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Journal

Vaccine, 21(21-22)

ISSN

0264-410X

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Publication Date

2003-06-01

DOI

10.1016/s0264-410x(03)00108-7

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Peer reviewed

Immunization with recombinant paraflagellar rod protein induces protective immunity against *Trypanosoma cruzi* infection

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Received 18 June 2002; received in revised form 9 January 2003; accepted 9 January 2003

Abstract

In the present study, we have produced recombinant paraflagellar rod proteins (PFR) and report their use for successful vaccination of mice against *Trypanosoma cruzi*. This protection is associated with a highly polarized type 1 cytokine production profile. Additionally, we have analyzed the gene sequence encoding PFR-2 to determine the degree of conservation among seven highly diverse strains of *T. cruzi*, and found it to be highly conserved. The results presented here indicate that the PFR antigens are highly conserved, and immunization with rPFR-1, PFR-2, or an equimolar mix of the PFR-1, -2, and -3 proteins provides protective immunity against *Trypanosoma cruzi*.

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Keywords: *Trypanosoma cruzi*; Vaccine; Paraflagellar rod; Th1; Chagas disease; Th1

1. Introduction

American trypanosomiasis, or Chagas' disease, is caused by the parasitic protozoan, *Trypanosoma cruzi*. Infection with this parasite affects 16–18 million people in South and Central America, where it causes significant mortality and morbidity, as well as exacting a large economic toll. To date, no vaccine is available to protect against infection and available chemotherapeutics are limited in effectiveness. Our laboratory has previously described successful vaccination of mice with paraflagellar rod proteins (PFR) purified from parasite extracts (pPFR). Vaccination with these proteins either emulsified with Freund's adjuvant [1] or co-adsorbed to alum with recombinant IL-12 (rIL-12) [2] resulted in greater than 90% reduction in circulating parasites and 100% survival of an otherwise lethal infection with *T. cruzi*. The parasite-derived preparation, pPFR, is composed of four distinct proteins, designated PFR-1 through PFR-4 (encoded by the *par1–4* genes) [3,4]. It is clear that the protective immunity elicited by this vaccine is strongly polarized to a T helper type 1 response [5]. Vaccine studies using knockout mice and antibody depletion of specific cell types have shown that B cell function is not required for protection; however, both CD4⁺ and CD8⁺ T cells play important roles.

In vivo, CD4⁺ and CD8⁺ T cells are required for reduction of parasitemia and survival, respectively. In vitro, CD4⁺, but not CD8⁺, splenic T cells from PFR-immune mice are able to produce IFN- γ and induce nitric oxide production by *T. cruzi*-infected macrophage cells [2,6]. IFN- γ has been shown to be critical for the protective immune response against *T. cruzi* in both experimental infection and vaccine models [7–10]. Likewise, nitric oxide has been shown to be a potent anti-microbial agent and highly effective against *T. cruzi* [11]. Consistent with these observations, activated macrophages, which produce NO in response to IFN- γ and other stimuli from activated T cells, play a key role in protective immunity against *T. cruzi* [12].

While our previous studies indicate that the protective antigen in the PFR vaccine is likely one or more of the four PFR proteins, they do not exclude the possibility that the protective antigen(s) may be a minor contaminant not previously recognized in the PFR preparation, nor do they identify which of the four PFR proteins might serve as a protective antigen. In order to unequivocally determine whether one or more of the PFR proteins are capable of protectively immunizing mice against *T. cruzi* challenge, we have cloned the genes encoding the four PFR proteins into fusion protein expression vectors for production in *E. coli*. Herein, we investigate the vaccine potential of individual recombinant PFR proteins (rPFR) as well as an equimolar mixture of rPFR-1–3 (rPFRmix).

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2. Materials and methods

2.1. Mice

Female C56BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 6–8 weeks of age.

2.2. Parasites

The Peru and Y clones of *T. cruzi* [13] were maintained by serial passage in female BALB/cByJ mice.

2.3. Construction and expression of recombinant PFR proteins in *E. coli*

Isolation of the *par2* [3], *par1*, *par3*, and *par4* [4] genes is described elsewhere. For production of rPFR-1, an 1787 bp product, representing the full length *par1* sequence (GenBank accession no. AF004380), was PCR amplified from a genomic DNA clone using the primers, 5'-CACGGATCCATGACGGTTTAC-3' and 5'-CACCTCGAGTCAATACTCCGGCG-3', designed to introduce *Bam*H I and *Xho* I restriction enzyme recognition sites, respectively. The resulting fragment was cloned into the *Bam*H I and *Xho* I sites of the pTrcHisA vector (Invitrogen, Carlsbad, CA). The entire coding region of PFR-2 (accession no. M97548) was amplified from a cDNA clone with the primers, 5'-CACGGATCCATGAGCTACAAG-3' and 5'-CACGTCGACTCAATACTCCGGCG-3', incorporating *Bam*H I and *Sal* I sites, respectively, which were cloned into the *Bam*H I and *Xho* I sites of pTrcHisA. The entire coding region of PFR-3 (accession no. AF005193) was amplified from a full length cDNA clone with the primers 5'-CACGGATCCTATGTCTGCCGAGG-3' and 5'-CACCTCGAGTCACTCCAGGCGGG-3'. This fragment was restricted with *Bam*H I and *Xho* I, which cut at the endogenous *Xho* I site found at position 1836, and was cloned into the pTrcHisB vector at the *Bam*H I and *Xho* I sites. Because this fragment does not contain a stop codon, the stop codon found 98 bp past the *Xho* I site is utilized, resulting in the addition of 28 amino acids to the carboxy terminus of the expressed rPFR-3 protein, accounting for the higher molecular weight observed for this recombinant protein. *par4* was subcloned by amplifying the full length coding sequence from a cDNA clone (accession no. AF045059) with the primers, 5'-CACGGATCCAATGCCTCCAAAGA-3' and 5'-CACCTCGAGTCATATCTCGTACGGGATA-3', the resulting product was restricted with *Bam*H I and *Xho* I and cloned into the corresponding sites of pTrcHisB. Each plasmid was used to transform DH5 α *E. coli* cells (Stratagene, La Jolla, CA). Cultures of each were grown in Terrific Broth (Sigma, St. Louis, MO) and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (Inalco Pharmaceuticals, San Luis Obispo, CA). Bacteria were pelleted by centrifugation at 4000 \times g for 10 min then lysed with B-PER reagent (Pierce, Rockford, IL) following the manufacturers

