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## VACCINATION WITH RECOMBINANT *LEISHMANIA DONOVANI* GAMMA-GLUTAMYL CYSTEINE SYNTHETASE FUSION PROTEIN PROTECTS AGAINST *L. DONOVANI* INFECTION

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**ABSTRACT:** Visceral leishmaniasis presents a serious health threat in many parts of the world. There is, therefore, an urgent need for an approved vaccine for clinical use to protect against infection. In this study, the ability of recombinant *Leishmania donovani* gamma-glutamyl cysteine synthetase protein (Ld $\gamma$ GCS) alone or incorporated into a non-ionic surfactant vesicle (NIV) delivery system to protect against *L. donovani* infection was evaluated in a BALB/c mouse model. Immunization with Ld $\gamma$ GCS alone or Ld $\gamma$ GCS-NIV induced specific IgG1 and IgG2a antibodies compared to controls, with Ld $\gamma$ GCS-NIV inducing significantly higher titers of both antibody classes ( $P < 0.05$ ). Both formulations induced similar increases in splenocyte IFN- $\gamma$  production following ex vivo antigen stimulation with Ld $\gamma$ GCS compared with cells from control mice ( $P < 0.05$ ). Similar levels of protection against infection were induced by Ld $\gamma$ GCS alone and Ld $\gamma$ GCS-NIV, based on their ability to suppress liver parasite burdens compared to control values ( $P < 0.01$ ), indicating that using a carrier system did not enhance the protective responses induced by the recombinant protein. The results of this study indicate that Ld $\gamma$ GCS may be a useful component in a vaccine against *L. donovani*.

*Leishmania* spp. the causative agent of leishmaniasis, are a serious health threat in 88 countries. *Leishmania donovani* causes the visceral leishmaniasis (VL) and is fatal if untreated. Resistance to antimonial drugs in certain areas, e.g., Bihar State, India, has severely reduced the options for clinical treatment. There are 500,000 new cases/yr and 50,000 reported deaths/yr, a figure that is likely to be an underestimate because VL is often not diagnosed or reported. Asymptomatic cases occur, with ratios varying from 1:2.6 up to 50:1 clinical cases, depending on the geographical location, indicating that it should be possible to immunize against infection (Chappuis et al., 2007). The prevalence of VL co-infection with human immunodeficiency virus (HIV) is increasing, and HIV infection can increase the risk of patients developing VL by 100–2,330 times, reduce their ability to respond to drug treatment, and increase the probability of disease relapse (Alvar et al., 2008). This highlights the potential importance and utility of a prophylactic vaccine to prevent infection. Different types of candidate vaccines have been tested in experimental systems, for example, sand fly antigens, defined parasite antigens, or bacteria-expressing parasite proteins (reviewed by de Oliveira et al., 2009). A number of candidates have been tested clinically, for example, a live vaccine for human use in Uzbekistan and a killed vaccine for human use in Brazil (Palatnik-de-Sousa, 2008). However, to date no vaccine has been approved for prophylactic use against leishmaniasis in clinics (Bhowmick and Ali, 2009).

In a previous study, we have shown that immunization with a plasmid containing the gene sequence for *L. donovani* gamma-glutamyl cysteine synthetase ( $\gamma$ GCS) protected BALB/c mice against infection with *L. donovani* (Carter et al., 2007), but the level of protection obtained was lower than likely to be clinically useful. It was possible that better protection would be achieved by vaccinating with the recombinant protein instead of the DNA sequence. Therefore, in the present study the ability of *L. donovani*

$\gamma$ GCS (Ld $\gamma$ GCS) to protect against infection was assessed. Adjuvants have been used to improve the efficacy of protein vaccines (O'Hagan and De Gregorio, 2009); therefore, in the present study, the ability of the non-ionic surfactant vesicles (NIV), which have adjuvant capabilities (Ferro et al., 2004), to increase the efficacy of Ld $\gamma$ GCS vaccination was also evaluated.

The effect of immunization on the production of both Th1- and Th2-associated immune responses was examined in pre- and post-infection to determine what type of immune response was associated with vaccination and whether it correlated with protection. Immunity to *L. donovani* is associated with an IL-12-driven Th1 response and the production of IFN- $\gamma$ , but Th2 responses are also important because the ability to produce the Th2 cytokine IL-4 is associated with resistance to infection (Alexander and McFarlane, 2008). It is, therefore, imperative to identify the immune parameters that correlate with vaccine-induced protection, as this would help in screening potential vaccine candidates (Bhowmick and Ali, 2009).

### MATERIALS AND METHODS

#### Reagents

Pharmingen capture and detection anti-IFN $\gamma$  antibodies, IFN $\gamma$  standard, and alkaline phosphatase conjugate were obtained from Insight Biotechnology (Wembley, U.K.). All other reagents were of analytical grade. LPS from *Escherichia coli* (0111:B4, Sigma-Aldrich, Poole, U.K.) was used.

#### Animals and parasites

Age-matched BALB/c female mice (20–25 g) in-house bred at University of Strathclyde were used in this study. Commercially obtained golden Syrian hamsters (*Mesocricetus auratus*; Harlan Olac, Bicester, U.K.) were used for maintenance of *L. donovani* strain 200016.

