57BL/6) and $\beta_2M^{-/-}$ (beta-2 microglobulin knockout) mice were immunized with P-8+C.parvum/P.acnes (adjuvant), adjuvant alone, or not immunized, and then infected with 1×10^5 *L. amazonensis* parasites and (A) development of the lesion monitored with time as the ration of the size of the infected/non-infected foot as measured with a micrometer. (B) Parasite burdens
30 were determined at 10 weeks post-infection. Each bar in the graph is an averaged value from 6 mice in panel A and 3 mice in panel B.

Figure 2. Wild type (C57BL/6) and $\text{IFN}\gamma^{-/-}$ (interferon-gamma knockout) mice were immunized with P-8+*C.parvum*/*P.acnes* (adjuvant) or adjuvant alone, or not immunized (Control). Mice were infected 5 weeks after the final immunization in the right hind foot with 1×10^5 *L. amazonensis* parasites. Parasite burdens were
 5 determined at 10 weeks post-infection. Each bar is the mean value from 3 mice.

Figure 3. Mice (5-6/group) were immunized 3 times at biweekly intervals with P-8+*C.parvum*/*P.acnes* (adjuvant), adjuvant alone, or not immunized, and 7 weeks after the final immunization mice were infected with 10^5 *L. amazonensis*. Lesion development was monitored, and 10 weeks post-infection parasite burdens (3
 10 mice/group) were determined.

Figure 4. Protection against visceral leishmaniasis in LACK DNA-LACK vaccinia virus-vaccinated mice. Shown are the results of parasite burden analyses of BALB/c mice vaccinated with a prime-boost regimen (DNA genes and recombinant vaccinia virus [VV]) using the LACK antigen and then infected intradermally with *L. infantum* promastigotes. Parasite burdens were determined using limiting dilution analyses and represent the averaged values for at least four mice/group. (A) Spleen; (B) liver; (C) lymph node. Statistical analyses were performed using Student's t test comparing vaccine groups to a vector control group (pCI-neo-WRLuc). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. PBS, phosphate-buffered saline.
 15

Figure 5. Antigen-specific $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, and IL-10 responses in prime-boost vaccinated mice prior to infection. Shown are the $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, and IL-10 responses found for the vaccinated and control groups of mice (as indicated) prior to infection. The results of (A) ELISAs of $\text{IFN-}\gamma$ from culture supernatants of splenic cells in response to recombinant LACK antigen, (B) corresponding enzyme-linked
 25 immunospot analyses of $\text{IFN-}\gamma$, and ELISAs of (C) $\text{TNF-}\alpha$ /LT and (D) IL-10 are shown. PBS, phosphate-buffered saline.

Figure 6. Shown are (A) the parasite burdens in the draining lymph nodes in LACK (DNA-VV) vaccinated mice infected with *L. major*, and (B) lesion development over 13 weeks of infection.

Figure 7. $\text{IFN-}\gamma$ production by LACK-stimulated splenocytes prior to infection with *Leishmania infantum*.
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Figure 8. Parasite burden five weeks post-infection in (A) the spleen and liver, and (B) the lymph node.

Figure 9. BALB/c mice were vaccinated with either D-13 or P-8 and infected 5 weeks after the last immunization with 10^4 *L. infantum* intravenously. Parasite burdens in the liver (panel A, left) or spleen (panel A, right) were evaluated one month after infection. Alternatively, BALB/c mice were infected with 10^5 *L. infantum* intravenously. Parasite burdens in the liver were evaluated 1 month after infection.

Figure 10. BALB/c mice were vaccinated with either D-13 or P-8 and infected 10 weeks after the last immunization with 10^5 *L. infantum* intravenously. Parasite burdens in the liver (panel A, left) or spleen (panel A, right) were evaluated 4.5 months after infection. Alternatively, BALB/c mice were infected with 10^4 *L. infantum* intravenously. Parasite burdens in the spleen were evaluated 4.5 months after infection.

Figure 11. Comparison of P4 amino acid sequences from six different *Leishmania* species. (SEQ ID Nos. 1-6). SEQ ID No. 1, labeled "453A1" is from *Leishmania infantum*. SEQ ID No. 2, labeled "Linf29" is from *Leishmania infantum*. SEQ ID No. 3, labeled "L.pif" is from *Leishmania pifanoi*. SEQ ID No. 4, labeled "LmajcI" is from *Leishmania major*. SEQ ID No. 5, labeled "Lama" is from *Leishmania amazonensis*. SEQ ID No. 6, labeled "LdcI" is from *Leishmania chagasi*.

Figure 12. Comparison of LACK amino acid sequences from six different *Leishmania* species. The sequences, obtained from public databases, are identical. (SEQ ID No. 7).

Figure 13. Comparison of GP46 amino acid sequences from six different *Leishmania* species. (SEQ ID Nos. 8-13). SEQ ID No. 8, labeled "MAQQP2" is from *Leishmania donovani*. SEQ ID No. 9, labeled "GP46/M2" is from *Leishmania amazonensis*. SEQ ID No. 10, labeled "PSA-2" is from *Leishmania major*. SEQ ID No. 11, labeled "PSA.1" is from *Leishmania tropica*. SEQ ID No. 12, labeled "Li PSA" is from *Leishmania infantum*. SEQ ID No. 13, labeled "Lch" is from *Leishmania chagasi*.

Figure 14. Comparison of GP63 amino acid sequences from six different *Leishmania* species. (SEQ ID Nos. 14-19). SEQ ID No. 14, labeled "C fasc" is from *Crithidia fasciculata*. SEQ ID No. 15, labeled "L amaz" is from *Leishmania amazonensis*. SEQ ID No. 16, labeled "L chag" is from *Leishmania chagasi*. SEQ ID No. 17, labeled "L don" is from *Leishmania donovani*. SEQ ID No. 18, labeled "L maj" is from *Leishmania major*. SEQ ID No. 19, labeled "L mex" is from *Leishmania mexicana*.

DETAILED DESCRIPTION OF THE INVENTION

1. Leishmania Antigens

In certain aspects, the disclosure provides leishmania antigens that may be used singly or in combination to provide effective vaccines or other formulations that elicit an immune response in a mammal, particularly a canine or a human. A vaccine will generally provide protection to the vaccinated mammal against future challenges with a leishmania parasite. A combination may include two or more of the following antigens: P4, P8, LACK, GP46 and GP63. Of these, P4, LACK, GP46 and GP63 are polypeptides. P8 is a glycolipid, the preparation of which is described below. Polypeptide antigens within the scope of the present invention include, but are not limited to, polypeptides comprising immunogenic portions of leishmania antigens comprising the sequences recited in SEQ ID Nos. 1-6 (P4), SEQ ID No. 7 (LACK), SEQ ID Nos. 8-13 (GP46) and SEQ ID Nos. 15-19 (GP63). Preferred antigens are those from a causative agent for visceral leishmaniasis, including, for example, SEQ ID Nos. 2, 7, 12 and 17. Polypeptide antigens may be used as full-length proteins or as truncated portions, so long as immunogenic activity is retained. For example, polypeptide antigens corresponding to 25%, 50%, 75%, 85%, 95% or more of the subject protein may be used.

An immunogenic portion of a leishmania antigen is a portion that is capable of eliciting an immune response (i.e., cellular and/or humoral) in a mammal (such as a human or a dog). The cells in which a response is elicited may comprise a mixture of cell types or may contain isolated component cells (including, but not limited to, T-cells, NK cells, macrophages, monocytes and/or B cells).

The compositions and methods of the present invention also encompass variants of the above polypeptides. A polypeptide "variant," as used herein, is a polypeptide that differs from a native protein in one or more substitutions, deletions, additions and/or insertions, such that the immunogenicity of the polypeptide is not substantially diminished. The ability of a variant to react with antigen-specific antisera may be enhanced or unchanged, relative to the native protein, or may be diminished by, for example, less than 50%, and preferably less than 20%, relative to the native protein. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide with antigen-specific antibodies or antisera as described herein. Certain variants include those in which one or more portions, such as an N-terminal leader sequence or transmembrane domain, have been removed. Other preferred variants include variants in which a small portion (e.g., 1-30 amino acids, preferably 5-15 amino acids) has been removed from the N- and/or C-terminal of the mature protein. With respect to GP46, one or more repeat portions (as indicated in Figure 13) may be removed or used in duplicate or higher multiples. Polypeptide variants encompassed by the present invention include those exhibiting at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity (determined as described below) to the polypeptides disclosed herein.

A variant may contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile,

leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. In a preferred embodiment, variant polypeptides differ from a native sequence by substitution, deletion or addition of five amino acids or fewer. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydrophobic nature of the polypeptide. Polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions, as further described below, preferably such that the immunogenicity of the encoded polypeptide is not diminished.

Immunogenic polypeptides may be produced as fusion proteins. A fusion protein comprises at least one of the immunogenic polypeptides disclosed herein and one or more additional sequences, particularly an immunogenic sequence from *Leishmania*, which are joined via a peptide linkage into a single amino acid chain. The sequences may be joined directly (i.e., with no intervening amino acids) or may be joined by way of a linker sequence (e.g., Gly-Cys-Gly) that does not significantly diminish the immunogenic properties of the component polypeptides.

Fusion proteins may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion protein is expressed as a recombinant protein, allowing the production of increased levels, relative to a non-fused protein, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in frame. This permits translation into a single fusion protein that retains the biological activity of both component polypeptides.

When comparing polynucleotide or polypeptide sequences, two sequences are said to be "identical" if the sequence of nucleotides or amino acids in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of

sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

5 Optimal alignment of sequences for comparison may be conducted using BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) Nucl. Acids Res. 25:3389-3402 and Altschul et al. (1990) J. Mol. Biol. 215:403-410, respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides and
10 polypeptides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

 The polypeptides to be used herein may be administered as polypeptides, or by introduction of the encoding nucleic acid into the individual. Mixtures of nucleic acids and proteins may also be prepared and administered, or nucleic acid and
15 protein components may be administered separately. Administration of DNA is advantageous because DNA itself acts as an adjuvant, stimulating certain immune responses. As demonstrated herein, DNA-vaccinia virus delivery systems are effective for producing the desired immune response. See Example 3. The polynucleotides encoding the polypeptide antigens disclosed herein, or fragments
20 thereof, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total
25 length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, illustrative DNA segments with total lengths of about 10,000, about 5000, about 3000, about 2,000, about 1,000, about 500, about 200, about 100, about 50 base pairs in length, and the like, (including all intermediate lengths) are contemplated to be useful in many implementations of this
30 invention. Moreover, it will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these

polynucleotides may bear limited homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that differ from one another as a result of one or more deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

10 In general, Leishmania antigens having immunogenic properties, and DNA sequences encoding such antigens, may be prepared using any of a variety of procedures from one or more Leishmania species including, but not limited to, *L. donovani*, *L. chagasi*, *L. infantum*, *L. major*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. mexicana*, *L. tropica*, and *L. guyanensis*. Such species are available, 15 for example, from the American Type Culture Collection (ATCC), Rockville, Md. The immunogenic properties of the Leishmania antigens may be confirmed by evaluating the ability of the preparation to elicit an immune response in cultures of lymph node cells and/or peripheral blood mononuclear cells (PBMC) isolated from presently or previously Leishmania-infected individuals. The response to be 20 measured may be the secretion of one or more cytokines (such as interferon-gamma; IFN- γ), and/or tumor necrosis factor-alpha (TNF- α) or the change in the level of mRNA encoding one or more specific cytokines.

Immunogenic portions of the antigens described herein may be prepared and identified using well known techniques, such as those summarized in Paul, 25 Fundamental Immunology, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides derived from the native antigen for immunogenic properties using, for example, the representative techniques described herein. An immunogenic portion of a polypeptide is a portion that, within such representative assays, generates an immune response (e.g., 30 proliferation and/or cytokine production) that is substantially similar to that generated by the full length antigen. For example, an immunogenic portion of an

antigen may generate at least about 25%, and preferably at least 25 about 50%, of the response generated by the full length antigen in the model assays described herein.

Portions and other variants of immunogenic Leishmania antigens may be generated by synthetic or recombinant means. Polypeptides may also be isolated directly from cultures of the parasitic organism. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. See Merrifield, J. Am. Chem. Soc. 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division, Foster City, Calif., and may be operated according to the manufacturer's instructions.

Recombinant polypeptides containing portions and/or variants of a native antigen may be readily prepared from a DNA sequence encoding the antigen. For example, supernatants from suitable host/vector systems which secrete recombinant protein into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant protein.

In general, any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a polynucleotide that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are E. coli, yeast or a mammalian cell line such as COS or CHO. The DNA sequences expressed in this manner may encode naturally occurring antigens, portions of naturally

occurring antigens, or other variants thereof. For example, variants of a native antigen may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides.

5 P4 has been purified as a membrane-associated *Leishmania pifanoi* amastigote protein and has been shown to induce protective immunity against infection and to elicit preferentially a T helper 1-like response in peripheral blood mononuclear cells of patients with American cutaneous leishmaniasis. Homologues of the P-4 gene are found in all other species of the genus *Leishmania* that have been
10 examined. The P-4 protein is related to the P1 zinc-dependent nuclease of *Penicillium citrinum* and the C-terminal domain of the 3' nucleotidase of *Leishmania donovani*. Purified *L. pifanoi* P-4 protein possesses single strand nuclease (DNA and RNA) and phosphomonoesterase activity. P-4 protein is localized in the endoplasmic reticulum of the amastigote. The gene is selectively expressed in the
15 intracellular amastigote stage (mammalian host) but not in the promastigote stage (insect) of the parasite. See, e.g., Kar et al., J Biol Chem. 2000 Dec 1;275(48):37789-97.

 P36-LACK Antigen (LACK), which is expressed by both promastigotes and amastigotes, has been shown to protect mice from infection. LACK is also the
20 major target for Th2 responses in susceptible BALB/c mice, and BALB/c mice made tolerant to LACK are resistant to infection.

 GP46 Antigen is also known as gp46/M2 or parasite surface antigen 2. As with gp63, PSA-2 belongs to a multigene family expressed in all *Leishmania* species except *L. braziliensis*. Similar but distinct gene products are found in amastigotes
25 and promastigotes of *L. major* and *L. donovani*, but in *L. mexicana* expression seems to be restricted to promastigotes. This antigen provides cross-protection between species. Immunization with the *L. donovani* GP46 protects mice against infection with *L. major* and that, conversely, immunization with the *L. major* proteins afforded partial protection against infection with *L. donovani*. Recombinant DNA-
30 derived GP46 protein is variable in its ability to confer protection, while the protein derived from the yeast *Pichia pastoris* provides good protection.

GP63 is also known as leishmaniolysin. It is a glycoprotein membrane protease with an Mr 65,000 that is present in promastigotes of all species. GP63 is one of the parasite receptors for host macrophages, and parasite mutants lacking the protein are avirulent.