recommendations for the purification of inclusion bodies. Lysates were purified by nickel affinity chromatography on HiTrap Chelating columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) charged with nickel chloride. Following affinity chromatography, samples were further purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Bio-Rad Prep Cell model 491 (Bio-Rad, Oakland, CA) and concentrated in a Centricon concentrator (Amicon, Beverly, MA). Both in vitro cell culture and adsorption of rPFR to alum necessitate the removal of the majority of the sodium dodecyl sulfate (SDS). However, in the absence of SDS, the PFR proteins were found to be highly insoluble. In order to achieve a balance between solubility of the rPFR proteins and compatibility with in vitro studies and adsorption to alum the following treatment was performed. The majority of SDS was removed by treatment with SDS-OUT reagent (Pierce, Rockford, IL), followed by extensive dialysis against 20 mM HEPES pH 7.4, 0.05% SDS and sterilization by 0.22 μ m filtration. Unfortunately, the rPFR-4 protein was insoluble at SDS concentrations less than 1.5%, which is incompatible with adsorption to alum and is toxic to cells in culture.

2.4. PFR Ag preparation

PFR proteins were isolated from epimastigote flagellar extracts, as previously described [14]. Briefly, 10¹¹ Peru strain epimastigotes were harvested by centrifugation, washed in PBS, and lysed in 0.1 M tricine, pH 8.5, containing 1% Nonidet P-40. The pellet was extracted with high salt buffer consisting of 0.1 M tricine, pH 8.5, 1 M NaCl, and 1% Triton X-100, using sonication. This crude flagellar pellet was successively extracted with 2.0 and 6.0 M urea in 10 mM tricine, pH 8.5. The resulting supernatant contains approximately 50% PFR and 50% tubulin. The PFR proteins were purified by preparative electrophoresis on a Bio-Rad Prep Cell, Richmond, CA. Fractions containing PFR were dialyzed extensively against 20 mM HEPES pH 7.4 with 0.05% SDS, concentrated by centrifugation in a Centricon and sterilized by 0.22 μ m pore filtration.

2.5. Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

Protein samples were adjusted to the composition of the electrophoresis sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 2% SDS, 0.001% bromophenol blue) and boiled for 5 min prior to analysis. One-dimensional SDS-PAGE in 0.75 mm slab gels was performed as previously described [4]. Prestained molecular weight markers (Amersham Pharmacia, Uppsala, Sweden) were included in the gels. Gels were either stained with Coomassie Brilliant Blue R or processed for Western blot analysis. Western blots were probed with polyclonal mouse serum against pPFR proteins. Visualization of rPFR-4 required a significantly longer exposure time, presumably due

to a lower titre of PFR-4-specific antibodies compared to those specific for the other PFR proteins in pPFR-immune serum. Additionally, rPFR-1 and rPFR-4 were reacted with the monoclonal antibodies 6B3 (anti-PFR-1) and 7C2 (anti-PFR-4) [4], respectively. While, rPFR-2 and rPFR-3 were reacted with mouse serum raised against a peptide sequence specific for PFR-2 and PFR-3, respectively [4].

2.6. Immunization of mice

Six-to-eight-week-old female C57BL/6 mice were immunized by subcutaneous (s.c.) injection of 40 µg of pPFR or rPFR proteins co-adsorbed to alum with 0.5 µg recombinant murine IL-12 (rIL-12; a generous gift from Genetics Institute, Cambridge, MA) and were boosted twice at 2-week intervals with 20 µg protein co-adsorbed to alum with 0.5 µg rIL-12. Alum-protein mixtures were prepared by combining equal volumes of Rehsorptor aluminum hydroxide adsorptive gel (Intergen Co., Purchase, NY) and antigen at 400 µg/ml and rIL-12 at 5 µg/ml in 0.9% saline with gentle mixing for 1 h at room temperature [2]. Control groups were injected with adjuvant (rIL-12 adsorbed to alum) plus saline. Two weeks after the last injection, mice were challenged with s.c. injection of 10² bloodstream Peru strain trypomastigotes.

2.7. Measurement of parasitemias

Parasitemia levels were determined as previously described [13] by removing a blood sample from the tail vein, diluting the sample in 0.9% ammonium chloride, and counting the trypomastigotes in a Neubauer hemocytometer (American Optical Corp., Buffalo, NY).

2.8. Macrophage and T cell cultures

Mice were immunized as described earlier. Seven to ten days following the last injection, spleens were removed and single-cell suspensions prepared in DMEM supplemented with 25 mM HEPES buffer (pH 7.2), 1 mM sodium pyruvate, non-essential amino acids, L-glutamine, 5 × 10⁻⁵ M 2-ME, 50 U/ml penicillin, 50 g streptomycin sulfate, and 10% FBS (C-DMEM). Spleen cell suspensions were enriched for T cells by passage over nylon wool columns [15]. IC-21 macrophages (ATCC TIB-186) were plated in C-DMEM at 1 × 10⁵ macrophages/well in 96-well plates. Macrophages were allowed to adhere overnight (37 °C at 8% CO₂). Adherent macrophages were either infected overnight with Y strain trypomastigotes at a 10:1 parasite:macrophage ratio then washed three times with DMEM to remove extracellular parasites or incubated with PFR protein antigen (5 µg/ml) at the time T cells were added to the culture. Control wells were incubated overnight with C-DMEM containing no parasites or antigen. Cells were cultured for 4 days

(37 °C, 8% CO₂), at which time supernatants were harvested and assayed for nitrite and cytokine production.

2.9. Nitrite assays

Nitrite levels in 4-day culture supernatants were measured using the Greiss reagent as previously described [5]. Briefly, 50 µl culture supernatants were combined in a 96-well plate with a 1:1 mixture of 1% sulfanilamide in 2.5% H₃PO₄ and 0.1% naphthylethylenediamide in 2.5% H₃PO₄. Plates were incubated for 10 min at room temperature and absorbance was determined at 550 nm using an automated microplate reader. Nitrite concentrations were determined in triplicate using a standard curve of sodium nitrite from 125 to 1 µM prepared in culture media.

