

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

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Abogada
LUZ CLEMENCIA DE PÁEZ

ASUNTO	Radicación	09 017 840
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	12

E.13059

Patente de Invención

☒

Patente de Modelo de Utilidad

☐

Vía Nacional

☐

Vía PCT (Fase Nacional)

☒

Capítulo I

☒

Capítulo II

☐

No. Solicitud Internacional

PCT/BR2007/000248

Fecha de Presentación Internacional

20/Julio/2007

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 40 a 67	23/Febrero/2009
Reivindicaciones:	Folios 68 a 69	23/Febrero/2009
Dibujos:	Folios 71 a 83	23/Febrero/2009
Prioridad:	BR PI0603490-0	21/Julio/2006

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Composición de Vacuna y Método de inmunización".

El problema planteado en esta solicitud consiste en la necesidad de desarrollar una vacuna que sea efectiva para proteger los perros y, consecuentemente, disminuir las posibilidades de transmisión a los humanos, que además, permita la distinción serológica entre perros vacunados e infectados empleando métodos de laboratorio de bajo costo que estén disponibles en la red publica.

Para resolver este problema técnico la solicitud en estudio proporciona una formulación de vacuna compuesta de la proteína A2 recombinante y saponina, como

Expediente 09 017 840 1er Art 45

adyuvante, que permite la distinción entre animales vacunados e infectados a través de las pruebas convencionales ELISA o de inmunofluorescencia que emplea antígenos de las formas promastigota de *Leishmania*.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/08.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 7 – 11 no es patentable de acuerdo con el Art citado.

5. De la Solicitud de Patente

Titulo

El título "Composición de Vacuna y Método de Inmunización" es general, se sugiere modificar el título haciéndolo más conciso según el objeto de la invención.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1, 3, 4 y 5 se refieren a una vacuna que: "permite la distinción serológica entre las especies caninas vacunadas e infectadas con *Leishmania*", se caracteriza por "inducir una respuesta inmune humoral en especies caninas a través de la producción de anticuerpos específicos contra el antígeno A2", se caracteriza por "inducir respuesta inmune celular en especies caninas a través de niveles elevados de IFN- γ ", se caracteriza por "mantenimiento de resultados negativos para las pruebas serológicas convencionales para leishmaniasis en especies caninas", que son resultados a alcanzar, no definen a la vacuna, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, que contribuyen a la obtención de ese efecto.

La reivindicación 2 nombra un antígeno A2 recombinante, sin definirlo adecuadamente, se sugiere referenciar el número de la secuencia correspondiente al antígeno.

En la reivindicación 6 se referencian los ingredientes de la vacuna como excipiente, portador, amortiguador, estabilizador, agente de isotonicidad, conservante o antioxidante farmacéuticamente aceptable, estos términos son generales y definen los ingredientes de la composición por su funcionalidad. Se le recuerda al solicitante que esta manera de reivindicar no es apropiada porque no se definen los ingredientes como tal y por lo tanto no permiten distinguir la solicitud del estado de la técnica. Se sugiere restringir la solicitud a una razonable generalización de lo que se encuentra debidamente soportado en el capítulo descriptivo, adicionalmente, se sugiere incluir los ingredientes de la vacuna en la reivindicación independiente 1.

Descripción (Art 28 D 486)

No es claro a que se refiere el solicitante con solución salina tamponada (Pág. 13, línea 14, Fl. 52)

Durante la lectura del capítulo descriptivo se utilizan las palabras imagen y fotografía (Pág. 17, línea 4, Fl. 56; Pág. 19, líneas 9 y 17, Fl. 58; Pág. 20, líneas 7 y 15, Fl. 59;

Pág. 21, línea 14, Fl. 60; Pág. 22, línea 4, Fl. 61; Pág. 23, línea 22, Fl. 62), se sugiere reemplazarlas por el término "figura" y utilizar solo un término durante el capítulo descriptivo.

En las descripciones de las figuras 1 y 2 se nombra la rIL-13 (Pág. 15, línea 18, Fl. 54; Pág. 16, línea 7, Fl. 55), sin embargo, en las figuras 1 y 2 no se encuentra ninguna referencia a la misma (Figura 1, Fl. 71; Figura 2, Fl. 72).

En el capítulo descriptivo se nombra el ADN A2 y pCDNA3 (Pág. 17, líneas 12 y 13, Fl. 56; Pág. 19, líneas 19 y 20, Fl. 58) para las figuras 3 y 6 (Figura 3, Fl. 73; Figura 6, Fl. 76), en las que no se encuentra ninguna referencia a las mismas, también se observa que estas figuras tienen para cada caso 6 barras y solo 4 se encuentran identificadas. Se sugiere realizar las correcciones o aclaraciones necesarias.

La palabra "meio" (Figura 13, Fl. 83) no hace parte del idioma castellano, se sugiere realizar la corrección necesaria.

Se requiere al Solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 21/Julio/2006 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO 95/06729	Differentially Expressed <i>Leishmania</i> Genes and Proteins	09/Marzo/1995
D2	GHOSH A., Vaccine, Vol. 20, Págs. 59 - 66	Immunization with A2 Protein Results in a Mixed Th1/Th2 and a Humoral Response which Protects Mice Against <i>Leishmania donovani</i> Infections	2002
D3	COLER R., Immunoparasitology Series, Vol. 21, No. 5, Págs. 244 - 249	Second-generation Vaccines Against Leishmaniasis	Mayo/2005
D4	WO 02/078735	Leishmania Vaccines	10/Octubre/2002
D5	US 2001/0034330	Innate Immunity-Stimulating Compositions of CPG and Saponin and Methods Thereof	25/Octubre/2001

D1 <http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=9506729A1&K=C=A1&FT=D&ND=3&date=19950309&DB=EPODOC&locale=en> EP divulga (Resumen; Pág. 31 reivindicación 24; Pág. 32, reivindicación 25) una vacuna que comprende la proteína antigénica recombinante A2 de *Leishmania* y un vehículo fisiológicamente aceptable y adyuvantes.

D2 divulga (Pág. 60, Fl. 180) la proteína recombinante A2 de *Leishmania donovani* su utilidad como vacuna.

D3 divulga (Pág. 246, Fl. 185) una vacuna con proteína recombinante A2 y IL-12 como adyuvante.

D4<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=02078735A2&KC=A2&FT=D&ND=3&date=20021010&DB=EPODOC&locale=en> EP divulga (Pág. 23, líneas 5 - 25) una vacuna que comprende la proteína recombinante A2.

D5<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2001034330A1&KC=A1&FT=D&ND=3&date=20011025&DB=EPODOC&locale=en> EP divulga (Pág. 6, párrafo 52; Pág. 7, párrafos 55 y 57; Pág. 9, párrafo 77) una vacuna recombinante para la leishmaniasis, que comprende saponina, tampón fosfato salino y timerosal.

