Vaccine 27 (2009) 1323–1332

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Identification of novel vaccine candidates for Chagas' disease by immunization with sequential fractions of a trypomastigote cDNA expression library

Valeria Tekiel[a,*,1], Catalina D. Alba-Soto[b,1], Stella M. González Cappa[b], Miriam Postan[c], Daniel O. Sánchez[a]

[a] Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín, CONICET, Buenos Aires, Argentina
[b] Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[c] Instituto Nacional de Parasitología Dr. M. Fatala Chaben, ANLIS-Malbrán, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 16 September 2008
Received in revised form
23 December 2008
Accepted 28 December 2008
Available online 20 January 2009

Keywords:
Trypanosoma cruzi
Vaccine
Trypomastigote antigens
Expression library immunization

ABSTRACT

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease, a major chronic infection in Latin America. Currently, there are neither effective drugs nor vaccines for the treatment or prevention of the disease. Several *T. cruzi* surface antigens are being tested as vaccines but none of them proved to be completely protective, probably because they represent only a limited repertoire of all the possible *T. cruzi* target molecules. Taking into account that the trypomastigote stage of the parasite must express genes that allow the parasite to disseminate into the tissues and invade cells, we reasoned that genes preferentially expressed in trypomastigotes represent potential targets for immunization. Here we screened an epimastigote-subtracted trypomastigote cDNA expression library by genetic immunization, in order to find new vaccine candidates for Chagas' disease. After two rounds of immunization and challenge with trypomastigotes, this approach led to the identification of a pool of 28 gene fragments that improved *in vivo* protection. Sequence analysis of these putative candidates revealed that 19 out of 28 (67.85%) of the genes were hypothetical proteins or unannotated *T. cruzi* open reading frames, which certainly would not have been identified by other methods of vaccine discovery.

1. Introduction

Chagas' disease currently affects ~18 million people and more than 100 million people in Latin America are potentially exposed to its causative agent, the protozoan *Trypanosoma cruzi*. Available treatments are limited to chemotherapies that exhibit high toxicity and display poor efficacy at the chronic stage of the disease [1]. Up to date, there is no effective vaccine against *T. cruzi* [2].

Several parasite antigens have been tested for their utility in controlling *T. cruzi* infection and/or disease (cruzipain, *trans*-sialidase (TS), amastigote surface protein-2, trypomastigote surface antigen-1, or mucins, among others) [3–10]. These antigens were chosen to be tested as vaccines mainly for their localization on the parasite surface and the induction of strong cellular and humoral responses against them during natural infection. However, they represent a limited repertoire of all the possible *T. cruzi* target molecules. Moreover, most of these antigens belong to large gene families whose size and variability are one of the parasite's mechanisms to evade the host immune system [11]. Accordingly, the magnitude of the immune response elicited solely by these antigens might be insufficient to control the infection. Previous experiences on vaccine discovery to others pathogenes that must be targeted by multiple immune responses as *Plasmodium* sp. indicate that protective immune responses could be better achieved by multivalent vaccines able to emulating the complexity of immune responses attained during natural infection, but given in formulation capable of increasing the immunogenicity of antigens [12,13]. Thus, additional target molecules remain to be identified for vaccine development against Chagas disease.

T. cruzi has multiple developmental stages cycling between a reduviid insect vector and a mammalian host. The replicative epimastigote and the non-replicative metacyclic trypomastigote are the parasite forms present in the vector; the latter is the infective stage when *T. cruzi* is transmitted in endemic areas. Focusing on the mammalian host, the circulating trypomastigote stage is a hallmark of the acute infection, when found in high numbers in the blood. The trypomastigote is responsible for the invasion of nucleate cells, where the parasite differentiates to amastigote, the stage that multiplies in the cytoplasm. Then, amastigotes differentiate to trypomastigotes, the infected host cell bursts, the parasites reach

* Corresponding author at: Instituto de Investigaciones Biotecnológicas, IIB-UNSAM, Av. Gral Paz 5445, Parque Tecnológico Miguelete, Edificios 19 and 24, B1650KNA, San Martín, Buenos Aires, Argentina. Tel.: +54 11 4580 7257; fax: +54 11 4580 7257.
E-mail addresses: valet@iib.unsam.edu.ar, vtekiel@gmail.com (V. Tekiel).
[1] These two authors contributed equally to this work.

