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The genetic immunization with paraflagellar rod protein-2 fused to the HSP70 confers protection against late *Trypanosoma cruzi* infection

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Abstract

The immunological properties of the *Trypanosoma cruzi* paraflagellar rod proteins (PFR2 and PFR3) administered alone as well as fused to HSP70 have been analyzed in mice in the context of genetic immunization. The immunization of mice with the DNA vectors containing the *PFRs* gene or *PFRs-HSP70* fused genes induced high level of IgG_{2a} anti-PFRs. However, only the immunization with the *PFR2-HSP70* fused genes triggers in spleen cells a statistically significant enhancement of expression of IL-12 and IFN-γ and a decrease in the percentage of cells expressing IL-4. Likewise, the *PFR2-HSP70* molecule elicits a statistically significant activation of PFR2 antigen specific CTLs. Immunization with the *PFR2-HSP70* chimeric gene provided a protective response against a *T. cruzi* experimental infection.

Keywords: *Trypanosoma cruzi*; DNA vaccine; Immunomodulator

1. Introduction

Trypanosoma cruzi is the etiologic agent of the Chagas' disease, a multisystemic chronic disorder that affects millions of people throughout the Americas. Approximately 30% of the South and Central America population is living in endemic areas and at risk of acquiring the infection. The overall incidence of the infection has been calculated to be 200,000 new cases a year [1]. Over 50,000 individuals die annually as a consequence of the disease. The infection is a zoonoses transmitted to humans by bloodsucking insects of the family Reduvidae when metacyclic trypomastigotes are deposited on the skin with the insect feces while taking a blood meal. The initiative to interrupt the transmission of Chagas' disease by controlling the insect vector in South American countries recently launched under the auspices of the PAHO/WHO has conducted to a substantial reduction of the number of persons newly infected [2]. However, it is a dangerous error to believe that the Chagas disease has been eliminated since, in fact, the frequencies of *T. cruzi* transmission by blood transfusion have increased in these countries. Moreover, high levels of vector-borne transmissions are still apparent even in areas where transmission has been declared to be zero. Thus, there is a continuing risk of recrudescence of vector-borne transmission [2].

Three clinical stages are known to occur in Chagas' disease: a short acute stage and a long-lasting chronic phase, separated by a long clinically asymptomatic indeterminate phase. Up to 30% of the chronically infected people will develop clinical Chagas' disease, particularly cardiac manifestations. Although the etiology of Chagas disease is controversial, a significant body of evidences has been accumulated supporting the hypothesis that the *T. cruzi* persists

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in host tissues being the stimulus for a chronic inflammatory response that results in tissue damage [3]. Since conventional chemotherapy has low efficacy viable parasites and subsequent chronic local inflammations may be detected during the whole life of the patient, making necessary the search for new alternatives to prevent or ameliorate the disease. Thus, vaccines probably constitute the most appropriate approach for its control. Although the immune response associated to protection is multiple and not completely known, several evidences have indicated that activation of $CD4^+$ and $CD8^+$ T cells and the presence of specific IgG_{2a} antibodies are required for protection [4–6].

For the immunotherapy against Chagas' disease DNA vaccines offer an advantage over other vaccination strategies since they induce cytotoxic T lymphocytes (CTL) and type 1 helper T lymphocytes (Th1) immune response. However, in order to increase the potency DNA vaccine frequently need to be associated with carrier molecules [7]. Thus, in an attempt to enhance the immune response generated by DNA vaccines the co-injection of plasmids encoding the foreign antigen fused to genes encoding also immune stimulatory molecules has been assayed [8].

In the present work we have studied the immunological response generated against PFR2 [9,10] and PFR3 (previously denominated PAR1) [11] *T. cruzi* proteins in BALB/c ($H2K^d$) and transgenic C57BL/6 (A2.1/K^b) mice immunized with DNA plasmids bearing either the *PFR2* and *PFR3* coding genes alone or fused in frame to the *T. cruzi HSP70* coding gene. We selected the gene coding for paraflagellar rod proteins because they are the major components of the paraxial structure of the flagellum which are structures critical for the mobility of the parasite and for its attachment to the host cell [12,13]. It has been recently reported that immunization with recombinant PFR-1, rPFR-2, or an equimolecular mix of the rPFR-1, 2 and 3 co-adsorbed to aluminum and rIL12 provides protective immunity against lethal challenge with *T. cruzi* [9]. Previous reports have shown that immunization of mice with a plasmid containing the KMP11 gene fused the *T. cruzi* HSP70 in the absence of adjuvant elicits an enhanced humoral and cellular KMP11 specific immune responses [14].

2. Materials and methods

2.1. Recombinant proteins, cloning and purification

The PFR2 and PRF3 genes were isolated by PCR amplification of genomic DNA of *T. cruzi* Y strain using oligonucleotides that correspond with the 5′ and 3′ end, respectively, of the mentioned genes. The DNA fragments were cloned into the pQE32 vector (Quiagen) using *Bam*HI and *Xba*I sites at 5′ and at 3′, respectively. The *Bam*HI and *Xba*I sites were generated *ad hoc* in the amplified fragment. The recombinant proteins were expressed in the *E. coli* M15 strain and purified through a Ni^{2+}-NTA-agarose affinity column (Quiagen).

2.2. Plasmid DNA constructs and purification

The *T. cruzi HSP70* gene was isolated from the pQE-70 vector [15] by *Kpn*I and *Ecl*I digestion and subsequently cloned into the *Kpn*I and *Not*I sites of pCMV4 to generate the vector pCMV4-H70. The *PFR2* and *PFR3* coding genes were PCR amplified using *T. cruzi* genomic DNA as template and primers corresponding to the genes 5′ and 3′ends which contained, respectively, the start and stops triplets and restriction sites for *Bam*HI and *Xba*I endonucleases (PFR2-5′(5′CGTAGGATCCAAACAACCAAACAAGCAA3′); PFR2-3′(5′CGTATCTAGACGCGCGACACAGGTTTAACT3′); PFR3-5′(5′CTTGGATCCTTATGACGG3′); PFR3-3′(5′ATTTCCTTCTAGAGTACACAC3′). The 1.8 kb amplified fragments were digested with *Bam*HI and *Xba*I enzymes and cloned into a pCMV4 vector digested with the same enzymes generating pCMV4-*PFR2* and pCMV4-*PFR3* vectors. The *PFR2* and *PFR3* coding genes were amplified by PCR using primers that contained *Kpn*I restriction sites and mapped respectively at the gene 5′ ends bearing the gene start codons and at the gene 3′ ends lacking the stop triplets, PFR2-5′-KpnI (5′GCAAAGGTACCCAATGAGCTACAAGG3′); PFR2-3′-KpnI (5′TTGGTACCGCTGTGTGATCTGCTG3′); PFR3-5′-KpnI (5′CTTGGTACCTTATGACGGTTTA3′); PFR3-3′-KpnI (5′GTAGGTACCACTCCGGCGCTG3′). After *Kpn*I digestion of the amplified DNA fragments they were directly in frame cloned into KpnI-digested pCMV4-H70 vector producing pCMV4-PFR2-H70 and pCMV4-PFR3-H70 constructs. The XL1-Blue *E. coli* strain was used as a host during the cloning experiments and to propagate plasmids. All the transformants were identified by restriction analysis and further confirmed by sequencing. Plasmid DNAs were purified using Endofree Plasmid Gigakit (Qiagen) according to the commercial protocol. DNA concentration was estimated at 260 nm.