2.10. Cytokine measurements

Culture supernatants were assayed for the cytokines IFN-γ and IL-4 by capture ELISA as previously described [6]. Briefly, culture supernatants and cytokine standards were diluted and incubated overnight in 96-well microtiter plates coated with cytokine-specific capture antibody (Pharmingen, San Diego, CA) according to the manufacturers' recommendations. Bound cytokine was detected using sequential incubations with biotinylated anti-cytokine detecting antibody, streptavidin-peroxidase, and 2,2'-azino-di-3-ethylbenzthiazoline (ABTS, Roche Diagnostics, Indianapolis, IN). All samples were tested in triplicate. Plates were read at 405 nm using an automated ELISA plate reader. Concentrations were calculated from the linear regions of cytokine standards, values for control wells were subtracted, and final concentrations were expressed in ng per ml.

2.11. In vitro blocking of CD4⁺ T cells

To block the activity of CD4⁺ T cells, purified antibody from culture supernatants of the hybridoma line GK1.5 (anti-CD4; ATCC TIB207) [16] were added to rPFR-specific splenic T cells. Normal rat Ig was used as a control. Triplicate wells of IC-21 macrophages at 2 × 10⁵ cells per well in 96-well plates were infected with tissue-culture trypomastigotes (100:1 ratio). After 16 h incubation, culture supernatants were aspirated, cell monolayers were washed three times with PBS, and T cells added. At 24 and 48 h, culture supernatants were collected and assayed for IFN-γ and IL-4 by capture ELISA as described earlier.

2.12. Inhibition of *T. cruzi* growth in vitro

Parasite titers in infected-IC-21 macrophage/T cell culture supernatants were determined by pipeting media up and down vigorously several times to resuspend trypomastigotes. Parasite number was determined by counting with a Neubauer hemocytometer.

2.13. DNA sequencing

The *par2* (Genbank accession number M97548) was amplified by the polymerase chain reaction (PCR) from genomic DNA isolated from the Peru, Y, and Esmeraldo strains of *T. cruzi* and zymodemes #19, 27, 36, and 43 [17] (kindly provided by Dr. Francisco Ayala, University of California, Irvine, CA) using the primers, 5'-GCAATGAGCTACAAGGAGGCATCTGG-3' (nt 46–71) and 5'-GGTACTGTGTGATCTGCTGCACCG-3' (nt 1829–1853). The resulting PCR product was directly sequenced by the dideoxy chain-termination method using Thermo Sequenase radiolabeled terminator cycle sequencing (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions using incremental primers. Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA).

3. Results

3.1. Production of recombinant PFR proteins

To directly investigate the vaccine potential of each of the four the PFR proteins, the *par* genes were cloned individually into the pTrcHis vector and expressed as polyhistidine fusion proteins in *E. coli*. Each recombinant plasmid was shown to produce an rPFR protein of the predicted molecular weight (ie. PFR protein + an additional 31 residues found in the amino-terminal fusion sequence, and in the case of rPFR-3 an additional 28 residues in the carboxy-terminal fusion sequence) when purified samples were analyzed by SDS-PAGE (Fig. 1A). To assure identity,

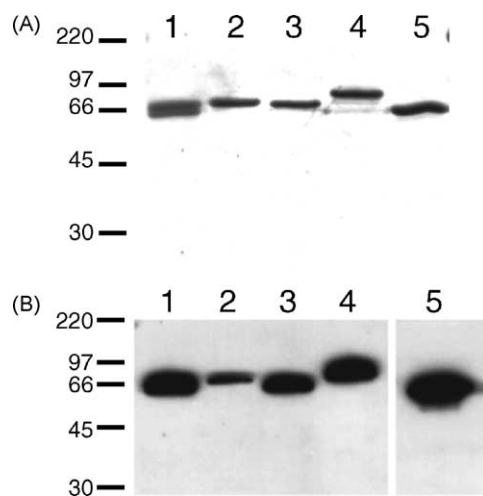


Fig. 1. SDS-PAGE and Western blot analysis of recombinant PFR proteins. Lanes are: (1) pPFR, (2) rPFR-1, (3) rPFR-2, (4) rPFR-3, (5) rPFR-4. Each protein sample (0.5 mcg) was electrophoresed on 7.5% SDS-PAGE gel under reducing conditions and visualized with Coomassie stain (A) or transferred to nitrocellulose and reacted against PFR-immune polyclonal mouse serum (B).

each rPFR was shown to be recognized by pPFR-immune mouse serum (Fig. 1B). Additionally, the identity of each rPFR was confirmed by positive reaction with a corresponding PFR-specific mAb or peptide-immune mouse serum. Several different culture, induction, and purification schemes were explored, in order to define the optimal conditions for protein production. The purified rPFR proteins were found to be highly insoluble in a variety of aqueous and organic solvents in the absence of SDS. Therefore, some SDS was required in the recombinant PFR protein solutions. For rPFR-1–3, a solution of 1 mg/ml PFR proteins in 20 mM HEPES buffer at pH 8.0 with 0.05% SDS was found to be optimal for solvation of rPFR to be used in further in vivo and in vitro studies. Unfortunately, SDS concentrations for solvation of rPFR-4 were incompatible with alum adsorption, thus excluding rPFR-4 from vaccine studies.