#### Ld $\gamma$ GCS recombinant protein production

The methods detailed in Carter (2007) were used to produce a his-tagged  $\gamma$ GCS recombinant protein. The full-length *L. donovani*  $\gamma$ GCS protein (GenBank AY371486) was cloned into pET45a (Novagen, Nottingham, U.K.) using primers designed for PCR:  $\gamma$ GCSfor (5'-GG GGT ACC GGG CTC TTG ACG ACT GGC GGC-3') containing a kpnI site (underlined) and  $\gamma$ GCSrev (GGG GGA TCC TCA CTT GGT GCT CTC TAC CTG containing a BamHI site (underlined). Recombinant protein was prepared from freshly transfected bacteria using LB broth containing ampicillin (100  $\mu$ g/ml), chloramphenicol (10  $\mu$ g/ml), and

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tetracycline (34 µg/ml) in Rosetta blue *E. coli*. Protein was induced using 1 mM IPTG, and cells were grown overnight at 30 °C. The insoluble material obtained after passing the bacteria through the French press was exposed to extraction buffer (0.15 M Tris, 0.2 M KCl, pH 7.4 containing 6 M urea, 20 ml/L culture) for 1 hr. Centrifugation was carried out at 12,000 *g* at 4 °C and the supernatant diluted 1:1 with extraction buffer, and 50 mM imidazole was added before applying to a His-binding agarose column (GraviTrap<sup>®</sup>; New England Biolabs, Hitchin, U.K.) as directed. The bound protein was eluted by adding 10 ml extraction buffer containing 0.5 M imidazole, and the absorbance of 3 ml fractions was measured at 280 nm using a GeneQuant Pro spectrophotometer to quantify the protein concentration. The purified protein aliquots were pooled and dialyzed against three 1 L batches of PBS, pH 7.4 at 4 °C over a 24 hr period. The protein concentration in sample was determined as directed using the Biorad reagent and BSA as the protein standard.

In some studies expression was carried out in the presence of protease inhibitors (cOmplete<sup>™</sup>; Roche Diagnostics, Burgess Hill, West Sussex, U.K.) to prevent protein degradation. Endotoxin was removed from the purified protein using a ProteoSpin Endotoxin Removal Maxi kit (GeneFlow, Fradley, U.K.), and the amount of endotoxin present in the sample before and after endotoxin removal was determined by Wickham Laboratories (Fareham, U.K.). The presence of recombinant LdγGCS protein was confirmed by carrying out a Western blot using an anti-his monoclonal antibody (NEB, diluted 1:3,000 with PBS, pH 7.4) for primary detection and an HRP-rat-anti-mouse IgG monoclonal antibody (NEB, diluted 1:3,000 with PBS, pH 7.4) for secondary detection. In addition, samples of major protein bands separated by gel electrophoresis were analyzed by mass spectrometry. Coomassie-stained protein bands were excised from SDS-PAGE gels and washed in 100 mM ammonium bicarbonate with shaking for 1 hr at room temp, followed by a second wash in 50% acetonitrile/100 mM ammonium bicarbonate. Proteins were reduced with 3 mM DTT in 100 mM ammonium bicarbonate for 30 min at 60 °C, followed by alkylation with 10 mM iodoacetamide for 30 min in the dark at room temperature. The gel pieces were washed with 50% acetonitrile/100 mM ammonium bicarbonate, shaking for 1 hr at room temp, then dehydrated by incubation with 0.1 ml acetonitrile for 10 min at room temperature. Gel pieces were dried to completion under vacuum, then rehydrated with a sufficient volume of trypsin (Promega sequencing grade, 2 mg/ml in 25 mM ammonium bicarbonate) to cover the gel pieces. Digest was performed at 37 °C overnight. Liquid was transferred to a fresh tube, and gel pieces washed 10 min with a similar volume of 50% acetonitrile. This wash was pooled with the first extract, and the tryptic peptides dried to completion. Tryptic peptides were solubilized in 0.5% formic acid and fractionated by nanoflow HPLC on a C18 reverse phase column, eluting with a continuous linear gradient to 40% acetonitrile over 20 min. Eluate was analyzed by online electrospray tandem mass spectrometry using a Qstar Pulsar (Applied Biosystems, Warrington, U.K.). Mass spectrometric analysis was performed in IDA mode (AnalystQS software, Applied Biosystems), selecting the 4 most intense ions for MSMS analysis. A survey scan of 400–1,500 Da was collected for 3 sec followed by 5 sec MSMS scans of 50–2,000 Da using the standard rolling collision energy settings. Masses were then added to the exclusion list for 3 min. Peaks were extracted using the Mascot script (BioAnalyst, Applied Biosystems) and automatically exported to the Mascot (Matrix Science, London, U.K.) search engine for searching against both *Leishmania major* and *E. coli* genome datasets.

### Production of NIV

Six hundred µmol vesicle constituents, consisting of 3:3:1 molar ratio of mono-*n*-hexadecyl ether tetraethylene glycol, cholesterol, and dicetyl phosphate, were 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 8,000 ± 100 *g* for 15 min at 70 °C, using a Silverson mixer, fitted with a 5/8-inch tubular work head. Two- or 1-ml aliquots of the NIV were stored at –70 °C before freeze-drying overnight then stored at –20 °C until required.

### Immunization studies

The day of infection was day 0 so that vaccination occurred pre-infection on day –28 and day –14. Animals (*n* = 5/treatment) were

immunized with either LPS (10 ng/ml equivalent to 5 EU/ml, 1 ng/dose), LdγGCS (2 or 50 µg, used as prepared or processed to remove endotoxin), or LdγGCS incorporated into NIV (50 µg) on days –28 and –14. The dose of LPS used was based on the lowest expected LPS contamination present in recombinant protein samples. Therefore, animals were exposed to the lowest dose of LPS, a known pyrogen (Dinareello, 2004). LdγGCS-NIV was produced by hydrating a 1- or 2-ml aliquot of freeze-dried NIV with the required amount of LdγGCS protein in PBS, pH 7.4, to the appropriate volume of 1 or 2 ml with shaking. On day 0 immunized mice and a control group (*n* = 4–10/treatment) were infected by intravenous injection (tail vein, no anaesthetic) with 1–2 × 10<sup>7</sup> *L. donovani* strain 200016 amastigotes harvested from the spleen of an infected hamster. Parasite burdens in the spleen, liver, and bone marrow were determined on day 40 post-infection (PI; Carter et al. 2007). All studies were carried out in accordance with U.K. Home Office regulations.