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.12.056

the bloodstream and disseminate the infection to diversal host tissues. Trypomastigotes are also the infective stage in congenital and transfusional modes of transmission, situations that take place even outside endemic areas. Therefore, this parasitic stage represents a promising target for the control of Chagas' disease.

Recently, with the purpose of generating information about genes preferentially expressed in trypomastigotes but not in epimastigotes, we have constructed a trypomastigote cDNA library subtracted with epimastigote cDNA (library TcT-E). We identified ~180 new *T. cruzi* expressed sequence tags, by means of automatic sequencing and database mining of a small portion of the TcT-E library, and confirmed experimentally that those clones were preferentially represented in the trypomastigote stage (Tekiel et al., unpublished results; http://genoma.unsam.edu.ar/projects/tct-e/tct-e.c.html). We consider that the product of some of these genes could be new potential vaccine candidates for Chagas' disease, but the lack of knowledge of their function makes it impossible to rationally select those suitable for vaccine trials.

The expression library immunization (ELI) is an attractive strategy for the identification of novel vaccine candidates, and, unlike traditional and *in silico*-based approaches, it makes no assumptions and requires no prior knowledge of antigenic targets [14]. Basically, expression libraries are used for genetic immunization and, after challenge with the pathogen, the host's immune system selects the immunoprotective clones. The method implies the fractioning of the library into smaller sub-libraries or pools, that are sequentially screened *in vivo* until a group or even single protective genes are identified [15–21]. ELI has the potential for screening the complete genome of a pathogen but, when the complexity of genomes is high, the coding density is low or when stage-specific vaccines are required – as is the case of *T. cruzi* – a modification of ELI starting from cDNA is more convenient [22].

We therefore used the subtractive trypomastigote cDNA library as starting point to allow the immune system of the murine host model of Chagas' disease to select protective antigens. After two rounds of genetic immunization and challenge, this approach led to the identification of novel – otherwise unpredictable – *T. cruzi* vaccine candidates.

2. Materials and methods

2.1. Animals, parasites and antigens

C3H/HeN^{k} mice were bred and housed in our animal facility (Department of Microbiology, Parasitology and Immunology, School of Medicine, University of Buenos Aires). All procedures requiring animals were performed in agreement with institutional guidelines.

The RA strain of *T. cruzi* was kept *in vivo* by weekly intraperitoneal passages of 10^5 bloodstream forms through mice [23].

For parasite lysate preparation, trypomastigotes from the CL Brener strain were obtained from supernatants of *in vitro* infected Vero cells. Cultured trypomastigotes (with less than 3% amastigote forms) were harvested, washed in PBS and subjected to five freeze–thawing cycles and sonication (10 cycles of 30 s at 40 Hz on ice). Protein concentration was determined by the Bradford method and the lysate was stored at −80 °C until use.