3.2. Immunization with rPFR proteins reduces acute parasitemia and protects mice against lethal challenge with *T. cruzi*

C57BL/6 mice were immunized s.c. with recombinant PFR proteins co-adsorbed to alum with rIL-12 either individually or as an equimolar mixture of rPFR-1–3. Control animals were similarly injected with saline containing rIL-12 adsorbed to alum. Immune and control animals were challenged with 100 Peru strain blood trypomastigotes. Parasitemias of adjuvant-control and immune animals were monitored from day 14 to day 25 post-infection (p.i.) and are plotted in Fig. 2. Without treatment, C57BL/6 mice infected with this strain fail to control parasite load in the blood, which surpasses 10^6 parasites/ml, and is 100% lethal by day 25 of infection [6,18]. Similarly, adjuvant control animals failed to decrease parasitemia levels, reaching a peak of $(17.6 \pm 4.6) \times 10^5$ on day 21. In contrast, rPFRmix, rPFR-1, and rPFR-2, immunized animals were able to successfully resolve parasitemia by day 30 p.i., with the peak parasitemia occurring between days 17 and 21 p.i. (Fig. 2B–D). Peak parasitemia levels of these groups were $(4.6 \pm 1.7) \times 10^5$ (74% reduction compared to control peak parasitemia), $(5.3 \pm 1.4) \times 10^5$ (70% reduction), and $(4.7 \pm 1.9) \times 10^5$ (73% reduction), respectively. Parasitemia levels of these groups were significantly lower ($P < 0.05$ by Mann–Whitney *U*-test) than in the adjuvant control group at all monitored days. In contrast to the success seen with the above antigens, immunization with rPFR-3 resulted in more modest reductions of acute parasitemia, peaking at $(9.0 \pm 8.1) \times 10^5$ (51% reduction compared to control).

As shown in Fig. 3, immunization with the rPFR-1, rPFR-2, and rPFRmix resulted in 100% protection against an otherwise lethal challenge with *T. cruzi*, demonstrating the ability of these recombinant PFR proteins to provide a level of protection equal to that seen with parasite-derived protein [2]. Consistent with the reduced ability to decrease parasitemia, immunization with rPFR-3 resulted in only 42%

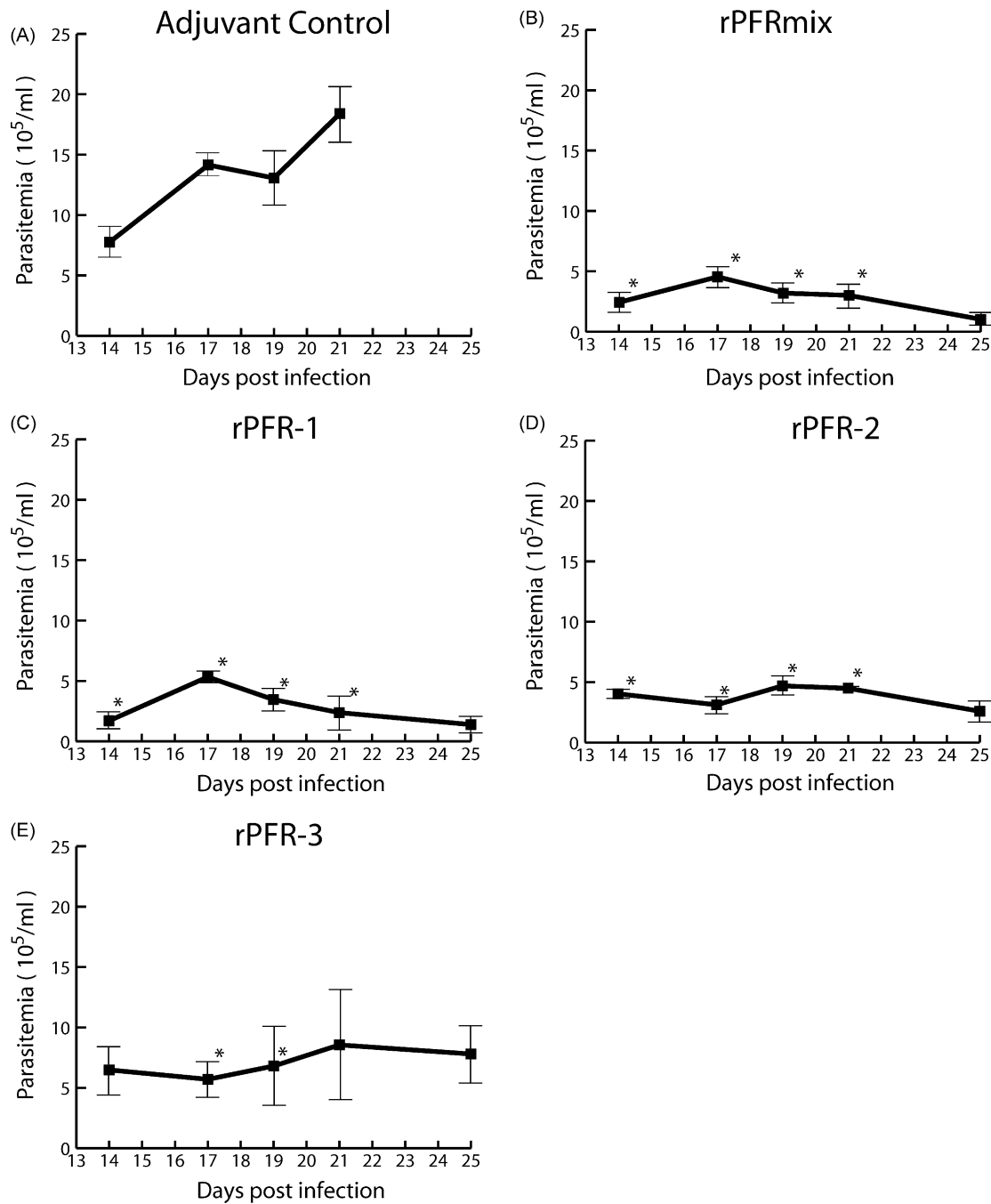


Fig. 2. Parasitemia time course during acute infection. Mice were immunized with alum/IL-12 only (A), rPFRmix/alum/IL-12 (B), rPFR-1/alum/IL-12 (C), rPFR-2/alum/IL-12 (D), or rPFR-3/alum/IL-12 (E). A total of three injections given at 2-week intervals were given to each animal. Ten days following the final boost, mice were infected s.c. with 100 Peru strain trypomastigotes. Parasitemia was monitored on days 14, 17, 19, 21, and 25 post-infection by counting trypomastigote forms in peripheral blood obtained from tail bleed. Mean values ($n = 6$) \pm S.D. are shown.

long-term survival (>60 days). Infection of adjuvant-control animals was 100% lethal by 25 days post-infection.