### Assessment of immunological responses

ELISA assays conducted to determine the end point titers of parasite-specific IgG1 and IgG2a present in the serum, against an *L. donovani*-soluble antigen (SAG) or the LdγGCS protein (Carter et al., 2007). At early time points, the lowest serum dilution tested was 1:100, while at later time points, when high antibody titers were obtained, the lowest dilution tested was 1:1,000. Single-cell suspensions, prepared from the spleens of mice, were used in proliferation assays and incubated with medium alone (unstimulated controls), Concanavalin A (ConA, 5 µg/ml, stimulated controls), or LdγGCS protein (25 µg/ml). Cell supernatants were stored at –20 °C until nitrite and cytokine were determined as previously described (Carter et al., 2007).

### Statistical analysis

Data from this study were analyzed as described by Carter et al. (2007). A 1-way ANOVA was used for log<sub>10</sub> transformed parasite data and a Kruskal-Wallis test followed by Dunn's ad hoc test for cytokine and nitrite data.

## RESULTS

### Expression of *L. donovani* LdγGCS in *E. coli* Rosetta blue bacteria resulted in the production of full-length and truncated LdγGCS protein

Purification of proteins from the insoluble bacterial extract from transformed bacteria using the GraviTrap column resulted in a reduction in the number of protein bands present in the sample (normally 4 main bands) (Fig. 1A, lane 8), with the highest molecular weight protein (80 kDa, also the most intense band) corresponding to the expected molecular weight for LdγGCS. Western blot analysis using antibodies recognising the histidine tag portion of the expressed protein demonstrated reactivity with the 4 main bands (lane 5, Fig. 1A). Proteins present in the 4 main bands were excised from a gel after separation by electrophoresis, and the proteins present determined by tandem MS analysis of tryptic peptides. Forty-seven tryptic peptides were matched to the 80 kDa band, giving 66% protein coverage and a MOWSE score of 1808. However, the smaller bands also gave rise to peptides, which matched to LdγGCS with very robust MOWSE scores, suggesting that either degradation of the full-length protein occurred during processing or that shorter forms of the protein were produced during expression. Consistent with this interpretation, peptide coverage was weaker in the carboxy-terminal region of LdγGCS for the smaller bands. Inclusion of protein inhibitors to prevent protein degradation during production of LdγGCS had no appreciable effect on the number of protein bands obtained or HIS-positive protein bands present in the soluble or insoluble fractions (data not shown). Importantly, none of the tandem MS data matched

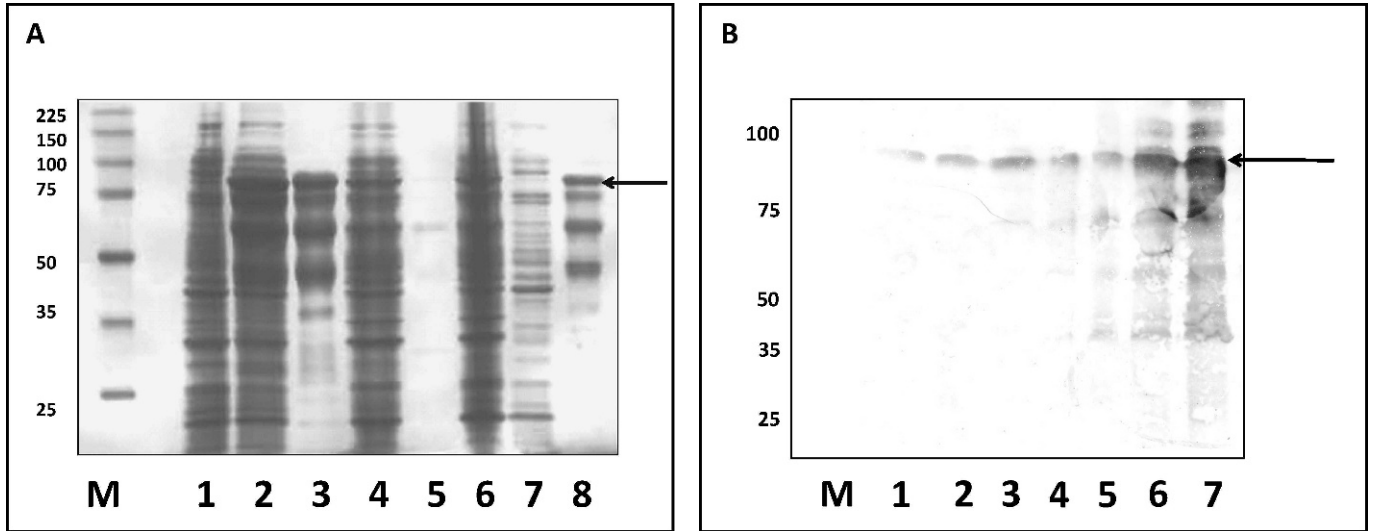


FIGURE 1. Proteins present in the different fractions collected during production of *Leishmania donovani* Ld $\gamma$ GCS S protein after Coomassie staining (A) or after staining for his-tagged proteins following Western blot studies (B). In Figure 1A, results for 3 batches of Ld $\gamma$ GCS protein are shown. M, lane for molecular weight markers (25–225 KDa), lane 1 uninduced protein sample; lanes 2, 4, and 6 induced protein samples; lanes 3, 5, and 8 protein samples after purification using GraviTrap column (Ld $\gamma$ GCS). Lane 7 induced a soluble protein sample, which contains very little Ld $\gamma$ GCS, indicating that the majority of the protein is in the insoluble fraction. The highest molecular weight band in lane 8 (arrow) correlates to full length Ld $\gamma$ GCS. The difference in intensity between Ld $\gamma$ GCS samples in lanes 3, 5, and 8 equates to differences in initial protein concentration. In Figure 1B, M lane for molecular weight markers (25–100 KDa), lanes 1–5 Ld $\gamma$ GCS, increasing amount of protein loaded to show that lower molecular weight bands become more apparent as the protein concentration increases, e.g., compare lanes 1 and 5, lanes 6 and 7 induced protein samples. Arrow shows full-length Ld $\gamma$ GCS.

