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Comparative assessment of a DNA and protein *Leishmania donovani* gamma glutamyl cysteine synthetase vaccine to cross-protect against murine cutaneous leishmaniasis caused by *L. major* or *L. mexicana* infection

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ABSTRACT

Leishmaniasis is a major health problem and it is estimated that 12 million people are currently infected. A vaccine which could cross-protect people against different *Leishmania* spp. would facilitate control of this disease as more than one species of *Leishmania* may be present. In this study the ability of a DNA vaccine, using the full gene sequence for *L. donovani* gamma glutamyl cysteine synthetase (γGCS) incorporated in the pVAX vector (pVAXγGCS), and a protein vaccine, using the corresponding recombinant *L. donovani* γGCS protein (LdγGCS), to protect against *L. major* or *L. mexicana* infection was evaluated. DNA vaccination gave transient protection against *L. major* and no protection against *L. mexicana* despite significantly enhancing specific antibody titres in vaccinated infected mice compared to infected controls. Vaccination with the LdγGCS protected against both species but only if the protein was incorporated into non-ionic surfactant vesicles for *L. mexicana*. The results of this study indicate that a *L. donovani* γGCS vaccine could be used to vaccinate against more than one *Leishmania* species but only if the recombinant protein is used.

1. Introduction

Leishmaniasis is a disease caused by infection with vector borne protozoan parasite *Leishmania*. The outcome of infection depends on a number of different factors, which include, the infecting species, the immune response, the genetics of the host, and the presence of other infections, *e.g.* human immunodeficiency virus (reviewed by [1]). The incidence of leishmaniasis is likely to be underestimated because it is not a reportable disease in many countries but figures published by the World Health Organisation indicate that 12 million people are currently infected with leishmaniasis, and that the cutaneous form is responsible for the majority of cases (http://www.who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/index.html). There are a limited number of drugs available for the treatment of leishmaniasis and the emergence of drug resistance, which is prevalent in some areas for antimonial drugs and likely to emerge for miltefosine, will limit the options even further [2,3]. Control of leishmaniasis is likely to involve a concerted effort involving treatment of existing cases, control of the vector and prevention of infection using an effective vaccine but at present there is no vaccine clinically available to treat the disease. A variety of different vaccines, based on killed promastigote parasites, recombinant proteins or using coding gene sequences for *Leishmania* proteins, have been tested in animal models. A limited number of these potential vaccines have been used in canine studies, since canine leishmaniasis is a veterinary disease and the dog can act as an animal reservoir, and even fewer have been used in clinical trials (reviewed by [4]). For example, Leish-111f, a recombinant polypeptide protein vaccine that consists of thiol-specific antioxidant (TSA), *Leishmania major* stress-inducible protein 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF), has been used in laboratory animals, dogs and Phase I and II human clinical trials. It is formulated with the adjuvant monophosphoryl lipid A as a stable emulsion (MPL-SE). In murine studies this vaccine protected against both visceral and cutaneous leishmaniasis but had variable results in subsequent canine studies. This vaccine failed to give any protection against *L. infantum* in one study [5] but did induce some protection in another, which was enhanced by co-treatment with Glucantime® [6]. In a more recent study, using a different vaccination protocol, dogs given adjuvant alone were as protected against natural *L. infantum* infection as dogs immunised with Leish-111f MPL-SE [7]. The authors did however

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report that in human studies the vaccine was more effective than the adjuvant alone against cutaneous leishmaniasis. It is likely that any effective vaccine would require the presence of more than one parasite antigen and therefore studies to identify potential vaccine candidates which could be used in a multi-component vaccine are useful. Ideally a vaccine that protects against all type of leishmaniasis is required as this would be more cost effective to produce and would be effective in areas where different species occur. We have shown in previous studies that vaccination with either a plasmid containing the gene sequence of *L. donovani* gamma glutamyl cysteine synthetase (pVAXγGCS [8]) or recombinant *L. donovani* gamma glutamyl cysteine synthetase protein (LdγGCS [9]) protected mice against infection with *L. donovani*. In this study the ability of both vaccines to protect against cutaneous leishmaniasis caused by *L. major* or *L. mexicana* was assessed to determine whether either vaccine was a candidate for a generic antileishmanial vaccine.

2. Materials and methods

2.1. Reagents

Pharmingen capture and detection antibodies and standards for IFN-γ and IL-4 and alkaline phosphatase conjugate were obtained from Insight Biotechnology (Wembley, UK). All other reagents were of analytical grade.