3.3. Vaccination with rPFR proteins with rIL-12 and alum generates a type 1 T cell response

Our previous studies have shown that the protective immune response generated by immunization with pPFR anti-

gen is characterized by a strong CD4⁺ type 1 T cell response [5,6]. It may be anticipated, therefore, that protective immunization with the rPFR proteins might also induce such a strong antigen-specific type 1 CD4⁺ T cell response. To determine if this is the case, mice were immunized s.c. with either rPFR-1–3, or rPFRmix, co-adsorbed to alum with rIL-12. Two booster immunizations were given at 14-day intervals. Ten days following the final boost, nylon wool

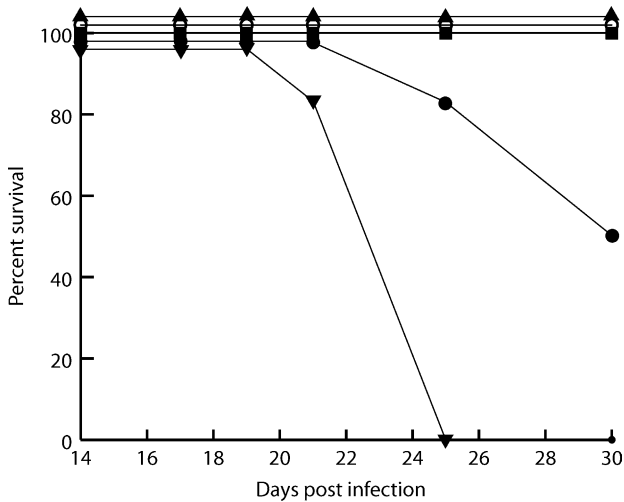


Fig. 3. Survival of rPFR-immune and adjuvant control mice. Mice were immunized as described with rPFRmix (▲), rPFR-1 (○), rPFR-2 (■), rPFR-3 (●), or adjuvant-only control (▼).

enriched splenic T cells were added to cultures of IC-21 macrophages. Cytokine production following stimulation of these cultures with pPFR or rPFR antigens was measured. As shown in Fig. 4, splenic T cells from mice immunized with rPFR-1–3, or rPFRmix produced high levels IFN- γ in response to stimulation with pPFR, with T cells from rPFRmix-immune mice producing the highest levels of IFN- γ . Cells from both pPFR- and rPFRmix-immune animals (data not shown, Fig. 4) produced IFN- γ in response to each of the three rPFR antigens, indicating that all three proteins are immunogenic in these mixtures. The highest level of IFN- γ production by rPFRmix T cells was in response to stimulation with rPFR-2, followed by rPFR-1,

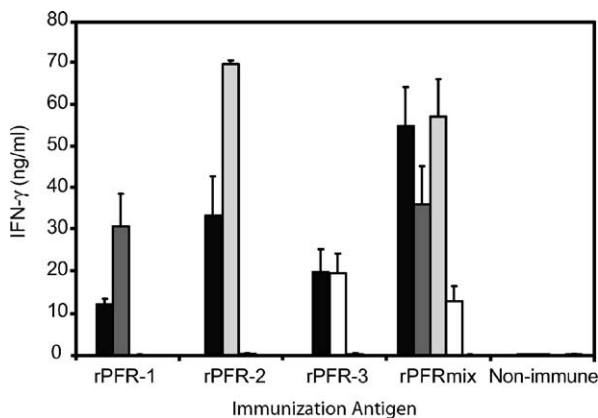


Fig. 4. IFN- γ production by T cells from rPFR-1–3, rPFRmix, and non-immune mice. Nylon wool-enriched T cells from immunized mice were cultured with IC-21 macrophage-like cells and pPFR (black bars), rPFR-1 (dark gray bars), rPFR-2 (light gray bars), rPFR-3 (white bars), or media alone (diagonal hatched bars) was added. Supernatants were collected after 5 days of culture and tested for IFN- γ by capture ELISA. Each value shown represents the mean value from triplicate cultures \pm the standard deviation.

then significantly less with rPFR-3. None of the rPFR or pPFR antigen preparations induced IFN- γ production in cultures of non-immune splenic T cells, verifying that the above responses are antigen specific (Fig. 4). None of the antigen-stimulated rPFR-immune cultures produced detectable levels of IL-4, indicating that this immune response is a highly polarized type I response (data not shown).

3.4. Immunization with recombinant PFR antigens produce CD4⁺ T cells capable of recognizing and activating *T. cruzi*-infected macrophages

It is well established that macrophages play a major role in resistance to *T. cruzi* infection [12,19,20]. Activated macrophages are highly competent to produce anti-microbial agents and kill *T. cruzi*, while unactivated macrophages are successfully parasitized [7]. This suggests that a major role for rPFR-immune CD4⁺ T cells is likely the recognition and subsequent activation of *T. cruzi* infected macrophages. To investigate this possibility, mice were immunized with rPFR antigens as described earlier and splenic T cells from these animals were co-cultured with *T. cruzi*-infected IC-21 macrophage-like cells. As shown in Fig. 5A, T cells from mice immunized with each of the rPFR formulations produced IFN- γ , but non-immune T cells did not (black bars). Treatment with an anti-CD4 antibody, which has been shown to block CD4⁺ T cell activity in vitro in the absence of complement [16], completely abrogated the ability of these cultures to produce IFN- γ , but treatment with control rat purified IgG had no effect. IL-4 was not detected in any cultures with rPFR-immune T cells reiterating that this immune response is highly polarized.

Production of IFN- γ by rPFR-immune T cells in response to infected macrophages was accompanied by the accumulation of nitrite, an indicator of NO production (Fig. 5B) High levels of nitrite were produced by cultures containing T cells from each of the rPFR formulations, with the rPFRmix producing the highest levels. Control cultures containing infected macrophages and non-immune T cells produced low levels of nitrite. Treatment with anti-CD4 antibody greatly reduced the amount of nitrite produced, but treatment with control rat IgG did not, verifying that this activity is mediated by CD4⁺ T cells.