5 In certain embodiments, the disclosure provides P-8 antigen for use as a component of a formulation for eliciting an immune response. As demonstrated herein, mice inoculated with P-8 are protected against infection with the VL causative agent *L. infantum*. Accordingly, P-8 may be used as the sole antigen, or in combination with other antigens, in a formulation for eliciting an immune response.
10 Additionally, P-8 has a surprisingly selective tendency to stimulate Natural Killer T cells (NKT). Thus, P-8 may be used in the role of an adjuvant in a formulation so as to assist in eliciting a protective immune response by eliciting an NKT response. While P-8 from a member of the *L. donovani* complex may be used as a preferred antigen in a formulation designed to elicit an immune response against VL causative
15 agents, P-8 from other forms of parasite, such as the *L. mexicana* complex, may also be used in such a formulation as a general NKT-active adjuvant.

 P-8 is a glycolipid complex from amastigote forms of *Leishmania spp.* P-8 may be prepared as described in Soong et al., *Infect Immun.* 1995 Sep;63(9):3559-66, and in Colmenares et al., *Infect Immun.* 2001 Nov;69(11):6776-84. In brief, P-8
20 antigen may be purified by obtaining surface membranes of *L. pifanoi* (or other *Leishmania spp.*) amastigotes. Membrane proteins are then solubilized, incubated with reducing agent (e.g., 2-mercaptoethanol) and subsequently alkylated by addition of iodoacetamide. The reduced and alkylated solubilized membranes may be fractionated by Sephadex G-25 (Pharmacia, Piscataway, N.J.) gel exclusion
25 chromatography to remove the excess of reagents. The sample may then be subjected to P-8 immunoaffinity chromatography and eluted. Any available P-8 antibody may be employed in affinity purification, including the CXVI-3G11-C10. See Pan et al. *J Immunol.* 1988 Apr 1;140(7):2406-14 and Traub-Cseko et al. *Mol Biochem Parasitol.* 1993 Jan;57(1):101-15.

30 In certain embodiments, the disclosure provides formulations for eliciting an immune response to a leishmania antigen in outbred canines, and particularly in

outbred domestic dogs. Antibodies that react with antigens termed D-13, D-2, D-6 and D-14 were previously identified in inbred dogs challenged with *L. donovani*. Jaffe et al., *J Immunol.* 1984 Jul;133(1):440-7. The antibodies disclosed therein can be used to prepare purified antigen by affinity purification from the membranes of *L. donovani* promastigotes. See, e.g., Ahmed et al., *Infect Immun.* 2003 Jan;71(1):401-10; Rachamim et al., *J. Immunol.* 150:2322-2331; White et al., *J. Infect. Dis.* 161:1313-1314. Applicants have determined that these antigens are also immunogenic in outbred dog populations, which is a more reliable predictor of the likelihood that such an antigen will produce a desired immune response in the general domestic dog population.

2. Leishmania Adjuvants

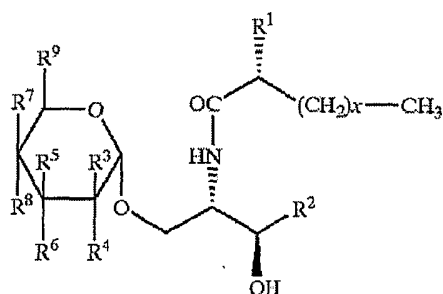
As shown in the Examples, adjuvants that stimulate a NKT cell reaction (“NKT-active adjuvants”) may be used as effective adjuvants in compositions for eliciting an immune response to a Leishmania antigen in a mammal. Examples of NKT-active adjuvants include P-8 and glycosyl ceramides. Given that alpha-GalCer is an agonist of the CD1d receptor found on NKT cells, it is expected that other agonists of this protein will be effective as adjuvants for eliciting an immune response to a Leishmania antigen.

It has been demonstrated that NKT cells can be activated both in vitro and in vivo by alpha-galactosyl-ceramide (alpha-GalCer), a glycolipid originally extracted from Okinawan marine sponges (Natori et al., *Tetrahedron*, 50:2771-2784, 1994) or its synthetic analog KRN 7000 [(2S,3 S,4R)-1-0-(.alpha.-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3-,4-octadecanetriol] which can be obtained from Pharmaceutical Research Laboratories, Kirin Brewery (Gumna, Japan) or synthesized as described previously (see, e.g., Kobayashi et al. 1995, *Onc. Res.* 7:529-534).

In one aspect, the disclosure provides compositions for eliciting an immune response to a Leishmania antigen in a mammal, comprising administering said antigen conjointly with an adjuvant composition comprising a glycosyl ceramide. For examples of glycosyl ceramides, see U.S. Pat. Pub. Nos. 20050222048 and

20050192248 and U.S. Pat. Nos. 6,747,010; 6,555,372; 6,531,453; 6,417,167; 6,071,884; 5,936,076; 5,849,716; 5,780,441; and 5,767,092. Preferred glycosyl ceramides are alpha-GalCer and the more hydrophobic form c-GalCer.

Various glycosyl ceramides are defined as follows (U.S. Pat. No. 6,747,010):



5

wherein:

R^1 represents H or OH,

X represents an integer between 7 and 27,

R^2 represents a substituent selected from the group consisting of the
 10 following (a) to (e) (wherein Y represents an integer between 5 and 17):

(a) $--CH_2 (CH_2)_Y CH_3$

(b) $--CH(OH)(CH_2)_Y CH_3$

(c) $--CH(OH)(CH_2)_Y CH(CH_3)_2$

(d) $--CH=CH(CH_2)_Y CH_3$

15 (e) $--CH(OH)(CH_2)_Y CH(CH_3)CH_2CH_3$, and

R^3 to R^9 represent substituents as defined in any one of the following i) to v):

i) when R^3 , R^6 and R^8 represent H,

R^4 represents H, OH, NH_2 , $NHCOCH_3$, or a sugar (preferably a pentose or hexose);

20 R^5 represents OH or a sugar (preferably a pentose or hexose derivative);

R^7 represents OH or a sugar (preferably a pentose or hexose);

R⁹ represents H, CH₃, CH₂OH or a sugar (preferably a pentose or hexose);

ii) when R^3, R^6 and R^7 represent H,

R⁴ represents H, OH, NH₂, NHCOCH₃, or a sugar (preferably a pentose or
5 hexose);

R⁵ represents OH or a sugar (preferably a pentose or hexose);

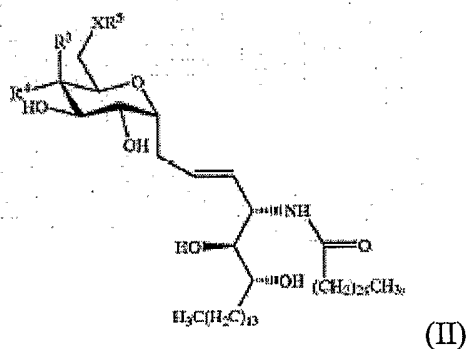
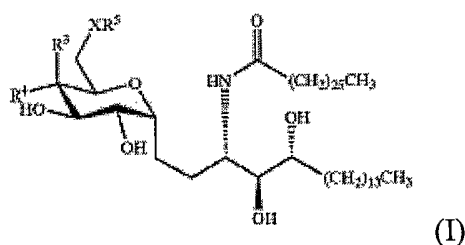
R⁸ represents OH or a sugar (preferably a pentose or hexose);

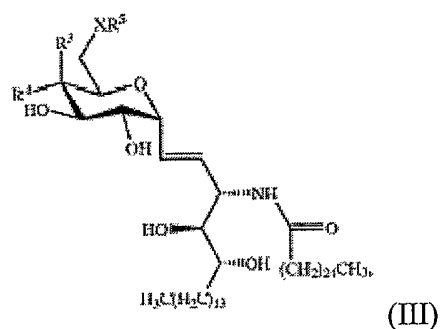
R⁹ represents H, CH₃, CH₂OH or a sugar (preferably a pentose or hexose);

and pharmaceutically acceptable salts or esters thereof.

10 In a preferred embodiment, a glycosyl ceramide is (2S,3S,4R)-1-(α D-galactopyranosyloxy)-2-hexacosanoylamino-3,4-octadecanediol.

Modified glycosyl ceramides that have increased hydrophobicity (generally, c-GalCer) are described in U.S. Pat. Pub. No. 20050222048 as compounds of formulae I, II and III:





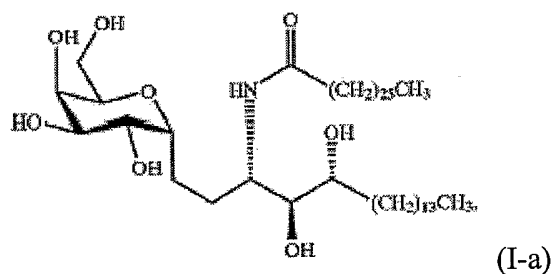
wherein X is O or NH;

R^3 is OH or a monosaccharide and R^4 is hydrogen, or R^3 is hydrogen and R^4 is OH or a monosaccharide;

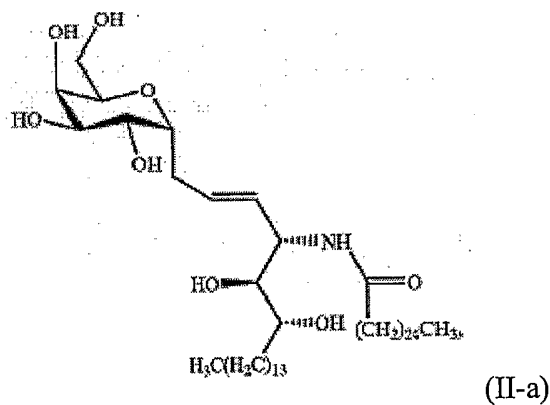
R^5 is hydrogen or a monosaccharide;

and pharmaceutically acceptable salts or esters thereof.

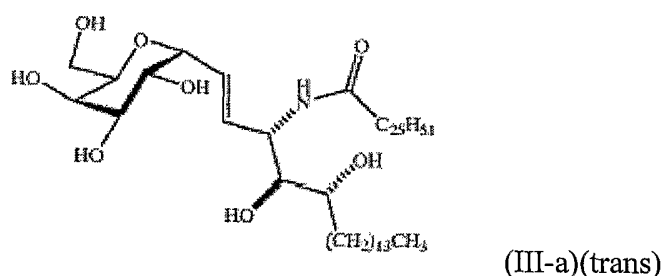
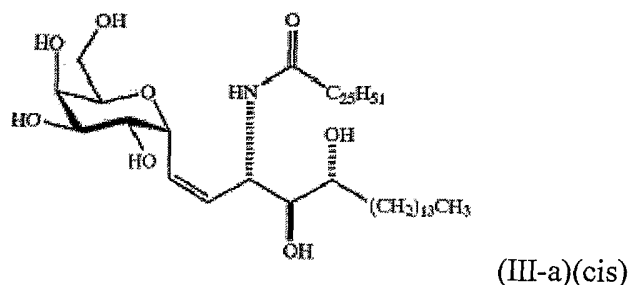
A compound of formula I is, for example, formula I-a:



A compound of formula II is, for example, formula II-a:



Compounds of formula III is, for example, are shown as III-a(cis) and III-a(trans).



- 5 A preferred c-GalCer is α -C-GalCer [(2S,3S,4R)-1-CH₂-(D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol] (see, e.g., Schmieg et al. *J Exp Med.* 2003 Dec 1;198(11):1631-41). According to the present invention, the use of an NKT-active adjuvant, such as P-8 or glycosyl ceramide, results in an enhancement and/or extension of the duration of the protective
- 10 immunity induced by the antigen, whether administered as a protein or as a nucleic acid encoding the protein. For example, as disclosed herein, conjoint administration of such adjuvants with polypeptide antigens or nucleic acid encoding polypeptide antigen increases the protection against leishmania challenge in many different tissues. An adjuvant and antigen can be administered either as two separate
- 15 formulations or as part of the same composition. If administered separately, the adjuvant and antigen can be administered either sequentially or simultaneously. Simultaneous administration of adjuvant with the antigen is preferred and generally permits the most efficient immunostimulation.

- 20 The adjuvant of the invention can be administered as part of a pharmaceutical or vaccine composition comprising an antigen or as a separate formulation, which is administered conjointly with a second composition containing

an antigen. In any of these compositions the compounds of the invention can be combined with other adjuvants and/or excipients/carriers, as described below.

3. Formulations

5 Pharmaceutical compositions for eliciting an immune response to a leishmania polypeptide may comprise one or more antigens, such as P-8, antigenic leishmania polypeptides and/or encoding nucleic acids, each of which may contain one or more of the above sequences (or variants thereof), and a physiologically acceptable carrier. Immunogenic compositions may comprise one or more of the
10 above antigens and an immunostimulant, such as an adjuvant, particularly an NKT-active adjuvant, such as a glycosyl ceramide or a P-8 antigen. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins.
15 Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Mich.); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.); AS-2 (SmithKline Beecham, Philadelphia, Pa.); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble
20 suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A, and cytokines, such as GM-CSF, interleukin-2,-7,-12, and other like growth factors, may also be used as adjuvants.

 Immunogenic compositions may additionally contain a delivery vehicle, such
25 as a biodegradable microsphere (disclosed, for example, in U.S. Pat. Nos. 4,897,268 and 5,075,109). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other Leishmania antigens, either incorporated into a combination polypeptide or present within one or more separate polypeptides.

 A pharmaceutical or immunogenic composition may contain an
30 immunostimulant, such as an adjuvant, and DNA encoding one or more of the polypeptides or fusion proteins described above, such that the polypeptide is

generated in situ. In such compositions, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for
5 expression in the individual (such as a suitable promoter and terminating signal). In a preferred embodiment, the DNA may be introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Techniques for incorporating DNA into such expression systems are well known to
10 those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., Science 259:1745-1749 (1993) and reviewed by Cohen, Science 259:1691-1692 (1993). The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

15 While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or
20 a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Pat. Nos. 4,897,268
25 and 5,075,109.

Routes and frequency of administration, as well as dosage, for the above aspects of the present invention will vary from individual to individual. In general, the pharmaceutical compositions and vaccines may be administered by injection (e.g., intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (e.g.,
30 by aspiration) or orally. Between 1 and 12 doses may be administered over a 1 year period. A suitable dose is an amount of polypeptide or DNA that, when administered as described above, is capable of raising an immune response in an immunized

patient sufficient to protect the patient from leishmaniasis for at least 1-2 years. In general, the amount of polypeptide present in a dose (or produced in situ by the DNA in a dose) ranges from about 100 ng to about 1 mg per kg of host, typically from about 10 μ g to about 100 μ g. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

The disclosure also provides a pharmaceutical pack or kit comprising one or more of the ingredients of the immunogenic formulations of the invention. In a related embodiment, the present invention provides a kit for the preparation of a pharmaceutical or vaccine composition comprising at least one antigen and an adjuvant compound, said kit comprising the antigen in a first container, and the adjuvant in a second container, and optionally instructions for admixing the antigen and the adjuvant and/or for administration of the composition. Each container of the kit may also optionally include one or more physiologically acceptable carriers and/or excipients and/or auxiliary substances. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human or canine administration.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient (i.e., an antigen and/or an adjuvant compound). The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

The application will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present application, and are not intended to limit the application.

EXAMPLES

Example 1. P-8 antigen protects against *L. amazonensis* in mice in an NK cell-dependent manner.