2.2. Animals and parasites

Age matched BALB/c male or female mice (20–25 g) in-house bred at University of Strathclyde were used in this study. *Leishmania major* (strain MRHO/IR/75/ER) and *L. mexicana* (strain MNYC/BZ/62/M379) were used in experiments and maintained by serial passage through BALB/c mice. Animals were infected with amastigotes, obtained from the lesion of an infected mouse, or promastigotes, obtained by culturing part of a lesion from an infected animal in RPMI-1640 medium containing 10% (v/v) heat-inactivated foetal calf serum (Gibco, UK). Studies were carried out in accordance with local ethical approval and under United Kingdom Home Office regulations.

2.3. LdγGCS recombinant protein and DNA vaccines

His-tagged recombinant *L. donovani* γGCS protein (LdγGCS), processed to remove endotoxin, was produced as described by Henriquez et al. [8] and endotoxin free plasmid as either empty vector or containing the full gene sequence for *L. donovani* γGCS (pVAXγGCS) was produced as described by Carter et al. [9]. Aliquots of LdγGCS, pVAXγGCS or plasmid alone (pVAX) were stored at −20 °C until required.

2.4. Production of NIV

One hundred and fifty micromolar vesicle constituents, consisting of 3:3:1 molar ratio of mono-n-hexadecyl ether tetraethylene glycol, cholesterol, and dicetyl phosphate was melted by heating at 130 °C for 5 min. The molten mixture was cooled to 70 °C, and hydrated with 5 ml of preheated (70 °C) water to form 'empty' non-ionic surfactant vesicles (NIV). Vesicular formulations were homogenized at 8000 ± 100 rpm for 15 min at 70 °C, using a Silverson mixer, fitted with a 5/8 in. tubular work head. One or 2 ml aliquots of the NIV were stored at −70 °C before freeze-drying and then storing at −20 °C until required. NIV were rehydrated with LdγGCS solution (0.5 mg/ml, PBS pH 7.4, LdγGCS-NIV) just prior to use.

2.5. Immunisation studies

The day of infection was day 0 so that vaccination occurred pre-infection on days −28 and −14. In studies using LdγGCS mice ($n = 4$ or 5/treatment) were immunised by subcutaneous injection with 50 μg LdγGCS solution (0.5 mg/ml PBS pH 7.4) or 50 μg LdγGCS-NIV (0.5 mg/ml PBS pH 7.4) In studies using the DNA vaccine mice were immunised by intramuscular injection into each thigh muscle with 25 μg pVAXγGCS (1 mg/ml, PBS pH 7.4) or 25 μg plasmid alone (pVAX, 1 mg/ml PBS pH 7.4). On day 0 immunised mice and an age and sex-matched control group were infected by subcutaneous injection into the shaven rump with 1×10^7 promastigotes or 2×10^6 amastigotes parasites. Amastigotes are more infective than promastigotes and this difference in inoculum results in similar rate of parasite growth in animals. Parasite growth was determined by measuring lesion size (mm) over the course of infection and experiments were terminated when the lesion size of any of the mice was greater than 12 mm. All studies were carried out in accordance with UK. Home Office regulations and repeated a minimum of twice so that results were confirmed.

2.6. Assessment of immunological responses

End point titres of parasite specific IgG1 and IgG2a present in the serum against the LdγGCS protein was determined by ELISA assays [9]. At early time points the lowest serum dilution tested was 1:100, while at later time points, when high antibody titres were obtained, the lowest dilution tested was 1:1000. Single cell suspensions, prepared from the spleens of mice, were used in *in vitro* assays where cells were incubated with medium alone (unstimulated controls), Concanavalin A (ConA, 5 μg/ml, stimulated controls) or LdγGCS protein (25 μg/ml) to stimulate the production of cytokines or nitrite. Cell supernatants were stored at −20 °C until nitrite or cytokine were determined as previously described [9].

2.7. Phylogenetic studies

Protein sequences for γGCS for *L. donovani* (AAQ73826.2) and *L. major* (XP_001682576.1) were obtained from NCBI and were used to search the *L. mexicana* database on GeneDB.org. GeneDB is part of the Sanger Institute Pathogen Genomics activities. The sequence LmxM18 1660.pep corresponds to the *L. mexicana* γGCS sequence. Protein sequences from all three species were aligned using the ClustalW2 Multiple sequence alignment tool at EMBL EBI [10] using the default web based parameters.

2.8. Statistical analysis of data

Lesion size data, cytokine, antibody and nitrite data were analysed using a non-parametric tests using the Statview® version 5.0.1 software package. Data was analysed using a Mann Whitney U test if two treatments were analysed a Kruskal Wallis test followed by Dunns *ad hoc* test was used to determine significant differences for three treatments. Results were considered significantly different if a P value of <0.05 was obtained.