2.3. Antibodies

The FITC and PE-anti-CD3ε (145-2C11), FITC-anti-CD4 (GK1.5), PE-anti-IL-2 (S4B6), PE-anti-IL-4 (BVD4-1D11), PE-anti-IFN-γ (XMG1.2), PE-anti-TNF-α (MP6-XT22) and PE-anti-IL12 (C15.6) antibodies were purchased from PharMingen (San Diego, CA), The 2.4G2 hybridoma, producing anti-FcγR mAbs was kindly provided by Dr. C. Terhorst (Beth Israel Deaccones Medical Center, Boston).

2.4. Cell lines and gene expression in eukaryotic cells

Complete medium, Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% FCS (Life Technologies), 2 mM L-glutamine (Gibco BRL), 50 μM 2-mercaptoethanol (Sigma), 100 IU/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) was used for all *in vitro* assays. For the cytotoxicity assays the spleen cells were stimulated with 10 U/ml of recombinant murine (rm) IL-2 (Boehringer Mannheim) added to the complete medium.

Jurkat-A2.1/K^b cells, human stable transfectants cells that express the product of the HLA-A2.1/K^b chimeric gene, were transfected with the pCMV4-*PFR2* and pCMV4-*PFR3* constructs, respectively. Cells were grown in the presence of 400 μg/ml G418 sulfate (Sigma) at 37 °C in a humidified 5% CO_2 incubator.

The expression of the PFRs and PFRs-HSP70 proteins was checked by plasmid transient transfections into COS-7 cells followed by immunoblot of the cell extracts. COS-7 cells transfection was performed by incubating the cells with lipofectin (Gibco BRL) and 2 μg of the mentioned plasmids for 6 h. Then, the transfected cells were lysed in lysis-buffer (0.1 M Tris–HCl pH 8.0, 0.1% Triton X-100), electrophoresed into 10% SDS-PAGE and transferred to PVDF membrane (Millipore) using the Miniprotean system (Bio-Rad). Western blot analysis was carried out according to standard methodology using rabbit antiserum against the PFR2 and PFR3 recombinant proteins. Blots were developed with anti-rabbit IgG alkaline phosphatase conjugate $F(ab')_2$ fragment (Sigma) at 1:5000 dilution. Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were used as substrate.

2.5. Mice and parasites

BALB/c (H2-K^d) mice were obtained from IFFA-CREDO (Saint Germain-sur-l'Abresh, France). C57BL/6-A2.1/K^b transgenic mice [16] were used. The transgenic mice express the product of the HLA-A2.1/K^b chimeric gene (α1 and α2 domains of human HLA-A2.1 and α3, transmembrane and cytoplasmic domains of murine H-2K^b). Mice were held under conventional conditions and females from 6 to 8 weeks of age were used. The *T. cruzi* Y Brazil strain was maintained *in vivo* in BALB/c mice by serial passages of 10^3 bloodstream trypomastigotes. The trypomastigote forms were obtained from blood of infected BALB/c mice. After 3 h of incubation at 37 °C followed by centrifugation the parasites were collected from the sera, washed twice in PBS and used to infect monolayers of LLC-MK2 cells at a proportion of 1:2 cell:parasite. The parasite virulence was slightly attenuate by two successive passes over LLC-MK2 cells. Trypomastigote forms were obtained according to the methodology described by Andrews et al. [17]. The parasite number was determined using a Neubauer chamber. Challenge with 10^3 trypomastigotes of the attenuated parasites produces 25% mortality of infected wild type BALB/c mice the fourth week post-infection. The remaining 75% enter into an infection chronic phase (laboratory data).

2.6. DNA immunization and T. cruzi challenge

Groups of nine BALB/c and of five C57BL/6-A2.1/K^b mice were intramuscularly injected with 100 μg of plasmids: pCMV4 (control), pCMV4-PFR2, and pCMV4-PFR3 (carrying *PFR2* and *PFR3* gene, respectively) and pCMV4-PFR2-H70 and pCMV4-PFR3-H70 (bearing the *PFR2-HSP70* and *PFR3-HSP70* fused genes, respectively) in 100 μl. Sterile 0.9% sodium chloride solution (Sigma) was injected to the control group. Each mouse was immunized four times at 3-week intervals. Groups of five immunized BALB/c and B6-A2/K^b mice were used to determine the induced cellular response (see paragraph 2.8 and 2.9). Groups of four immunized BALB/c mice were challenged with 2.5×10^3 of attenuated trypomastigote forms 10 weeks after the last immunization. The protection assays were carried in two of the three experiments done to analyze the immune response.

2.7. ELISA measurements

PFR2 and PFR3 specific antibodies were measured by ELISA using 0.5 μg/well of each recombinant protein and sera at 1:800 dilution. Affinity isolated goat anti-mouse IgG (Fab specific) (Sigma), IgG_1 (subclass specific) and IgG_{2a} (subclass specific) (Nordic), peroxidase conjugate antibodies, were used at dilution 1:2000, 1:4000 and 1:2000, respectively as secondary antibodies. The optical density (OD) was determined at a wavelength of 492 nm using a Multiskan Plus plate reader (Labsystem). The results are expressed as the mean of triplicate wells minus the background level.

2.8. Intracytoplasmic cytokine analyses and lymphoproliferation assays

Eight weeks after the last immunization the spleens from two mice per group were removed and pooled. A single cell suspension was prepared for each group in complete medium at 2.5×10^6 cells/ml in a 24-well plate and stimulated with 4 μg/ml of the PFR2 and PFR3 recombinant proteins for 48 h. For the last 8 h 10 μg/ml brefeldin A (Sigma) was added to the wells in order to block Golgi protein transport. Aliquots of 0.5×10^6 cells/well were washed with PBS and resuspended in Fc block. After 10 min in ice the cells were fixed with 4% paraformaldehide (PFA) and permeabilized (0.2% saponine in PBS). Afterwards the cells were stained with the anti-cytokine labeled antibodies and washed twice in permeabilizing buffer and resuspended in PBS. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

For the cell proliferation assays the spleen cells from the immunized and control mice were split in triplicate in flat bottom 96-well plates (4×10^5 cells/well) in the presence of 1.25, 2.5, 5 and 10 μg/ml of PFR2 and PFR3 recombinant proteins or 2–10 μg/ml of ConA. Plates were incubated at 37 °C in a CO_2 atmosphere for 72 h. After addition of [methyl-^{3}H] thymidine (0.5 μCi/well) the cells were incubated for another 6 h at 37 °C. The DNA was immobilized in glass fiber filtermats using an Inothech harvester. The ^{3}H incorporation was measured in a Wallac 1450 Microbeta counter device.