significantly to *E. coli* proteins, suggesting that the detected bands are not contaminants deriving from the recombinant expression system. Endotoxin contamination was variable between different batches of recombinant protein, and processing removed >85% contamination (mean bacterial endotoxin levels: batch 1, pre-processing 2,576 EU/ml; post-processing <5 EU/ml; batch 2 pre-processing 24,140 EU/ml; post-processing 3,430 EU/ml). To eliminate the possibility that endotoxin contamination could have

been responsible for the anti-leishmanial efficacy of the recombinant protein, Ld $\gamma$ GCS was treated to remove any endotoxin.

#### Immunization with Ld $\gamma$ GCS protein induced a mixed Th1/Th2 response

Mice were immunized with Ld $\gamma$ GCS protein alone or Ld $\gamma$ GCS protein incorporated into NIV to determine whether using a

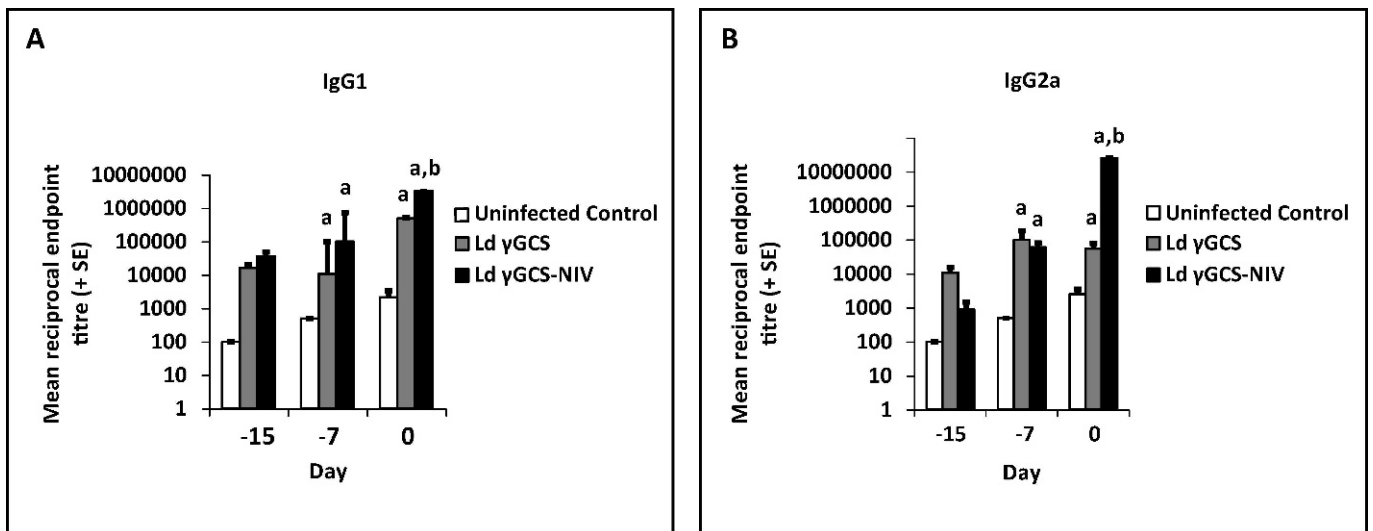


FIGURE 2. The effect of vaccination with Ld $\gamma$ GCS on pre-infection specific antibody responses of mice. Mice were immunized by subcutaneous injection with PBS (uninfected control) or 50  $\mu$ g Ld $\gamma$ GCS antigen alone (Ld $\gamma$ GCS) or 50  $\mu$ g Ld $\gamma$ GCS incorporated in NIV (Ld $\gamma$ GCS-NIV) on days –28 and –14. Specific IgG1 (A) and IgG2a (B) antibody titers to Ld $\gamma$ GCS were determined by ELISA on the day before the second immunisation, i.e., day –15, 7 days after the second immunisation, i.e., day –7, and on the day mice were infected, i.e., day 0. Immunization with either formulation induced significant antibody production compared to control values ( $^aP < 0.05$ ). Immunization with Ld $\gamma$ GCS-NIV induced significantly higher antibody levels compared to Ld $\gamma$ GCS alone on day 0 ( $^bP < 0.04$ ).