Nitric oxide has been shown to be effective against *T. cruzi*, both in vitro and in vivo [21]. Extracellular trypomastigote-stage *T. cruzi* from the T cell/infected IC-21 cultures described earlier were counted and percent inhibition of parasite replication was calculated. Control wells contained non-immune T cells cultured with infected IC-21 macrophages. As shown in Fig. 6, T cells from mice immunized with each individual rPFR or rPFRmix reduced parasite numbers in vitro by 75–90%. The addition of non-immune T cells had very little impact on parasite replication (12% reduction) compared to cultures without T cells. These data demonstrate that immunization with rPFR proteins generates an immune response capable of

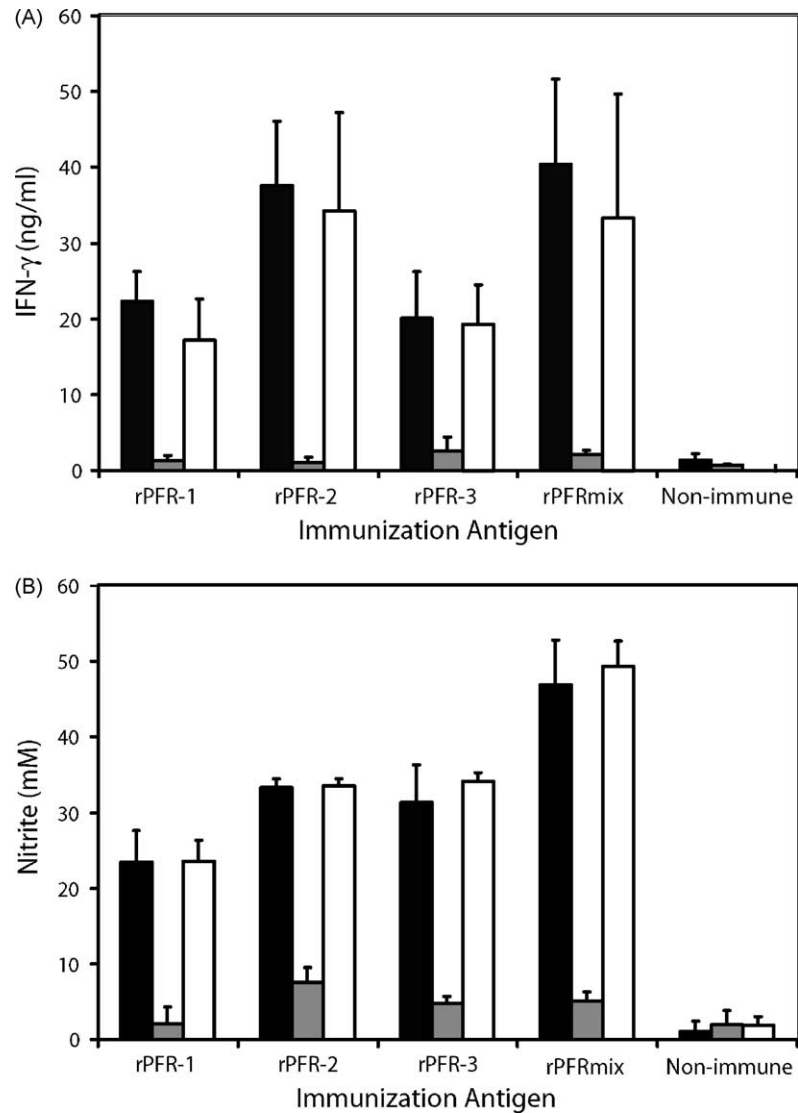


Fig. 5. IFN- γ and nitrite production by cultures of Y strain *T. cruzi*-infected IC-21 cultured with rPFR-immune or non-immune T cells. Cultures containing rPFR-1–3, rPFRmix, or non-immune T cells and infected IC-21 cells were treated with either media alone (black bars), neutralizing anti-CD4 antibody (gray bars), or control purified rat IgG (white bars). (A) On day 5 of culture, supernatants were collected and IFN- γ was measured by capture ELISA. (B) On day 4 of culture, supernatants were collected and nitrite was measured using the Greiss reagent. Each value shown represents the mean value from triplicate cultures \pm the standard deviation.

activating effector mechanisms thought to play a protective role in vivo against *T. cruzi* infection.

3.5. The PFR-2 protein is highly conserved among diverse *T. cruzi* isolates

Extensive genetic and biological diversity exists between different *T. cruzi* strains [22,23]. This is likely the consequence of the clonal population structure of this organism, in which individuals evolve independently of each other [24]. This high degree of genetic variability poses a special problem for vaccine design, because an effective vaccine must be protective against each of the highly divergent *T. cruzi* clones found to infect human beings. To investigate the amount of

divergence within the *par* genes, we have examined the nucleotide sequence of the *par2* gene from seven clones that represent about 85% of the genetic diversity found within the *T. cruzi* population [25]. This sampling includes members of the two major subgroups of *T. cruzi* thought to represent evolution in either North American placentals or South American primates [26]. Since *T. cruzi* is putatively a diploid organism [22] and the *par2* gene is present in multiple copies (30–40 per haploid genome) [3], it was important to obtain sequence data from several members of the gene family in each clonal isolate. To this end, we selected the gene encoding the protective antigen, PFR-2, and amplified by PCR the complete coding sequence of the *par2* gene using total genomic DNA from each of the seven different *T. cruzi*

Table 1
Summary of nucleotide differences within the *par2* gene of diverse strains of *T. cruzi*

| Strain | Nucleotide position | | | | | | | | | | | | | | | | | | | | | | | | |
|-----------|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|------|------|---|
| | 213 | 231 | 238 | 333 | 384 | 561 | 621 | 636 | 645 | 660 | 663 | 666 | 742 | 1092 | 1098 | 1122 | 1221 | 1272 | 1298 | 1302 | 1512 | 1578 | 1599 | 1604 | |
| Peru | T | A | T | G | A | G | G/T | C | G | C | G | G | C | C | C | G | C | C | G | C | C | C | C | C/T | C |
| Esmeraldo | T | A | T | G | A/G | A | A | C | A | C | G | G | C | T | C | G | T | C | C | C | T | C | T | C | C |
| Y | T | G | T | G | G | A | A | C | A | T | G | G | C | T | C | G | T | C | C | C | T | C | T | C | C |
| 19 | G | G | G/A | A | G | A | A | C | A | T | G | G | C | T | C | G | T | C | C | C | T | C | C | C | C |
| 27 | T | G | A | G | G | G | G | T | A | C | T | G | C | C | C | G | C | C | C | T | C | C | C | C | T |
| 36 | T | G | A | G | G | G | G | C | A | C | T | G/A | C | C | C | A | C | C/T | C | C | C | T | C | C | C |
| 43 | T | G | A | G | G | G | G | C | G/A | C | G/T | G | C/T | C | C/T | G/A | C | C | G/C | C | C | C | C/T | C | C |

For each site at which differences were found, the nucleotide(s) occupying that position for each strain is shown. When two different nucleotides separated by a “/” are given, both nucleotides were observed.