2.9. CTL assay

Spleens from three mice per group were removed 9 weeks post-last immunization, homogenized, pooled and grown in complete medium in a humidified 5% CO_2 incubator at 37 °C. Jurkat-A2/K^b cells expressing PFR2 and PFR3 proteins were treated with 50 μg/ml mytomicin C (Sigma) for 1 h at 37 °C and used to weekly stimulate splenocytes. Spleen cell bulk cultures were replenished each 3 days with fresh complete medium supplemented with 10 U/ml rmIL2 (Roche).

The cytotoxic activity was determined on day 6 post-stimulation by a 4 h ^{51}Cr release assay as described previously [15]. Jurkat-A2/K^b cells lanes expressing PFR2 and PFR3 were labeled with 200 μCi of $Na^{51}Cr$ (Amersham) and mixed with effector cells at a effector:target ratios from 2.5:1 to 20:1. The liberated radioactivity was measured in supernatants using a 1450 Microbeta liquid scintillation counter (Wallac). Determinations were conducted in triplicate. Specific lysis was calculated using the following formula: % specific lysis = (experimental release (cpm) − spontaneous release (cpm)/(total release (cpm) − spontaneous release (cpm) × 100. Spontaneous release represents the counts obtained when the target cells where incubated in culture medium without effectors. Total ^{51}Cr release was measured after treatment of target cells with 1 M ClH.

2.10. Histology

Ten weeks post-challenge hearts of mice were fixed in neutral 10% formalin, embedded in paraffin, dehydrated and sectioned by standard procedures. Random samples of pericardium, myocardium and endocardium were assessed for the degree of inflammation using a three-point grading system as described Morris et al. [18]. Four mice per group were analyzed.

2.11. Statistical analysis

The statistical analysis was carried out using the Student's *t*-test performed with the STATISTICA software. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Purification of PFR2 and PFR3 T. cruzi recombinant proteins

Fig. 1A shows the analysis by SDS-PAGE of the purified recombinant proteins after passing the soluble fraction of the total expressed proteins through a Ni^{2+} affinity chromatography column. In the PFR3 and PFR2 labeled lanes an intense stained band both of approximately 70 kDa was observed. The purity was >95% as assessed by Coomassie Blue staining.

3.2. Expression of PFR2 and PFR3 genes and the fused genes PFR2-HSP70 and PFR3-HSP70 in COS-7 cells

To study the modulation of the immune response induced by the HSP70 protein in the context of a genetic immunization, the *PFR3* and *PFR2* genes and the *PFR3-HSP70* and *PFR2-HSP70* fused genes were subcloned into the pCMV4 plasmid. The recombinant plasmids express the PFR3 and PFR2 proteins and the fusion proteins as demonstrated by Western blotting in SDS/PAGE of transfected COS-7 cells. The immunoblots, using polyclonal anti-PFR3 and anti-PFR2 antibodies showed four intensive bands, two of approximately 70 kDa in the PFR3 and PFR2 lanes and two of approximately 140 kDa in the PFR3-H70 and PFR2-H70 lanes, respectively (Fig. 1B). The size of the bands corresponds to the molecular weights expected for the recombinant proteins. The pCMV4 lane, corresponding to the expression

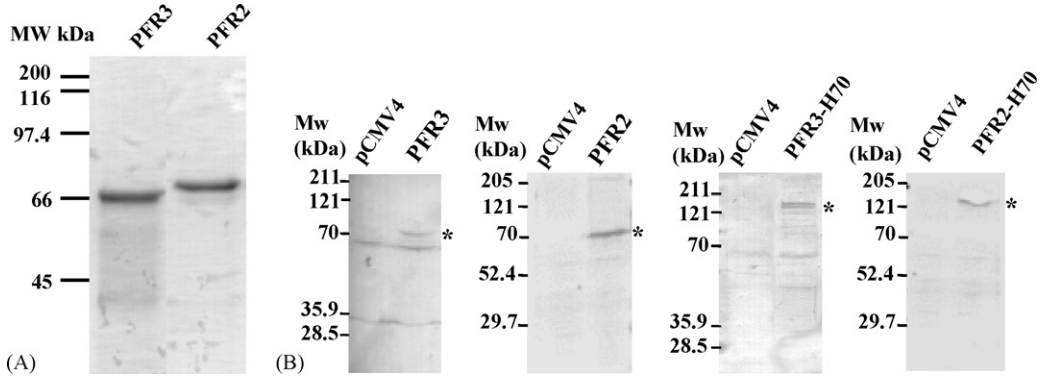

Fig. 1. (A) Purified PFR2 and PFR3 recombinant proteins (lanes PFR2 and PFR3, respectively) were electrophoresed in a 10% SDS-PAGE gel and visualized by coomassie blue staining. MW, molecular weight marker. (B) Western blot analysis of PFRs proteins expression in COS-7 cells transfected with the empty vector (pCMV4), PFRs genes (PFR3 and PFR2) and *PFRs-HSP70* fused genes (PFR3-H70 and PFR2-H70) 60 h post-transfection. The asterisk indicates the location and molecular weight of the expressed proteins.

product of cells transfected with the empty pCMV4 vector did not show any of the two above-mentioned bands.

3.3. Antibody response against PFR2 and PFR3 antigens

We investigated whether mice of different haplotypes ($H2K^d$ and $A2.1/K^b$) elicited a humoral response against PFR3 and PFR2 antigens after inoculation of the vector containing the correspondent PFRs coding genes isolated or fused to the *HSP70* gene. High antibody titers were present 2 weeks after the third dose (Fig. 2A) in the sera of the BALB/c mice immunized with the constructs containing the *PFR2* gene alone or fused to *HSP70* gene. The mice immunized with the plasmid bearing only the *PFR3* gene or fused to the *HSP70* gene also reached significant anti-PFR3 reactivity after the fourth immunization. Enhancement of the humoral immune response against the PFRs antigens occurred in a dose-dependent manner achieving a maximum level 6 weeks post the fourth immunization. Fourteen weeks after the last immunization a positive response against the PFR3 and PFR2 proteins could still be detected. The analysis of the IgG isotypes revealed that immunization with the construct containing the isolated *PFRs* genes or the *PFRs-HSP70* fused genes induced a clear IgG_{2a} antibody bias (Fig. 2B and C). An analogous antibody response was obtained in C57BL/6-$A2.1/K^b$ mice (data not shown).