2.2. Epimastigote-subtracted trypomastigote cDNA library (subtractive TcT-E library) construction

The subtractive TcT-E library was constructed by using the PCR-Select cDNA Subtraction kit following the selective subtractive hybridization protocol provided by the manufacturers (CLONTECH, USA) [24]. First-strand cDNA synthesis was performed with 2 μg of polyA+ of each *T. cruzi* stage (trypomastigote and epimastigote, CL Brener strain), oligo dT primer with a 5′ RsaI site and Superscript II reverse transcriptase (Gibco-BRL, USA). Second-strand cDNA synthesis was performed with T4 DNA polymerase. After RsaI digestion of double-stranded cDNA, two different sets of adaptors were ligated to the tester cDNA (trypomastigotes) but not to the driver cDNA (epimastigotes). Two rounds of subtractive hybridization in the presence of an excess of epimastigote cDNA were performed, leading to the enrichment of differentially expressed sequences in the trypomastigote cDNA population. This subtracted sample was the template for further suppression PCR amplification performed with adaptor-specific primers. The subtraction efficiency was verified by monitoring the PCR amplification of *T. cruzi* histone 2A transcript in subtracted and unsubtracted samples (H2_3′: tcttggacgccttcttcgct; H2_5′: gtgatgccgagcctgaacaa) and by reverse Northern blot. PCR products enriched for trypomastigote differentially expressed sequences – higher than 100 bp – were cloned into the pGEM-T Easy vector (Promega, USA).

2.3. Construction of the expression library ELI_TcT-E

2.3.1. Vector construction

Eukaryotic expression vectors pCI30, pCI31 and pCI32 constructed as described by Moore et al. (2002) were a kind gift of Dr. D. Comerci and Dr. J.E. Ugalde [25]. For the present project, the NotI site of pCI30-32 was removed and a new NotI site added next to the BamHI site. Each of the three new vectors – pCI-Not30, pCI-Not31, pCI-Not32 – has the NotI cloning site in a different reading frame. This site was used for the subcloning of inserts released from the subtractive TcT-E library using the same restriction enzyme.

2.3.2. Preparation of inserts

Plasmid DNA from the original TcT-E library, cloned in pGEM-T Easy vector, was purified by the alkaline lysis method and digested with NotI. Released inserts were gel-purified according to their size (<400 bp, ~500 bp, 500–700 bp and >700 bp) using QiaexII (QIAGEN Inc., GmbH, Germany) and separately cloned into the pCI-Not30, pCI-Not31 and pCI-Not32 vectors. Each of the 12 ligations (pCI-Not30–32 ligated with each of the four different size-range inserts) was used to transform *E. coli* DH5α by standard procedures [26].

2.4. Selection of polyHis-positive clones

Colonies lifts were taken from the transformation plates onto nitrocellulose filters. To induce the expression of polyHis recombinant peptides, the filters were placed onto LB agar containing 2 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and incubated for 4 h. Then, filters were treated with denaturing and neutralization solutions, blocked with 3% BSA in TBS and incubated with an anti-polyHis monoclonal antibody (Penta-His Antibody, 1/1000 dilution, Qiagen). The detection of expressing clones was carried out with a secondary antibody coupled to peroxidase and developed with a staining solution containing 4-chloro-1-naphthol and H_2O_2. Filters were aligned with the original transformation plates and the polyHis-positive clones representing the ELI_TcT-E library were picked into 384 plates in *Hogness Modified Freezing Medium* (*HMFM*). After overnight growth at 37 °C, replicates were made for long-term storage at −80 °C.

2.5. Expression library arrangement and deconvultion

A first master plate containing the productive clones for each cloning vector (pCI-Not30, 31 and 32) was made in 384-well plates to further easily reorder the clones. From these initial master plates, we subsequently picked the clones to construct the different sub-libraries used for the immunization assays. The

first sub-library arrangement was made by joining columns 1–8 (sub-library pELI_Tc1), 9–16 (sub-library pELI_Tc2) and 17–24 (sub-library pELI_Tc3) of the original master plates, into 384-well plates. For the second deconvultion, clones were picked from the library pELI_Tc2 master plate in a way that assured equilibrium among the original pCI-Not vectors (i.e. reading frames). Clones from columns 1–8 contained inserts cloned into pCI-Not30, those from columns 9–16 contained inserts cloned into pCI-Not31 and those from columns 17–24 of the pELI_Tc2 master plate contained inserts cloned into pCI-Not32. Therefore, sub-library pELI_Tc-2A (68 clones) was made up with clones from columns 1, 2, 9, 10, 17, 18; sub-library pELI_Tc-2B (68 clones) with those from columns 3, 4, 11, 12, 19, 20; sub-library pELI_Tc-2C (65 clones) with those from columns 5, 6, 13, 14, 21, 22; and sub-library pELI_Tc-2D (63 clones) with those from columns 7, 8, 15, 16, 23, 24.