This example confirms that the P-8 antigen protects against *L. amazonensis* infection in mice. The mechanism of P-8 action was investigated in various knockout mouse strains. The data indicate that P-8 stimulates an NKT cell response which is necessary for protection against subsequent challenge with *L. amazonensis*.

NK cells are generally characterized by their ability to kill certain tumor cells without prior sensitization and to produce pro-inflammatory cytokines, especially interferon gamma (IFN γ), following activation. A unique subset of T cells, designated NKT cells, express the NK1.1 marker, as well as other typical NK receptors and upon stimulation through their TCR, rapidly produce substantial amounts of cytokines especially IL4. Unlike NK cells, NKT cells develop in the thymus and express a rearranged TCR. In contrast to typical T cells, NKT cells respond to antigen presented by the atypical MHC Class I molecule, CD1D, and express intermediate levels of TCR. In addition, NKT cells are either CD4+ or CD4-CD8-, in contrast to typical CD8+ Class I restricted T cells.

Transgenic mice which exhibit decreases in the level or function of NK or NK T cells can be used to probe the contribution of these cell types to certain immune responses. Cell surface expression of MHC class I (including CD1D) is dependent on association with Beta 2 microglobulin (β 2M). Since Class I proteins are essential for the maturation of conventional Class I restricted CD8+ cytolytic T cells and Cd1d restricted NKT cells, mice homozygous for the null mutation of B2M have severe depletion of both conventional CD8+ T cells, as well as NKT cells. In this case there is an almost complete deletion of NKT cells, but the deletion is not specific. Complete and specific deletion of the NKT population is obtained in mice that are homozygous for the Cd1d targeted mutation. The CD1d mutation leaves NK cells unaffected. Incomplete loss of function is also found in perforin-deficient mice in which natural killing capability is absent, but other functions are likely to be intact. By using mice deficient for β 2M, IFN- γ , TAP-1, CD1d and

perforin, it was possible to demonstrate that NKT cells play a critical role in the protective effect of P-8 immunization.

Wild type (C57BL/6) and $\beta_2M^{-/-}$ (beta-2 microglobulin knockout) mice were immunized with P-8+C.parvum/P.acnes (adjuvant), adjuvant alone, or not
5 immunized, and then infected with 1×10^5 *L. amazonensis* parasites. As shown in Fig. 1, P-8 provided substantial protection against parasite in wild type mice. In $\beta_2M^{-/-}$ mice, P-8-mediated protection was attenuated. Cell surface expression of MHC class I receptors is dependent on β_2 -microglobulin. Thus, the effectiveness of P-8 immunization depends upon MHC class I receptor activity.

10 Wild type (C57BL/6) and $IFN\gamma^{-/-}$ (interferon-gamma knockout) mice were immunized with P-8+C.parvum/P.acnes (adjuvant) or adjuvant alone, or not immunized, and then infected with 1×10^5 *L. amazonensis* parasites. As shown in Fig. 2, the protective effect of P-8 immunization was abolished in the $IFN\gamma^{-/-}$ mice.

Finally, mice with deficiencies in various proteins of the immune system
15 were immunized with P-8+C.parvum/P.acnes (adjuvant) or adjuvant alone, or not immunized, and infected with 1×10^5 *L. amazonensis* parasites. P-8 immunization was not dependent on TAP-1. TAP-1 deficient mice have severely reduced levels of surface class I molecules, a reduced ability to present cytosolic antigens to class I - restricted cytotoxic T cells and a reduced number of CD4-8+ T cells. Accordingly,
20 the P-8 protection appears to be independent of these components. By contrast, CD1d and perforin deficiencies caused substantial reductions in the effectiveness of the P-8 immunization. CD1d is an essential component of the NKT cell response. Perforin is a component of the cell lysis machinery of NKT cells and other cytotoxic cells. See Fig. 3.

25 Taken together, these data indicate that the P-8 antigen stimulates an NKT response against leishmania parasites. Therefore, it is expected that other agents that stimulate an NKT response will provide protective effects and may be useful as adjuvants in leishmania vaccines. This conclusion is further supported by experiments with the NKT-stimulating agents, glycosyl ceramides, presented in
30 Example 3, below.

Example 2. Heterologous Prime-Boost Vaccination with the LACK Antigen Protects against Murine Visceral Leishmaniasis

This example demonstrates the efficacy of a heterologous prime-boost vaccination using DNA and vaccinia viruses (Western Reserve [WR] virus and
5 modified [attenuated] vaccinia virus Ankara [MVA]) expressing the LACK antigen (*Leishmania* homologue of receptors for activated C kinase) and an intradermal murine infection model employing *Leishmania infantum*.

Visceral leishmaniasis (VL) is a protozoan parasitic disease, fatal in the absence of treatment. Although drug treatment exists for VL, alternative approaches
10 for the control of this disease (vector control, immunotherapeutic, chemotherapeutic, and vaccine) are still needed. Vaccine studies of VL have been less extensive, and the level of protection found is generally poorer than those found for murine cutaneous leishmaniasis. However, studies utilizing a murine intradermal infection model of VL indicate that this is, in part, due to the animal model employed.
15 Immunological studies of the mechanisms of pathogenesis as well as immunotherapeutic studies of VL indicate tissue site-specific mechanisms (for the spleen, liver, and lymph node). Consequently, one of the challenges in the development of a vaccine against VL is the induction of protection at multiple and distinct tissue sites.

20 The LACK antigen (*Leishmania* analogue of the receptors of activated C kinase) (36 kDa) is highly conserved among *Leishmania* species and expressed by both the promastigote and amastigote forms of the parasite. Studies indicate that DNA coding for the LACK antigen provides protection against *Leishmania major*. However, a LACK DNA vaccine failed to protect against *L. mexicana*. Further, a
25 LACK DNA vaccine, although highly immunogenic, failed to protect against murine VL in either intradermal or intravenous infection, suggesting that LACK may not be a useful antigen for a general DNA-based vaccine against leishmaniasis.

A heterologous prime-boost regimen using DNA and vaccinia viruses expressing the LACK antigen has been shown to be highly immunogenic and
30 protective against murine *L. major* infection. A heterologous prime-boost regimen using DNA and the replication-competent Western Reserve (WR) strain of vaccinia

virus expressing the LACK antigen was recently explored in canine VL. However, the immune response in the canine model is known to differ significantly from those in the murine and human hosts of leishmaniae in terms of their regulation by interleukin-13 (IL-13), IL-12, and IL-10. Previous leishmaniasis vaccine studies have demonstrated that the murine model can be predictive for vaccine outcomes in nonhuman-primate models. Therefore, in the current study, the potential of a prime-boost regimen using DNA-vaccinia virus was explored using the murine intradermal model for VL. In order to assess the potential use of this vaccination regimen against murine VL, the efficacies of priming were examined using an *L. infantum* DNA-LACK construct (previously employed for vaccine studies against cutaneous leishmaniasis caused by *L. major*) and the highly attenuated modified vaccinia virus Ankara (MVA) strain as well as the replication-competent WR strain, given the abilities of these viruses to induce both strong Th1 and CD8+ T-cell responses.

BALB/c mice (4 to 6 weeks of age) were vaccinated intradermally with 100 µg of DNA encoding the LACK antigen (DNAp36) and then boosted 2 weeks later intraperitoneally with 1×10^7 or 5×10^7 PFU of either recombinant Western Reserve-wild-type (WR-LACK or WRp36) or Ankara-MVA (MVA-LACK or MVAp36) vaccinia viruses expressing the LACK antigen. Three and one-half weeks after boosting, mice were infected intradermally in the ear pinnae using 10^7 metacyclic promastigotes of *L. infantum*. One month after infection, the parasite burdens were evaluated by limiting dilution analysis in vaccinated and control groups of mice. This evaluation of protection in the spleen, the liver, and the draining lymph node demonstrated that the mice receiving a prime-boost vaccination using the LACK (p36) antigen were significantly protected against infection (Fig. 4). The levels of protection at each tissue site were comparable among the various vaccinated groups of mice and did not statistically differ between mice receiving the WRp36 or the MVAp36 virus. However, the level of protection did vary with the target organ site, with the highest levels of protection achieved in the draining lymph node (Fig. 4C). The level of protection in the draining lymph node was evidenced by a 144- to 244-fold reduction in the parasite burdens in comparison to those of control mice. Lower levels of protection were achieved when the parasite burdens were evaluated in the spleen and the liver. These results ranged from 6- to 9-fold

reductions in parasite burdens in the liver and 9- to 30-fold reductions in the spleen. In the spleen, a slight protective effect was also observed for the mice receiving control DNA and vaccinia virus (WR-Luc), which may be due to the low gamma interferon (IFN- γ) response observed for these mice (Fig. 5). However, in other vaccine experiments employing WR-Luc (107 PFU) an IFN- γ response and a reduction of the splenic parasite burden were not consistently observed, nor were reductions in parasite burdens observed in the livers and lymph nodes of the WR-Luc-vaccinated mice (Fig. 4). However, the differences between the parasite burdens observed for the WR-Luc-vaccinated mice and those for mice receiving WR-LACK or MVA-LACK were significant ($P < 0.02$ to 0.05) (Fig. 4A), demonstrating a LACK antigen-specific effect.

IFN- γ and tumor necrosis factor alpha/lymphotoxin (TNF- α /LT) have been found to be involved in resistance to infection in murine VL, while IL-10 correlates with susceptibility. The levels of IFN- γ , IL-10, and TNF- α /LT produced by spleen cells of vaccinated and nonvaccinated mice in response to LACK antigen were evaluated before infection and at 1 month after infection (Fig. 5). Before infection, mice receiving 10^7 WRp36 or 5×10^7 MVAp36 PFU appeared to produce somewhat higher levels of IFN- γ (100 to 113 ng/ml) than mice boosted with either 5×10^7 WRp36 or 10^7 MVAp36 PFU (55 to 67 ng/ml) (Fig. 5A). As shown in Fig. 5B, enzyme-linked immunospot analyses indicated that the number of IFN- γ -secreting cells correlated with the levels of IFN- γ found by enzyme-linked immunosorbent assay (ELISA), with the frequency of IFN- γ -producing cells ranging from 380 to $640/10^6$ spleen cells. In addition, significant levels of TNF- α /LT (58 and 134 pg/ml) were observed for mice boosted with recombinant wild-type WRp36, while lower levels of TNF- α /LT were produced in response to LACK antigen by mice receiving MVAp36 (27 pg/ml and 8 pg/ml, respectively). These differences in the levels of induction of TNF- α may reflect, in part, the different abilities of WR and attenuated MVA vaccinia viruses to induce an inflammatory response and NF- κ B activation, which result in distinct cytokine profiles. MVA has been shown to enhance NF- κ B activation, while WR appears to inhibit it.

The amounts of LACK-specific IL-10 produced by splenocytes before challenge varied from 0.1 ng/ml in mice boosted with 5×10^7 WRp36 PFU to 0.7 ng/ml in those receiving 10^7 WRp36 or MVAp36 PFU (Fig. 5D).

- The cytokine responses at 1 month postinfection paralleled but were somewhat higher than those found prior to infection. IFN- γ levels ranged from 20 ng/ml in mice receiving 5×10^7 WRp36 PFU to 204 ng/ml in those boosted with 5×10^7 MVAp36 PFU. The levels of TNF- α /LT in response to LACK antigen stimulation in vaccinated mice ranged from 64 pg/ml in mice boosted with 10^7 MVAp36 PFU to 120 pg/ml in the group boosted with 5×10^7 WRp36 PFU. Significant levels of IL-10 (0.04 ng/ml to 0.54 ng/ml) were also produced in response to LACK antigen at 1 month postinfection. Both the level of IFN- γ and the IFN- γ /IL-10 ratio found at 1 month postinfection appeared to correlate with the protection levels found (Table 1).

TABLE 1. Cytokine levels and corresponding reductions (*n*-fold) in parasite burden at 1 month postinfection

Expl group	IFN- γ / IL-10 ratio	Amt of IFN- γ (ng/ml)	Fold reduction in parasite burden, by tissue site		
			Spleen	Liver	Lymph node
DNAp36 + 5×10^7 MVA PFU	4,633	204	31	9	244
DNAp36 + 10^7 WR PFU	720	153	18	6.5	244
DNAp36 + 10^7 MVA PFU	271	147	12.8	6.7	203
DNAp36 + 5×10^7 WR PFU	92	20	9.2	6	144

- Both IFN- γ and TNF- α have been implicated in the macrophage killing of intracellular *L. donovani*, through the up-regulation of inducible NO synthase

(iNOS) and production of nitrite oxide. Nitric oxide has been demonstrated to be critical for the leishmanicidal activity of murine macrophages (4, 16, 19) and also has been shown to enhance, at low levels, a Th1 response. However, IL-10 is known to down-regulate macrophage NO production. To further determine the ongoing
5 immune response dynamics in the vaccinated versus control mice, the induction of NO/nitrite at 1 month postchallenge was examined. Significant amounts of this antimicrobial agent were observed and ranged from 6 to 7 μ M in the vaccinated mice, with control group NO/nitrite levels ranging from 0.6 μ M to 1 μ M. Therefore, the vaccinated mice, consistent with the findings for levels of IFN- γ (Fig. 5; Table
10 1), had higher levels of nitric oxide induction and potential leishmanicidal activity. These results were consistent with the protection found in the LACK-DNA-LACK-WR- or MVA-vaccinated mice.

In conclusion, this study demonstrated that a heterologous prime-boost regimen using DNA and vaccinia virus, both expressing the same antigen, LACK, is
15 highly immunogenic and confers protection against murine *L. infantum* infection. Notably, the highly attenuated MVA and the replication-competent WR strain vaccinia viruses achieved comparable levels of protection. This heterologous prime-boost approach resulted in higher levels of IFN- γ (up to 200 ng/ml) than those that have been reported for DNA-DNA vaccination (6 to 12 ng/ml) (23), where
20 protection was not achieved. This observation prompts a question as to the biologically effective amount of IFN- γ required to induce protection against VL. Although additional effector mechanisms may be involved, these results suggest that higher levels of IFN- γ may be required for protection against visceral disease than are needed against cutaneous leishmaniasis.

25

Materials and Methods for Example 2:

Parasite strains and animals:

L. infantum was provided by Dr. Jorge Alvar (Centro Nacional de Microbiologia Instituto de Salud Carlos III, Majadahonda, Spain). Promastigotes
30 were grown at 24°C in Schneider's *Drosophila* medium (Sigma, St. Louis, MO) supplemented with 20% heat inactivated fetal bovine serum and antibiotics. The

virulence of the strain was preserved by periodic passage through BALB/c mice. All mice used in this study were female BALB/c mice of 4-6 weeks of age purchased from the NIH.

5 Plasmids, recombinant p36 protein and recombinant vaccinia viruses:

The gene encoding *L. infantum* p36 protein was obtained and inserted downstream of the cytomegalovirus (CMV) promoter into the SmaI site of the pCI-neo vector (Promega, Madison, WI). The empty plasmid pCI-neo (Promega) was used as control (DNA control). Plasmid DNA was purified using the Qiagen
10 Endofree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) using pyrogen-free material and eluted in pyrogen-free deionized water. The endotoxin level of the purified plasmid was tested using LAL Kt (Bio-Wittaker, Walkersville, MD) before immunization. Preparations had a maximum of 0.1 ng LPS/ μ g DNA.

rVVs were derived from the wild-type WR and MVA strains; MVA-LACK
15 (MVAp36) and WR_LACK (WRp36) were prepared using standard methods.