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una vacuna recombinante para leishmaniasis que permite la distinción serológica entre especies caninas vacunadas e infectadas con *Leishmania*.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2	D3	D4
Vacuna recombinante para leishmaniasis	Vacuna recombinante para leishmaniasis	Vacuna recombinante para leishmaniasis	Vacuna recombinante para leishmaniasis	Vacuna recombinante para leishmaniasis

Las características **esenciales** de la vacuna de la reivindicación 1 habían sido divulgadas previamente en los documentos D1 (Resumen) D2 (Págs. 60 y 61, Fls. 180 y 181), D3 (Págs. 244 y 246, Fls. 184 y 185) y D4 (Pág. 23, líneas 5 - 25) los cuales revelan vacunas recombinantes para leishmaniasis.

El documento D1 también enseña que la vacuna contiene un antígeno A2 recombinante, un vehículo fisiológicamente aceptable y adyuvantes (Resumen; Pág. 31 reivindicación 24).

El documento D2 da a conocer que la proteína recombinante A2 de *Leishmania donovani* su utilidad como vacuna (Pág. 60, Fl. 180).

El documento D3 revela que la vacuna comprende proteína recombinante A2 (Pág. 246, Fl. 185), y el uso de adyuvantes y sistemas de entrega (Págs. 245 y 246, Fls. 184 y 185).

El documento D4 divulga una vacuna que comprende proteína recombinante A2 (Pág. 23, líneas 5 - 25).

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1, D2, D3 o D4.

Las reivindicaciones dependientes 2 – 6 no mencionan alguna característica que haga nueva a la vacuna de la reivindicación 1, por lo cual se considera que no son nuevas.

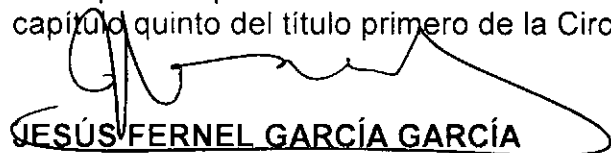
8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO 95/06729	1 – 6	Novedad
D2	GHOSH A., Vaccine, Vol. 20, Págs. 59 - 66	1 – 6	Novedad
D3	COLER R., Immunoparasitology Series, Vol. 21, No. 5, Págs. 244 - 249	1 – 6	Novedad
D4	WO 02/078735	1 – 6	Novedad
D5	US 2001/0034330	1 – 6	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JESÚS FERNEL GARCÍA GARCÍA
 Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha
27 JUL. 2012

Elaborado por: Jeny Paola Villate Becerra *P. Villate*
 Revisado por: Consuelo Leguizamón *CL*



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Vaccine 20 (2002) 94–66

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www.elsevier.com/locate/vaccine

Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections

Anirban Ghosh, Wen Wei Zhang, Greg Matlashewski*

Department of Microbiology and Immunology, McGill University, 3857 University Street, Montreal, Que., Canada H3A 2B4

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Abstract

The A2 genes of *Leishmania donovani* encode amastigote-specific A2 proteins, which are considered to be virulence factors required for the survival of this protozoan parasite in the mammalian host. The A2 genes are present within a multigene family and corresponding A2 proteins are composed predominantly of multiple copies of a 10 amino acid repeat sequences. A2 specific antibodies have been detected in the sera of patients suffering from visceral leishmaniasis (VL) and it has been shown that generation of A2 deficient *L. donovani* resulted in an avirulent phenotype. In this report, we show that immunization of mice with recombinant A2 protein conferred significant protection against challenge infection with *L. donovani*. The protection correlated with *in vitro* splenocyte proliferation, production of IFN- γ in response to A2 protein and the presence of A2-specific antibodies in the sera of immunized mice. These data demonstrate that A2 represents a potential antigen for protection against infection with *L. donovani* and VL. © 2001 Published by Elsevier Science Ltd.

Keywords: Vaccine; *Leishmania donovani*; Macrophage

1. Introduction

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan parasite *Leishmania* [1–4]. These diseases range from self-limiting cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL), also known as kala-azar, which is a fatal infection if not treated successfully. Leishmaniasis affects over 12 million people in 88 countries, over 350 million are at risk, and over 2 million new cases emerge every year [1–3]. Different species of sandfly transmit *Leishmania* and reservoirs include canine, wild rodents, and human. Within the insect host, *Leishmania* is present as flagellated promastigote form and upon infecting the mammalian host it differentiates into the smaller aflagellated round amastigote stage and multiplies in the phagolysosomal vacuole of macrophages. Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs [5]. New disease foci are identified every year in different parts of the world and this may be due to the emerging resistance of sandflies towards insecticides [6] and resistance of the parasite to the existing chemotherapy. In developing and underdeveloped parts of the world, acquired immunosuppressive syndromes (including AIDS) add to the

higher risk of leishmaniasis [7]. Based on these and other observations, there is clearly an urgent need for vaccine development against this disease and in particular against fatal kala-azar.

Several vaccine clinical trials against CL have been undertaken [8–10] and more recently a trial has been carried out against VL in the Sudan [11]. Most experimental vaccines against leishmaniasis have been either live strains [12–14], defined subunit vaccines [15–18] or crude fractions of the parasite [19]. Recently, DNA vaccines have resulted in protection in experimental CL and have appeared to potentially induce a Th1 immune response [20]. There are considerably more protective antigens described for *L. major* infections (example [21–23]) as compared to *Leishmania donovani* in this respect [24–27]. All of the experimental vaccine candidates for *L. donovani* are thought to produce T-cell mediated responses and high antigen-specific antibody titres [24,25,27]. Since few experimental vaccination studies have been carried out against VL, we have therefore examined the possibility of using the A2 antigen of *L. donovani* as a vaccine candidate against VL.

A2 genes are expressed specifically in *L. donovani* amastigotes [28]. The A2 genes are present within a multigene family and the corresponding A2 proteins are composed of predominantly multiple copies of a 10 amino acid repeat sequence. The A2 proteins are abundant in amastigotes and range in molecular weight from 42 to 100 kDa

depending on the number of repeats within each protein species [29,30]. A2 specific antibodies have been identified in the sera of patients suffering from VL [31]. It has been shown that generation of A2 deficient *L. donovani* resulted in avirulent amastigotes, which could not survive in a mouse model but were able to multiply in axenic culture as promastigotes [32]. All these above findings tempted us to investigate the potential of using the A2 antigen as a vaccine candidate against *L. donovani* infection.

2. Materials and methods

2.1. *Leishmania strain and mice*

L. donovani Sudanese (SD) promastigotes and amastigotes were cultured as described previously [32]. Female BALB/c (18h⁺, 11–20) and C57BL/6 mice (4–6 weeks old) were obtained from Charles River, Canada.