2.6. Plasmid DNA preparation and vaccination

For the preparation of plasmid DNA used in immunization, clones were grown individually in 1 ml of LB containing ampicillin (LB-ampi) in 96-well plates (2.2 ml-deep well) for 24 h with agitation (sub-libraries pELI_Tc1, pELI_Tc2 and pELI_Tc3 were grown in four 96-well plates each). Then, the clones corresponding to each 96-well plate were pooled and grown for another 8 h in 200 ml of LB-ampi at 37 °C and 6 additional hours at 28 °C in the presence of chloramphenicol (170 μg/ml) for the amplification of plasmid copy number. The plasmid DNA from each preparation was purified using the QIAGEN EndoFree Plasmid Mega Kit (QIAGEN, GmbH, Germany) according to manufacturer's instructions.

Groups of 6-week-old male C3H/HeN mice were immunized three times with 100 μg of plasmid DNA (in a volume of 100 μl of sterile PBS) from each sub-library by the intramuscular route in quadriceps muscle at 20-day intervals. Control mice were inoculated with empty vectors pCINot-30, pCINot-31 and pCINot-32 and with the whole library pELI_TcT-E (first immunization screening) or the sub-library pELI_Tc2 (second immunization screening).

2.7. Antibody and cytokine measurements

Levels of *T. cruzi*-specific antibodies in serum were determined by ELISA using standard procedures. ELISA plates (Nunc Immunoplate™, USA) were coated with *T. cruzi* homogenates (epimastigotes 15 μg/well, equivalent to 2×10^6 parasites or trypomastigotes 0.5 μg/well, equivalent to 5×10^5 parasites) in carbonate/bicarbonate buffer (pH 9.6). After overnight incubation at 4 °C, nonspecific binding was blocked with 5% low fat milk for 1 h at 37 °C. After three washes in PBS-0.01% Tween 20™ (Sigma), sera were diluted and incubated for 2 h at 37 °C. Plates were washed five times with PBS-0.01% Tween 20™ to remove unbound antibodies, and a polyclonal goat anti-mouse IgG-peroxidase conjugate (Sigma, St. Louis, MO, USA) was applied at 1/5000 in PBS for 1 h at 37 °C. For isotype determinations, biotinilated goat anti-mouse IgG1 or IgG2a (Caltag Laboratories, Invitrogen, CA, USA) were added at 1/10,000, incubated for 1 h, washed, and incubated with streptavidin-peroxidase (1/1000, Sigma). After being washed with PBS-0.01% Tween 20™, the reaction plates were developed with *o*-phenylenediamine (Sigma) 0.4% in citrate buffer (24 mM citrate, 58 mM $Na_2HPO_4 \cdot 2H_2O$, pH 5.2) and 5 μl hydrogen peroxide 0.4 mg/ml. The reaction was stopped by addition of 1N H_2SO_4 and optical densities were read at 492 nm in an ELISA microplate reader (Costar).

For cytokine measurements, spleens were harvested and single-cell suspensions obtained by tissue homogenization. The erythrocytes were lysed and the cells cultured in 24-well plates at 5×10^6 cells/ml in RPMI 1640 medium (Gibco, BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM L-glutamin (Sigma), 100 UI/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. Cells were cultured for 72 h in medium alone or stimulated with concanavalin A (5 μg/ml) or *T. cruzi* trypomastigote lysate (10 μg/ml). The level of IL-10, and IFN-γ production was assessed by two-site sandwich ELISA using unlabeled (capture) and biotin-labeled (detection) monoclonal antibodies following the manufacturer's protocol (R&D Systems, USA).

2.8. Challenge of mice

Twenty days after the last vaccination, mice were challenged by the intraperitoneal route with 500 trypomastigotes from the RA strain of *T. cruzi*. Mortality was monitored daily and parasitemia levels were assessed three times a week by counting the number of bloodstream trypomastigotes from the tail vein of infected mice in a hemocytometer.