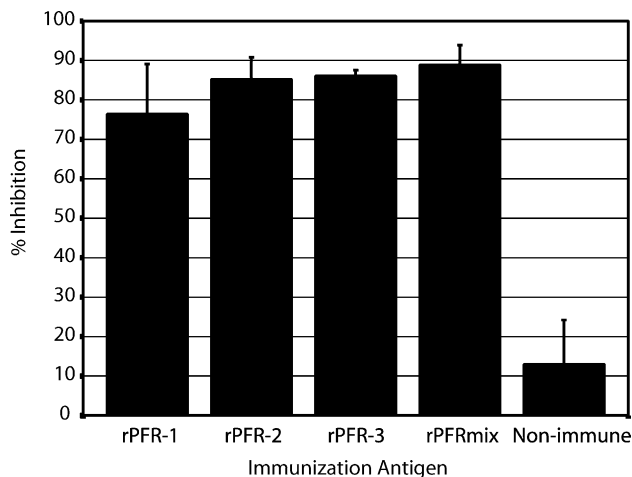


Fig. 6. Inhibition of parasite replication in IC-21 cells cultured with rPFR-immune or non-immune T cells. IC-21 cells were infected overnight with tissue-culture-derived Y strain trypomastigotes. Following overnight incubation, wells were washed three times with media to remove free parasites and T cells were added to the cultures. On day 4 of culture with T cells, supernatants were thoroughly mixed, removed, and parasites counted. The % inhibition was calculated using the following formula: $100 \times (1 - \# \text{parasites in experimental well} / \# \text{parasites in wells without T cells})$. Each value shown represents the mean value from triplicate cultures \pm the standard deviation.

clones and directly sequenced the product. This approach is expected to provide representative sampling of the *par2* gene family within each individual clone. Satisfactory data was collected for nucleotides 211–1620 of the *par2* gene sequence, which represents 78% of the total coding region of this gene. As shown in Table 1, several nucleotide differences are observed within this region at a total of 24 sites. Several sites were found to be polymorphic within individual strains, which can either be due to heterozygosity or differences between distinct copies of the gene within the *par2* family; however, only one of the clones examined in this study was found to theoretically encode two PFR-2 proteins with different primary amino acid sequences. This was observed in isoenzyme strain 19, which is predicted to encode both a serine and a threonine due to polymorphism at nucleotide 238 (aa 80). The greatest number of intra-strain polymorphisms are found in isoenzyme clone 43, consistent with the recent finding by Machado and Ayala [27] that this isolate is a hybrid of two other isoenzyme strains. Their study failed to find evidence that any of the other clones that we have examined in this work are hybrids.

In addition to intra-strain polymorphisms, a number of inter-strain differences were observed, with the most distantly related strains, by the isoenzyme analysis done by Tibayrenc et al. [28], 43 and Peru, having a nucleotide substitution rate of 0.8% per base pair. Despite the multiple nucleotide differences found between strains, only two, nucleotides 238 and 1604 (aa position 535), are predicted to result in amino acid substitutions. As mentioned earlier,

isoenzyme strain 19 encodes both a threonine and serine at this position. Isoenzyme strains 27, 36, and 43 encode only threonine, and the Peru, Esmeraldo, and Y clones only serine. The other difference, at nucleotide position 1599, indicates a valine at residue 533 for isoenzyme strain 27, but an alanine in each of the other clones examined. Interestingly, at most of the sites where nucleotide substitutions are found in *par2*, differences are seen in not just one, but in several different strains of *T. cruzi*, suggesting that many of the nucleotide changes occurred prior to the divergence of these isolates.

4. Discussion

Immunization with the paraflagellar rod proteins has previously been shown to be highly effective at reducing acute parasitemia and providing 100% protection against an otherwise lethal challenge with a highly virulent strain of *T. cruzi* [1,2]. Although potent immune responses against the previously identified PFR proteins have been observed, it was impossible to entirely rule out the possibility that the protective antigen in this preparation was a minor, unidentified component. In this study, recombinant PFR proteins were produced and successfully used to vaccinate mice against *T. cruzi* infection. Consistent with previous findings using pPFR antigen purified from the parasite, s.c. immunization with rPFR-1, rPFR-2, or a mixture of rPFR-1–3 is capable of dramatically reducing acute parasitemia and providing 100% survival of *T. cruzi* infection, indicating that the PFR proteins are protective antigens.

Similar to previous studies with pPFR, this protective immunity is characterized by a highly polarized Th type 1 immune response, demonstrated by the production of high levels IFN- γ and undetectable levels of IL-4 by PFR-specific CD4⁺ T cells in response to PFR antigen in the presence of antigen presenting cells. Our vaccine is not unique in this respect, as other experimental *T. cruzi* vaccines that rely on cell-mediated immunity have also described an association between IFN- γ production and protection [10,29]. This is not surprising given the critical role for IFN- γ in protective immunity against *T. cruzi*. IFN- γ knockout mice and mice that have been depleted of IFN- γ show markedly increased susceptibility to infection [6,8]. Conversely, addition of exogenous IFN- γ during infection has been shown to ameliorate disease [7]. More recently, it has been shown that mice incapable of developing Th2 immune responses exhibit reduced cardiac pathology from *T. cruzi* infection compared to animals that may develop a mixed Th1/Th2 response [30], suggesting that a highly polarized Th1 immune response, such as that induced by rPFR immunization, is preferable.