2.2. A2 immunization and challenge infection

A2 was purified from *E. coli* BL-21 containing pET16bA2 plasmid. Endotoxin free recombinant A2 protein was used for vaccination and other studies. Mice were injected i.p. with A2 protein combined with 100 μ g heat killed *Propionibacterium acnes* (Elkins, Sinn, Cherry Hill, NJ) as the adjuvant for the first injection and subsequent boosts were with A2 protein in PBS in the absence of adjuvants. For the vaccination studies, the antibody response experiments, and for passive immunization studies, each mouse received 10 μ g of recombinant A2 protein for the first injection and 5 μ g each for the two boosts with 3-week intervals between each injection. Control mice received only 100 μ g heat killed *P. acnes* as the adjuvant for the first injection and subsequent boosts were with PBS. Mice were bled 3 weeks following the final injections and serum from the mice in each group ($n = 4$) were pooled. For the vaccination experiment, mice were immunized as above and then challenged 3 weeks after the final boost and euthanized for liver biopsies 4 weeks following challenge. For challenge infection, 2×10^8 stationary phase cultured promastigotes of *L. donovani* (152D) were injected in the tail vein in 100 μ l PBS per mice. For passive immunization, 3 weeks after the final boost 8×10^8 splenocytes were collected and transferred to naive mice by tail iv. One week after the transfer mice were challenged with 2×10^8 *L. donovani* promastigotes and 4 weeks after the challenge infection mice were killed and parasite burden were measured by liver touch biopsy.

For the cell proliferation and cytokine production assays, mice were immunized with 10 μ g recombinant A2 protein and 100 μ g heat killed *P. acnes* in the first injection and 5 μ g of A2 protein in PBS for 1 boost injection at 2-week intervals. Control mice received only 100 μ g heat killed *P.*

acnes for the first injection and the subsequent boost was with PBS. Two weeks after the boost, mice were euthanized and spleens isolated. Spleens from mice in the same group (four per group) were pooled together.

2.3. Vaccination analysis

Four weeks following challenge infection, mice were euthanized and liver touch biopsies were microscopically examined after fixing and staining the slides with Giemsa [33]. Leishman Donovan unit (LDU) were calculated as LDU (number of amastigotes/number of liver nuclei) $\times$ weight of liver in milligrams [34]. Protection studies were performed in 4 mice per group and the experiment was repeated three times with similar results.

2.4. ELISA

The method for end point titration was described elsewhere [35]. In brief, 5 ng of recombinant A2 protein was coated per well in a 96 well plate at 4°C overnight in 50 μ l binding buffer (0.1 M NaPO₄, pH 9.0). The wells were washed three times with PBS-T (PBS, 0.1% Tween 20) and blocked with 200 μ l of 3% bovine serum albumin (BSA) in PBS-T for 2 h at 37°C. Wells were then washed three times with PBS-T and incubated with 100 μ l of diluted serum (serially diluted at 2-fold starting at 1:20 in PBS-T; 1% BSA) for 2 h at 37°C. Wells were then washed three times with PBS-T and incubated with 1:2000 diluted HRP-conjugated anti-mouse goat antibody in PBS-T; 1% BSA for 1 h at 37°C. After washing three times with PBS-T, the color was developed with TMB-ELISA (Life Technology) as manufacturers recommendation. The cut-off was determined as: $3 \times$ (mean blank OD $\pm$ S.D. of blanks).

For cytokine capture ELISA of IL-4 and IFN- γ , 5×10^6 per single spleen cell suspensions in RPMI-1640 were stimulated with 50 ng/ml recombinant A2 antigen and culture supernatant were collected after 96 h. The concentration of IFN- γ and IL-4 in the resulting supernatant was determined as described previously [36] using hirtinylated capture antibody followed by streptavidin conjugated to HRP (Pharmingen).

Isotype-specific antibodies were purchased from Sigma and antigen mediated ELISA were performed according to suppliers instructions. In brief, 100 ng of recombinant A2 protein in 100 μ l were coated over night at 4°C in 0.1 M phosphate buffer pH 9.0 and blocked with 200 μ l of 3% BSA in PBS-T for 1 h at room temperature and washed three times with PBS-T. Mouse sera (100 ml) diluted to 1:100 in PBS-T was added to the wells and incubated at room temperature for 2 h then washed three times with PBS-T. Goat anti-mouse isotype antibodies were incubated at 1:1000 dilution for 1 h washed again and rabbit anti-goat-HRP conjugated at 1:5000 dilution was incubated for 0.5 h and the color was developed with TMB-ELISA. All samples were run in triplicates.

*Corresponding author. Tel.: 1-514-398 7327; fax: 1-514-398-5072.
E-mail address: greg.matlashewski@macdon.mcgill.ca (G. Matlashewski).

2.5. Cell proliferation assay

Single cell suspensions of isolated splenocytes (4×10^6 cells/ml) were stimulated with 0.5 µg/ml of recombinant A2 in 240 µl in a 96 well plate at 37 °C, 5% CO₂ for 12 h and pulsed for additional 18 h with 1 µCi of [³H] thymidine per well. The plate was harvested and the amount of incorporated [³H] thymidine was measured in a β-counter. Results are represented as the difference in counts obtained between the A2-stimulated and non-stimulated controls.

2.6. Western blot analysis of A2

The SDS-PAGE (12%) was run with 1 µg of recombinant A2 protein in each lane. The resolved proteins were then transferred to a nitrocellulose filter in the presence of 20% v/v methanol, 25 mM Tris, pH 8.2, 190 mM glycine at 30 V for 12 h. Filters were washed then incubated directly in anti-A2 C9 hybridoma supernatant [30] with 5% milk in PBS-T for 2 h at 22 °C then washed and incubated in the presence of horse radish peroxidase labeled anti-mouse IgG in PBS-T at room temperature for 1 h. The membrane was then incubated in Amersham ECL detection solution for 1 min and then exposed to X-ray film followed by autoradiography.

2.7. Infection of macrophages with amastigotes

Bone marrow derived macrophages (BMMs) were obtained from femurs of 6–8-week-old female BALB/c mice as previously described [19]. Quiescent BMM (10^6 cells/ml) were infected with cultured amastigotes at a ratio of 1:1 amastigote per macrophage for 24 h in polystyrene tubes. The infected BMMs were washed extensively for four times with 50 volume PBS at 900 rpm for 10 min. Internalization of parasites was measured by microscopic count of Giemsa-stained cytocentrifuged slides. The sera were decanted by incubating at 65 °C for 2 h in a water bath.

2.8. Statistical analysis

Significance of difference was examined by Student's *t*-test using "GraphPad PRISM" (version 3.02) software with 99% confidence intervals and a value of $P < 0.05$ was considered statistically significant. The *P*-values and S.E. values reported were determined from the replicate measurements (minimum 3) within each experiment. Each experiment was repeated two or more times with similar outcomes.