2.9. Hystopathological analysis

Liver, heart and musculoskeletal tissues (quadriceps) were fixed in 10% formol. Paraffin-embedded tissue sections were stained with hematoxilin–eosin and evaluated for the presence of tissue damage, parasite nests and the extension of tissue inflammatory infiltrates under a light microscope (Leica DMLA) with an image data analyzer (Leica Q550IW).

2.10. DNA sequencing and in silico analysis of the sequence information

Template preparation of clones was carried out as previously described [27]. Sequencing reactions were performed in a PerkinElmer 9600 thermal cycler by using a BigDye Terminator 3.1 Cycle sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the primer T7pCI (taatacgactcactataggc). Sequencing was performed in ABI 377 or ABI 3130 automated sequencers. Chromatograms were edited with FinchTV (version 1.4.0) and the sequence of recombinant peptides was conceptually determined by using the EditSeq program. BlastP analysis was performed at http://www.tcruzidb.org against databases hosting predicted proteins and open reading frames (ORFs) of *T. cruzi* [28]. Matches were considered significant by inspecting the identity in the alignments; *E*-values were not considered for the cut-off since the small length of some pELI_Tc-2D peptides (~20 amino acids corresponding to *T. cruzi*) might result in underestimated *E*-values, even in cases of good alignments. Coding sequences (CDSs) at TcruziDB with the best hit to each pELI_Tc-2D clone were used to search the NR database at NCBI by BlastP.

The presence of 9-mer CTL epitopes using neural networks was determined by NetMHC v.3.0 at http://www.cbs.dtu.dk/services/NetMHC/ [29,30].

2.11. Statistical analysis

Differences between the paired groups were determined by the Mann–Whitney *U* test. In survival analysis, cumulative survival between groups was compared by the Log-rank test. In all cases, Graph Pad Prism version 4.00 (GraphPad Software, USA) was used and a *P* value below 0.05 was considered significant.

3. Results

3.1. Construction and first screening of the expression library ELI_TcT-E

With the aim of identifying new putative vaccine antigens for *T. cruzi* we reasoned that an innovative starting point would be an epimastigote-subtracted trypomastigote cDNA library (library TcT-E), representing cDNAs preferentially expressed in

the trypomastigote stage (Tekiel et al., unpublished results; http://genoma.unsam.edu.ar/projects/tct-e). To examine the TcT-E library for the presence of protective clones, we used a modification of the original expression library immunization technique. Therefore, the original library was subcloned into the eukaryotic expression vectors pCINot-30-32. These vectors derived from the pCI30-32 plasmids [25] and were chosen because they provided a translation start codon that allowed the expression of peptides with open reading frames but without the amino terminal portion of the protein. This might be the case for most of the clones in the TcT-E library as inserts comprise trypomastigote-specific expressed tags cut by RsaI instead of complete coding sequences of genes (see Section 2). Besides, pCI30-32 vectors provide a histidine-tag sequence that makes possible the *in vitro* screening for clones expressing histidine-tagged proteins, thus avoiding immunization of animals with clones that do not express a recombinant protein. Additionally, the presence of unmethylated CpGs motifs in the ampicilin resistance gene confers these plasmids with their own adjuvant activity [25,31].

Inserts released from the original TcT-E library were subcloned into each of the pCINot-30-32 vectors and plated out separately. We obtained a total of ~5000 clones that were screened by colony dot with an anti-histidine antibody. By this first screening we selected 772 productive clones (i.e. clones producing a polyHis-fusion protein), which represents a ~85% reduction of the initial library ELI_TcT-E. The productive ELI_TcT-E library (pELI_TcT-E) was picked in 384-well plates, ordered according to their cloning vector (30, 31 or 32; i.e. reading frame) and insert size to facilitate its further partition into sub-libraries. The master plate of each cloning vector contained 268, 292 and 212 clones for pCINot-30, pCINot-31 and pCINot-32, respectively.