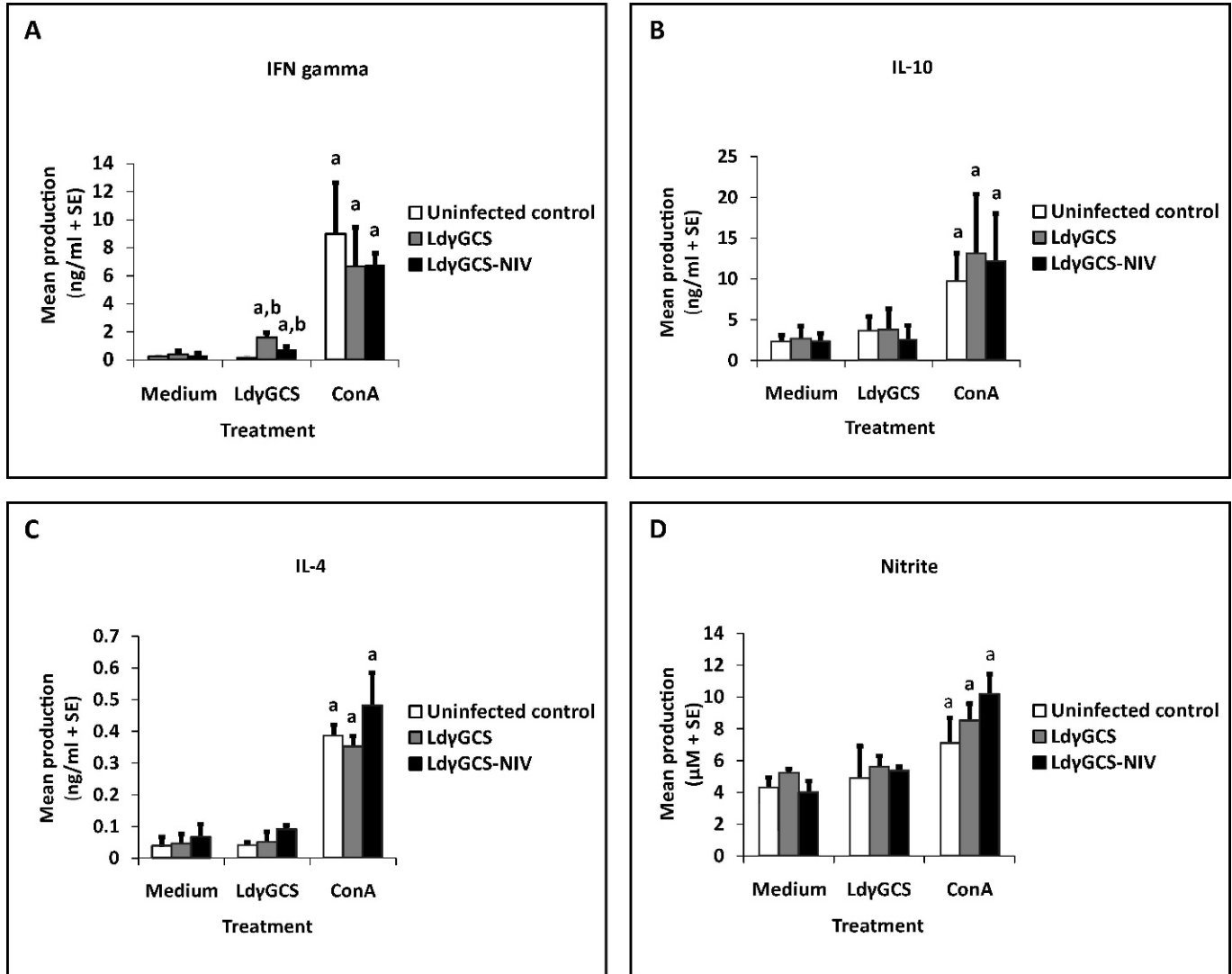


FIGURE 3. Mean IFN- $\gamma$  (A), IL-10 (B), IL-4 (C), and nitrite (D) production by splenocytes from mice taken on day 0 from mice immunized by subcutaneous injection on days -28 and -14 with PBS (uninfected control), Ld $\gamma$ GCS alone (50  $\mu$ g protein), or Ld $\gamma$ GCS-NIV (50  $\mu$ g protein). Cells were incubated with medium alone (control), Ld $\gamma$ GCS protein (Ld $\gamma$ GCS, 25  $\mu$ g/ml), or Con A (5  $\mu$ g/ml), and 72 hr later cell supernatants stored until nitrite or cytokine levels could be determined by ELISA. <sup>a</sup> $P < 0.05$  compared to corresponding unstimulated control (unstim. control) value, <sup>b</sup> $P < 0.05$  compared to stimulated cells from uninfected mice. Specific stimulation did not induce any significant increase in IL-4, IL-10, or nitrite compared to the relevant unstimulated control for each group of mice. ConA stimulation induced similar cytokine and nitrite production by cells from each group of mice.

carrier system altered the type of immune response generated. Initial studies showed that immunization with 2 doses of 2  $\mu$ g Ld $\gamma$ GCS did not protect against infection (mean liver parasite burdens  $\pm$  SE: control 276  $\pm$  71, Ld $\gamma$ GCS vaccinated 167  $\pm$  41); therefore, in subsequent studies, mice were immunized with 50  $\mu$ g recombinant protein. Immunization with this higher dose of protein resulted in significant production of both IgG1- and IgG2a-specific antibodies with the vesicular formulation inducing significantly higher levels on the day of infection (day 0,  $P < 0.04$ ; Fig. 2). Specific stimulation of splenocytes from immunized mice resulted in significant production of IFN- $\gamma$  ( $P < 0.05$ ), but not IL-10, IL-4, or nitrite, compared to corresponding unstimulated controls with similar results for both vaccine formulations (Fig. 3). In a second experiment, cells from immunized mice produced significantly more IFN- $\gamma$  and IL-10 in response to specific antigen stimulation compared with cells from non-

immunized mice ( $P < 0.05$ ). IL-10 and IFN- $\gamma$  production by cells from mice immunized with Ld $\gamma$ GCS-NIV (mean IFN- $\gamma$  production ng/ml  $\pm$  SE; unstimulated cells 0.66  $\pm$  0.66, antigen-stimulated cells 77.47  $\pm$  18.25, mean IL-10 production ng/ml  $\pm$  SE; unstimulated cells 0.38  $\pm$  0.17, antigen-stimulated cells 1.81  $\pm$  0.51) were significantly higher than that of cells from mice immunized with Ld $\gamma$ GCS alone (mean IFN- $\gamma$  production ng/ml  $\pm$  SE; unstimulated cells 0  $\pm$  0, antigen-stimulated cells 33.18  $\pm$  4.55; mean IL-10 production ng/ml  $\pm$  SE; unstimulated cells 0.21  $\pm$  0.14, antigen-stimulated cells 0.76  $\pm$  0.09,  $P < 0.05$ ). IL-4 and nitrite production by cells from immunized mice were similar to control values. Stimulation of cells from uninfected mice with parasite antigen did not result in any increased production of cytokines or nitrite over corresponding unstimulated control (Fig. 3). ConA stimulation resulted in similar levels of IFN- $\gamma$ , IL-4, IL-10, and nitrite production from immunized mice and