3. Results

3.1. Immunization with A2 protein protects mice from *L. donovani* infection

We initially determined whether immunization with the recombinant A2 protein was protective against in

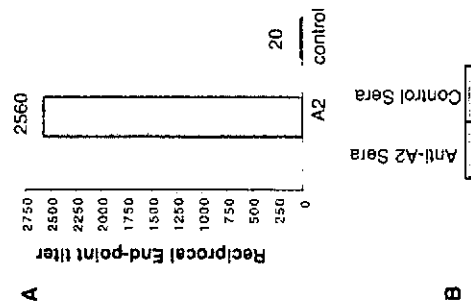


Fig. 1. Infection levels following A2 protein vaccination as determined by LDU. BALB/c mice were immunized with recombinant A2 or recombinant GST protein three times at 3 week intervals as described in Section 2. Three weeks following the final injection, the mice were challenged i.v. with 2×10^6 *L. donovani* promastigotes. Four weeks after the challenge infection, mice were killed and LDU was calculated from liver biopsies. The mean LDU $\pm$ S.E. is shown in $\pm$ 4 mice per group. This result is the representative of three independent experiments.

fection from *L. donovani* in BALB/c mice. As described in the introduction, the A2 protein is a *L. donovani* amastigote-specific gene product which is highly expressed in infected macrophages. Mice were immunized with recombinant A2 protein as described in Section 2 and 3 weeks after the final injection, BALB/c mice were challenged with *L. donovani* promastigotes. The degree of protection against infection was evaluated by amastigote levels in the liver touch biopsies represented as LDU. As shown in Fig. 1, A2 protein immunization had reduced the LDU by 89% over the control mice or recombinant GST protein-immunized mice ($P < 0.0001$). These data demonstrate that vaccination with the recombinant A2 antigen provided a significant level of protection against infection.

3.2. High-specific antibody titer generated in mice immunized with A2

The above observations demonstrated that the recombinant A2 protein immunization provided a significant level of protection against infection. It was therefore necessary to characterize the immune response generated against the A2 antigen. To determine the titer of anti-A2 antibodies in each immunized group of mice, an ELISA end point titration was performed. As shown in Fig. 2A, the antibody response against A2 was much higher in the mice immunized with A2 antigen with a reciprocal end point titre reaching 2560 as compared to mice immunized with adjuvant only.

To confirm that the antibody response was generated against A2, the sera (1:500 dilution) were also tested by Western blot analysis against recombinant A2 protein. As shown in the Fig. 2B, the sera from the mice immunized with recombinant A2 protein demonstrated a specific anti-A2 antibody response. These Western blot data confirmed the ELISA results in demonstrating that A2 vaccination did generate a strong anti A2 antibody response.

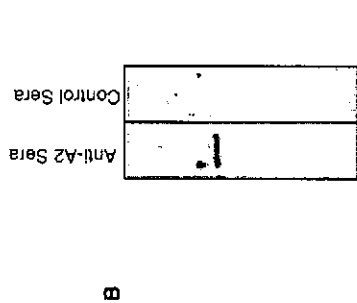
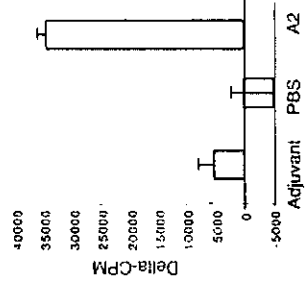


Fig. 2. Relative anti-A2 antibody levels in mice following A2 protein vaccination. Panel A. BALB/c mice were immunized as described in Fig. 1 and anti-A2 antibody levels determined by reciprocal end point titer. This result is the representative of two independent experiments and triplicates were used for each sample. Panel B. Western blot analysis of serum for specificity against A2 protein. Serum were used at 1:500 dilution on 1 µg of recombinant A2 protein per lane.

3.3. Antigen-specific splenocyte proliferation in the mice immunized with recombinant A2 antigen

We next examined the lymphocyte proliferation response to A2 antigen in a mixed splenocyte reaction as described in Section 2. Lymphocytes from a mixed splenocyte preparation were stimulated with recombinant A2 protein in vitro and thymidine incorporation measured. As shown in Fig. 3, thymidine uptake was much higher in splenocytes collected from mice vaccinated with the recombinant A2 antigen. Immunization with the adjuvant alone or PBS resulted in minimal splenocyte proliferation in response to stimulation with A2 protein. Thymidine incorporation was also negligible over background in the former groups when stimulated with an irrelevant recombinant GST antigen (data not shown).

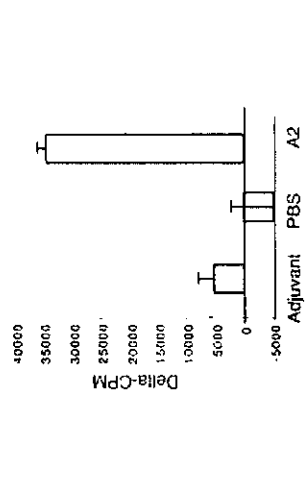


Fig. 3. Proliferation response of splenocytes from mice receiving A2 protein immunization. Mice were immunized with A2 and spleens were collected following the final immunization. Splenocytes were stimulated with recombinant A2 and thymidine incorporation was measured. Delta CPM represents the difference in counts compared with the corresponding non-stimulated cells. Control mice received either adjuvant or PBS.

3.4. Induction of IFN-γ production in response to A2 protein stimulation in splenocytes of immunized mice

It has been established that protection against *L. donovani* infection requires an IFN-γ-activated immune response generated against the parasite [37,38] and production of IFN-γ rather than IL-4 determines the degree of resistance of *L. donovani* infection [39]. We therefore determined whether immunization with the recombinant A2 protein resulted in increased IFN-γ or IL-4 production in response to A2 challenge. As demonstrated in Fig. 4A, splenocytes from mice vaccinated with A2 secreted significantly higher level of IFN-γ ($P < 0.0001$) when stimulated with A2 than splenocytes collected from control mice. Moreover, the release of IL-4 was not significantly higher in the recombinant A2 antigen-immunized mice than control mice following stimulation with A2.

It has been previously shown that IFN-γ production, a marker of Th1 cellular response, directly correlates with a higher IgG2a antibody subclass against the antigen [40], whereas IL-4, a Th2 marker is associated with generation of IgG1 [41]. We therefore investigated the A2 antigen-specific IgG subclass antibody levels in immunized mice as described in Section 2. As shown in Fig. 4B, all of the A2 antigen-specific IgG subclass titres were significantly higher in mice immunized with recombinant A2 protein than in the control group. These data argue that A2 immunization resulted in stimulating both Th1 and Th2 response against the A2 protein.

Taken together, the A2 antigen immunization data show that the A2 is protective against *L. donovani* infection and was able to stimulate both an antibody response as well as induce IFN-γ production in response to recombinant A2 protein. These data strongly argue that the A2 antigen has the prerequisite characteristics for delivering a protective immune response against *L. donovani* infection.

to *Leishmania* infection have considerable importance for their potential as vaccines against leishmaniasis. The major observation in this study is that immunization with recombinant A2 protein resulted in significant protection of BALB/c mice against *L. donovani* infection. The protective response generated by recombinant A2 protein immunization was associated with a mixed Th1/Th2 response, production of IFN- γ in response to A2 antigen, and an anti-A2 humoral response. These results argue that protection against *L. donovani* infection by A2 immunization did not appear to be biased the Th response towards either Th1 or Th2. Protection of passively-immunized mice against challenge infection with splenocytes from A2-immunized mice indicated that protection is mainly due to the cellular immune response and this was observed in both BALB/c and C57BL/6 mice.