3.2. Secondary screen by immunization and challenge

The pELI_TcT-E library was partitioned into three sub-libraries: pELI_Tc1 (276 clones), pELI_Tc2 (264 clones), pELI_Tc3 (232 clones). Mice were immunized with plasmid DNA of each of the three sub-libraries ($n = 8$), with the whole pELI_TcT-E library (complete, $n = 6$) or with empty vectors (sham, $n = 6$).

The stimulation of a specific humoral response against *T. cruzi* epimastigotes (Fig. 1A) and trypomastigotes (Fig. 1B) upon immunization was evaluated 15 days after the last dose. Specific antibodies were detected in all groups of mice immunized with plasmids bearing *T. cruzi* inserts, although optical densities and titers were low. All immunized mice presented higher reactivity values than those sham-vaccinated both against trypomastigote and epimastigote antigens indicating that this response is specific to parasite antigens. Attempts to determine IgG1 and IgG2a profiles in these sera were unsuccessful.

The development of a cellular immune response was evaluated in two animals per group, at 15 days after the last immunization. Parasite-specific release of IFN-γ was only noticed in splenocytes obtained from mice vaccinated with sub-library pELI_Tc2 and with the complete pELI_TcT-E library (comp), but not in mice vaccinated with sub-libraries pELI_Tc1, pELI_Tc3 or empty vectors (sham) (Fig. 1C). In contrast, IL-10 production was observed in all immunized mice, but its production decreased by vaccination with pELI_Tc1 and pELI_Tc2 (Fig. 1D). The ratio between IFN-γ and IL-10 was determined (Fig. 1E) because it has been reported as a good correlation of protection and/or disease resolution for other intracellular pathogens [32,33]. Mice immunized with sub-library pELI_Tc2 displayed the pattern of IFN-γ vs. IL-10 production that associates most with a Th1-biased response (Fig. 1E).

Twenty days after immunization, animals were challenged with *T. cruzi* trypomastigotes and the level of circulating parasites and survival rates were evaluated (Fig. 2). As shown in Fig. 2B, 66% of mice immunized with sub-library pELI_Tc2 kept their parasitemia levels below 1×10^6/ml. In general, parasitemia levels above this value are achieved from day 20 p.i. on and are usually lethal, as depicted among mice immunized with sub-library pELI_Tc1, empty vectors and with the complete library (Fig. 2A,