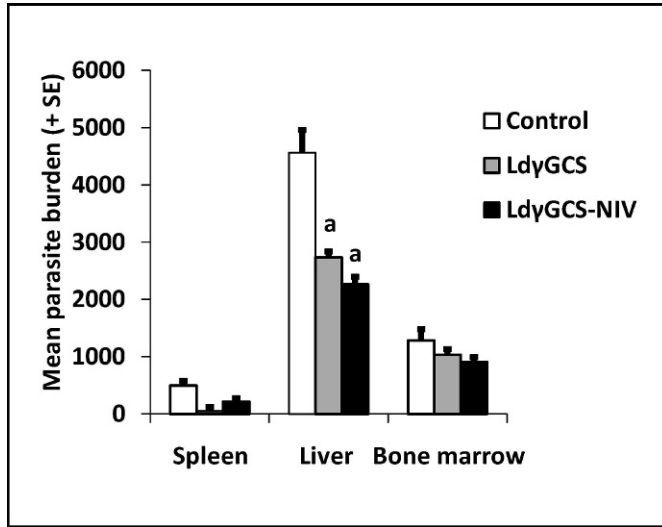


FIGURE 4. The effect of vaccination with LdγGCS formulations on the *L. donovani* spleen, liver, and bone marrow burdens of control and immunized mice. Mice were immunized by subcutaneous injection with PBS (uninfected control) or 50 μg LdγGCS alone (LdγGCS) or 50 μg LdγGCS-NIV (LdγGCS-NIV) on days -28 and -14. On day 0, mice were infected with *L. donovani*, and parasite burdens in the spleen, liver, and bone marrow determined on day 33 PI. <sup>a</sup> $P < 0.01$  compared to control values.

uninfected control mice, a finding that was consistent in the second experiment (Fig. 3).

#### Immunization with LdγGCS protected against infection with *L. donovani*

An initial study was carried out to compare the effect of vaccinating with LdγGCS and LPS to eliminate the possibility that endotoxin present in LdγGCS preparations could be responsible for any protection associated with vaccination. Vaccination with LdγGCS gave protection in mice against

challenge infection, resulting in a significant reduction in liver parasite burdens relative to control animals ( $P < 0.01$ ), whereas injection with LPS did not protect animals against *L. donovani* infection (mean liver parasite burden  $\pm$  SE, control  $1,352 \pm 151$ , LdγGCS immunization  $819 \pm 62$ , LPS treatment  $1,231 \pm 150$ ).

The effect of using a delivery system during immunization on the outcome of infection was subsequently determined by comparing the efficacy of immunization with LdγGCS protein alone with that of LdγGCS incorporated in NIV. Both formulations induced a similar significant reduction in liver parasite burdens compared to control values ( $P < 0.01$ ; Fig. 4), indicating that using a carrier system did not enhance the ability of the recombinant protein to protect against infection. Both vaccines had no suppressive effect on splenic or bone marrow parasite burdens because similar numbers were present in control and vaccinated mice (Fig. 4).

#### Protection against infection was associated with a mixed Th1:Th2 response post-infection

Specific IgG1 and IgG2a titers against the LdγGCS antigen were similar in mice given either immunization type, but were significantly higher than control values ( $P < 0.02$ ; Fig. 5). In contrast, IgG1 and IgG2a antibody titers against a parasite lysate antigen (SAG) had a much lower titer and were similar for all 3 groups of mice.

Similar levels of IFN-γ were induced by in vitro antigenic stimulation of splenocytes derived from for all 3 groups of infected mice. These levels were significantly greater than produced by unstimulated splenocytes from the same sources ( $P < 0.02$ ) or from splenocytes derived from uninfected animals. ConA stimulation resulted in the production of similar levels of IFN-γ by cells from uninfected and infected mice (Fig. 6A). Infection with *L. donovani* was associated with IL-10 production because significantly higher levels were produced by splenocytes derived from all 3 groups of infected mice compared with uninfected controls ( $P < 0.05$ ; Fig. 6B). Immunization was not associated with an increase in IL-10 production as splenocytes