Prime-boost vaccination and infection of mice with *L. infantum*:

Six experimental groups of BALB/c mice ($n=7$) were constituted. Mice were primed intradermally with p36 (LACK) plasmid DNA or control vector DNA (100
20 μ g/mouse in 100:1 of phosphate buffered saline). Mice were boosted intraperitoneally two weeks later (14 d.p.i.) with two different doses of WRp36 or MVAp36 (1×10^7 or 5×10^7 PFU). Mice immunized earlier with the control DNA were boosted with 5×10^7 PFU of the control virus encoding for the luciferase gene (WR-Luc). Infection control mice received PBS alone. Three weeks after boosting
25 (35 days after the initial immunization), three mice per group were sacrificed and serum, spleen and draining lymph node were collected for immunological analysis. Three and a half weeks after viral boosting, the mice were infected intradermally on the ear pinnae with 1×10^7 metacyclic *L. infantum* promastigotes. This method of infection, which mimics the situations in vivo where ingestion is transmitted through
30 the bite of a phlebotomine sand fly, has been previously described and the results

in successful visceralization of the parasite. Mice were sacrificed at one month post-challenge and draining lymph node, spleen, liver and serum were harvested for parasitological and immunological assays.

5 Antibody Response:

Antibody levels to LACK antigen were assessed by ELISA. Total IgG as well as IgG1 and IgG2a isotype responses were evaluated for sera collected from each group of animals before challenge and at one month post-challenge. The ELISA was performed as previously described with some modifications. Briefly,
10 96-well Maxisorp plates (Nunc, USA) were coated overnight at 4°C with 100:1 of purified recombinant p36 proteins (5 µg/ml) or unstimulated. Supernatants were collected after 72 h of incubation and stored at -20 °C until used. Cytokine levels were measured from culture supernatant by sandwich ELISA, according to manufacturers' specifications. Background cytokine levels were determined using
15 supernatant from unstimulated cell populations.

ELISPOT Assay:

The ELISPOT assay to quantify LACK-specific IFN-γ producing cells from the spleen was conducted. The 96-well cellulose plats (Millipore, Bedford, MA) were coated with 6 µg/ml of anti-mouse IFN (BD-Pharmingen, San Diego, CA)
20 monoclonal antibody and incubated overnight at room temperature. Subsequently, two-fold serial dilution of splenic cells (beginning with 10⁶) were added to each well in RPMI medium supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin. Recombinant p36 antigen was added (5 µg/ml) and the plates were incubated at 37°C with 5% CO₂. After 72 h, the plates were washed and then
25 incubated for 2 h at room temperature with 2 µg/ml of biotinylated anti-mouse IFN-γ (BD_Pharmingen, San Diego, CA) followed by incubation with 1/800 dilution streptavidin-horseradish peroxidase conjugate (Pharmingen, Palo Alto, CA). After 1 h at room temperature, plates were washed and the presence of IFN-γ producing cells was revealed by adding 1 mg/ml of fluorescent substrate.

30 Measurement of nitrite/nitric oxide

Nitric oxide (NO) production was measured as nitrite using the Greiss reaction. Briefly to each 96-well flat bottom microtiter plate (Nunc, USA) containing 50:1 of culture supernatant equal volumes of the Greiss reagents (1% sulfanilamide and 0.1% naphthylethylenediamine dichloride in 2.5% phosphoric acid) were added and incubated at room temperature for 10 minutes. Standards of NaNO₂ using concentrations from 1.56 to 100 μ M NaNO₂ were employed with each assay. Absorbance was measured at 540 nm with the Bio-tek plate reader (Bio-tek Instruments, Winooski, VT). Background concentrations were assessed using supernatant from unstimulated splenocytes.

10 Evaluation of parasite burden:

The total number of parasites in the various infected tissues (spleen, liver and draining lymph node) was evaluated by limiting dilution analysis using Schneider's *Drosophila* medium. The individual parasite burdens from four mice per group were determined; results are expressed at the average $\pm$ standard errors of the parasite burdens obtained from each group.

Example 3. Glycosyl ceramides are effective adjuvants for immunization with leishmania antigens.

It has been demonstrated that NKT cells can be activated both in vitro and in vivo by alpha-galactosyl-ceramide (alpha-GalCer), a glycolipid originally extracted from Okinawan marine sponges (Natori et al., Tetrahedron, 50:2771-2784, 1994) or its synthetic analog KRN 7000 [(2S,3 S,4R)-1-0-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3-,4-octadecanetriol] which can be obtained from Pharmaceutical Research Laboratories, Kirin Brewery (Gumna, Japan) or synthesized as described previously (see, e.g., Kobayashi et al. 1995, Onc. Res. 7:529-534). Other glycosyl ceramides, particularly c-GalCer, have similar effects on NKT cells.

The data presented in this Example demonstrate that glycosyl ceramides act as effective adjuvants in the immunization of mice against antigen from the causative agents for cutaneous and visceral leishmaniasis. In particular, the data

demonstrate that the protection elicited using a DNA-vaccinia virus prime-boost system with the LACK antigen (see Example 2) can be substantially increased using alpha-GalCer or c-GalCer during the DNA priming.

BALB/c mice were primed either with DNA-LACK (100 µg/mouse) alone or
5 DNA-LACK (100 µg/mouse) and alpha-GalCer (2µg/mouse). They were boosted
two weeks later with vaccinia virus expressing LACK (1×10^7 pfu/mouse). Control
mice received either PBS twice or control DNA and alpha-GalCer, and boosted with
the control virus (VVluc). Mice were infected three weeks later in the right hind
footpad with 5×10^4 stationary phase promastigotes of *L. major*. Prior to challenge,
10 immunological data were collected from immunized and control mice (3
mice/group). Parasite burden in the draining lymph nodes evaluated by limiting
dilution assay 13 weeks post-infection showed significantly higher protection in the
vaccinated mice primed with DNA-LACK and alpha-GalCer (2000-fold reduction in
parasite burden versus controls) than those primed with DNA-LACK alone (258-
15 fold reduction. See Fig. 6A. The footpad lesion size was monitored weekly.
Although smaller compared to control groups, lesions of mice primed with DNA-
LACK alone appeared progressive while those of mice primed with DNA-LACK
and alpha-GalCer were healing at 13 weeks post-infection.

Cytokines such as IFN-γ, IL-10 and TNF-α were measured from in vitro
20 LACK- and SLA- (soluble leishmanial antigen) stimulated spleen and lymph node
cells. The pre-challenge and post-challenge levels of IFN-γ were significantly
higher in the protectively vaccinated groups. An enhanced production of these
cytokines was observed in the vaccinated mice receiving alpha-GalCer as adjuvant.
Consequently, the enhanced protection correlates with the increased levels of IFN-γ
25 and TNF-α produced in response to the antigen. Notably, the enhanced cytokine
levels persist for as long as five months post-boost. Therefore these experiments
suggest that activation of NKT cells results in enhanced effector (protection) and
memory T cells and should be useful as an adjuvant for vaccine against cutaneous
leishmaniasis.

30 The effectiveness of alpha-GalCer and c-GalCer as adjuvants for a vaccine
against visceral leishmaniasis was also examined. This disease is particularly

challenging because the parasite disseminates to multiple tissue sites. In this experiment, BALB/c mice were primed with DNA-LACK (100 µg/mouse) alone, DNA-LACK (100 µg/mouse) and alpha-GalCer (2µg/mouse), DNA-LACK (100 µg/mouse) and c-GalCer diluted with either polysorbate (Tween20) or DMSO (2µg/mouse). Mice were boosted two weeks later with vaccinia virus expressing LACK (1x10⁷ pfu/mouse). Control mice received either PBS twice or control DNA and alpha-GalCer, and boosted with the control virus (VVluc). Mice were infected three weeks later intradermally with 1x10⁷ metacyclic promastigotes of *L. infantum*. This method of infection allows visceralization of the parasite and mimics the natural course of infection, caused by the bite of a phlebotomine sandfly.

Pre- and post-challenge data showed higher levels of IFN-γ produced by spleen cells stimulated with LACK antigen. See Fig. 7. However, these levels of IFN-γ did not consistently correlate to the level of protection observed at 5 weeks post-infection. See Fig. 8. Note that the enhanced protection caused by c-GalCer is tissue-site specific (evident in the liver and spleen but not in the draining lymph node). In a similar experiment using only alpha-GalCer and a different intradermal site for vaccination, enhanced protection resulted in the draining lymph node and spleen but not the liver. It should be noted that the LACK-DNA vaccine alone fails to provide protection in this model, and therefore the effects of glycosyl ceramides are substantial.

Data from a subsequent experiment in the *L. infantum* system are shown in Table 2. These data confirm that c-GalCer improves the protective effect of immunization in multiple tissues, including the spleen and the liver.

Table 2: Intradermal Infection *Leishmania infantum* – 1 Month Post-Infection

Vaccine Group	Lymph Node	Spleen	Liver
PBS	148.2 $\pm$ 22	5.5 $\pm$ 2.1	12.3 $\pm$ 2.1
pCIneo+VVp36 (2X)	135.6 $\pm$ 12.7	4.04 $\pm$ 1	11.3 $\pm$ 2.2
DNAp36+VVLuc (2X)	134 $\pm$ 13.3	3.63 $\pm$ 1.5	12.2 $\pm$ 4.4
DNAp36+VVp36 (2X)	1.2 $\pm$ 0.16 (124)	0.21 $\pm$ 0.05 (26)	0.7 $\pm$ 0.06 (16)
DNAp36+cGalCer (2X) +VVp36	0.12 $\pm$ 0.02 (1235)	0.033 $\pm$ 0.02 (168)	0.05 $\pm$ 0.03 (246)
DNAp36+cGalCer (2X) +VVLuc	1.03 $\pm$ 0.34 (143)	0.1 $\pm$ 0.05 (55)	0.42 $\pm$ 0.22 (30)

* Values are parasite burdens $\times 10^{-3}$ (fold reduction).

Example 4. P-8 and D-13 Antigens Protect Against *L. infantum* infection.

BALB/c mice were vaccinated with either D-13 or P-8 and infected 5 weeks after the last immunization with 10^4 or 10^5 *L. infantum* intravenously. See figures 9 and 10. Parasite burdens in the liver and spleen were evaluated one month and 4.5 months after infection. The immunization diminished the degree of infection in both the liver and the spleen.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Fuertes-Lopez et al. U.S. Pat. Pub. No. 2004-0235772-A1

Larraga Rodriguez De Vera et al. U.S. Pat. Pub. No. 2004-0156866-A1

Bedate et al. U.S. Pat. No. 6,458,359

Gonzalez-Aseguinolaza et al. 2003-0157135-A1

Taniguchi et al. U.S. Pat. No. 6,747,010

Taniguchi et al. U.S. Pat. No. 6,531,453

Bachmann et al. U.S. Pat. Pub. No. 2003-0099668-A1

Jaffe et al. J Immunol. 1984 Jul;133(1):440-7.

5 Belli, et al., 1999, Exp. Parasitol. 91:151-160.

Campos-Neto et al., 2001, Infect. Immun. 69:4103-4108.

Dondji et al. Infection and Immunity, August 2005, p. 5286-5289, Vol. 73, No. 8.

Dumonteil et al., 2003, Vaccine 21:2161-2168.

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Quinnell, et al., 2001, J. Infect. Dis. 183:1421-1424.

15 Ramiro et al., 2003, Vaccine 21:2474-2484.

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Santos-Gomes, et al., 2002, Vet. Immunol. Immunopathol. 88:21-30.

EQUIVALENTS

20 While specific embodiments of the subject inventions are explicitly disclosed herein, the above specification is illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the inventions should be determined by reference to the claims, along with their full scope of equivalents, and
25 the specification, along with such variations.

WE CLAIM:

1. A formulation for eliciting an immune response to a Leishmania antigen
5 in a mammal, the formulation comprising at least two antigens selected from the group consisting of:
 - (a) GP46 or an antigenic portion thereof;
 - (b) P4 or an antigenic portion thereof;
 - (c) GP63 or an antigenic portion thereof;
 - 10 (d) P36-LACK or an antigenic portion thereof; and
 - (e) P8.
2. A formulation for eliciting an immune response to a Leishmania antigen
in a mammal, the formulation comprising two or more nucleic acids
encoding at least two antigens selected from the group consisting of:
 - 15 (a) GP46 or an antigenic portion thereof;
 - (b) P4 or an antigenic portion thereof;
 - (c) GP63 or an antigenic portion thereof;
 - (d) P36-LACK or an antigenic portion thereof.
3. A formulation for eliciting an immune response to a Leishmania antigen
20 in a mammal, the formulation comprising
 - (a) an antigen selected from the group consisting of:
 - i. GP46 or an antigenic portion thereof;
 - ii. P4 or an antigenic portion thereof;
 - iii. GP63 or an antigenic portion thereof;
 - 25 iv. P36-LACK or an antigenic portion thereof; and
 - v. P8; and

- (b) A nucleic acid encoding an antigen selected from the group consisting of:
- i. GP46 or an antigenic portion thereof;
 - ii. P4 or an antigenic portion thereof;
 - 5 iii. GP63 or an antigenic portion thereof; and
 - iv. P36-LACK or an antigenic portion thereof.
4. The formulation of any of claims 1-3, wherein each antigen is derived from a causative agent of visceral leishmaniasis.
 5. The formulation of any of claims 1-3, wherein each antigen is derived from an organism selected from the group consisting of: *Leishmania*
10 *donovani*, *Leishmania pifanoi*, *Leishmania chagasi* and *Leishmania infantum*.
 6. The formulation of any of claims 1-3, further comprising an adjuvant.
 7. The formulation of claim 6, wherein the adjuvant is an NKT-active
15 adjuvant.
 8. The formulation of claim 6, wherein the adjuvant is selected from the group consisting of: a glycosyl ceramide, a P-8 antigen and P.acnes/C. parvum.
 9. A formulation for eliciting an immune response to a Leishmania antigen
20 in a mammal, the formulation comprising an NKT-active adjuvant and an antigen from a causative agent of leishmaniasis.
 10. The formulation of claim 9, wherein the adjuvant is a glycosyl ceramide.
 11. The formulation of claim 10, wherein the glycosyl ceramide is selected from the group consisting of: a c-GalCer and an alpha-GalCer.
 - 25 12. The formulation of claim 10, wherein the adjuvant is a P-8 antigen.
 13. A formulation for eliciting an immune response to a Leishmania antigen in an outbred canine, the formulation comprising an antigen selected from the group consisting of: D-13, D-2, D-6/D-15, and D-14.