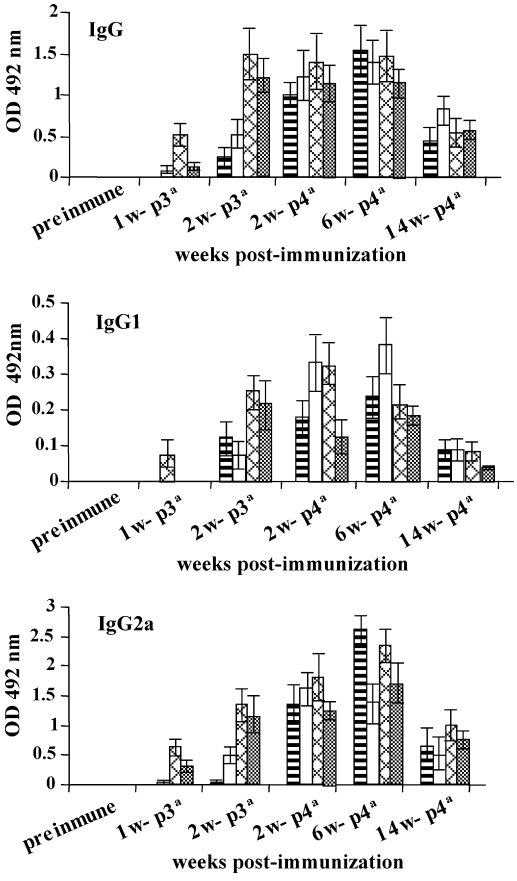

Fig. 2. Anti-PFR2 and anti-PFR3 antibody response (A, IgG; B, IgG_1; C, IgG_{2a}) in sera from BALB/c mice immunized with the pCMV4-PFR3 (▤), pCMV4-PAR3-H70 (□) pCMV4-PFR2 (▨), and pCMV4-PFR2-H70 (▩) plasmids. The bars indicate the optical density (OD) of the pooled sera from six mice. The results represent the mean and standard deviation of three independent immunization experiments.

3.4. Effect of the HSP70 protein on the cell-mediated immune response elicited against the PFR2 and PFR3 antigens

A significant cellular proliferation index was obtained when the splenocytes from the BALB/c and C57BL/6-$A2.1/K^b$ mice immunized with the *PFRs* genes or with the *PFRs-HSP70* fused genes were stimulated with the correspondent PFR proteins. The proliferation index was somewhat lower in mice immunized with the *PFRs* genes linked to the *T. cruzi* HSP70 protein (Fig. 3).

The pattern of cytokine expression of spleen cells from the C57BL/6-$A2.1/K^b$ mice immunized with PFRs coding genes alone and fused to *HSP70* gene are shown in Fig. 4. The percentage of spleen cells expressing IFN-γ and IL12

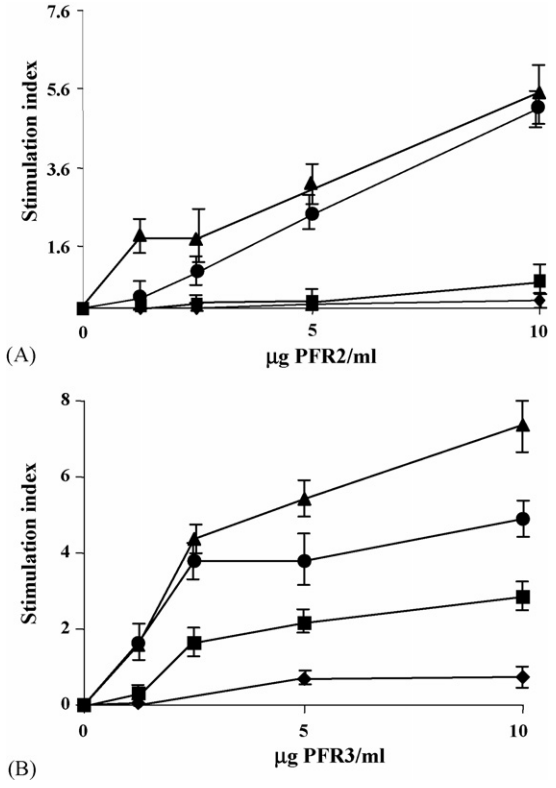

Fig. 3. Lymphoproliferative response to PFR2 (panel A) and PFR3 (panel B) proteins in BALB/c mice immunized with *PFR2* or *PFR3* isolated gene (triangles), *PFR2-H70* or *PFR3-H70* fused genes (circles), pCMV4 empty vector (square) and saline solution (rhombus). Stimulation index was calculated as [arithmetic mean of cpm (stimulated culture) – arithmetic mean of cpm (control culture)/arithmetic mean of cpm]. The results represent the mean and standard deviation of three independent immunization experiments.

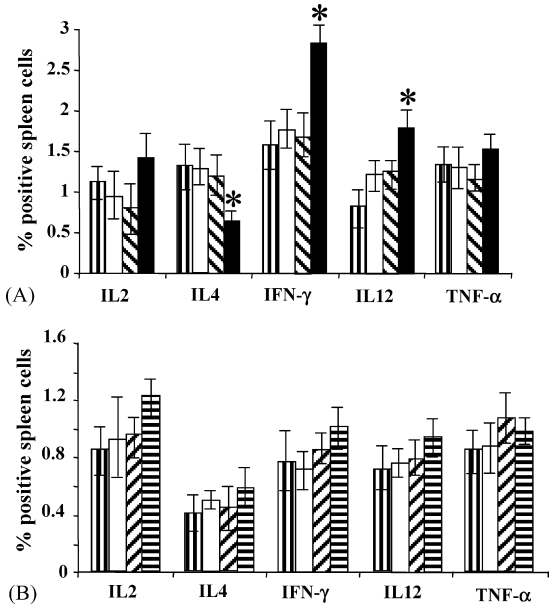

Fig. 4. Intracellular synthesis of cytokines by BALB/c splenocytes stimulated with 4 μg/ml of PFR2 (panel A) and PFR3 (panel B) *Trypanosoma cruzi* recombinant proteins from mice immunized with pCMV4 empty vector (▥), inoculated with saline solution (□), immunized with isolated gene *PFR2* (▩) or *PFR3* (▨) and immunized with *PFR2-H70* (■) or *PFR3-H70* fused genes (▤). The results represent the mean values and the standard deviation of three independent immunization experiments. Asterisks indicate significant differences ($P < 0.05$).