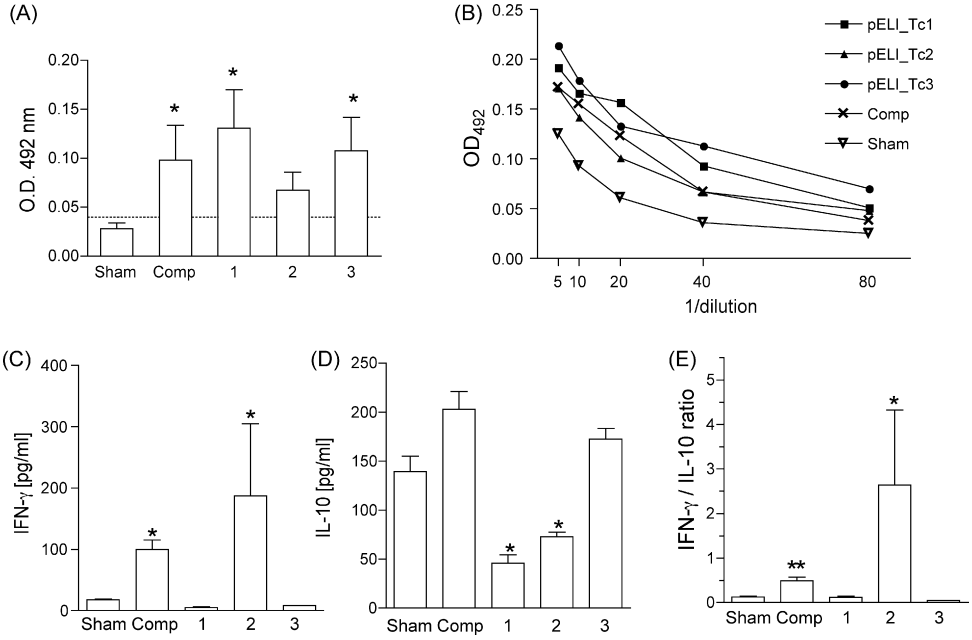

Fig. 1. Parasite-specific humoral and cellular immune responses following the first vaccination assay. Mice were genetically immunized with libraries pELI_Tc1 (1), pELI_Tc2 (2), pELI_Tc3 (3), empty vectors (sham), or the complete pELI_TcT-E library (comp). (A and B) IgG antibodies production against epimastigote (A) or trypomastigote (B) antigens was evaluated by ELISA on sera from immunized mice obtained 15 days after the last dose. (C, D and E) Splenocytes were cultured in triplicate for 72 h in the presence of *T. cruzi* trypomastigote lysate. Supernatants were collected and the levels of IFN-γ (C) and IL-10 (D) were measured by capture ELISA. The ratio of IFN-γ vs. IL-10 from supernatants of cultured splenocytes was calculated (E). Results represent the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.001$ when pairwise compared to sham-vaccinated group by Mann–Whitney test.