14. A method for eliciting an immune response to a Leishmania antigen in a mammal, the method comprising administering to a mammal at least two antigens selected from the group consisting of:
- 5 (a) GP46 or an antigenic portion thereof;
- (b) P4 or an antigenic portion thereof;
- (c) GP63 or an antigenic portion thereof;
- (d) P36-LACK or an antigenic portion thereof; and
- (e) P8.
- 10 15. A method for eliciting an immune response to a Leishmania antigen in a mammal, the method comprising administering to the mammal one or more nucleic acids encoding at least two antigens selected from the group consisting of:
- (a) GP46 or an antigenic portion thereof;
- (b) P4 or an antigenic portion thereof;
- 15 (c) GP63 or an antigenic portion thereof;
- (d) P36-LACK or an antigenic portion thereof; and
16. A method for eliciting an immune response to a Leishmania antigen in a mammal, the method comprising administering to the mammal:
- (a) an antigen selected from the group consisting of:
- 20 i. GP46 or an antigenic portion thereof;
- ii. P4 or an antigenic portion thereof;
- iii. GP63 or an antigenic portion thereof;
- iv. P36-LACK or an antigenic portion thereof; and
- v. P8; and
- 25 (b) A nucleic acid encoding an antigen selected from the group consisting of:
- i. GP46 or an antigenic portion thereof;

- ii. P4 or an antigenic portion thereof;
 - iii. GP63 or an antigenic portion thereof; and
 - iv. P36-LACK or an antigenic portion thereof.
- 5 17. The method of any of claims 14-16, wherein antigen and/or nucleic acid are administered as a single formulation.
18. The method of any of claims 14-16, wherein antigen and/or nucleic acid are administered separately.
- 10 19. A method for eliciting an immune response to a *Leishmania* antigen in a mammal, the method comprising administering to the mammal an NKT-active adjuvant and an antigen from a causative agent for leishmaniasis.
20. A purified P4 polypeptide, comprising an amino acid sequence selected from the group consisting of: SEQ ID No. 1, an antigenic portion of SEQ ID No. 1, and SEQ ID No. 2, an antigenic portion of SEQ ID No. 2.
- 15 21. A formulation for eliciting an immune response to a *Leishmania* antigen in a mammal comprising a purified polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID No. 1 and SEQ ID No. 2.
22. A formulation for eliciting an immune response to a *Leishmania* antigen in a mammal, the formulation comprising P-8 antigen.
- 20 23. The formulation of claim 22, wherein the P-8 antigen is derived from a causative agent for visceral leishmaniasis.
24. The formulation of claim 22, wherein the P-8 antigen is derived from an organism selected from the group consisting of: *Leishmania donovani*, *Leishmania pifanoi*, *Leishmania chagasi*, *Leishmania amazonensis*,
25 *Leishmania mexicana*, and *Leishmania infantum*.
25. The formulation of any of claims 22-24, further comprising an adjuvant.
26. A vaccine for protecting a mammal from visceral leishmaniasis, the vaccine comprising at least two antigens selected from the group consisting of:

- (a) GP46 or an antigenic portion thereof;
 - (b) P4 or an antigenic portion thereof;
 - (c) GP63 or an antigenic portion thereof;
 - (d) P36-LACK or an antigenic portion thereof; and
 - 5 (e) P8.
27. A vaccine for protecting a mammal from visceral leishmaniasis, the vaccine comprising two or more nucleic acids encoding at least two antigens selected from the group consisting of:
- (a) GP46 or an antigenic portion thereof;
 - 10 (b) P4 or an antigenic portion thereof;
 - (c) GP63 or an antigenic portion thereof;
 - (d) P36-LACK or an antigenic portion thereof.
28. A vaccine for protecting a mammal from visceral leishmaniasis, the vaccine comprising
- 15 (a) an antigen selected from the group consisting of:
 - i. GP46 or an antigenic portion thereof;
 - ii. P4 or an antigenic portion thereof;
 - iii. GP63 or an antigenic portion thereof;
 - iv. P36-LACK or an antigenic portion thereof; and
 - 20 v. P8; and
 - (b) A nucleic acid encoding an antigen selected from the group consisting of:
 - i. GP46 or an antigenic portion thereof;
 - ii. P4 or an antigenic portion thereof;
 - 25 iii. GP63 or an antigenic portion thereof; and
 - iv. P36-LACK or an antigenic portion thereof.

29. The vaccine of any of claims 26-28, wherein each antigen is derived from a causative agent of visceral leishmaniasis.
30. The formulation of any of claims 26-28, further comprising an adjuvant.
31. The formulation of claim 30, wherein the adjuvant is an NKT-active adjuvant.
32. The formulation of claim 30, wherein the adjuvant is selected from the group consisting of: a glycosyl ceramide, a P-8 antigen and *P.acnes/C. parvum*.
33. A vaccine for protecting a mammal from visceral leishmaniasis, the vaccine comprising an NKT-active adjuvant and an antigen from a causative agent of visceral leishmaniasis.
34. The formulation of claim 9, wherein the adjuvant is a glycosyl ceramide.
35. The formulation of claim 10, wherein the glycosyl ceramide is selected from the group consisting of: a c-GalCer and an alpha-GalCer.
36. The formulation of claim 10, wherein the adjuvant is a P-8 antigen.
37. A formulation for eliciting an immune response to a Leishmania antigen in a mammal comprising a purified polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID No. 1 and SEQ ID No. 2.
38. A formulation for eliciting an immune response to a Leishmania antigen in a mammal, the formulation comprising P-8 antigen.
39. The formulation of claim 22, wherein the P-8 antigen is derived from a causative agent for visceral leishmaniasis.
40. The formulation of claim 22, wherein the P-8 antigen is derived from an organism selected from the group consisting of: *Leishmania donovani*, *Leishmania pifanoi*, *Leishmania chagasi*, *Leishmania amazonensis*, *Leishmania mexicana*, and *Leishmania infantum*.