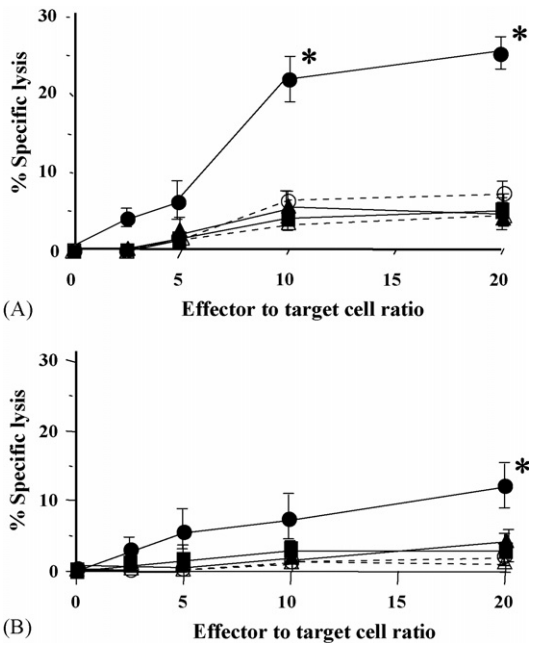

Fig. 5. CTL activity against Jurkat-A2/K^b cells (at various effector/target cell ratios) transfected with *PFR2* gene (panel A) and *PFR3* gene (panel B) in splenocytes from C57BL/6-A2.1/K^b transgenic mice immunized with isolated *PFRs* genes (full triangle), *PFRs-HSP70* fused genes (full circles) and pCMV4 empty vector (square). In both panels, the cellular lysis obtained in control assays using spleen cells from BALB/c H2/k^d immunized mice with the *PFRs* genes (empty triangle and discontinues lane) or the *PFRs-HSP70* fused genes (empty circles and discontinues lane) is shown. Data show the mean and standard deviation of three independent immunization experiments. The asterisks indicate values with significant differences ($P < 0.05$).

was significantly higher ($p < 0.05$) in mice immunized with the *PFR2-HSP70* fused genes than that observed in mice that received saline solution, empty pCMV4 vector or the *PFR2* coding gene alone (Fig. 4A). In the mice immunized with the *PFR2-HSP70* fused genes a lower percentage of cells expressing IL4 was also observed relative to that observed in mice that received saline solution, empty pCMV4 vector or the *PFR2* coding gene alone. In contrast, an increase in the number of cells expressing IL-4 was observed in mice immunized with the *PFR3-HSP70* fused genes, although it was not statistically significant (Fig. 4B). Thus, immunization with the *PFR2-HSP70* genes enhances a type 1 cytokine expression pattern. The pattern of cytokine expression by spleen cells from BALB/c mice was similar to that observed in C57BL/6-A2.1/K^b mice.

The presence of CTLs was determined in C57BL/6-A2.1/K^b transgenic mice immunized with DNA constructs bearing the *PFR2* and *PFR3* genes and with constructs containing the genes fused to the *HSP70* gene. The results are shown in Fig. 5. The presence of CTLs against the Jurkat-A2/K^b target cells overexpressing the PFR2 and PFR3 proteins was only observed in mice immunized with the *PFRs-HSP70* fused genes and not in those immunized with the *PFRs* genes alone. In addition, cellular lysis of Jurkat-A2/K^b target cells was not observed in control assays (spleen cells from BALB/c H2/k^d immunized mice). The highest level of cellular lysis (27%) was detected in spleen cells from *PFR2-HSP70* immunized mice (Fig. 5B).

3.5. Protection against T. cruzi infection

In order to determine whether the genetic immunization with the *PFR2* and *PFR3* genes alone or fused to *HSP70* gene provides protection against late *T. cruzi* infection, BALB/c immunized mice were infected with the attenuated trypomastigote forms described in Section 2 10 weeks after the last immunization. As indicated above using this challenge procedure 25% of infected wild type BALB/c mice die the fourth week post-infection (laboratory data). Fig. 6 and Table 1 show the results of the protection assays. Two out of eight control

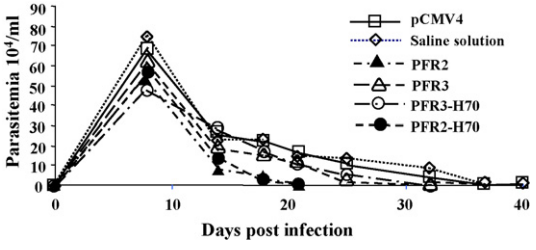

Fig. 6. Circulating levels of parasites for immunized mice after challenge with *T. cruzi*. The level of parasites in the bloodstream was determined individually in four mice per group and data show the mean of values obtained. The results of a single representative experiment, out of two independent ones, are shown.

Table 1
Inflammation degree in cardiac tissue from infected mice[a]

Experiment A				Experiment B			
Mice	Pericardium area	Myocardium area	Endocardium area	Mice	Pericardium area	Myocardium area	Endocardium area
Saline sol	+++	+++	+++	Saline sol	+++	+++	+++
Saline sol	++	+	−	Saline sol	Dead day 27 post-challenger		
Saline sol	++	++	−	Saline sol	++	++	−
Saline sol	Dead day 26 post-challenger			Saline sol	++	++	−
pCMV4	+	++	+++	pCMV4	+	+	+
pCMV4	+	+	++	pCMV4	+	++	+
pCMV4	+	−	++	pCMV4	+	−	−
pCMV4	Dead day 30 post-challenger			pCMV4	+	++	++
pPFR2	+	+	−	pPFR2	+	−	−
pPFR2	+	+	−	pPFR2	+	+	−
pPFR2	+	+	−	pPFR2	+	−	−
pPFR2	+	−	−	pPFR2	++	−	−
pPFR3	+	+++	−	pPFR3	++	++	−
pPFR3	++	++	−	pPFR3	++	+	+
pPFR3	+	−	−	pPFR3	−	−	−
pPFR3	+	++	−	pPFR3	+	+	+
pPFR2-H70	−	−	−	pPFR2-H70	−	−	−
pPFR2-H70	−	−	−	pPFR2-H70	+	−	−
pPFR2-H70	−	−	−	pPFR2-H70	+	−	−
pPFR2-H70	−	−	−	pPFR2-H70	−	−	−
pPFR3-H70	−	−	−	pPFR3-H70	++	++	−
pPFR3-H70	++	++	−	pPFR3-H70	−	−	−
pPFR3-H70	+	+	+	pPFR3-H70	++	++	++
pPFR3-H70	++	++	−	pPFR3-H70	+	−	−
Uninfected	−	−	−	Uninfected	−	−	−
Uninfected	−	−	−	Uninfected	−	−	−