VL disease progression in human and experimental mice is associated with an increased titre of polyclonal and parasite-specific antibodies [43–46], absence of delayed type of hypersensitivity (DTH), and increased IFN- γ production by PBMCs [37]. Various experimental and clinical studies have shown that parasite-specific cellular responses are required to protect against Kala-azar [47]. With respect to CL, it has been documented that susceptible to infection in susceptible BALB/c mice is associated with proliferation of CD4 $^{+}$ Th2 cells, which result the secretion of IL-4, IL-5, IL-6 and IL-10, leading to a humoral response [48–51]. In contrast, CL infection in resistant C57BL/6 mice results in proliferation of the CD4 $^{+}$ Th1 subpopulation of T cells, leading to a cell mediated immune response via release of IFN- γ , TNF- α and IL-2, which results in a recovery from infection [48–51].

Unlike for CL infections, vaccine induced protection against VL in experimental murine model does not correlate with the differential production of Th1 and Th2 cytokines [52] as we observed in this report. Moreover, both Th1 and Th2 responses coexisted in cured VL patients [53,54] and a consistent correlation between IFN- γ production and disease protection in human and experimental VL is observed [38]. These previous findings are consistent with our observations that protection with A2 antigen is due to IFN- γ production where both Th1 and Th2 response coexist.

As described in Section 1, the A2 anastigote-specific protein of *L. donovani* is a virulence factor [32]. Electron microscopy of *L. donovani* anastigotes stained with gold beads conjugated with an anti-A2 monoclonal antibody shows A2 protein throughout the anastigote and also is present on or along the anastigote surface as concentrated patches [J Chos, personal communication]. Results from the in vitro macrophage infection with anastigotes in presence of anti-A2 sera (Fig. 6) may indicate a potential role of A2 in the binding and internalization of anastigotes by macrophage. The internalization of promastigotes into macrophages has been shown to mediate by mannose receptor [55], fibronectin receptor [56], complement receptor CR1 and CR3 [55,57]. However, internalization of anastigotes into the macrophages remains controversial. Some

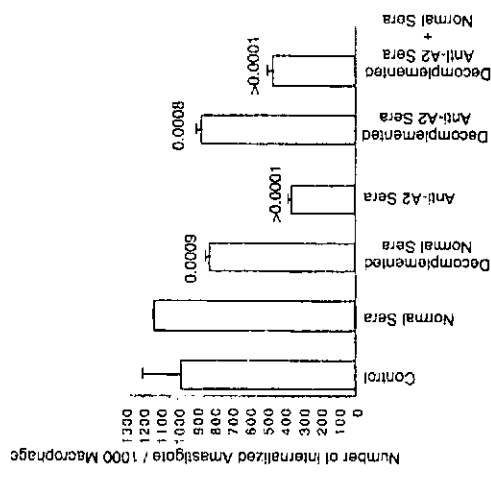


Fig. 6. Internalization of anastigotes in the presence of anti-A2 sera. BMaMs (10^6 cells/ml) were infected with anastigotes for 24 h and internalization of parasites were measured. Prior to infection, the anastigotes were incubated with indicated sera samples or control (no-sera). The result is represented as number of internalized anastigotes per 1000 macrophages. The P-values of Student's t-test indicated on each bar are in comparison with the values obtained from normal sera treatment. The mean $\pm$ S.E. is shown ($n = 3$). This result is the representative of three independent experiments.

therefore used this in vitro model system to measure infection with *L. donovani* anastigotes in macrophages in the presence of anti-A2 antibodies. This was carried out both in the presence and absence of viable complement. BMaMs were incubated with the same number of *L. donovani* anastigotes in the presence of 1:50 dilution of the various sera combinations. As shown in Fig. 6, there was a significant reduction in *L. donovani* infection in the presence of anti-A2 sera. However, when the anti-A2 sera was decomplemented, the internalization of anastigotes was significantly increased to levels similar to the control. When decomplemented anti-A2 sera was reconstituted with normal mouse sera as a source of complement the internalization was again significantly reduced. Similar observations were made using anti-A2 monoclonal antibodies where the addition of complement to these antibodies also reduced the levels of infection (data not shown). These data argue that the A2 antisera in the presence of complement can reduce the viability of anastigotes resulting in a reduction in infection of macrophages.

4. Discussion

L. donovani complex is the lethal causative agent of VL in humans. Antigens stimulating cellular immune response

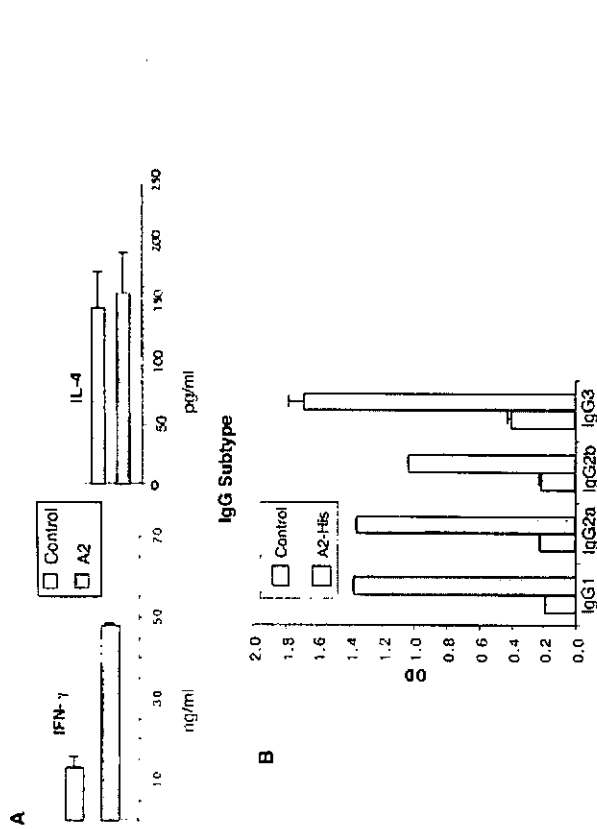


Fig. 4. Panel A, IFN- γ and IL-4 release assay in spleenocytes from A2 protein-immunized mice. Mice were immunized with A2. Splenocytes were stimulated with recombinant A2 for 96 h and concentrations of IFN- γ and IL-4 in the culture supernatants was determined. The data is represented as the mean $\pm$ S.E. Each sample was examined in triplicate and these results are representative of two experiments. Note that the IFN- γ and IL-4 are represented on different scales. Panel B, IgG isotype assay. The A2-specific IgG isotype titre was determined by ELISA. The relative subclass titre is represented as OD values and the data is representative of two experiments. Control mice received only adjuvant as described in Section 2.

3.5. Adoptive transfer of splenocytes from A2-vaccinated mice protects against *L. donovani* infection

Protection against *L. donovani* infection is thought to be predominantly T-cell mediated as demonstrated by adoptive transfer of immune spleen cells to naive mice [42]. Thus, adoptive transfer of spleen cells from A2-immunized mice was carried out in both BALB/c and C57BL/6 mice. As shown in the Fig. 5, mice demonstrated a significant level of protection when passively immunized with spleen cells from A2-vaccinated mice in comparison to the control group of mice which received spleen cells from adjuvant-immunized mice. The IDU was reduced by 50% ($P = 0.0215$) and 55% ($P = 0.0044$) for BALB/c and C57BL/6 mice, respectively. These results confirm that irrespective of the strain of mice, A2 antigen passive immunization imparts significant protection against challenge infection.