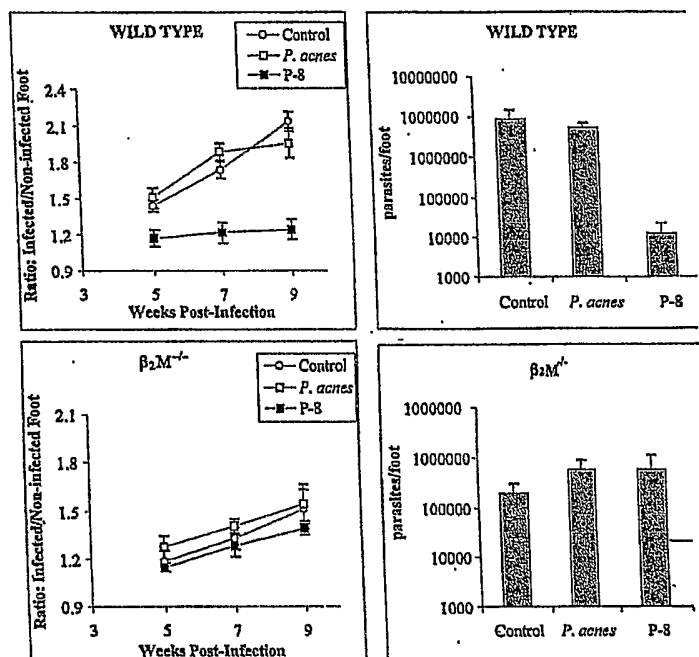


FIGURE 1

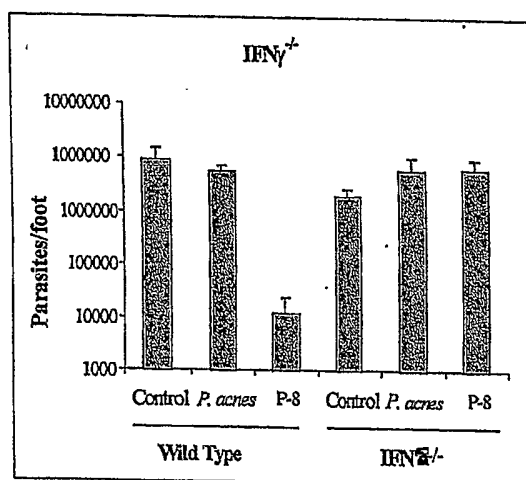


FIGURE 2

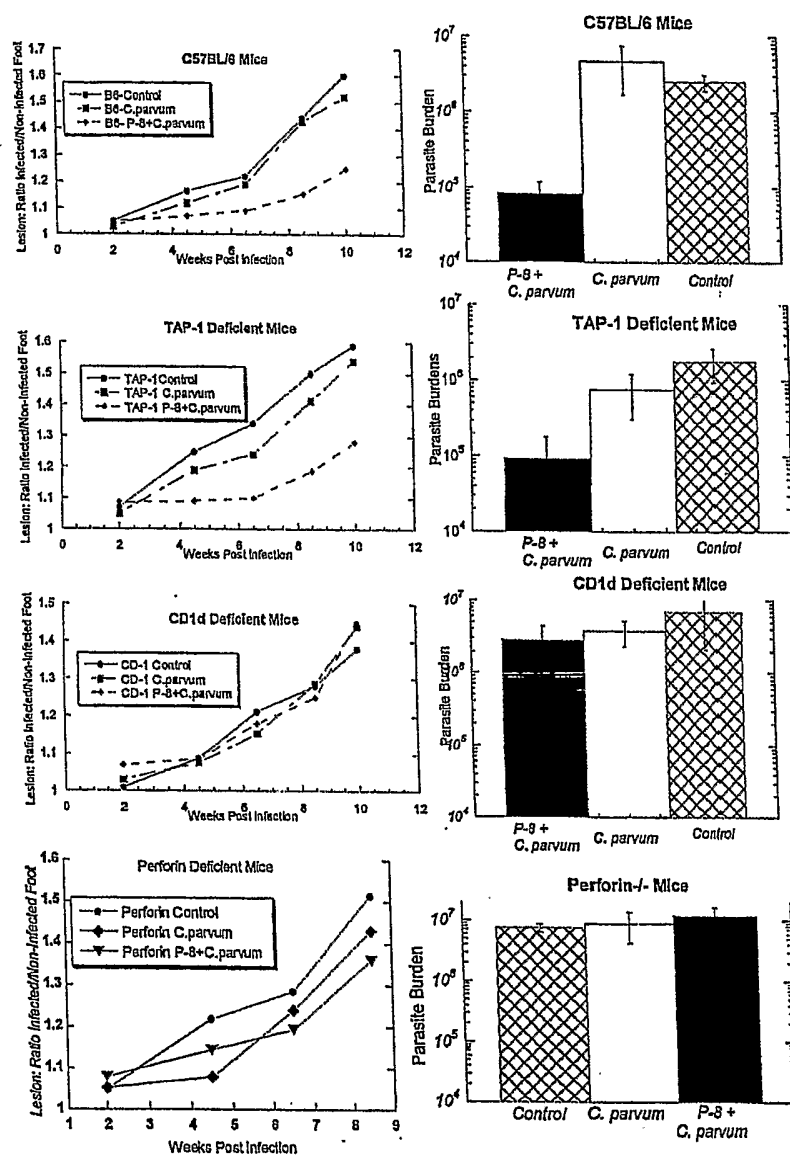


FIGURE 3

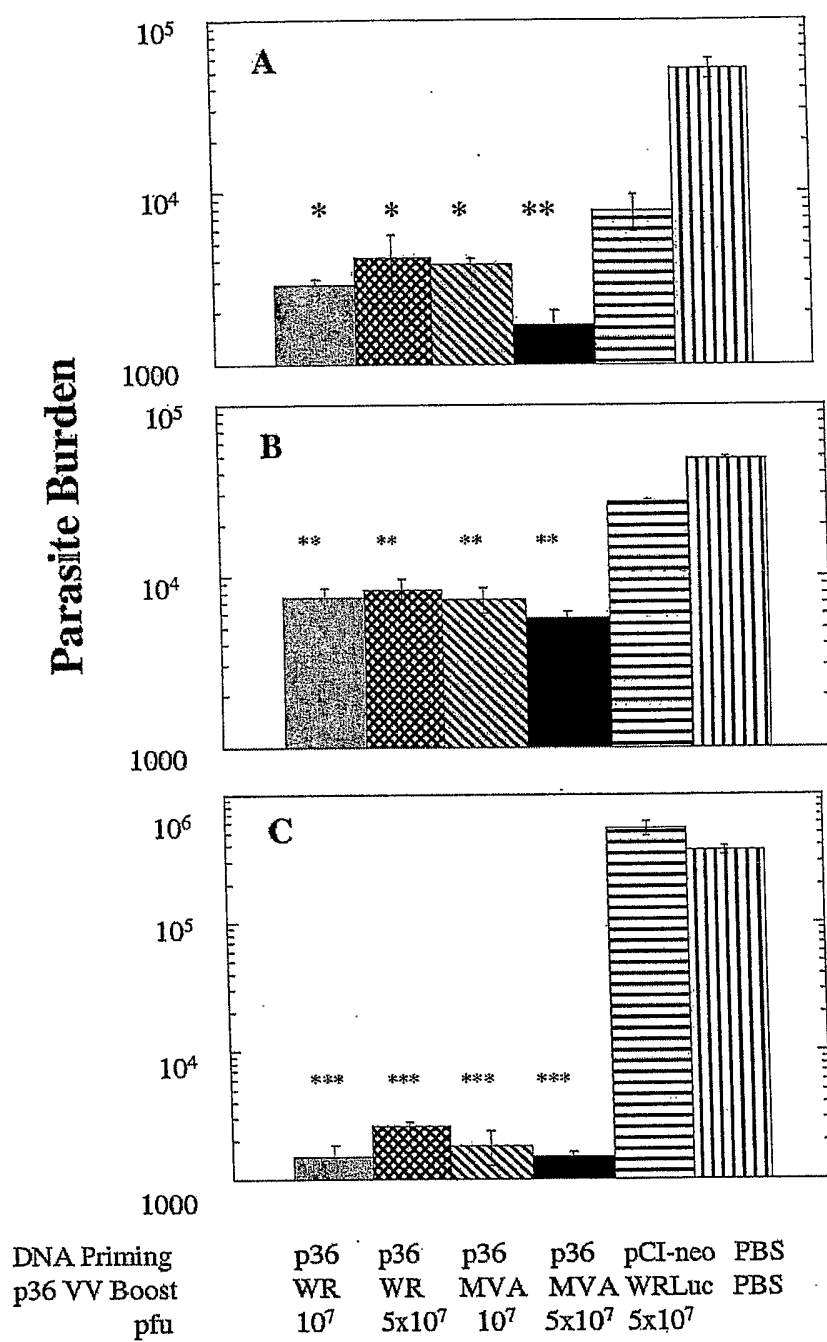


Figure 4

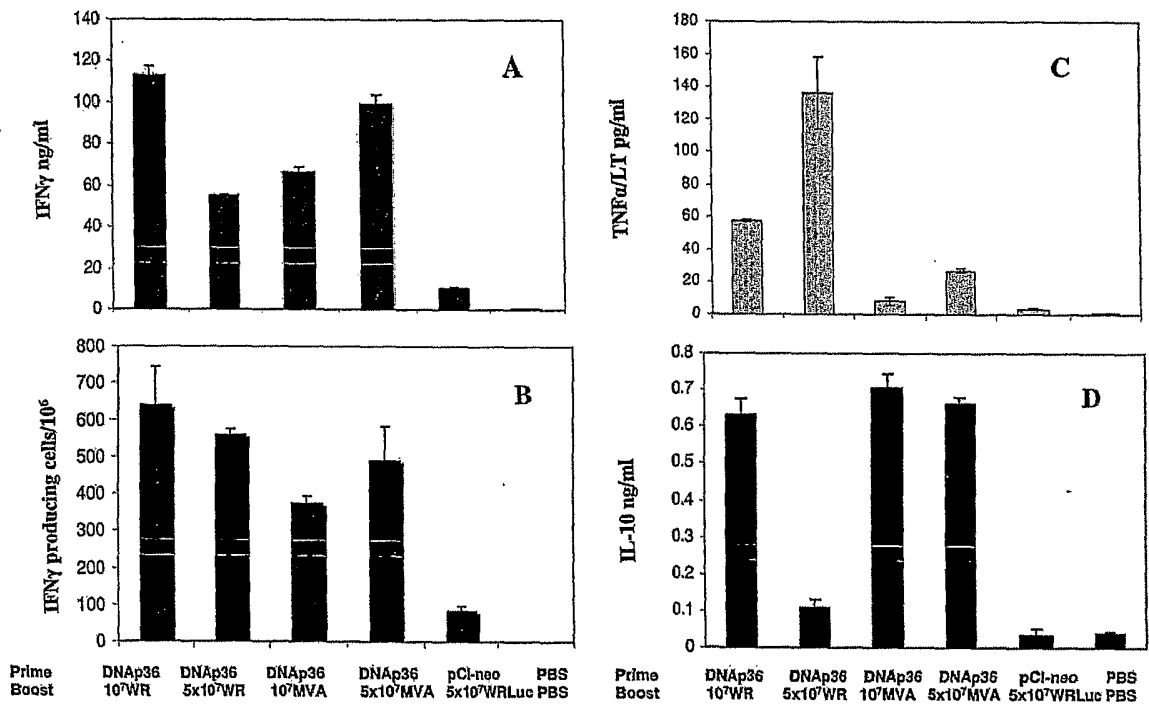


Figure 5

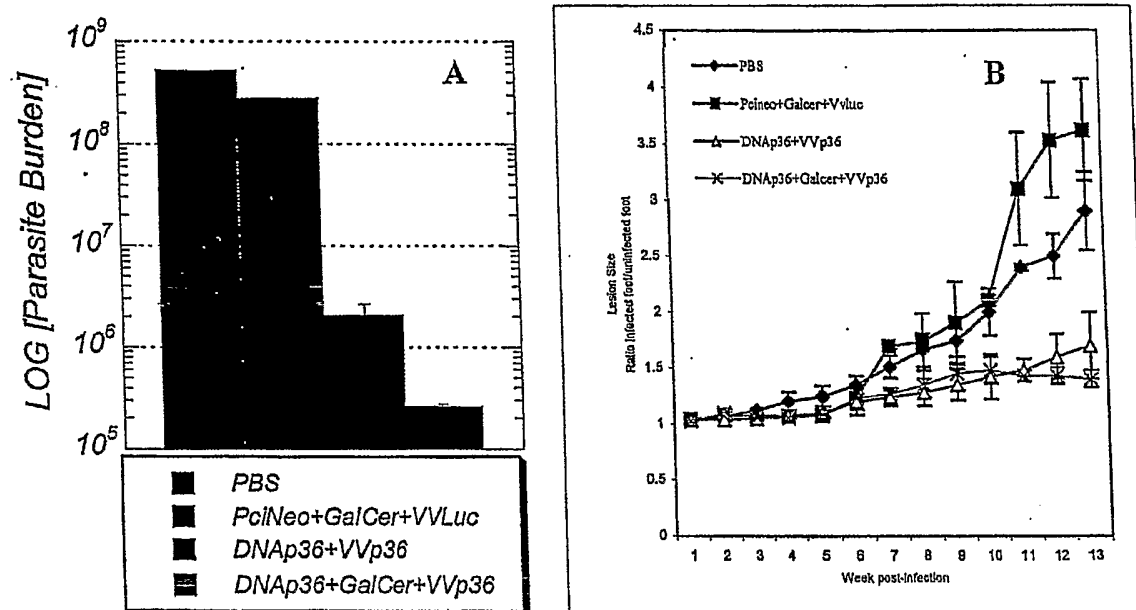


FIGURE 6

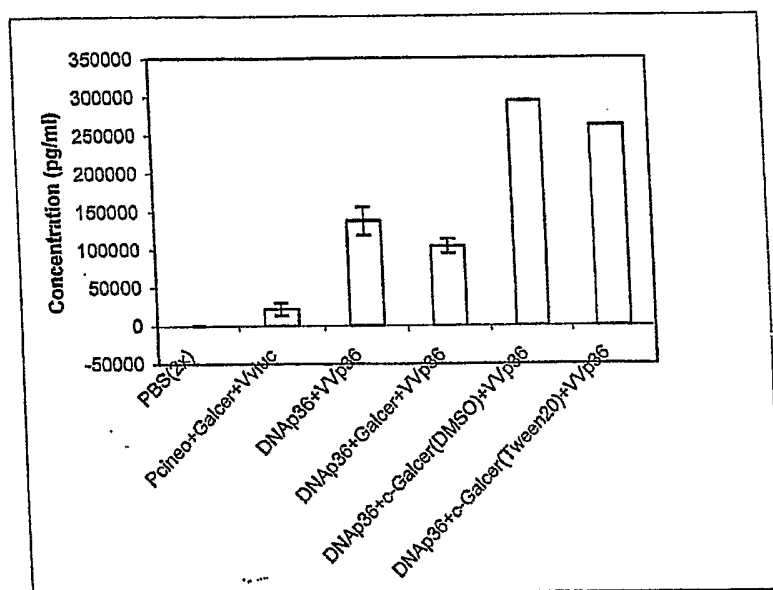


FIGURE 7

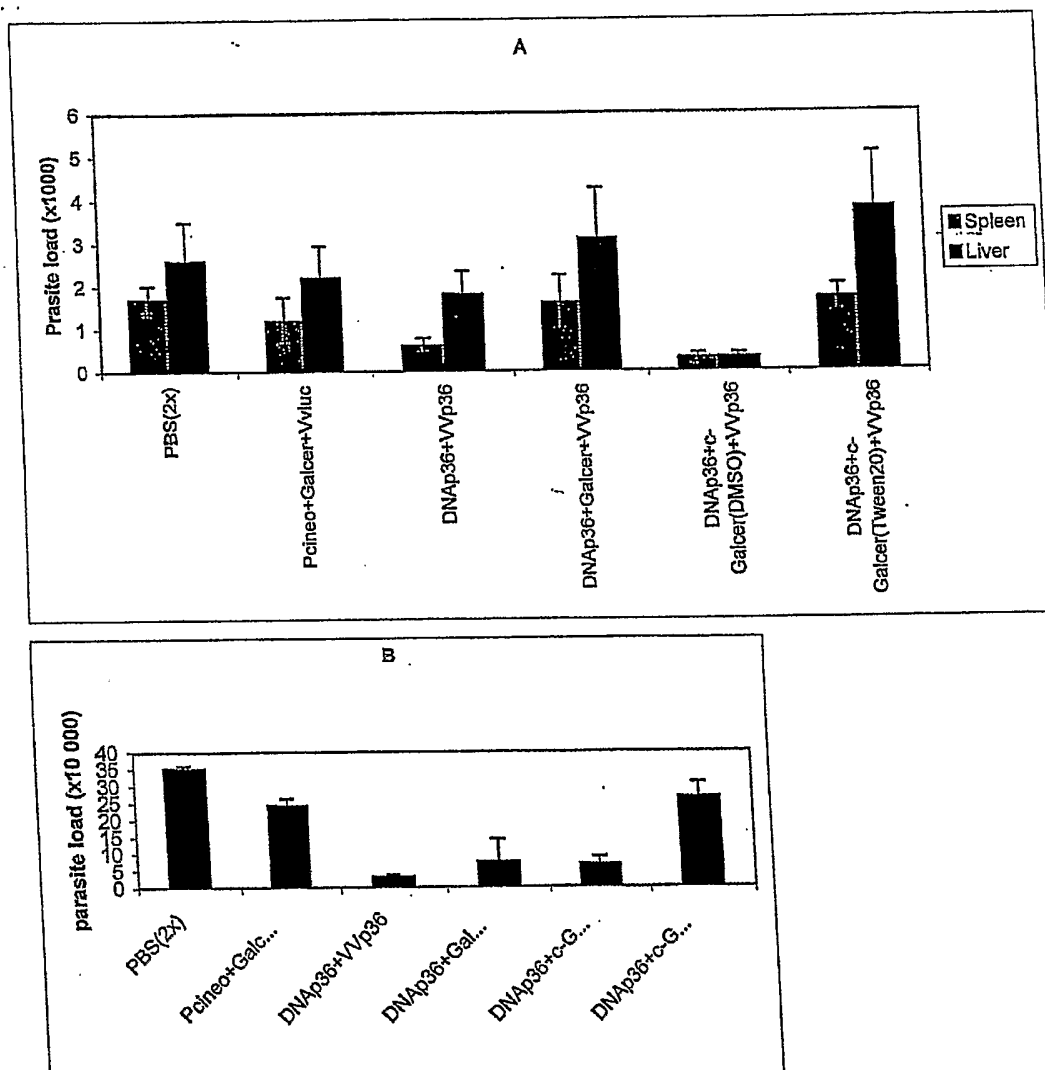


FIGURE 8

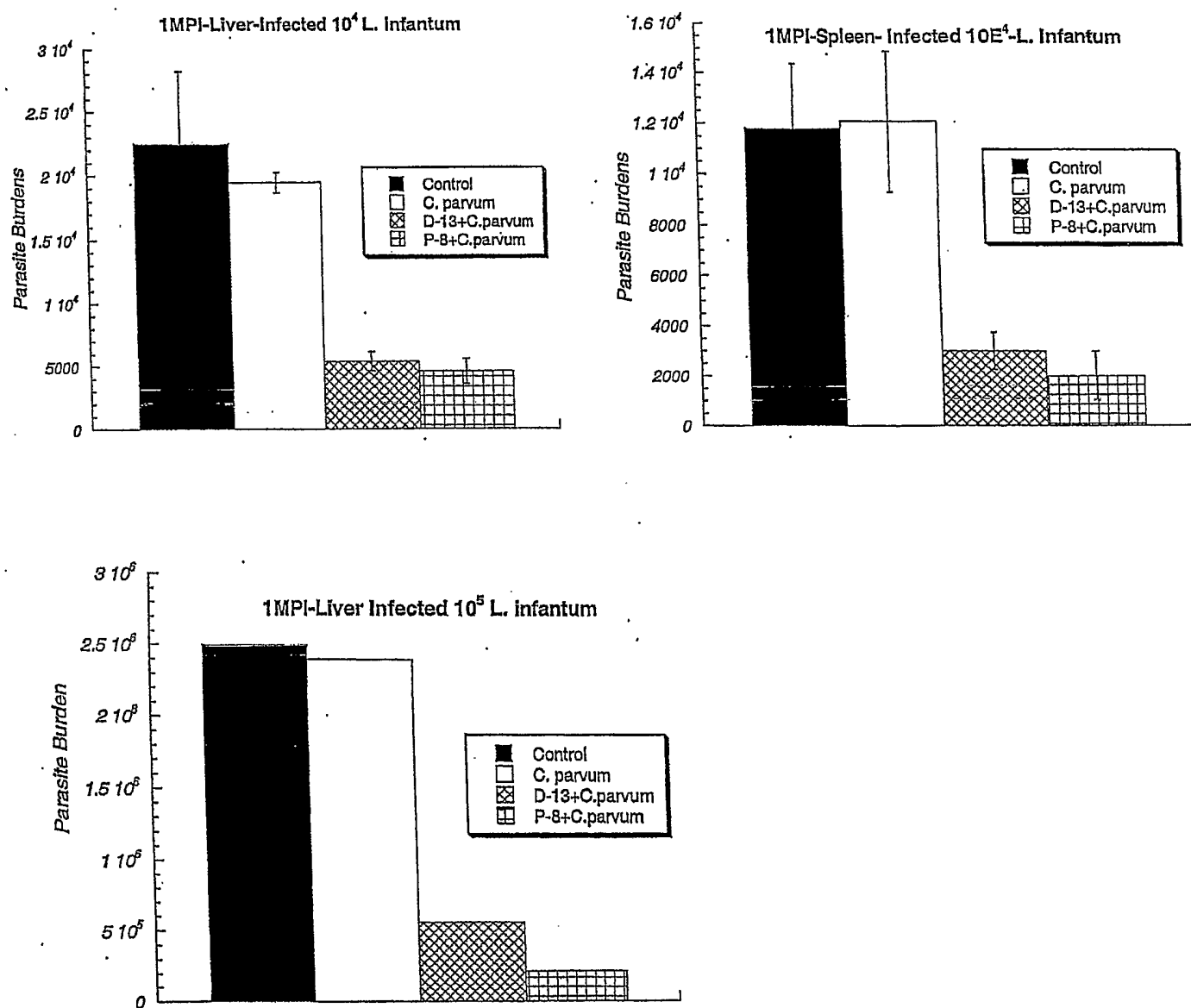


FIGURE 9

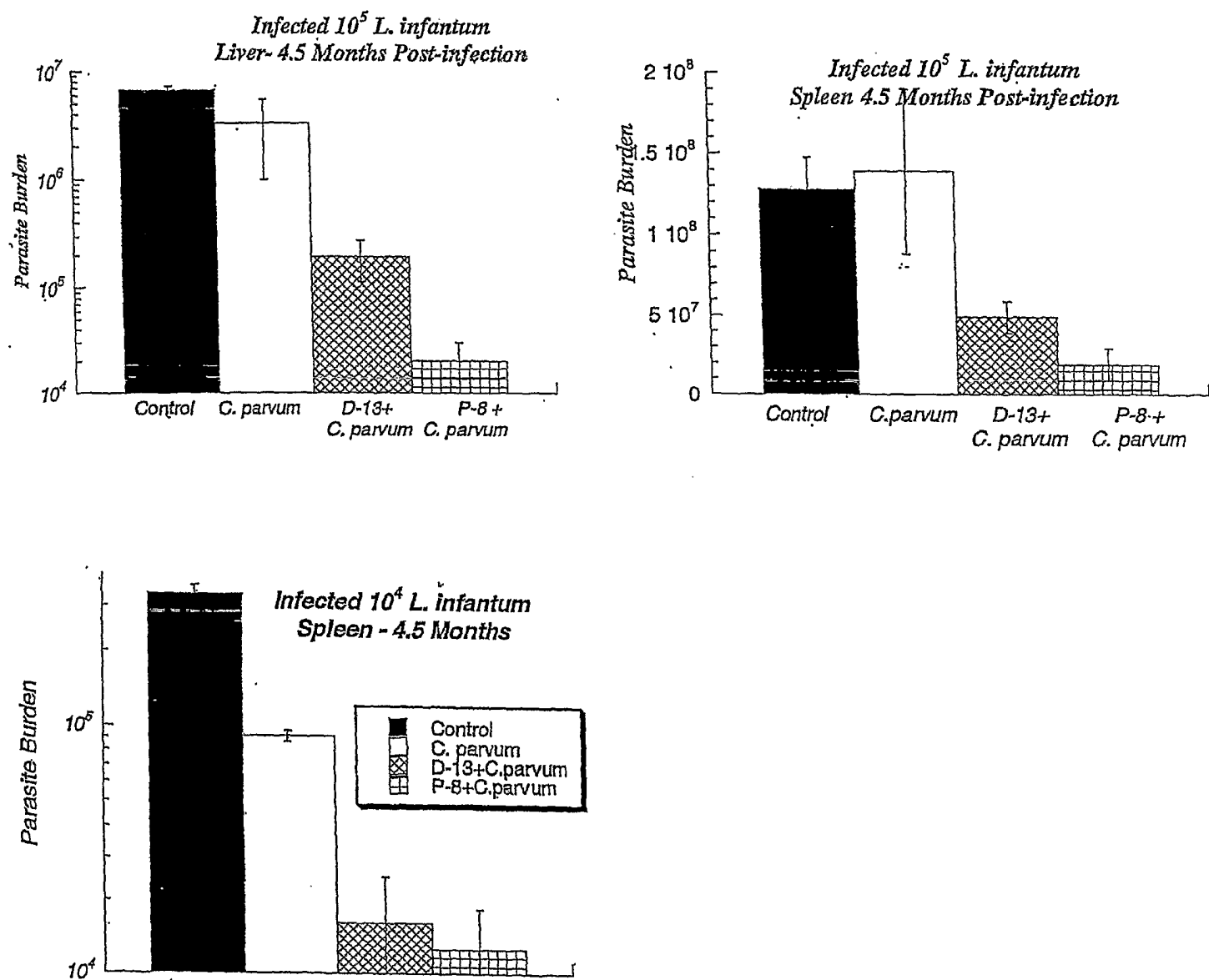


FIGURE 10

P4 Zoo Comparison

453A1	MPALVGLRLPLTLVLCLLVLSSALCVTEALGWGCVGLMLLAEIAHRQLDDKNKEKIDAMAE
Linf29	MPALVGLRLPLTLVLCLLVLSSALCVTEALGWGCVGHMLLAEIAHRQLDDKNKEKIDAMAE
L.pif	MPAPVSLRLPLTALCCLLVLSALCVTEVLGWGCVGHMLLAEIARRQLDDKNKEKIQKMAA
LmajcI	MPAFVGLRLPLTLVLCCLLVLSALCVTEVLGWGCVGHMLLAEIAHRQLNDENKEKLDAMAH
Lama	MPAPVSLRLPLTALCCLLVLSALCVTEVLGWGCVGHMLLAEIARRQLDLENEEKIELMAA
LdcI	MLALVGLRLPLTLVLCCLLVLSALCVTEALGWGCVGHMLLAEIAHRQLDDKNKEKIDAMAE
453A1	VFAQSGFPSPSPDMVQAACWADDVKRWRQYAMATWHFFAAPYNPENINITDAIDTVNAV
Linf29	VFAQSGFPSPSPDMVQAACWADDVKRWRQYAMGSHFFDAPYNPENINITDAIDTVNAV
L.pif	VFSDSGFPSPSPDMVQAACWADDVKLRQRGMSTWHYYDKVYNPENINITDRVNTVNALT
LmajcI	AFAQSGFPSPSPDMVQAACWADDVKRWGQYAMATWHFFATPYNPEDINITDPVATVNAV
Lama	VFSGSGFPSPSPDMVQAACWADDVKLRQYAMSTWHFYAMPYNPGNINITDPVNTVNAV
LdcI	VFAQSGFPSPSPDMVQAACWADDVKRWRQYAMATWHFFAAPYNPENINITDAIDTVNAV
453A1	VSLDMISALKNTKAPLYMLNFAWANLVHIFGDLHQPLHTISRYSSSEYPHGDKGNGNAVEVR
Linf29	VSLDMISAFKNTKAPLYMLNFWWANLVHIFGDDHQPLHTISRYSSSEYPHGDNGGNLIQVM
L.pif	ASRNMVTSLKKSAPLYLLNFAWVNLVHIFGDLHQPLHTISRYTTAYPHGDQGGNAVSVR
LmajcI	VSRNMVTSLRRTNAPLHLLNFAWVNLVHILGDLHQPLHTISRYSSSEYPHGDRGNGKVEVR
Lama	VCLDMVTSLKNSKAPLYLLNFAWVNLVHIFGDLHQPLHTISRYTTAYPHGDQGGNAISVR
LdcI	VSLDMISALKNTKAPLYMLNFAWANLVHIFGDLHQPLHTISRYSSSEYPHGDKGGNLIQVM
453A1	AGRRKVKLHALWDNICTGAPPRYQRPLSYTDLFALAATADRLLLETYKFPEALRTLVDIRE
Linf29	VGRKWLRLHALWDNICTGAPPRYQRPLSYTDLFALAATADRLLLETYIFPEALRTLVDVMA
L.pif	AGRRKVK-HALWDNICTGAPPRYQRPLSYTDLFALSATADRIVETTYTFSEELRTLVSVA
LmajcI	VRKRKVNLAHAWDNICSGTPPRYKRPLSYTDLFALAATADRLLLETYTFPEALRTLVDVVA
Lama	VGGKKVKLHALWDNICSATPPRYQRPLSHTDLFALSATADGLVETTYTFSEALETLVDVMA
LdcI	VGRKSLRLHALWDNICTGAPPRYQRPFSYTDLFALAATADRLLLETYIFPEALRTLVDVMA
453A1	VHEESHMFVANTSYPGVTPGATLSEAYLARCKRVAEARLTLGGYRLGYLLNTLLSSIHVD
Linf29	IHEESHMFVANTSYPGVTPGATLSEAYLARCKRVAEARLTLGGSRLGYLLNTLLSSIHL
L.pif	IHEESYMFVANTSYPGVTPGATLSDAYLDKCKRVAEARLTLGGYRLGYLLNQLLSGITVD
LmajcI	IHEESHMFVANTSYPGVTPGATLSDDYLARCKRVAEARLTLGGYRLGYLLNELLSSIIPVD
Lama	IHEESYMFVANTSYPGVTPGGTLSRAYLDKCKRVAEARLTLGGYRLGYLLNQLLSGITVD
LdcI	IHEESHMFVANTSYPGVTPGATLSEAYLARCKRVAEARLTLGGYRLGYLLNTLLSSIHVD
453A1	EATLEAYRAARPKRGA
Linf29	EATLEAYRAARPKRGA
L.pif	EAALEAHRAARPKWSA
LmajcI	EATLEAYRAARWKWSA
Lama	KAALEAHRAARPKWSA
LdcI	EATLEAYRAARPKRGA
453A1, Linf29 -	Two different P-4 genes from <i>Leishmania infantum</i>
L.pif	<i>Leishmania pifanoi</i> P-4 gene
LmajcI	<i>Leishmania major</i> genebank sequence
Lama	<i>Leishmania amazonensis</i> - genebank sequence
LdcI	<i>Leishmania chagasi</i> - genebank sequence

Figure 11

LACK Antigen Comparison

Lamaz	MNYEGHLKGH	RGWVTSACP	QQAGSYIKVV	STSRDGTAS	WKPANPRHSV	50
Lmaj	MNYEGHLKGH	RGWVTSACP	QQAGSYIKVV	STSRDGTAS	WKPANPRHSV	
Lmaj2	MNYEGHLKGH	RGWVTSACP	QQAGSYIKVV	STSRDGTAS	WKPANPRHSV	
Linf	MNYEGHLKGH	RGWVTSACP	QQAGSYIKVV	STSRDGTAS	WKPANPRHSV	
Lchag	MNYEGHLKGH	RGWVTSACP	QQAGSYIKVV	STSRDGTAS	WKPANPRHSV	