3. Results

3.1. Comparison of L. donovani, L. major and L. mexicana γGCS gene sequence

The alignment for the gene sequence for γGCS for the three *Leishmania* species is shown in Fig. 1. There is considerable homology (87.6%) in the sequence of the protein for all three species. There was 95% identity and 97% similarity between *L. donovani* and *L. major*, 91% identity and 95% similarity between *L. donovani* and

```
L.donovani      MGLLTTGGAPIQWGTDANRKAIPHVREHGIQQFLNVFKNKKDLHGMPFLWGEELEHQLIQ 60
L.major         MGLLTTGGAPIQWGTDANSKAIPHVREHGIQQFLNVFKSKKDLHGMPFFWGEELEHQLIQ 60
L.mexicana      MVFLTDGGAAIQWGTDAHSKAIPHVREHGIQQFLNVFKNKKDLHGMPFLWGEEVEHQLIQ 60
                * :** ***.*******: ******************.*********:****:******

L.donovani      IHDNTVTLSTESAMVMNKLRARPDNCAVWNPEYGSFMIESTPDHPYSLSVESLDSVQDNI 120
L.major         LHDDTVTLSTEGAEVMNKLRARPDNCAVWNPEYGSFMVESTPDHPYTLSVESLDSVQDNI 120
L.mexicana      IHDNTVTLSTESEMVINKLRARPDSCAVWNFEYGSFMVESTPDHPYNLSVESLDSVQDNI 120
                :**:*******.  *:********.***** ******:********.*************

L.donovani      ERRYDMLNKEAPPGVVGTTFVTFPLMGQGNFVHCSDKSSPYSQSLFVPDACINQTHPRFA 180
L.major         ARRYHMLNEEAPPGVVGTTFVTFPLMGQGNFVHCSDKSSPYSQSLFVPDACINQTHPRFA 180
L.mexicana      ARRYDMLNKEAPPGVVGTTFVTFPLMGQGNFVHCSSKSSPYSQSLFVPDACINQTHPRFA 180
                 ***.***:************************.***********************

L.donovani      NLTANIRLRRGQKVCVLVPLYMDSRTMQDTVDPQLNIDLTPHNKDIFYSMRENGRNMTDE 240
L.major         NLTANIRLRRGQKVCILVPLYVDSRTMQDTVDPRLNIDLTPHNKDIFHSRRENGRSMTDE 240
L.mexicana      NLTANIRLRRGQKVCILVPLYMDTRTMENTVDPRLNIDLTPRNNDIFYSMRENGRNTTDE 240
                ***************:*****:*:***::****:*******:*:***:* *****. ***

L.donovani      LYAETDASAALLVPSSSLDPREDYPVTETLKQLFTPATLYYYAQYFTGQRREHMQERYNA 300
L.major         LYAHTDASAALLVPSSSLDPREDYPVTETLKQLFTPAALYYYAQYFTGQHREHMQERYNA 300
L.mexicana      LYAETDAFAAPLVPRSSIDPREDYPATETLRQLFTPATLRYYAQYFTEEHREHMQELYNA 300
                ***.*** ** *** **:*******.****:******:* ******* ::****** ***

L.donovani      CNCPVTLVSHPCIYMDCMAFGMGNSALQVTMQLDNIHEARHVYDQLAILCPAFLALSSAT 360
L.major         CNYPVTLVSHPCIYMDCMAFGMGNSALQVTMQLDNIHEARHVYDQLAILCPALLALSSAT 360
L.mexicana      CPCPVPLVSHPCIYMDCMAFGMGSSALQVTMQLDNIHEARHVYDQLAILCPAFLALSSAT 360
                *  **.*****************.***************************:*******

L.donovani      PFQKGLLCDTDVRWLTIAGAVDDRRVEEVPRILKSRYDSISVFISDRTENLEEFNDSQIA 420
L.major         PFQKGLLCDTDVRWLTIAGAVDDRRVEEVPHILKSRYDSISVFISDRTENLEEFNDSQIA 420
L.mexicana      PFQKGLLCDTDVRWLTIAGAVDDRRAEEVPRILKSRYDSISVFISDRTENLEEFNDSHIE 420
                ************************.****:**************************:*

L.donovani      INRSYCELLKDSGVDVRLANHIAHLFIRDPLVMYDKMIDIDDTTHTEHFDNIQSTNWQTV 480
L.major         INRSYYELLKDSGVDVRLANHIAHLFIRDPLVMYDKMIDIDDTTHTEHFDNIQSTNWQTV 480
L.mexicana      VNRSYCELLKDSGVDVRLANHIAHLFIRDPLVMYDKMIDIDDTTHTEHFDNIQSTNWQTM 480
                :**** **************************************************:

L.donovani      RFKPPPIGNDIGWRVEFRVMDIQPTPFENAAFAVFIPLLTKAIITYKPCFYTKISIVDEN 540
L.major         RFKPPPLGNDIGWRVEFRVMDIQPTPFENAAFAVFIPLLTKAIVNYKPCFYTKISIVDEN 540
L.mexicana      RFKPPPIGSDIGWRVEFRVMDIQPTPFENAAFAVFIPLLTKAIVNYKPCFYTKISIVEEN 540
                ******:*.*********************************:.************:**

L.donovani      MGRAHRINPCGEQYIMRKDIFADKCTASDEETARMSIDEIFNGKEGGFYGLIPLVCRYLD 600
L.major         MGRAHRINPCGEQYIMRKDIFAHKCTASDEETARMSIDEIFNGKEGGFYGLIPLVCRYLD 600
L.mexicana      MSRAHRINPCGEQYVMRKDIFANKCTASDEETARMSIDEIFNGKEDGFYGLIPLVCRYLD 600