3.6. Anti-A2 antibodies and complements block anastigote internalization by macrophages in vitro

BMaMs from BALB/c mice represent an appropriate cell type to measure infection by *Leishmania* in vitro. We

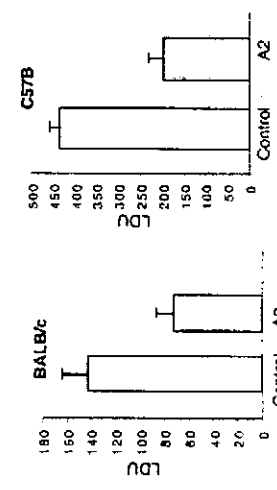


Fig. 5. Infection levels in mice challenged with *L. donovani* following adoptive transfer of splenocytes from A2-vaccinated mice. BALB/c and C57BL/6 mice were immunized with A2 protein and 3 weeks following the final boost, spleen cells were collected and transferred to naive mice. One week after the transfer, mice were challenged with *L. donovani* promastigotes and 4 weeks after the challenge infection, mice were killed and IDU was calculated from liver biopsies. The mean IDU $\pm$ S.E. is shown ($n = 4$ mice per group). This result is the representative of two independent experiments.

reports showed no contribution of opsonins to the infection of amastigotes [58], whereas other studies have demonstrated role of both Fc receptor and CR3 for macrophage infection [59,60]. However, the *in vitro* internalization experiment reported within this study may indicate a possible role of A2 in internalization process and thus argue that A2 immunization could protect against enter macrophage infection and disease progression.

In summary, there are a number of factors which support the argument that A2 proteins could represent an effective vaccine against VL. These proteins contain multiple repeat subunits and are expressed at much higher levels in amastigotes than in promastigotes [30,32]; A2 deficient *L. donovani* resulted in a avirulent phenotype [32]; A2-specific antibodies are present in kala-azar patients' sera [31]. Taken together, the A2 immunization (both direct and passive) shows protection against visceral infection which is associated with IFN- γ production, coexistence of both Th1 and Th2, strong humoral response and reduced internalization of amastigotes into macrophages. These data argue that A2 is a strong vaccine candidate against VL or kala-azar.

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Second-generation vaccines against leishmaniasis

Rhea N. Coler^{1,2} and Steven G. Reed^{1,2}

¹The Infectious Disease Research Institute, 1124 Columbia Street, Suite 600, Seattle, WA 98104, USA
²TheraVax, 1124 Columbia Street, Suite 600, Seattle, WA 98104, USA

Several species of *Leishmania* cause human diseases that range from self-healing cutaneous lesions to fatal visceral leishmaniasis, mucosal leishmaniasis and diffuse cutaneous leishmaniasis. Drug resistance and toxicities associated with chemotherapy emphasize the need for a safe, effective vaccine. Studies of the immunopathogenesis and mechanisms of protective immunity define several features that should be met by an effective vaccine. The leishmaniasis are unique among parasitic diseases because a single vaccine has the potential to protect against more than one species (disease) and be successful at both treating and preventing disease. In addition, several antigens have been identified and characterized that might be potential vaccine candidates. In this article, we focus on advances made with second-generation vaccines against leishmaniasis.

Immunology of leishmaniasis

Leishmaniasis is caused by several species of protozoan parasites that are transmitted by the bite of the female phlebotomine sand fly. Leishmaniasis is currently endemic in 88 countries, and is a threat to 350 million people with a worldwide prevalence of 12 million cases.

Acquired resistance to leishmaniasis is mediated by T cells [1]. T-cell-deficient mice succumb rapidly after infection with *Leishmania* and adoptive transfer of normal T cells confers resistance to these animals. Moreover, patients with AIDS are highly susceptible to leishmaniasis either as a result of concurrent infection or reactivation of older, sub-clinical infection [2]. Among T cells, CD4+ T cells are crucial for resistance, whereas CD8+ T cells participate more in the memory events of the immune response than as effector cells involved in parasite elimination. More recently, a role for CD4+ CD25+ regulatory T cells has been identified in the persistence of *Leishmania major* infections [3].

In humans, there is a good correlation between Thelper 1 cell (Th1) responses and resistance to cutaneous leishmaniasis (CL). Generally, a predominance of cells that produce interferon γ (IFN- γ) occurs in healing, cutaneous lesions, whereas in chronic cutaneous and mucosal lesions there is a mixture of Th1 and Th2 cytokines with an abundance of interleukin 4 (IL-4) and

IL-10 [4]. In visceral leishmaniasis (VL) however, no association between increased IL-4 and active disease has been identified. In the spleen, mRNAs that encode IFN- γ and IL-4 are elevated during active disease and decline significantly after cure. The same cytokine profile occurs after antigenic stimulation of peripheral blood monocytes from patients [5]. However, a direct correlation between production of IL-10 and active disease is reported in patients with VL [6].

Innate immunity has an important role in the control of *Leishmania* infections. To respond rapidly to organisms that have not been encountered previously, a highly effective system of innate immunity has developed that enables pattern-recognition receptors to respond to conserved, pathogen-associated molecular patterns. Toll-like receptors (TLRs) have a central role in orchestrating the type and strength of this response. Recently, important immunomodulatory roles of natural killer cells [7,8], IL-1 α and myeloid differentiation factor 88 [9] have been identified in early resistance to infection, development of acquired immunity and the pathology observed during infection with *Leishmania* parasites.

Vaccine studies

Vaccination against human cutaneous leishmaniasis has been practiced for centuries. Deliberate inoculation of virulent organisms from the pus of an active lesion is an ancient practice [10]. Promastigotes of *L. major* grown in culture were first used in Russia in 1937 to induce protection against natural infection [11]. More recently, standardized inoculums of culture promastigotes have been developed by Israeli scientists and used in several trials [11]. This process, known as 'leishmanization', is still used in some countries, notably Uzbekistan [12]. Leishmanization been is efficacious against Old World CL [13]. However, several basic, logistical problems preclude the widespread use of this procedure to prevent CL, including difficulty in standardizing the virulence of the vaccine and occasional severe, persistent lesions resulting from the inoculum [14]. Vaccination using a crude antigen preparation obtained from promastigote forms of various species of *Leishmania*, either with or without BCG (bacillus of Calmette and Guérin) as adjuvant, has been tested against CL and VL in human clinical trials in both the Old and New World. Generally, there is no evidence

that two injections of autoclaved *L. major* results in significant protection compared with BCG alone [15].

Vaccination trials that demonstrate that either a cocktail of five, killed *Leishmania* stocks or a single strain of *Leishmania antenensis* induces significant protection from disease caused by natural infection [16], has led to the registration in Brazil of a vaccine for leishmaniasis. These studies also indicate that delayed-type hypersensitivity (DTH) conversion might be used as a surrogate marker for protective immunity.