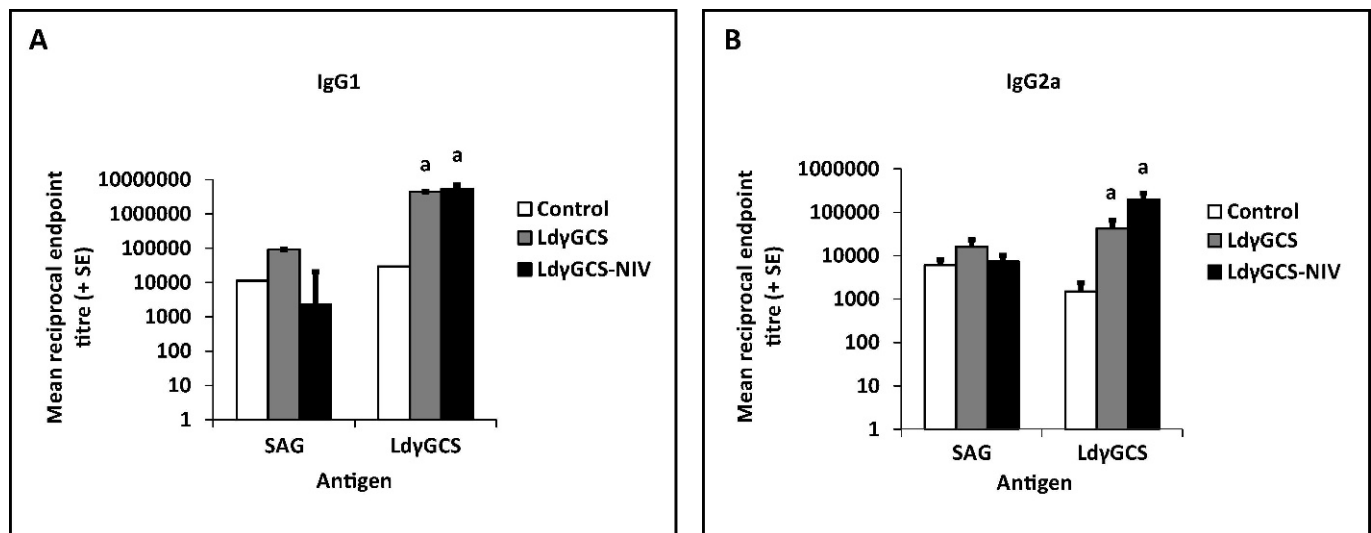


FIGURE 5. The effect of vaccination with LdγGCS on IgG1 (A) and IgG2a (B) specific antibody responses of mice from Fig. 4 to 2 different *L. donovani* antigens (LdγGCS and SAG). <sup>a</sup> $P < 0.02$  compared to infected control value.

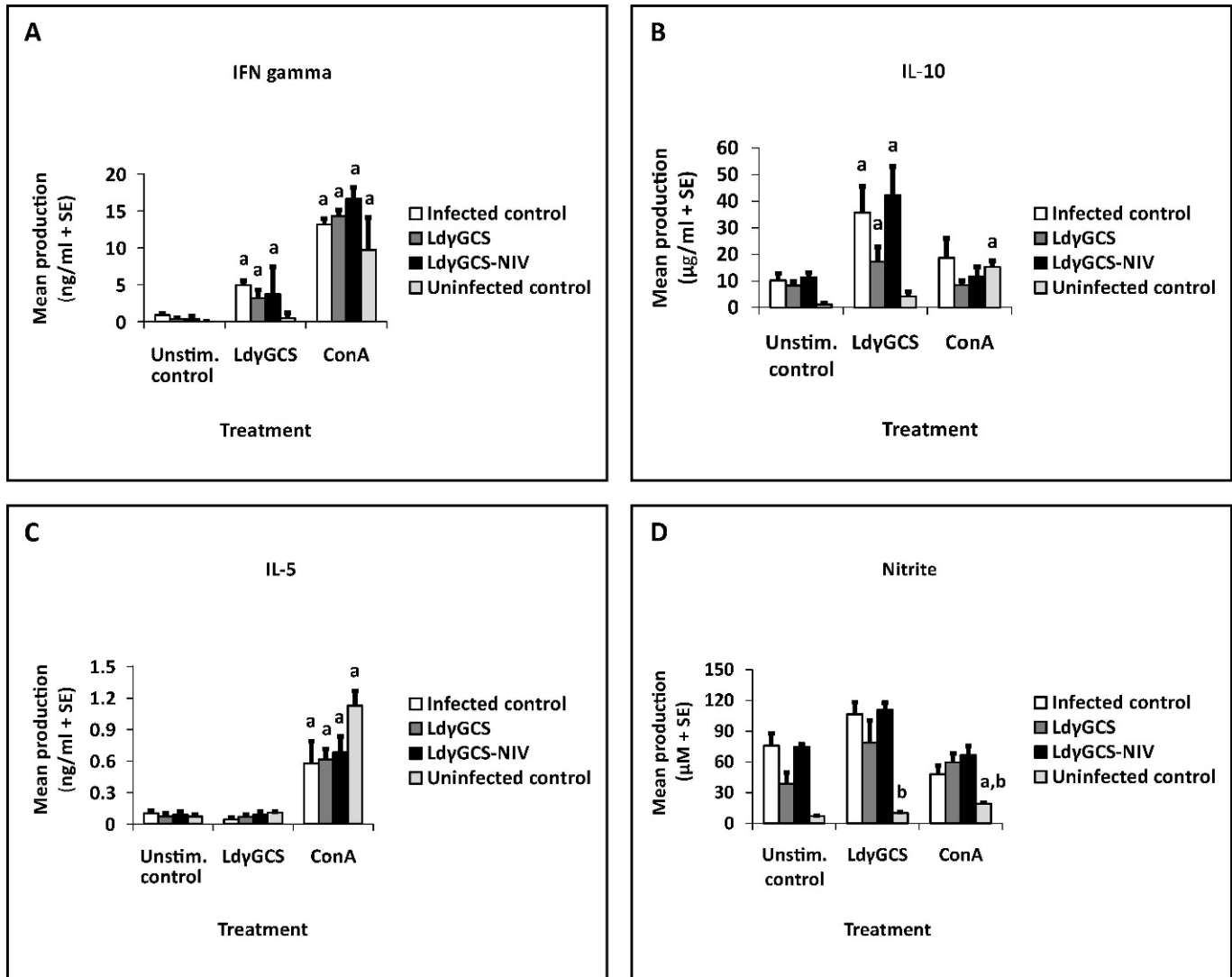


FIGURE 6. Mean IFN- $\gamma$  (A), IL-10 (B), IL-5 (C), and nitrite (D) production by splenocytes of mice from Fig. 4. <sup>a</sup> $P < 0.02$  for stimulated cells compared to corresponding unstimulated control cells; <sup>b</sup> $P < 0.02$  compared to infected group.