                *.************:*******.**********************.***************

L.donovani      DEGKRSPLVNSYLKFLSMRASGRIPTPAQYMRKFVTTHPDYKHDSRLTDSIARDLVQRMH 660
L.major         DEGKRSPLVNSYLKFLSMRASGRIPTPAQYMRKFVTTHPDYKHDSRLTDSIARDLVQRMH 660
L.mexicana      SEGKRSPLINSYLKLLSMRASGSIPTPAQYMRRFVTTHPDYKHDSRLTDSIARDLVQHMH 660
                .*******:*****:******* *********:************************:**

L.donovani      GLAANQIHDDDYLPMSFFTADTVESTK 687
L.major         GLASNQIHDDDYLPISVFKATTRESVK 687
L.mexicana      SLASNQIHDDDYLPMSIFRVDSVESTK 687
                .**:**********:*.*  .  : **.*
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Fig. 1. Alignment for γGCS gene sequence from *L. donovani*, *L. major* and *L. mexicana*. Sequences for γGCS from *L. donovani* (AQQ73826.2), *L. major* (XP_001682576.1), and *L. mexicana* (GeneDB LmxM18 1660.pep) were aligned using the ClustalW2 Multiple Sequence Alignment Tool and show 87.6% homology across all three species. Residues marked (*) are conserved across all three species. Residues marked (:) are conserved amino acid substitutions and those marked (.) are semi-conserved substitutions. Blank spaces designate a lack of conservation.

L. mexicana and 89% identity and 94% similarity between *L. major* and *L. mexicana*.

3.2. Immunisation with pVAXγGCS induced transient protection against L. major but no protection against L. mexicana

Immunisation with pVAXγGCS significantly delayed lesion growth in mice infected with *L. major* ($P < 0.05$) at one time point post-infection but failed to influence lesion development in *L. mexicana* infected mice (Fig. 2). Specific antibody levels and cytokine production by splenocytes stimulated *in vitro* with specific antigen were determined at the end of the experiment to determine the type of immune response present in infected mice. Immunisation with pVAXγGCS significantly enhanced specific IgG1 ($P < 0.01$) but not IgG2a titres in *L. major* infected mice and enhanced both IgG1 ($P < 0.01$) and IgG2a ($P < 0.05$) levels in *L. mexicana* infected mice compared to control values (Fig. 3). Similar amounts of IFN-γ, IL-4 and nitrite were produced by antigen stimulated and unstimulated control splenocytes from vaccinated or control mice infected with *L. major* or *L. mexicana* (Tables 1 and 2). ConA stimulation of cells from all three groups of mice given *L. major* or *L. mexicana* produced similar enhanced levels of IFN-γ and nitrite compared to corresponding unstimulated controls ($P < 0.05$, Tables 1 and 2). IL-4 levels in cell supernatants were also significantly higher than unstimulated controls in mice given *L. major* ($P < 0.05$, Table 1) whereas IL-4 production by ConA stimulated cells from all three groups of mice

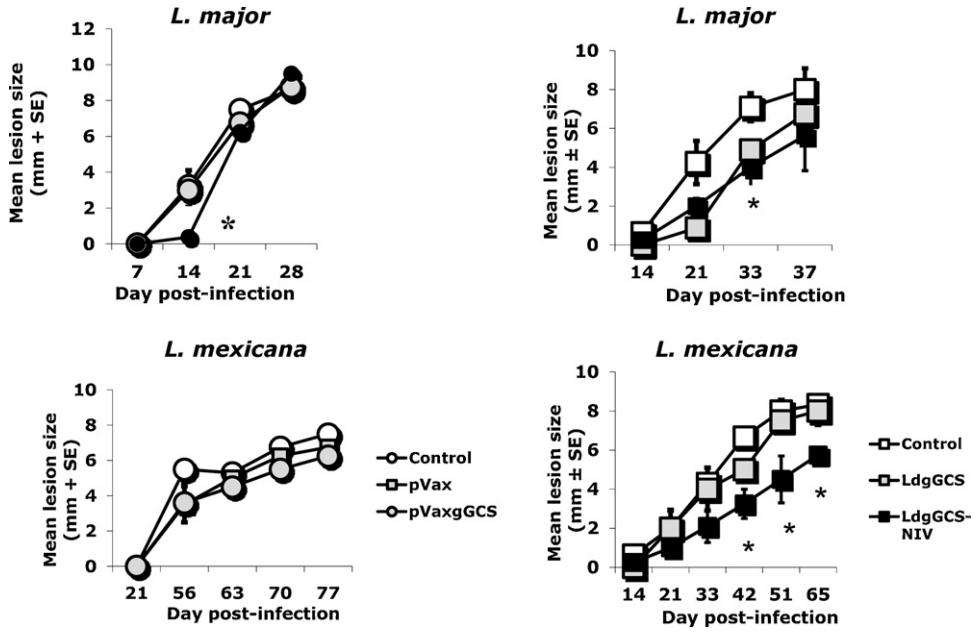

Fig. 2. The effect of vaccination with different vaccines on the progression of *L. major* and *L. mexicana* infection. Mice were vaccinated intramuscularly on days −28 and −14 with 50 μg pVAX (plasmid alone, pVAX), 50 μg pVAXγGCS (pVAXgGCS), 50 μg LdγGCS protein (gGCS) or LdγGCS protein incorporated into NIV (gGCS-NIV). On day 0 vaccinated or uninfected control mice were infected by subcutaneous injection in the shaven rump with *L. major* (1×10^7 promastigotes in both protein and DNA vaccine studies) or *L. mexicana* (data for mice were infected with 1×10^7 promastigotes shown for DNA vaccine; data for mice infected with 2×10^6 amastigotes shown for protein vaccine). Parasite growth was monitored over the course of infection by measuring lesion diameter. $^*P < 0.05$ compared to relevant infected control value.