Overall, the results from several clinical trials using whole parasite antigens vary from 0–75% efficacy against CL and little (<6%) or no protection against VL [15,17,19]. Although these crude vaccine approaches are not ideal, they confirm some of the animal data that indicates that induction of protection against leishmaniasis is feasible and can be achieved with either a viable vaccine or with parasite components.

It is certain that a consistently effective, stable and reliable source of vaccine against leishmaniasis is unlikely to arise from whole-parasite approaches. The manufacturing, quality control, potency and stability issues are likely to be insurmountable for any but extremely local situations with relaxed regulatory requirements. Indeed, the variability seen with the whole-parasite-vaccine trials is reminiscent of the experience with BCG. Efforts must be made to produce one or more consistent, safe and effective vaccines against leishmaniasis. Although several new approaches used in modern vaccinology are being investigated in models of experimental leishmaniasis, only one defined second-generation vaccine has reached clinical trials in humans [20].

Second-generation vaccine development

An ideal vaccine against leishmaniasis should have several properties, including (i) it must be safe; (ii) a minimum number of immunizations must induce long-term protection against most or all human pathogens that cause leishmaniasis; (iii) it must be free of animal products that are used to manufacture the product; (iv) it must be produced as cost-effectively as possible; and (v) it should be effective in both treating and preventing leishmaniasis. Other attributes (e.g. a needle-free, single-dose vaccine) could also be listed. To develop such a vaccine, it is essential to characterize protective antigens and to deliver them in creative systems that are optimized to meet both scientific and regulatory standards.

Candidates for second-generation vaccines

From experimental vaccine studies in the mouse model it is evident that solid protection against either syringe or sand-fly challenge can be achieved with defined proteins. It is also clear that not all antigens protect against leishmaniasis in the mouse model, which has enabled the selection of a few antigens as candidate vaccines. Several *Leishmania* proteins have been identified, based on abundance and surface localization [21]. T-cell clones, screening of antigen pools [22] and screening expression libraries with sera from infected animals and humans [23–30]. These include glycoprotein 63 (gp63) [31], membrane glycoprotein 46 (gp46), also known as M-2) [32], *Leishmania* homolog of

receptors activated for C kinase (p36/LACK) [33], cysteine protease (CP13) and CPA [34], LDI antigens [35], hydrophilic acylated surface protein B1 (HASPBL) [36], LCR1 [37], salivary protein 15 (SP15) [38], M-2 [39], promastigote surface antigen 2 (PSA-2) [40], histone H1 [41], *Leishmania* elongation and initiation factor (LeIF) [42], *L. major* homolog of the eukaryotic stress-inducible protein-1 (ImST1) [30], and the *L. major* homolog of the eukaryotic thiol-specific-antioxidant (TSA) [43].

The ideal vaccine is a pan-*Leishmania* vaccine that includes several molecules that are, preferably, conserved among different species and expressed abundantly on the tissue amastigote stage. Many of the antigens mentioned above meet these criteria, but few protect against more than one species in animal models. Apart from protection, another important consideration is safety. Many of the protective antigens of *Leishmania* are highly conserved with mammalian proteins, so stops must be taken to ensure that anti-host responses are not induced, particularly with prophylactic application.

Adjuvant and delivery selection

Most vaccine studies aim to limit parasite replication in the vertebrate host. Over the past decade several investigators have searched for genes encoding leishmanial proteins that induce protection against CL and VL in experimental models [22,29,33,35,36,42–47]. However, identifying candidate antigens is not enough. Appropriate antigen delivery to induce the right type of immune response against leishmaniasis (i.e. induction of a strong antigen-specific Th1 response) is another crucial component of an effective vaccine. The two adjuvants that are approved in human vaccines, alum and squalene, induce potent antibody responses but are poor inducers of antigen-specific Th1 responses. Several strategies, including IL-12, live vectors, naked DNA, DNA vaccines encapsulated in microspheres and oligonucleotides (CpG sequences), have been evaluated extensively in pre-clinical models.

Subcutaneous injection of IL-12 and soluble leishmanial antigens (SLAs), induces a strong, anti-SLA Th1 response with no detectable Th2 response to this antigen mix [48]. IL-12 has been used successfully as a Th1 adjuvant for antigens in both murine and non-human-primate models of several infectious diseases, including leishmaniasis [49–51]. One drawback of IL-12 is its inability to stimulate strong immunological memory to the immunizing antigen. Thus, vaccination of BALB/c mice with the leishmanial homologue of receptors for activated C kinase (LACK) protein mixed with IL-12 as adjuvant results in short-term protection against challenge with *L. major* [50]. By contrast, vaccination with either LACK DNA or LACK protein and IL-12 DNA induces long-term protection [52].

Vaccines that encode gp63 and gp46 have also been tested as DNA vaccines, either alone or in combination, in BALB/c mice challenged with *Leishmania mexicana* [53]. These studies demonstrated that mice immunized with a combination of high-dose plasmids (50 μ g of each antigen) have higher levels of protection than mice immunized

with either individual plasmids or low-dose plasmids (20 µg of each plasmid) [53].

The A2 genes are anastaxite-stage-specific, form part of a multigene family of at least 11 genes and are considered to be virulence factors that are required for the survival of *Leishmania* parasites in the mammalian host. A2 has been tested both as a recombinant protein (with IL-12 as adjuvant) and as a DNA vaccine in the murine model of VL. These studies demonstrate that immunization of mice with A2 confers significant protection against challenge infection with *L. donovani* [54].

DNA vaccines that encode histone H1, CPB and CPA, either alone or in combination, encapsulated in micro-spheres, have been also tested in mice. These studies demonstrate that CPA DNA is not protective, and that CPB DNA and a combination of both DNA vaccines are only partially protective for up to 14 weeks after challenge with *L. major* [53]. The addition of alum and IL-12 as adjuvant did not increase either the immunogenicity or protection of these DNA vaccines. Recombinant CPA and CPB proteins mixed with polyoxamer as adjuvant have also been tested in the *L. major* mouse model of CL, demonstrating that CPA is not protective and that CPB elicits only partial protection. More recently, a construct has been developed in which the CPA and CPB genes are fused to give rise to a single hybrid protein. Protection studies in BALB/c mice indicate that the hybrid CPA/B elicits a protective immune response against *L. major* challenge. Similar studies using a cocktail of *Leishmania infantum* type I and II cysteine proteinases have been performed in mixed-breed dogs.

Vaccination studies using either recombinant histone H1 antigen or a long synthetic peptide representing the complete *L. major* histone H1 sequence, each formulated with Montanide ISA 721 as adjuvant, have also been performed in an African green monkey model of CL [53].

The fucose mannose ligand (FML) is a complex glycoprotein fraction that has been used to immunize both mice and dogs against *L. donovani* and *L. infantum*. Adjuvants used in combination with FML, or the gp36 component of the FML antigen complex include alum, BCG, IL-12, saponin and QuilA. These studies indicate that vaccines that contain FML/gp36 provide significant protection (reduction in parasite burden and increased survival rates) in the murine model of *L. donovani*. Another study that has monitored naturally exposed dogs for 2 years demonstrates that this antigen induces significant protection against canine VL [53].