An important function of IFN- γ in PFR-immune mice is likely activation of infected macrophages to produce nitric oxide and reactive oxygen species known to be effective against *T. cruzi* [11,31]. Interestingly, the selective depletion

of macrophages results in increased cardiac pathology in the rat model, suggesting a direct role for macrophages in protection against Chagas' disease pathology [12]. The role of nitric oxide in human immunity is an item of some contention; however, human macrophages have been shown to kill *T. cruzi* in vitro through NO-dependent mechanisms when activated by IFN- γ and TNF- α , or CC chemokines [32,33]. In the current study, we have demonstrated the ability of recombinant PFR-immune T cells to activate infected macrophages to produce NO, resulting in a reduction of parasite replication in vitro. This mechanism is apparently important in vivo in the mouse model because iNOS deficient mice are not protected from *T. cruzi* infection by immunization with PFR antigen. Furthermore, parasitemias of these animals are dramatically increased, an effect that is also observed in CD4 depleted animals [6], consistent with the hypothesis that CD4⁺ T cell mediated activation of macrophage effector function is largely responsible for the clearance of parasites from the blood of PFR-immune mice infected with *T. cruzi*.

Interestingly not all rPFR proteins were able to provide 100% survival. Despite the fact that immunization with each of the three individual rPFR proteins elicited a highly polarized Th1 immune response to PFR antigens and infected macrophages, immunization with rPFR-3 failed to provide the same level of protection as rPFR-1, rPFR-2, or rPFRmix. This less protective immune response is not correlated with decreased levels of in vitro cytokine production, as IFN- γ and nitrite concentrations were not significantly different ($P < 0.02$, (student's *t*-test) from those seen with immunization conditions that were protective, indicating that there is no gross impairment in either the immunogenicity of this protein or the ability to be processed from intact parasites and presented by antigen presenting cells.

The use of rPFRmix, which contains three rPFR proteins, may have an advantage over immunization with an individual rPFR. One of the challenges to the application of recombinant protein vaccines is the problem of MHC restriction [34]. Due to the reduced amount of protein sequence present in many subunit vaccines, compared to complex antigen preparations or whole organisms, the potential arises that a significant portion of the target population will be unable to present protective T cell epitopes with a vaccine containing a limited antigen repertoire. Each of the rPFR proteins used in the study is able to be processed from live parasites and presented through the MHC class II antigen presentation pathway. While the current study was limited to a single mouse MHC haplotype (H-2^b), the amount of protein sequence contained within these three proteins is large, increasing the probability that this mixture will contain epitopes capable of binding to a variety of MHC class II haplotypes, thus enhancing the vaccine potential of the PFR proteins.

The biology of *T. cruzi* presents several challenges to the design of an effective vaccine. Of particular importance is the extraordinary genetic distance observed between differ-

ent strains, which is estimated to be four times that seen between the human and chimpanzee [22]. This divergence has resulted from the clonal evolution of *T. cruzi*, characterized by a structured population where several discrete genotypes persist with little or no genetic exchange between groups [35], although hybrid genotypes have been identified suggesting that genetic exchange can occur in rare instances [27]. Of particular relevance to vaccine design, mixed infections with different clones are frequently observed in both the insect vector and humans [36,37]. Thus, it is imperative that any vaccine designed to protect against *T. cruzi* be composed of antigens that are highly conserved among the diverse strains of *T. cruzi* known to infect man. In the present study, sequence analysis of the gene encoding PFR-2, a protein that we have identified as a protective antigen, displays high conservation between strains representing roughly 85% of the total genetic diversity of *T. cruzi*. Comparison of the genes encoding trypanothione reductase (TR) and dihydrofolate reductase-thymidylate synthase (DHFR-TS) between similarly diverse strains, reveal nucleotide substitution rates of 1.6 and 1.4%, respectively, between the most highly divergent clades (Clade A, containing the isozyme clone 19 used in this study, versus Clade C, containing (isozyme strain 43) of *T. cruzi* [27]. In the current study of the *par2* gene, comparison of isozyme clones 19 and 43 reveals a 1.1% nucleotide substitution rate, indicating that the conservation of the *par2* gene is similar to that seen in single copy genes encoding proteins with important enzymatic function, such as TR and DHFR-TS. This implies that stringent structural requirements, important for either macromolecular organization or for critical function(s), have likely resulted in the primary sequence of this protein being highly conserved. Consistent with this belief, the *Leishmania mexicana* homologues of PFR-1 and PFR-2 have both been shown to be required for flagellar movement [38]. We have recently identified the dominant PFR-2 CD4⁺ T cell epitopes (haplotype H-2^b) and found the putative amino acid sequence of these regions to be absolutely conserved (unpublished data). Additionally, we have shown that the dominant CD8⁺ T cell epitopes found within the PFR-1 and PFR-3 proteins are also 100% conserved among *T. cruzi* isolates we have analyzed [39]. The high degree of identity observed in PFR-2 and those regions that we have examined in the PFR-1 and PFR-3 proteins suggest that these antigens are highly conserved and have the potential to protect against divergent *T. cruzi* populations.

In summary, the principal findings of this work are: (1) that immunization with recombinant PFR-1, PFR-2, or a equimolar mix of rPFR-1–3 co-adsorbed to alum with rIL-12 is 100% protective against an otherwise lethal challenge with *T. cruzi*; (2) this immune response is characterized by the antigen-specific production of IFN- γ by CD4⁺ T lymphocytes in response to macrophage cells that have been exposed to soluble PFR antigen or infected with *T. cruzi*, similar to that seen by immunization with pPFR antigen; and (3) the sequence of the *par2* gene is highly conserved, suggesting that

it may be an effective antigen against the highly divergent strains of *T. cruzi* known to infect man. We have recently identified the dominant H-2^b T cell epitopes of the PFR-2 protein and no amino acid polymorphisms were observed in the sequences encoding these peptides (unpublished data). These results demonstrate the utility of recombinant PFR proteins as candidate vaccine antigens and represent an important step in the exploration of an experimental vaccine against *T. cruzi* suitable for further development for use in humans.

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