was similar to corresponding unstimulated control values in mice infected with *L. mexicana* (Table 2).

3.3. Immunisation with LdγGCS-NIV induced protection against both L. major and L. mexicana whereas LdγGCS alone only protected against L. major

It was possible that vaccination with the whole gene sequence of *L. donovani* γGCS did not induce any protection against *L. mexicana* and limited protection against *L. major* infection because *L. donovani* γGCS protein translated from the plasmid vector had low similarity to the native γGCS produced by the other *Leishmania* spp. Therefore the ability of LdγGCS alone or LdγGCS formulated into non-ionic surfactant vesicles (LdγGCS-NIV) to confer cross-protection against *L. major* and *L. mexicana* infection was determined. Both formulation of LdγGCS gave a similar delay in lesion growth in *L. major* infected mice with the mean lesion size in vaccinated mice being lower at all time points compared to control values. However lesion size in vaccinated mice was only significantly lower than control values at day 33 post-infection (Fig. 2). Only vaccination with LdγGCS-NIV significantly enhanced specific antibody titres against the recombinant antigen in *L. major* infected

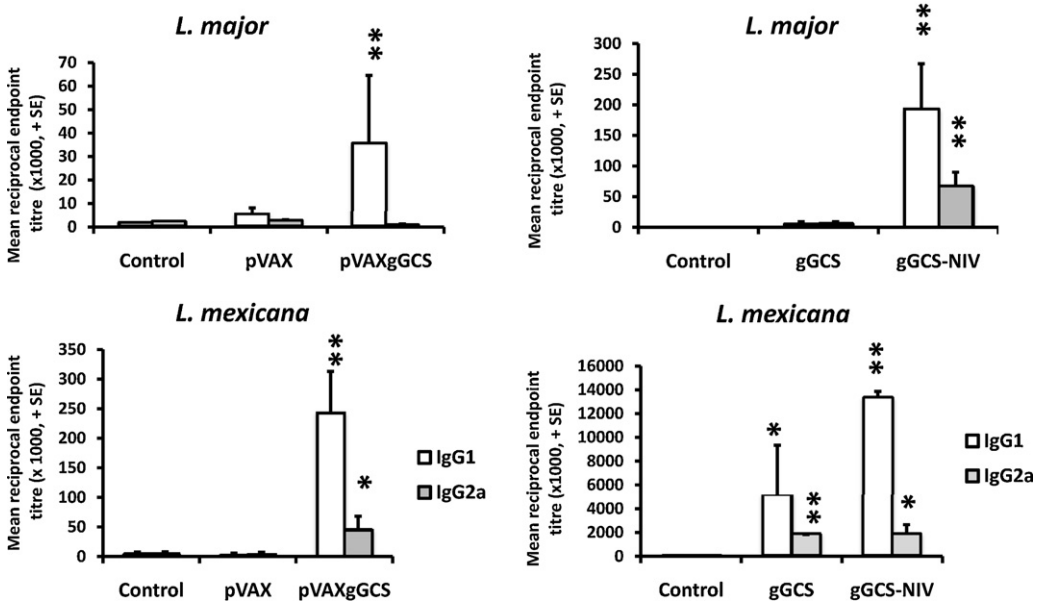

Fig. 3. The effect of vaccination with different vaccines on the antibody responses of *L. major* and *L. mexicana* infected mice. Specific IgG1 and IgG2a titres at termination of the experiments for mice from Fig. 2. $^{**}P < 0.01$, $^*P < 0.05$ compared to relevant infected control value.

Table 1
The effect of vaccination with different vaccines on the immune responses of *L. major* infected mice. Mice were vaccinated intramuscularly on days −28 and −14 with 50 μg pVAX (plasmid alone), 50 μg pVAXγGCS, 50 μg LdγGCS protein or 50 μg LdγGCS protein incorporated into NIV (LdγGCS-NIV) and infected on day 0, along with infection controls, with 1×10^7 *L. major* promastigotes. Splenocytes isolated from infected animals at the end of the experiment were incubated with medium alone (unstimulated), γGCS protein (antigen, 25 μg/ml) or ConA (5 μg/ml) for 72 h and IFN-γ, IL-4 and nitrite levels in cell supernatants determined by ELISA. Mean values ± SE are shown.

DNA vaccine	Stimulus	Treatment		
		pVAXγGCS	Plasmid alone	Infected control
IFN-γ production (ng/ml)	Unstimulated	0.34 ± 0.31	0.21 ± 0.11	0.06 ± 0.06
	Antigen	1.18 ± 0.47	2.07 ± 0.74	2.35 ± 0.25
	ConA	67.06 ± 8.66***	66.45 ± 7.56***	67.75 ± 10.67***
IL-4 production (ng/ml)	Unstimulated	0.04 ± 0.02	0 .04 ± 0.02	0.06 ± 0.04
	Antigen	0.16 ± 0.09	0.03 ± 0.01	0.10 ± 0.04
	ConA	3.48 ± 1.00**	1.18 ± 0.91*	1.92 ± 0.76*
Nitrite (μM)	Unstimulated	3.67 ± 0.40	4.86 ± 0.83	3.71 ± 0.17
	Antigen	4.88 ± 0.39	6.15 ± 1.98	3.70 ± 0.44
	ConA	26.21 ± 1.29***	25.96 ± 2.12***	21.96 ± 2.21***
Recombinant protein vaccine	Stimulus	Treatment		
		LdγGCS	LdγGCS-NIV	Infected control
IFN-γ production (ng/ml)	Unstimulated	1.18 ± 0.44	1.35 ± 0.26	0.90 ± 0.33
	Antigen	1.68 ± 0.29	2.79 ± 0.80	2.15 ± 0.76
	ConA	9.71 ± 1.50**	13.45 ± 2.39***	13.26 ± 1.14***
IL-4 production (ng/ml)	Unstimulated	0.72 ± 0.60	0.83 ± 0.34	0.22 ± 0.18
	Antigen	0.31 ± 0.27	0.89 ± 0.30	0.30 ± 0.19
	ConA	1.97 ± 0.35	2.06 ± 0.17	0.15 ± 0.08
Nitrite (μM)	Unstimulated	15.3 ± 3.0	12.8 ± 1.3	7.2 ± 0.7
	Antigen	9.9 ± 3.4	9.7 ± 1.7	6.8 ± 0.8