Lmex	MNYEGHLKGH	RGWVTSACP	QQAGSYIKVV	STSRDGTAS	WKPANPRHSV	
Lamaz	DSDYGLPSHR	LEGHTGFVSC	VSLAHATDYA	LTASWDRSIR	MWDLRNGQCQ	100
Lmaj	DSDYGLPNHR	LEGHTGFVSC	VSLAHATDYA	LTASWDRSIR	MWDLRNGQCQ	
Lmaj2	DSDYGLPSHR	LEGHTGFVSC	VSLAHATDYA	LTASWDRSIR	MWDLRNGQCQ	
Linf	DSDYGLPSHR	LEGHTGFVSC	VSLAHATDYA	LTASWDRSIR	MWDLRNGQCQ	
Lchag	DSDYGLPSHR	LEGHTGFVSC	VSLAHATDYA	LTASWDRSIR	MWDLRNGQCQ	
Lmex	DSDYGLPSHR	LEGHTGFVSC	VSLAHATDYA	LTASWDRSIR	MWDLRNGQCQ	
Lamaz	RKFLKHTKDV	LAVAFSPDDR	LIVSAGRDNV	IRVWNVAGEC	MHEFLRDSHE	150
Lmaj	RKFLKHTKDV	LAVAFSPDDR	LIVSAGRDNV	IRVWNVAGEC	MHEFLRDGHE	
Lmaj2	RKFLKHTKDV	LAVAFSPDDR	LIVSAGRDNV	IRVWNVAGEC	MHEFLRDSHE	
Linf	RKFLKHTKDV	LAVAFSPDDR	LIVSAGRDNV	IRVWNVAGEC	MHEFLRDGHE	
Lchag	RKFLKHTKDV	LAVAFSPDDR	LIVSAGRDNV	IRVWNVAGEC	MHEFLRDGHE	
Lmex	RKFLKHTKDV	LAVAFSPDDR	LIVSAGRDNV	IRVWNVAGEC	MHEFLRDSHE	
Lamaz	DWVSSICFSP	SLEHPIVVSG	SWDNTIKVWN	VNGGKCERTL	KGHSNYVSTV	200
Lmaj	DWVSSICFSP	SLEHPIVVSG	SWDNTIKVWN	VNGGKCERTL	KGHSNYVSTV	
Lmaj2	DWVSSICFSP	SLEHPIVVSG	SWDNTIKVWN	VNGGKCERTL	KGHSNYVSTV	
Linf	DWVSSICFSP	SLEHPIVVSG	SWDNTIKVWN	VNGGKCERTL	KGHSNYVSTV	
Lchag	DWVSSICFSP	SLEHPIVVSG	SWDNTIKVWN	VNGGKCERTL	KGHSNYVSTV	
Lmex	DWVSSICFSP	SLEHPIVVSG	SWDNTIKVWN	VNGGKCERTL	KGHSNYVSTV	
Lamaz	TVSPDGSLCA	SGGKDGAALL	WDLSTGEQLF	KINVESPINQ	IAFSPNRFWM	250
Lmaj	TVSPDGSLCA	SGGKDGAALL	WDLSTGEQLF	KINVESPINQ	IAFSPNRFWM	
Lmaj2	TVSPDGSLCA	SGGKDGAALL	WDLSTGEQLF	KINVESPINQ	IAFSPNRFWM	
Linf	TVSPDGSLCA	SGGKDGAALL	WDLSTGEQLF	KINVESPINQ	IAFSPNRFWM	
Lchag	TVSPDGSLCA	SGGKDGAALL	WDLSTGEQLF	KINVESPINQ	IAFSPNRFWM	
Lmex	TVSPDGSLCA	SGGKDGAALL	WDLSTGEQLF	KINVESPINQ	IAFSPNRFWM	
Lamaz	CVATERSLSV	YDLESKAVIA	ELTPDGAKPS	ECISIAWSAD	GNTLYSGHKD	300
Lmaj	CVATERSLSV	YDLESKAVIA	ELTPDGAKPS	ECISIAWSAD	GNTLYSGHKD	
Lmaj2	CVATERSLSV	YDLESKAVIA	ELTPDGAKPS	ECISIAWSAD	GNTLYSGHKD	
Linf	CVATERSLSV	YDLESKAVIA	ELTPDGAKPS	ECISIAWSAD	GNTLYSGHKD	
Lchag	CVATERSLSV	YDLESKAVIA	ELTPDGAKPS	ECISIAWSAD	GNTLYSGHKD	
Lmex	CVATERSLSV	YDLESKAVIA	ELTPDGAKPS	ECISIAWSAD	GNTLYSGHKD	
Lamaz	NLIRVWSISD	AE				312
Lmaj	NLIRVWSISD	AE				
Lmaj2	NLIRVWSISD	AE				
Linf	NLIRVWSISD	AE				
Lchag	NLIRVWSISD	AE				
Lmex	NLIRVWSISD	AE				

Figure 12

GP46 COMPARISON

MAQQP2	MAQCVRRRLVL	A*PTLWLVAL	LLCTSSAGPA	VKEDNLTETQ
GP46/M2	MAQCVRRRLVL	AAPLAAVVAL	LLCTSSAPVA	RAAGTSDFTG
PSA-2	MAQCVRRRLVL	GDARRCGGAA	AAVHEQARVA	RAAGTGDFTA
PSA.1	MAQCVRRRLVL	AATLAAVAL	LLCTSSAPVA	RAAGTGDFTD
Li PSA	MAQCVRRRLVL	AATLAAVAL	LLCTSSAPVA	RAAGTGDFTD
Lch	MALCVRRRLVL	AATLAAVVAL	LLCTSSAPVA	RAVKEDNLTE

MAQQP2	KS**NTRLVL	QAFGRAIL**	GRTVKAH*FC	SVEVRVLLPL
GP46/M2	AQQKNTLTVL	QAFARAIPAL	GDTWTS*DFC	SWEHIICYSS
PSA-2	AQRTNTLAVL	QAFGRAIPKL	GEKWAGNDFC	SWEAVLCNAP
PSA.1	EQRTHTLAVL	QAFGRAIPEL	GKNWTGDDFC	SWDHVTCAPL
Li PSA	EQRTHTLAVL	QAFGRAIPEL	GKNWTGDDFC	SWDHVTCAPL
Lch	TQKSNTLAVL	QAFGRAIPEV	KKLWKGDDFC	SWKYVVCYPS

MAQQP2	*VGVLMDSVD	YAGSLPEMLR	AVDYTNV
GP46/M2	GVGVMHNVND	YTGTLPEMPA	SVDYKDV
PSA-2	DVYVSGISPT	YAGTLPEMPN	*VDYRHV
PSA.1	SVAVGLDVST	YAGTLPEIPV	GVDYRY*
Li PSA	SVAVGLDVST	YAGTLPEIPV	GVDYRY*
Lch	AVGVLMDSVD	YAGSLPEMPA	GVDYTNV

REPEATS

MAQQP2	VITQLDFSAGQ	RLSGTVPASWSS
GP46/M2	MILALDFGAMQ	GLSGTLPPSWSS
PSA-2	VIRRLDFSEMGP	GLSGTVPASWHS
PSA.1	VMIRDLGFWNMP	LLVGTLPDSWSN
Li PSA	VMIRDLGFWNMP	LLVGTLPDSWSN
Lch	VITQLDFSAGQ	RLSGTLPASWSS

MAQQP2	MTSLVSLWLEG	ERVGTLPASWSS
GP46/M2	MKHLIVLDLEGT	KVSGT*LPPEWSE
PSA-2	MTSLESLSIEKC	ESISGSVPPEWGS
PSA.1	LIRLSFLTLSGC	GVSGT*LPASWSS
Li PSA	LIRLSFLTLSGC	GVSGT*LPASWSS
Lch	MTSLVSLWLEG	ERVGTLPASWSS