from infected, non-immunized animals stimulated with the recombinant antigen produced as much cytokine as immunized mice. Stimulation with ConA did not induce a significant increase in IL-10 production compared with unstimulated levels for all 3 groups of infected mice, whereas splenocytes from uninfected mice produced significantly higher levels compared to their unstimulated controls ( $P < 0.05$ , Fig. 6B). IL-4 production by cells from infected mice in all 3 groups was similarly low, even after ConA stimulation ( $< 0.04$  ng/ml for all treatment groups). Only cells from uninfected, non-immunized animals stimulated with ConA produced enhanced levels of IL-4 compared with unstimulated controls values (mean IL-4 production, ng/ml  $\pm$  SE: unstimulated control  $0.031 \pm 0.031$ , ConA stimulated  $0.125 \pm 0.04$ ). Cells from all 4 groups of mice incubated with medium, antigen, or ConA had similar levels of IL-5 present in their supernatants (Fig. 6C). Unstimulated cells from all 3 groups of *L. donovani* infected mice had high levels of nitrite in their supernatants, and stimulation with antigen or Con A did not

modify their nitrite production. In contrast, cells from uninfected mice produced very little nitrite, and ConA stimulation did not induce the levels present in unstimulated controls from infected mice (Fig. 6D).

## DISCUSSION

The results of this study demonstrate that vaccination with the recombinant fusion protein Ld $\gamma$ GCS protected against *L. donovani* infection. The level of protection obtained after immunization with 2 doses of 50- $\mu$ g Ld $\gamma$ GCS protein/dose, based on reduction in liver parasite burdens, was not appreciably different from that achieved in previous studies using an Ld $\gamma$ GCS DNA-based vaccine (Carter et al., 2007). This perhaps is not surprising since studies have shown that DNA vaccines are more effective than their respective recombinant protein vaccine (Dumontel, 2007). It may be possible to increase the efficacy of vaccination by increasing the amount of Ld $\gamma$ GCS protein used to

immunize mice, or by using a 'prime-boost' approach in which mice are primed with the Ld $\gamma$ GCS DNA vaccine and boosted with recombinant Ld $\gamma$ GCS protein, or by using another *Leishmania* sp. antigen in the vaccine formulation, or by including other agents, such as cytokines, in the vaccine formulation to boost the immune response. Immunizing with multiple antigens is now regarded by many researchers as being inevitable, and DNA constructs that contain the gene sequence for multiple *Leishmania* sp. proteins are being developed as potential vaccines, e.g., Leish-111f encodes for 3 leishmanial proteins (Ghalib and Modabber, 2007).

Incorporating Ld $\gamma$ GCS into a NIV formulation was more effective than immunization with Ld $\gamma$ GCS alone based on its ability to induce specific antibody pre- and post-infection. However, the vesicular formulation gave a similar level of protection as immunization with Ld $\gamma$ GCS alone. In previous studies, we have shown that immunization with modified gonadotrophin hormone-NIV formulation to induce castration of male rats (4 doses, 10  $\mu$ g/dose) induced a predominant Th2 response (Ferro et al., 2004), whereas in the present study immunization with Ld $\gamma$ GCS induced a mixed Th1/Th2 response. The difference in results could be due to the different species, antigen used, or dosing regimen. Using vesicular systems to deliver antigen can increase the efficacy of a protein vaccine in a number of ways, e.g., by improving uptake by antigen presenting cells or providing an antigen depot (Perrie et al., 2008). Protection against *L. donovani* infection is ultimately dependent on the ability to stimulate macrophage killing of the intracellular parasite, and many studies have shown that susceptibility to *L. donovani* is associated with a reduced ability to produce IFN- $\gamma$  and an increased ability to produce IL-10 (Nylén and Sacks, 2007; Ganguly et al., 2008). Therefore the ability of vaccination with Ld $\gamma$ GCS alone or Ld $\gamma$ GCS-NIV to protect against infection and enhance IFN- $\gamma$  production by splenocytes from immunized mice pre-infection is not surprising. The present study and a previous one using the DNA vaccine (Carter et al., 2007) have indicated that vaccine formulations can be screened on the basis of their ability to induce specific IgG1 and IgG2a antibodies and their ability to prime IFN- $\gamma$  production by splenocytes. The inability of either formulation to modify immune responses after infection despite causing a reduction in parasite burdens may be a feature of the experimental model used in the present study, i.e., the parasite strain used or that mice were infected with a large number of amastigote parasites. In previous studies, an alteration in immune responses of mice cured of their *L. donovani* infection by drug treatment did not occur until after day 31 post-treatment (Banduwardene et al., 1997). Thus, cured mice had a positive DTH response instead of a negative DTH response to promastigote parasites injected into the footpad by day 31 post-treatment and increased IFN- $\gamma$  production by ex vivo antigen-stimulated splenocytes compared to infected controls by splenocytes was not obtained until day 42 post-treatment. These results perhaps indicate that changes in immune responses require removal of dead parasites or antigens before protective T cell responses associated with protection in *L. donovani* can be expressed. Here, there was only partial parasite clearance, and it is not surprising that the immune responses induced by infection predominate.

Expression of Ld $\gamma$ GCS resulted in production of full-length and truncated forms of the protein. Inclusion of protein inhibitors during production did not increase the recovery of full-length

protein in the soluble fraction, suggesting that truncated versions were made after induction. The his-tag has been cloned into the 'N-terminus' of the protein so that when the protein was purified the truncated versions were also selected. Protein refolding occurred when the protein was dialyzed because it showed activity in assays carried out to determine the enzymatic activity of Ld $\gamma$ GCS (data not shown). Similar problems with protein expression are common (Qoronfleh et al., 2007). Endotoxin contamination is inevitable as the protein is expressed in bacteria, but it can be removed using a variety of methods whose success is dependent on the characteristics of the protein (Magalhães et al., 2007). The maximum level of endotoxin contamination for parenteral products is 5 EU/kg/hr in most countries (Magalhães et al., 2007), which was easily avoided here where mice were immunized with <0.5 EU each.

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