	ConA	21.6 ± 4.9**	21.6 ± 2.4*	17.2 ± 0.7**

* $P < 0.05$ compared to unstimulated control values.
** $P < 0.01$ compared to unstimulated control values.
*** $P < 0.001$ compared to unstimulated control values.

Table 2
The effect of vaccination with different vaccines on the immune responses of *L. mexicana* infected mice. Mice were vaccinated intramuscularly on days −28 and −14 with 50 μg pVAX (plasmid alone), 50 μg pVAXγGCS, 50 μg LdγGCS protein or 50 μg LdγGCS protein incorporated into NIV (LdγGCS-NIV) and infected on day 0, along with infection controls, with 1×10^7 promastigotes (DNA vaccine) or 2×10^6 amastigotes (protein vaccine) *L. mexicana*. Splenocytes isolated from infected animals at the end of the experiment were incubated with medium alone (unstimulated), γGCS protein (antigen, 25 μg/ml) or ConA (5 μg/ml) for 72 h and IFN-γ IL-4 and nitrite levels in cell supernatants were determined by ELISA. Mean values ± SE are shown.

DNA vaccine	Stimulus	Treatment		
		pVAXγGCS	Plasmid alone	Infected control
IFN-γ production (ng/ml)	Unstimulated	0.16 ± 0.12	0 ± 0	0.04 ± 0.04
	Antigen	3.06 ± 1.49	2.68 ± 0.61	1.48 ± 0.83
	ConA	41.36 ± 3.75***	42.44 ± 3.8***	33.22 ± 3.41***
IL-4 production (ng/ml)	Unstimulated	0 ± 0	0 ± 0	0 ± 0
	Antigen	0.02 ± 0.02	0.01 ± 0.01	0 ± 0
	ConA	0.28 ± 0.06	0.14 ± 0.02	1.17 ± 0.05
Nitrite (μM)	Unstimulated	3.67 ± 0.40	4.86 ± 0.83	3.71 ± 0.17
	Antigen	4.88 ± 0.39	6.15 ± 1.98	3.70 ± 0.44
	ConA	26.21 ± 1.29***	25.96 ± 2.12***	21.96 ± 2.21***
Recombinant protein vaccine	Stimulus	Treatment		
		LdγGCS	LdγGCS-NIV	Infected control
IFN-γ production (ng/ml)	Unstimulated	0.19 ± 0.08	0.29 ± 0.05	2.59 ± 1.30
	Antigen	0.57 ± 0.3	0.63 ± 0.09	1.43 ± 0.96
	ConA	2.46 ± 2.1	15.21 ± 0.47***	5.64 ± 3.75
IL-4 production (ng/ml)	Unstimulated	2.38 ± 0.92	2.19 ± 0.23	2.44 ± 1.55
	Antigen	2.72 ± 1.38	2.93 ± 0.63	2.48 ± 1.42
	ConA	3.58 ± 1.41	4.02 ± 0.89	2.52 ± 1.59
Nitrite (μM)	Unstimulated	12.79 ± 3.97	8.32 ± 1.22	16.88 ± 2.66
	Antigen	24.83 ± 6.67	18.07 ± 3.47	29.01 ± 5.78
	ConA	35.90 ± 6.96	26.37 ± 7.52*	38.66 ± 5.78

* $P < 0.05$ compared to unstimulated control value.
*** $P < 0.001$ compared to unstimulated control values.

mice compared to control values ($P < 0.01$), with titres being higher for IgG1 (Fig. 3). Splenocytes from mice immunised with LdγGCS or LdγGCS-NIV and infected with *L. major* and stimulated with specific antigen produced similar amounts of IFN-γ, IL-4 and nitrite as corresponding unstimulated control cells (Table 1). ConA stimulation of the same cells significantly enhanced IFN-γ ($P < 0.01$) and nitrite ($P < 0.05$) levels compared to unstimulated control values but did not enhance IL-4 production (Table 1).

Only vaccination with LdγGCS-NIV protected mice against infection with *L. mexicana* ($P < 0.05$) and in this case lesions were significantly lower than control values at three of the six time points assessed (Fig. 2). Specific IgG1 and IgG2a antibody titres were enhanced in *L. mexicana* infected mice immunised with either LdγGCS or LdγGCS-NIV compared to infected control values ($P < 0.05$), with IgG1 titres being higher than IgG2a titres (Fig. 3). IFN-γ, IL-4 and nitrite concentrations in cells supernatants of antigen-stimulated cells and unstimulated controls were similar for splenocytes from vaccinated infected mice and infection controls (Table 2). ConA stimulation of splenocytes from mice immunised with LdγGCS-NIV and infected with *L. mexicana* produced significantly higher amount of IFN-γ ($P < 0.001$) and nitrite ($P < 0.05$) but not IL-4 compared to unstimulated control cells (Table 2). In contrast similar levels of IL-4, IFN-γ or nitrite production by splenocytes from mice immunised with LdγGCS and infected with *L. mexicana* and unstimulated control cells (Table 2).