[a]Inflammation degree are expressed using a three-point grading system: (−) absent; (+) two to five inflammatory foci; (++) multiple inflammatory foci that occupied 1/4 field at 40×; (+++) inflammatory foci that occupied one or more fields of 40×.

mice that received saline solution died at days 26 and 27 post-infection. One mouse of the group of eight mice inoculated with pCMV4 died at day 30 post-infection. In contrast, in mice immunized with the *PFR2* gene or the *PFR2-HSP70* construct circulating parasites could be detected only the first 3 weeks post-infection. Parasites could not be detected afterwards. Mice immunized with the *PFR3* gene or the *PFR3-HSP70* construct were able to control infection at week fourth post-infection. Controls mice controlled infection at week fifth post-infection. In order to know whether timely differences in controlling infection has a counterpart in disease we performed a histological examination of the hearts of the infected mice. A significant reduction of inflammatory foci in all *PFR2* immunized mice relative to the infected control mice inoculated with the saline solution or the empty vector was observed. Moreover, in two out of the eight mice immunized with the *PFR3-HSP70* fused genes no inflammatory foci could be detected. Interestingly, in six out of the eight mice immunized with the plasmid containing the *PFR2-HSP70* fused gene no inflammatory foci could be detected. Rather they presented a cardiac morphology compatible with a healthy tissue. The other two mice of this group presented few and slight inflammatory foci. A representative image of the tissue of one mouse per group is shown in Fig. 7.

4. Discussion

Vaccination is the most efficacious medical treatment for preventing mortality and morbidity due to infectious agents. While no vaccine is yet available for the Chagas' disease, some antigens with potential use in immunotherapy have been identified. It is generally accepted that DNA vaccines, though dependent on the type of antigen that they carry and the immunization route, induce a T type cellular response ($CD4^+$ and $CD8^+$) and a low but predominant, preferably of the IgG_{2a} type, antibody response [19]. It has been suggested that this immunological response may be necessary to induce protection against Chagas' disease [4,5]. Genetic vaccines may, thus, be an excellent tool for inducing effective protection against *T. cruzi* infection.

In this report we analyze the potential of the PFR2 and PFR3 paraflagellar rod proteins as candidates for vaccine development against the *T. cruzi* infection chronic-phase and the adjuvant capacity of the *T. cruzi* HSP70 protein in the context of genetic fusions. Previous results, using KMP11 as antigen, showed that HSP70 from *T. cruzi* behaves as an immunomodulator capable of directing the immune response towards a Th1 type and of inducing antigen-specific CTLs [14]. Recent reports have shown that PFR antigens induce protection against the acute-phase of *T. cruzi* infection [9].

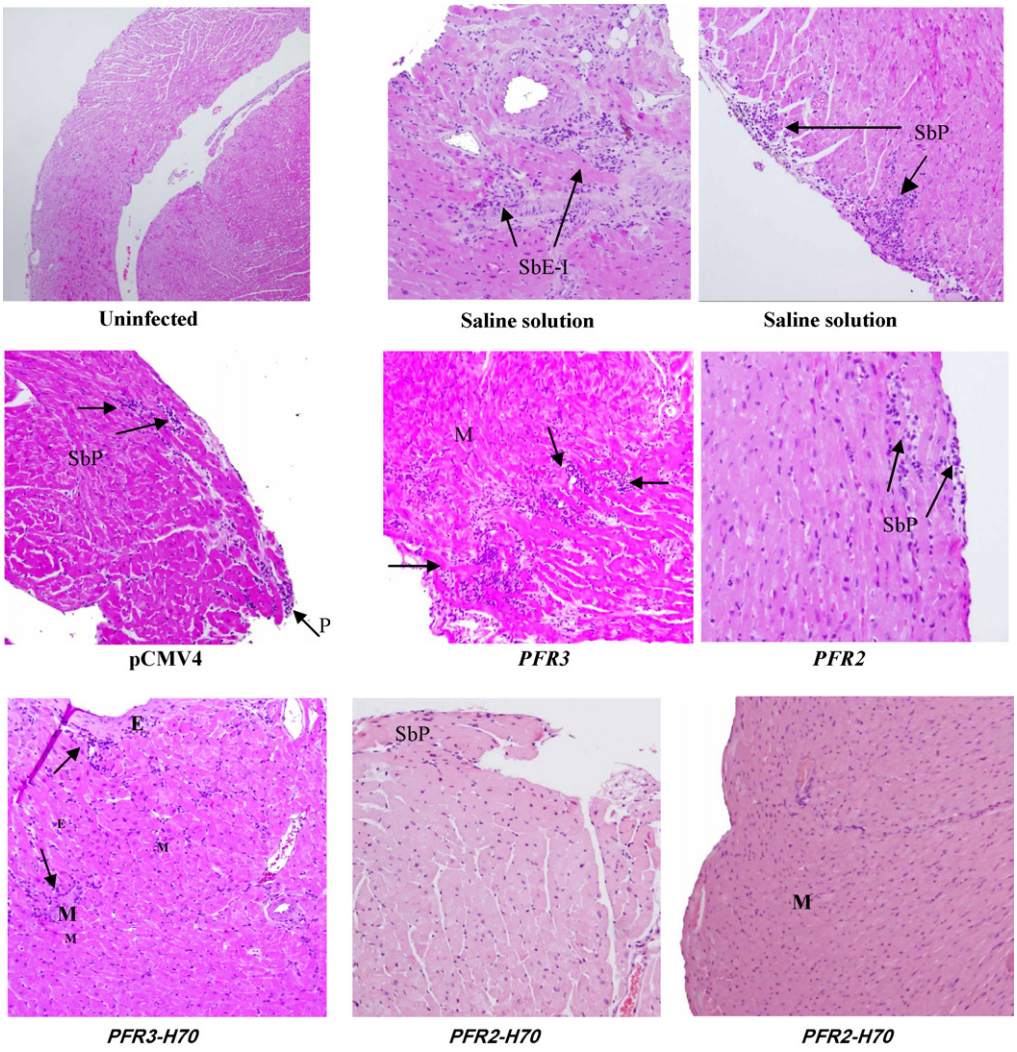

Fig. 7. Heart sections of mice immunized with isolated *PFRs* genes and *PFRs-HSP70* fused genes and infected with *T. cruzi*. The tissue sections were stained with haematoxylin–eosin (H&E) and observed with an optical microscope (10× to 20×). The inoculated molecule is indicated below each panel. A representative photograph of one mouse per group is shown.