MAQQP2	MTSLTDLNLHGT	QVS*GSLRPQWSS
GP46/M2	MTSAEALQLENC	GLS*GSLPTSWSS
PSA-2	MTSLSVLNLRG	GIS*GTLPPQWSG
PSA.1	MAFLRELTVGNC	GRLTGSLPPEWGS
Li PSA	MAFLRELTVGNC	GRLTGSLPPEWGS
Lch	MTSLSDLNLHGT	QVS*GSLPPQWSS

MAQQP2	MTSLATLSLRLT	KISGSLPSEWSS
GP46/M2	MPKLRIVSLSGN	HFCGCVPDWRE
PSA-2	MSKARSLQLQDC	DLSGSLPSSWSA
PSA.1	MPNLSRLVLRQL	QLSGTLPAEWSS

Figure 13A

Li PSA	MPNLSRLVLRQL	QLSGTLPSEWSR
Lch	MTSLATLSLRLT	KVSGSLPSEWSS
MAQQP2	MTSLTLDDLQGT	KVSGSLPSEWSG
PSA-2	IPMLASVSLKGN	KFCGCVPDSDWQ
PSA.1	MRLVLTLEIAAAG	SFTGTLPSEWSS (25 RESIDUES)
Li PSA	MRLVLTLEIAAAG	SFTGTLPSEWSS
Lch	MTSLTLDDLQGT	KVSGSLPSEWSG
MAQQP2	MKSAEVLQLQHG	DLSGSLPSSWSA
PSA.1	<u>IKSLANLYLDDL</u>	<u>PIGGSLPPEWGS</u>
Li PSA	IKSLANLYLDDL	PIGGSLPPEWGS
Lch	MKSAEVLQLQDC	DLSGSLPSSWSA
MAQQP2	MPKLREVL LSGN	HFCGCVPESWTL
PSA.1	MANLRELKIWEL	PLTGPLPPQWGS
Li PSA	MANLRELKIWEL	PLTGPLPPQWGS
Lch	MPKLREVL LSGN	HFCGCVPESWTL
PSA.1	IAGLRVLSLRGT	SISGTLPPEWSS
Li PSA	IAGLRVLSLRGT	SISGTLPPEWSS
PSA.1	MKFLTNLDLGA	NISGSLPPQWGS
Li PSA	MKFLTNLDLGA	NISGSLPPQWGS
PSA.1	LEFLRGLDLQGT	KVSGTLPPEWSE
Li PSA	LEFLRGLDLQGT	KVSGTLPPEWSE
PSA.1	MKWMTFLYLELT	QLSGTLPSEWSS
Li PSA	MKWMTFLYLELT	QLSGTLPSEWSS
PSA.1	MTRLRDLNLHGA	NISGSLPPEWTS
Li PSA	MTRLRDLNLHGA	NISGSLPPEWTS
PSA.1	MAALNFLDLGGT	QVSGSLPPQWST
Li PSA	MAALNFLDLGGT	QVSGSLPPQWST
PSA.1	MLSAEILQLEHC	DLSGSLPPEWSA
Li PSA	MLSAEILQLEHC	DLSGSLPPEWSA
PSA.1	MPKLRLVGLKGN	HFCGCVPDSDWAQ
Li PSA	MPKLRLVGLKGN	HFCGCVPDSDWAQ

HINGE

Figure 13B

MAQQP2 KRKLMVTIEADHEGR
 GP46/M2 KDRLDVTIEEWHMGE
 PSA-2 KAGLVVDIEDKHKGS
 PSA.1 NAGLVVRIEDEHKGK
 Li PSA NAGLVVRIEDEHKGK
 Lch KRKLMVTIEADHEGR

C-TERMINUS

MAQQP2 (GAP)HRWSLR RVERTARAAA VCMVLLSVG LAA
 GP46/M2 TECACP ALFDGARLRC CALVVCAGAA PAG
 PSA-2 DGGVAA VSSGVAAA VCVAVLFSVG LAA
 PSA.1 YCRL GNDCRTTEPT TTATPRGTPT PAP
 Li PSA YCRL GNDCRTTEPT TTATPRGTPT PAP
 Lch RNCKLENKCR PAAPTTTTTT TSTTTKRATA STSTTTTTTV
 PPTLPSTTAT PTATPTPAPE TECEVDGCEV CEEDSAARCA
 RCREGYFVTS EKTCLMTTDG GLAAALSGAA AAAAVCMALL LSVGLAA

Figure 13C

GP63 Sequence Comparison –Leishmania species (zoo compare)

C fasc.	MHAPPTATRR	SGPRRTHGIM	ARLVRLAAGV	LVVTLVIGAL	TALSADDAKT	50
L amaz	MSVDSSSTHR	HRCVAARLVP	LAAAGAAVTV	AVGTAAAWAH	AGAVQHRCIH	
L chag	MSVDSSSTHR	HRSVAARLVR	LAAAGAAVIA	AVGTAAAWAH	AGAVQHRCIH	
L don	MSVDSSSTHR	HRSVAARLVR	LAAAGAAVIA	AVGTAAAWAH	AGAVQHRCIH	
L maj	MSVDSSSTHR	RRCVAARLVR	LAAAGAAVTV	AVGTAAAWAH	AGALQHRCVH	
L mex	MPVDSSSTHR	HRCVAAPLVR	LAAAGAAVTV	AVGTAAAWAH	AGAPQHRCIH	
C fasc.	HPHKVCIHDE	LQQSL LDSVA	QQGLAPQRVS	RVGLPYVASA	TAAPAAQVGG	
L amaz	DAMQARVRQS	VAAQRMAPS	VSAVGLPHVT	LDAGNTAAGA	DPSTGTANVV	
L chag	DAMQARVRQS	VARHHTAPGA	VSAVGLPYVT	LDTAAADRR	PGSAPTIVRA	
L don	DAMQARVRQS	VARHHTAPGA	VSAVGLSYVT	LGAAPTIVRA	ANWGALRIAV	
L maj	DAMQARVRQS	VADHHKAPGA	VSAVGLPYVT	LDAHTAAAA	DPRPGSARSV	
L mex	DAMQARVLQS	VAAQRMAPS	VSAVGLPYVS	VVPVENASTL	DYSLSDSTSP	
C fasc.	VDFALAGDSA	PDVTRSAEWG	ERITVSAEEL	TDPAYHCATV	GQVISNHIDD	150
L amaz	RAANW GALRI	AVSAEDLTDP	AYH CARVGQR	VNNHVGDI VT	CTAEDILTDE	
L chag	ANWGALRIAV	STEDLTDPAY	H CARVGQHIK	RRLGGVDICT	AEDILTDEKR	
L don	STEDLTDSAY	H CARVGQRI S	TRDGRFAICT	AEDILTDEKR	DILVKYLIPQ	
L maj	VRDVNW GALR	IAVSTEDLTD	PAYH CARVGQ	HVKDHAGAI V	TCTAEDILT N	
L mex	GVVRAANWGA	LRVAVSAEDL	TDPAYH CARV	GQQVNNHAGD	IVTCTAEDIL	
C fasc.	YVTCTADDIM	TAEKLDILMN	YLIPEALQMH	KDRLQVQQVQ	GTWKVARMTS	200
L amaz	KRDILVKHLV	PQALQLHRER	LKVQQVQGW	KVTGMTADV C	RYFKVPPAHV	
L chag	DILVKHLIPQ	ALQLHTERLK	VRQVQDKWKV	TGMGDDVCSD	FKVPPAHITD	
L don	ALQLHTERLK	VRQVQDKWKV	TGMGNEICGH	FKVPPAHITD	GLSNTDFVMY	
L maj	EKRDILVKHL	IPQAVQLHTE	RLKVQQVQGW	WKVTD MVGDI	CGDFKVPQAH	
L mex	TDEKRD TLVK	HLVPQALQLH	RERLKV RQVQ	GKWKVTGMAD	VICGDFKVP P	
C fasc.	YCGRFKVP EE	HFTTGLSNTD	FVLYVASVPT	SPGVLAWANT	CQVFSNDQPA	250
L amaz	TGGVTNTDFV	LYVASVPSEE	SVLAWATTCQ	VFADGHPAVG	VINIPAANIA	
L chag	GLSNTDFVMY	VASVPSEEGV	LAWATTCQVF	SDGHPAVGVI	NIPAANIASR	
L don	VASVPSEGDV	LAWATTCQVF	SDGHPAVGVI	NIPAANIASR	YDQLVTRVVT	
L maj	ITEGFSNTDF	VMYVASVPSE	EGVLAWATTC	QTFSDGHPAV	GVINIPAANI	
L mex	EHITEGVTNT	DFVLYVASVP	SEESVLAWAT	TCQVFPDGH P	AVGVINIPAA	
C fasc.	VGVINIPAAT	ITERYDHLMV	HAVTHEIAHS	LGFSNAFFTN	TGIGQFVTGV	300
L amaz	SRYDQLVTRV	VAHEMAHALG	FSGTFFDRVG	IVQKVPDVRG	KPYFTPMINS	
L chag	YDQLVTRVVT	HEMAHALGFS	VGFFEGARIL	ESISNVRHKD	FDVPVINSST	
L don	HEMAHALGFS	VVFFRDARIL	ESISNVRHKD	FDVPVINSST	AVAKAREQYG	
L maj	ASRYDQLVTR	VVTHEMAHAL	GFGSPFFEDA	RIVANVPNVR	GKNFDVPVIN	
L mex	NIASRYDQLV	TRVVTHEMAH	AVGFSGTFFG	AVGIVQEVPH	LRRKDFNVSV	
C fasc.	RGNPDTPVPI	NSPTVVAKAR	EHYGCDDVTY	VELEDAGGSG	TMGSHWKIRN	350
L amaz	STAVAKAREQ	YGCNSLEYLE	MEDQGSAA PG	SHIKANAQDE	LMAPTASAGY	
L chag	AVAKAREQYG	CDTLEYLEIE	DQGGAGSAGS	HIKMRNAQDE	LMAPAAAAGY	
L don	CGTLEYLEME	DQGGAGSAGS	HIKMRNAQDE	LMAPASDAGY	YSALTMAIFQ	
L maj	SSTAVAKARE	QYGCDTLEYL	EVEDQGGAGS	AGSHIKMRNA	QDELMAPAAA	
L mex	ITSSTVVAKA	REQYGCNSLE	YLEIEDQGGA	GSAGSHIKMR	NAKDELMAPA	
C fasc.	AQDELMAGIS	GVAYYTS LTL	SAFEDLGYYK	ANY SNAETMK	WGKDVGC AFL	400
L amaz	YTALTM AVFQ	DLGFYQADFS	KAEAMPWGRN	AACAFLSEKC	MANGITKWPA	
L chag	YSALTMAIFQ	DLGFYQADFS	KAEVMPWGRN	AGCAFLSEKC	MERNITKWPA	

Figure 14A

L don	DLGFYQADFS	KAEEMPWGRN	AGCAFLSEKC	MEDGITKWPA	MFCNENEVTM	
L maj	AGYYTALTMA	IFQDLGFYQA	DFSKAEVMPW	GQNAGCAFLT	NKCMEQSVTQ	
L mex	ASAGYYTALT	MAVFQDLGFY	QADFSKAEEM	PWGRNVGCAF	LSEKCMAKNV	
C fasc.	TGKCVVDNVT	QFPSMYCDKD	ENVYRCHTAR	LNLSGCEVTD	YTFDLDPDYLQ	450
L amaz	MFCNESADAI	RCPTSRLGVG	MCDVTPYQAL	PPYLQYFTDP	FLAGSSAFMD	
L chag	MFCNENEVTM	RCPTSRLSLG	KCGVTRHPDL	PPYWQYFTDP	SLAGISAFMD	
L don	RCHTGRLSLG	VCGLSSSDIP	LPPYWQYFTD	PLLAGISAFM	DYCPVVVPFG	
L maj	WPAMFCNESE	DAIRCPTSRL	SLGACGVTRH	PGLPPYWQYF	TDPSLAGVSA	
L mex	TKWPAMFCNE	SAATIRCPTD	RLRVGTCGIT	AYNTSLATYW	QYFTNASLGG	
C fasc.	YFTVPSVGGG	ADYYDYCPYI	VRSPIGSCTQ	AASSASPFVS	AFNTFSMASR	
L amaz	YCPVVVPYAD	GSCAQSASEA	DAAFKAFNVF	SDAAACIDGA	FRPKTTHGLI	
L chag	CCPVVEPYGD	GSCAQRASEA	GAPFKGFNVF	SDAARCIDGA	FRPKTSHGII	
L don	DGSCAQRASE	AGAPFKGFNV	FSDAARCIDG	AFRPKTTETV	TNSYAGLCAN	
L maj	FMDYCPVVVP	YSDGSCTQRA	SEAHASLLPF	NVFSDAARCI	DGAFRPKATD	
L mex	YSPFLDYCPF	VVGYRNGSCN	QDASTTPDLL	AAFNVFSEAA	RCIDGAFTPK	
C fasc.	CIDGTFTPKS	TGGATVTAHL	GMCTNVACNT	ADKTYSIQVY	GNGAYIPCTP	550
L amaz	KSYAALCANV	KCDTAARTYS	VQVRGSSGYA	NCTPGLRFDL	STVSDAFEKG	
L chag	KSYAGLCANV	RCDTATRTYS	VQVHGGSGYA	NCTPGLRVEL	STVSSAFEEG	
L don	VRCDTATRTY	SVQVHGGSGY	ANCTPGLRVE	LSTVSSAFEE	GGYITCPPYV	
L maj	GIVKSYAGLC	ANVQCDTATR	TYSVQVHGSN	DYTNCTPGLR	VELSTVSNAF	
L mex	NRTAADGYT	ALCANVKCDT	ATRRTYSVQVR	GTNGYANCTP	GLRVKLSSVS	
C fasc.	GATISLDTVS	DAFEAGGNIT	CPPYLEVCQS	NVKGAMDYES	MTNSGSGSSR	600
L amaz	GYVTCPPYVE	VCQGNQAQAIK	DGGNAAGR	PRAATALVVA	ALLAVAL	597
L chag	GYITCPPYVE	VCQGNVQAQ	DGGNAAAGR	GPRAAATALL	VAALLAVAL	599
L don	EVCQGNVQAA	KDGGNAAAGR	RGPRAAATALL	LVAALLAVAL		590
L maj	EGGGYITCPP	YVEVCQGNVQ	AAKDGGNTAA	GRRGPRAAAT	ALLVAALLAV	600
L mex	DAFEKGGYVT	CPPYVEVCQG	NVKAAKDFAG	DTDSSSSADD	AADKEAMQRW	600
C fasc.	PAPVEPSGSG	SGSSAATTAP	SPTRDGSAAA	DRIAPRTAAV	ALLALAVAAA	650
L amaz						
L chag						
L don						
L maj	AL					602
L mex	SDRMAALATA	TLLLLGMVLS	LMALLVVRL	LTSSPWCCCR	LGGLPT	646
C fasc.	CV					652
L amaz						
L chag						
L don						
L maj						
L mex						

Figure 14B