4. Discussion

In this study the ability of a *L. donovani* γGCS DNA or LdγGCS recombinant protein vaccine to cross-protect against *L. major* or *L. mexicana* was determined. LdγGCS was the most effective at protecting against both species but only if LdγGCS was incorporated into NIV for *L. mexicana*. NIV would have acted as an adjuvant and boosted immune responses perhaps by acting as an antigen depot at the site of injection or by enhancing antigen uptake and subsequent presentation by phagocytic cells. Adjuvants are well known for their ability to enhance the efficacy of *Leishmania* vaccines [11]. Differences in the native γGCS expressed by the three *Leishmania* spp. may explain the inability (*L. mexicana*) or relative low protective capability (*L. major*) of the *L. donovani* pVAXγGCS vaccine construct compared to LdγGCS treatment. At the genetic level there is considerable homology between the γGCS gene sequence for the three species (86.7%). However there may be qualitative species-specific differences in the secondary or tertiary structure of γGCS expressed or quantitative species-specific differences in antigenic exposure, based on differential protein expression during infection or antigen availability for priming immune cells. Studies have shown that *Leishmania* spp. have differences in gene expression in infected individuals [12]. For example, comparison of gene expression in *L. infantum* and *L. major* showed that only 10.5% of genes expressed in the amastigote stage were common and regulation of gene expression in *Leishmania* can occur at translational and post-transcriptional levels, *e.g.* differences in mRNA stability [13]. Differences in antigen availability for priming immune cells may reflect the different tropisms of the parasites within the host or different relationships between the parasite and host immune responses. The results of this study indicate that LdγGCS is not normally antigenic in either *L. major* or *L. mexicana* infection as infected controls had very low specific IgG1 and IgG2a antibody titres to LdγGCS over the course of infection.

It is possible to characterise murine specific immune responses as having a Th1 phenotype (enhanced specific IgG2a titres, enhanced IFN-γ production in lymphocyte proliferative assays after antigenic stimulation) or a Th2 phenotype (enhanced specific IgG1 titres, enhanced IL-4 production in lymphocyte proliferative assays after antigenic stimulation) based on cytokine and IgG subtype production [14]. In this study protection in *L. major* and *L. mexicana* was associated with an enhanced Th1 and Th2 response based on antibody data, with the Th2 response being predominant, but characterising immune responses on the basis of cytokine production was difficult. Protection was not associated with IL-4 production in either *L. mexicana* or *L. major* nor did cells from protected animals produce significantly higher amounts of IL-4 after stimulation with the mitogen ConA. The inability of the protein vaccine to enhance IL-4 production may be significant. *L. major* studies have shown that IL-4 has a role in disease exacerbation, which is mediated by Th2 cells rather than by induction of alternatively