The data reported in this study show that the humoral response induced by plasmids containing *PFR* genes, whether alone or fused to HSP70, is independent of haplotype and particularly high compared with that induced by other *T. cruzi* antigens, such as KMP11 protein [14], complement regulatory protein [20], ASPs amastigote surface proteins [21] or trans-sialidase [22]. Plasmids containing the *PFR2* gene proved to be more immunogenic than those that carry the *PFR3* gene. The IgG response appears earlier in time in the BALB/c than in the C57BL/6 strain. Remarkably, the antibodies generated are long lasting, particularly in mice immunized with *PFRs-HSP70* fused genes. Furthermore, the isotype of the anti-PFR antibodies elicited are mainly of IgG_{2a} type.

In addition to inducing a humoral response the immunization with both the *PFR2* and *PFR3* genes triggers significant cellular proliferation, in a haplotype independent manner. The cellular stimulation indexes recorded in cases where HSP70-containing plasmids were used as immunogens were slightly lower than in cases in which the genes were administered non fused to HSP70. This situation has likewise been also observed when mice were immunized with the *T. cruzi KMP11* gene [23] fused to *HSP70* [14] as well as when the L14 ribosomal protein from *Leishmania braziliensis* [24] was administered to mice fused to HSP70.

In infection caused by *T. cruzi* T cells are reported to contribute to the elimination of the parasite by secretion of Th1 type cytokines such as IFN-γ, TNF-α or IL12 [25–27]. Thus, it has been postulated that the induction of a strong Th1 type response is crucial for the control of infection and that inhibition or even delay in the production of Th1 type cytokines will, therefore, induce higher acute-phase mortality rates [28,29]. Our data showed that the expression level of IFN-γ and IL-12 was higher in splenocytes of mice immunized with the *PFR2* gene fused to *HSP70* than in those immunized with the *PFR2* and *PFR3* genes alone. The IL-4, a Th2

type cytokine, which has been shown to be related to higher disease susceptibility [29,30], produced by splenocytes from mice immunized with the *PFR* genes alone was higher than that produced by splenocytes from mice immunized with the fused genes in agreement with the observation that the HSP70 protein, in association with antigens, enhances an antigen-specific response of the Th1 type [14]. In genetic vaccines, the modulator effect of the HSP70-induced Th1 response is most likely heightened by the parallel adjuvant effect exerted by the CpG motifs present in the plasmids used as gene vectors. Such unmethylated sequences are capable of inducing activation of antigen-presenting cells and secretion of IL-12 [31], factors that are essential to trigger a Th1 type response. Thus, the immunostimulatory effect of these sequences can account for the higher values of Th1 type cytokine expression observed in mice inoculated with the control plasmid versus controls inoculated with saline solution.

The presence of specific $CD8^+$ T lymphocytes against specific antigens of the parasite seems to be essential for control of *T. cruzi* infection [6]. In spleen cells from mice that survived to *T. cruzi* infection $CD8^+$ T cells specific against epitopes contained in the TSA-1 and ASP-1 and ASP-2 [32,33] and KMP11 [14] antigens have been described. Our data indicate that also the genetic vaccination with the *PFRs-HSP70* fused genes leads to the induction of *PFR*-proteins-specific $CD8^+$ cytotoxic T lymphocytes. PFR-proteins-specific $CD8^+$ cytotoxic T cells were not present in mice immunized with the *PFR*s genes alone. The protection efficiency of the fused genes may also be explained by the fact that the percentage of cellular lysis induced by the cells of mice immunized with the PFR2-HSP70 plasmid on Jurkat-A2-K^b cells overexpressing the PFR2 protein was about 27%.

There is strong evidence that, after intramuscular immunization with recombinant plasmid, the encoded protein is expressed in myocytes, released into the medium and captured by the APCs for processing and presentation [7,19]. The observation that *T. cruzi* HSP70, is able to induce maturation of murine dendritic cells [34] would explain why antigen-specific CD8 cytotoxic T lymphocytes are only elicited in response to inoculation with the antigen fused to HSP70. Indeed, the existence of HSP receptors in APCs has been described [35]. In all likelihood, once the PFR-HSP70 fusion protein is captured by the receptors present in APCs, the presence of HSP70 would enhance its processing through the class I pathway, as it has been described for receptor-mediated uptake of other antigen-HSP70 complexes [36]. In the context of genetic vaccination, similar results have been reported when the KMP11 antigen is fused to HSP70 [14].

In the present paper the protective capacity against chronic *T. cruzi* infection was analyzed using attenuated forms of the strain, inoculated 10 weeks after the fourth immunization. While non attenuated strains are lethal for BALB/c mice [29], 75% of mice receiving the attenuated strain survive to the challenge and entered into a chronic phase, characterized by inflammation of the cardiac tissue. A number of authors have used cardiac tissue to assess the damage caused by the parasite in murine and canine models [37,38] as well as in human patients [39]. The data presented show that immunization with the plasmids containing *PFRs* genes reduces cardiac damage and the number of lesions associated with the chronic stage of the disease. Interestingly, the hearts of 75% of the mice immunized with the *PFR2-HSP70* fusion gene displayed lesion-free cardiac tissues. The remaining 25% of the mice from this group presented few and slight inflammatory foci. In our view the data presented does not only highlight the protective capacity of genetic vaccines containing the *PFR2* gene fused to the HSP70-protein encoding gene against chronic *T. cruzi* infection but support the potential use of the HSP70 molecule in genetic vaccines, especially when induction of a Th1 type cellular response with CTL activation and IgG_{2a} antibody induction is needed.

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References

[1] World Health Organization. Control of Chagas' Disease. WHO Technical Report Series. 2002;905:82–3.
[2] Dias JC, Silveira AC, Schofield CJ. The impact of Chagas disease control in Latin America: a review. Mem Inst Oswaldo Cruz 2002;97:603–12.
[3] Tarleton RL, Zhang L. Chagas disease etiology: autoimmunity or parasite persistence? Parasitol Today 1999;15:94–9.
[4] Brodskyn CI, Silva AM, Takehara HA, Mota I. IgG subclasses responsible for immune clearance in mice infected with *Trypanosoma cruzi*. Immunol Cell Biol 1989;67:343–8.
[5] Tarleton RL, Grusby MJ, Postan M, Glimcher LH. *Trypanosoma cruzi* infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. Int Immunol 1996;8:13.
[6] Martin D, Tarleton RL. Generation, specificity, and function of CD8 T cells in *Trypanosoma cruzi* infection. Immunol Rev 2004;201:304–7.
[7] Liu MA. DNA vaccines: a review. J Int Med 2003;253:402–10.
[8] Flo J, Tisminetzky S, Baralle F. Modulation of the immune response to DNA vaccine by co-delivery of costimulatory molecules. Immunology 2000;100:259–67.
[9] Luhrs KA, Fouts DL, Manning JE. Immunization with recombinant paraflagellar rod protein induces protective immunity against *Trypanosoma cruzi* infection. Vaccine 2003;21:3058–69.
[10] Gadelha C, LeBowitz JH, Manning J, Seebeck T, Gull K. Relationships between the major kinetoplastid paraflagellar rod proteins: a consolidating nomenclature. Mol Biochem Parasitol 2004;136:113–5.