Several other adjuvant and delivery systems deserve attention. These include methods to deliver antigen such as vectored DNA (adenovirus and pox virus), peptides that target APC, water-in-oil emulsions (mineral oil and squalene), oil-in-water emulsions (MF59) and TLR agonists including monophosphoryl lipid A (MPL[®]) and CpG.

Until recently, a major hurdle in the development of vaccines against infectious diseases was the availability of safe, effective, T-cell adjuvants. Previously, only IL-12, with antigen afforded consistent prophylactic efficacy in animal models of CL. Given that IL-12 does not provide long-term immunity and is not being developed as an adjuvant for use in human vaccines, we have evaluated

candidate leishmanial antigens in the presence of several adjuvants, including those that contain MPL[®], and might be more suitable for human use.

MPL[®] is a detoxified derivative of 4'-monophosphoryl lipid A of lipopolysaccharide (LPS) obtained from *Salmonella minnesota*. Several studies demonstrate that it is a potent immunostimulant through activation of TLR4 [55] that lacks the toxic properties of LPS. MPL[®] has been used as an adjuvant in several safety and immunogenicity human clinical trials, including vaccines for malaria, hepatitis B, genital herpes, allergy desensitization and human papilloma virus. These studies find that MPL[®] is well tolerated with no evidence of systemic toxicity and is likely to be included in vaccines for several human indications. MPL[®] is likely to be the first T-cell adjuvant to be approved for human use.

Thus, the use of MPL[®] as a vaccine adjuvant for leishmaniasis is a practical solution. In addition, MPL[®] is a logical adjuvant because it activates antigen-presenting cells through TLR-4, a receptor that contributes to the control of parasite growth in both the innate and acquired immune response to *Leishmania* infection [56].

Development of Leish-111f as a vaccine candidate

An ideal vaccine against leishmaniasis is unlikely to consist of a single antigen. Our approach (research and preclinical validation) is to select antigens with demonstrated ability to protect mice and non-human primates, and to test antigen combinations in both prophylactic and therapeutic models (Figure 1).

TSA

The *L. major* homologue of eukaryotic TSA was discovered by screening expression libraries [43] to characterize the immune responses elicited by proteins isolated from filtrates of *L. major* promastigote cultures. Immunizing BALB/c mice with recombinant TSA protein formulated with either IL-12 or TSA DNA results in the development of strong cellular immune responses and confers protective immune responses against infection with *L. major* [43,49,57].

LmST11

LmST11 was identified by screening an *L. major* amastigote cDNA library with sera from BALB/c mice infected with *L. major* [29]. Vaccination experiments with recombinant LmST11 protein plus either IL-12 or LmST11 DNA elicit a mixed cellular response that is skewed toward a Th1 phenotype, and protects BALB/c mice [29,30,49,57].

LefF

LefF was identified by screening a *Leishmania braziliensis* genomic library with sera from a patient with mucosal leishmaniasis (ML). LefF stimulates the innate immune system to produce IL-12, IL-18 and IFN-γ, and, therefore, is a Th1 inducer. LefF has immunotherapeutic properties in mice [8,27,42,58].

The protective efficacy of LmST11 and TSA has also been tested in rhesus monkeys [49]. Although used less than the mouse, this model is accepted as a system that mirrors human immunity more closely [51,59]. Monkeys

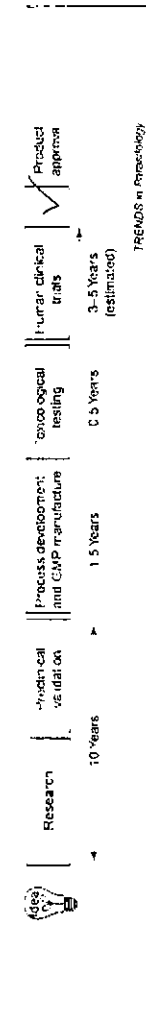


Figure 1 Proposed timelines for the development of a defined vaccine against human leishmaniasis

immunized with a preparation containing LmST11 and TSA with the recombinant human IL-12 and alum as adjuvant mount excellent protection against challenge with *L. major* [49].

Recent studies using the mouse model point to high immunogenicity and protective efficacy of the antigens LmST11, TSA and LefF formulated with the adjuvant MPL[®]-SE.

Constructing Leish-111f

The concept of producing a polypeptide containing several *Leishmania* antigens is based on the practical consideration of producing a multi-antigen vaccine as inexpensive as possible. For this reason, a polypeptide was made that comprises the three priority candidate antigens, TSA, LmST11 and LefF, fused in tandem. This polypeptide is called Leish-111f (Figure 2). In prophylactic immunization studies, long-term protection in BALB/c mice is achieved using 2 µg of Leish-111f. The most effective combination of Leish-111f, which is formulated with 20 µg of MPL[®]-SE, affords protection for > 14 weeks [55,60]. No decrease in immunogenicity, diversity of epitope recognition and protection is noted with Leish-111f compared with protein mixtures. The Leish-111f-MPL[®]-SE vaccine has been evaluated extensively in preclinical safety and toxicology studies in five animal species, with no adverse effects observed.

Post-infection vaccine studies

Whole-parasite vaccines have been used therapeutically in leishmaniasis for decades, with mixed results. In general, this approach is applied to cases of drug-refractory disease. Overall, post-infection immunization is safe. We have reported that immunotherapy with recombinant leishmanial antigens can have therapeutic effects in humans with chemotherapy-refractive ML [61]. In these studies, patients were treated with a mixture of antigens including TSA, LmST11 and LefF. The results of

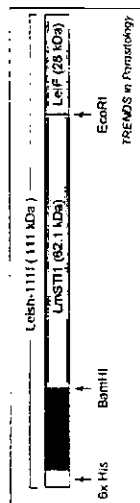


Figure 2 Generation of the recombinant Leish-111f construct. Leish-111f was generated by the sequential linkage in tandem of the open reading frames (ORFs) of the full-length sequences of TSA and LmST11, and the 28-kDa N-terminal portion of LefF. This was accomplished using sequence-specific oligonucleotides that include unique restriction sites (BamHI and EcoRI) and that (in the case of TSA and LmST11) lack stop codons at the C terminus to PCR amplify and link the ORFs. The final construct encodes a single polypeptide, Leish-111f. The size of the predicted ORF is 2946 nucleotides, which encodes 982 amino acids, with a predicted molecular weight of ~111 kDa.



The combination of these antigens has been used successfully to treat patients with drug-resistant ML [61]. These results in patients give us confidence in the ability of animal models to predict successful candidates for prophylactic and therapeutic vaccines in humans. The vaccine formulation that contains Leish-111f and MPLTM-SE confers protection in animal models against leishmaniasis caused by *L. major*, *L. amazonensis* and *L. infantum*. Although completion of a Phase I study in the USA is an important milestone in developing a second-generation leishmaniasis vaccine, it is only the beginning of a long process and we hope that there will be clinical trials of other candidates during the coming years.

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