activated macrophages [15]. IL-4 also has an exacerbatory role in *L. mexicana* infection as IL-4 receptor alpha deficient mice fail to develop lesions. IL-13 was not responsible for mediating susceptibility in *L. mexicana* since IL-13 deficient mice were as susceptible to infection as wild-type mice [16]. $CD4^+$ T cells were found to be responsible for long term control of lesion growth but a non-T cells population was found to control initial lesion development since female mice with IL-4 receptor alpha $CD4^+$ deficient T cells had smaller lesions than their wild-type counterparts [17]. In this study protection in *L. mexicana* was associated with a Th1 responses as ConA stimulated cells from LdγGCS-NIV vaccinated mice infected with *L. mexicana* produced significantly higher amounts of IFN-γ compared to unstimulated controls and the values were three higher than similar treated cells from infected control animals. Studies using other leishmanial antigens have correlated cross-protection with the ability to produce IFN-γ in *L. major* [2,11] and *L. mexicana* [18]. All species of *Leishmania* reside in macrophages in the mammalian host therefore parasite clearance can only occur if the infected macrophage is stimulated to kill the intracellular pathogen. IFN-γ is a potent stimulator of macrophage microbial killing mechanisms and nitric oxide is an important antileishmanial mediator [19]. In this study nitrite production was used as an indirect measure of nitric oxide production in lymphocyte proliferation assays and protection in LdγGCS-NIV vaccinated mice infected with *L. mexicana* was also associated with the ability to produce significantly more nitrite than unstimulated controls after ConA stimulation. The inability to correlate protection with the type of immune responses associated with protection in *L. major* or *L. mexicana* may be related to the inability of immunisation to produce sterile cure in the animals. In addition the time when immune responses are determined can also influence results. In this study cytokine/nitrite production was only determined by antigen stimulated splenocytes at the end of an experiment and it is well known that immune responses show temporal changes during a normal course of infection for *L. major* [20] and *L. mexicana* [21].

In summary the results of this study indicate that a γGCS protein vaccine would more be suitable than a DNA based vaccine for protection against *L. major* and *L. mexicana*. The level of protection is too low for clinical use but γGCS could be considered as a potential candidate for a multicomponent vaccine, an approach now recognised as a more likely option for vaccination against *Leishmania* [4]. It is possible that a different immunisation schedule or using a 'prime-boost' approach [22], where the DNA vaccine is used to prime host immune responses and then the recombinant protein is used to boost the immune response induced, may increase the efficacy of the protection awarded by a vaccine using *L. donovani* γGCS. In our studies we have only assessed the protective capabilities of LdγGCS against three *Leishmania* spp. but it would be interesting to assess its activity against *L. infantum* as there is 87.2% homology in its γGCS gene sequence and that of *L. donovani*, *L. major*, *L. mexicana*. It may not protect against *L. brasiliensis* as inclusion of this species reduces the overall homology to 68.9%.

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