[11] Michailowsky V, Luhrs K, Rocha MO, Fouts D, Gazzinelli RT, Manning JE. Humoral and cellular immune responses to *Trypanosoma cruzi*-derived paraflagellar rod proteins in patients with Chagas' disease. Infect Immun 2003;71:3165–71.
[12] de Souza W, Souto-Padron T. The paraxial structure of the flagellum of Trypanosomatidae. J Parasitol 1980;66:229–36.
[13] Saborio JL, Manuel Hernandez J, Narayanswami S, Wrightsman R, Palmer E, Manning J. Isolation and characterization of paraflagellar proteins from *Trypanosoma cruzi*. J Biol Chem 1989;264:4071–5.
[14] Planelles L, Thomas MC, Alonso C, López MC. DNA immunization with the *T. cruzi* HSP70 fused to KMP11 protein elicits a cytotoxic and humoral immune response against the antigen and leads to protection. Infect Immun 2001;69:6558–63.
[15] Marañón C, Planelles L, Alonso C, López MC. HSP70 from *Trypanosoma cruzi* is endowed with specific cell proliferation potential leading to apoptosis. Int Immunol 2000;12:1685–93.
[16] Vitiello A, Marchestni D, Furze J, Sherman LA, Chesnut RW. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human–mouse class I major histocompatibility complex. J Exp Med 1991;173:1007–15.
[17] Andrews NW, Hong KS, Robbins ES, Nussenzweig V. Stage-specific surface antigens expressed during the morphogenesis of vertebrate forms of *Trypanosoma cruzi*. Exp Parasitol 1987;64:474–84.
[18] Morris SA, Weiss LM, Factor S, Bilezikian JP, Tanowitz H, Wittner M. Verapamil ameliorates clinical, pathologic and biochemical manifestations of experimental chagasic cardiomyopathy in mice. J Am Coll Cardiol 1989;4:782–9.
[19] Srivastava IK, Liu MA. Gene vaccines. Ann Int Med 2003;138: 550–9.
[20] Sepulveda P, Hontebeyrie M, Liegeard P, Mascilli A, Norris KA. DNA-Based immunization with *Trypanosoma cruzi* complement regulatory protein elicits complement lytic antibodies and confers protection against *Trypanosoma cruzi* infection. Infect Immun 2001;68:4986–91.
[21] Garg N, Tarleton RL. Genetic immunization elicits antigen-specific protective immune responses and decreases disease severity in *Trypanosoma cruzi* infection. Infect Immun 2002;70:5547–55.
[22] Costa F, Franchin G, Pereira-Chioccola VL, Ribeirao M, Schenkman S, Rodrigues MM. Immunization with a plasmid DNA containing the gene of trans-sialidase reduces *Trypanosoma cruzi* infection in mice. Vaccine 1998;16:768–74.
[23] Thomas MC, García-Pérez JL, Alonso C, López MC. Molecular characterization of KMP11 from *Trypanosoma cruzi.* A cytoskeleton associated protein regulated at a translational level. DNA Cell Biol 2000;19:47–57.
[24] Gonzalez AC, Thomas MC, Martinez-Carretero E, Carmelo E, López MC, Valladares B. Molecular and immunological characterization of L14 ribosomal protein from *Leishmania braziliensis*. Parasitol 2004;128:139–47.
[25] Aliberti JC, Cardoso MA, Martins GA, Gazzinelli RT, Vieira LQ, Silva JS. Interleukin-12 mediates resistance to *Trypanosoma cruzi* in mice is produced by murine macrophages in response to live trypomastigotes. Infect Immun 1996;64:1961–7.
[26] Abrahamsohn IA, Coffman RL. *Trypanosoma cruzi*: IL-10, TNF, IFN-gamma, and IL12 regulate innate and acquired immunity to infection. Exp Parasitol 1996;84:231–44.
[27] Reed SG. Immunology of *Trypanosoma cruzi* infections. Chem Immunol 1997;114:103–10.
[28] Silva JS, Vespa GN, Cardoso MA, Aliberti JC, Cunha FQ. Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. Infect Immun 1995;63:4862–7.
[29] Planelles L, Thomas MC, Marañón C, Morell M, López MC. Differential CD86 and CD40 co-stimulatory molecules and cytokine expression pattern induced by *Trypanosoma cruzi* in APCs from resistant or susceptible mice. Clin Exp Immunol 2003;131:41–7.
[30] Petray PB, Rottenber ME, Bertot G, Diaz A, Orn A, Grinstein S. Effect of anti-gamma-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of *Trypanosoma cruzi*. Immunol Lett 1993;35:77–80.
[31] Mender P, Thomas R. Immunology of DNA vaccines: CpG motifs and antigen presentation. Inflamm Res 2000;49:199–205.
[32] Wizel B, Garg N, Tarleton RL. Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* infection. Infect Immun 1998;66:5073–81.
[33] Low HP, Tarleton RL. Molecular cloning of the gene encoding the 83 kDa amastigote surface protein its identification as a member of the *Trypanosoma cruzi* sialidase superfamily. Mol Biochem Parasitol 1997;88:137–49.
[34] Planelles L, Thomas MC, Pulgar M, Marañón C, Grabbe S, López MC. The *T. cruzi* heat shock protein-70 kDa alone or fused to parasite KMP11 antigen induces functional maturation of mouse dendritic cells. Immunol Cell Biol 2002;80:241–7.
[35] Binder RJ, Han DK, Srivastava PK. CD91: a receptor for heat shock protein gp96. Nat Immunol 2000;1:151–5.
[36] Castellino F, Boucher PE, Eichelberg K, Mayhew M, Rothman JE, Houghton AN, et al. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J Exp Med 2000;191:1957–64.
[37] Andrade ZA, Andrade SG, Sadigursky M, Wenthold Jr RJ, Hilbert SL, Ferrans VJ. The indeterminate phase of Chagas' disease: ultrastructural characterization of cardiac changes in the canine model. Am J Trop Med Hyg 1997;57:328–36.
[38] Kumar S, Tarleton RL. Antigen-specific Th1 but not Th2 cells provide protection from lethal *Trypanosoma cruzi* infection in mice. J Immunol 2001;166:4596–603.
[39] Palomino SA, Aiello VD. Higuchi ML Systematic mapping of hearts from chronic chagasic patients: the association between the occurrence of histopathological lesions and *Trypanosoma cruzi* antigens. Ann Trop Med Parasitol 2000;94:571–9.