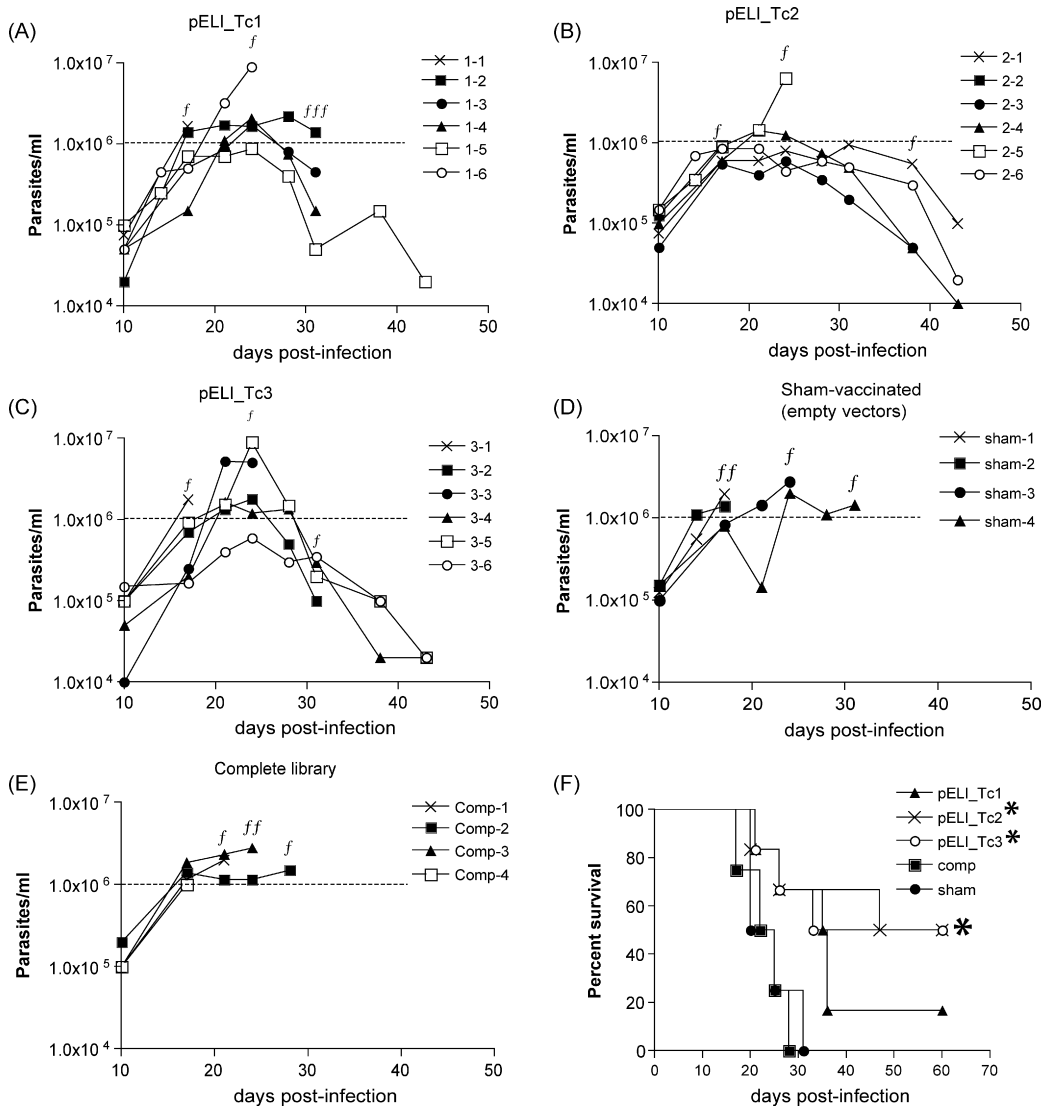

Fig. 2. Parasitemia and survival rates in genetically immunized mice after *T. cruzi* challenge. Mice immunized with plasmid DNA from each sub-library pELI_Tc1 ($n=6$), pELI_Tc2 ($n=6$), pELI_Tc3 ($n=6$), with empty vectors (sham, $n=4$) or the complete pELI_TcT-E library (comp, $n=4$) were challenged with 500 trypomastigotes of the RA strain 20 days after the last immunization. Individual parasitemias from mice immunized with sub-libraries pELI_Tc1 (A), pELI_Tc2 (B), pELI_Tc3 (C), empty vectors (sham; D) or complete pELI_TcT-E library (E) and survival rates (F) are shown. *f* indicates animal death. $^{*}P<0.05$ when pairwise compared to sham-vaccinated group by Log-rank test.

D and E). Interestingly, mice inoculated with sub-library pELI_Tc3 were able to survive even after reaching parasite levels above 1×10^6/ml.

In line with parasitemia results, 50% of mice immunized with sub-libraries pELI_Tc2 and pELI_Tc3 survived until the end of the experiment (90 days post-infection). In contrast, immunization with sub-library pELI_Tc1 showed lower protection levels as only one mouse survived and all mice vaccinated both with empty vectors and the complete pELI_TcT-E library were dead by day 35 after infection (Fig. 2F).

After day 50 post-infection, no deaths were recorded. Surviving mice were euthanized 90 days post-infection and tissues collected for immunopathological studies. No signs of liver tissue damage were detected, thus ruling out toxicity as an effect of plasmid DNA immunization. Cardiac tissues displayed only mild infiltrates whose extension did not differ between infected groups (data not shown). In musculoskeletal tissues, the extension of infiltrates was smaller in mice vaccinated with the sub-library pELI_Tc2 than in those immunized with sub-library pELI_Tc3, which also exhibited amastigote nests (Table 1 and Fig. 3).

Table 1
Score of target tissue damage from vaccinated mice that survived to the lethal challenge with *T. cruzi*.

Immunization group	Skeletal muscle	Heart	Amastigote nests
Not immunized, infected ($n=2$; 180 d.p.i.)	++++	+++	+
Not immunized, not infected ($n=2$)	–	–	–
Sub-library pELI_Tc1 ($n=1$)	+++	++	–
Sub-library pELI_Tc2 ($n=3$)	+ +	++	–
Sub-library pELI_Tc3 ($n=3$)	++++	+	+

Tissue observation was scored on a gradual scale from (–) to (++++), representing normal and maximal injury, respectively. The presence (+) or absence (–) of amastigote nests was also recorded. d.p.i = days post-infection.

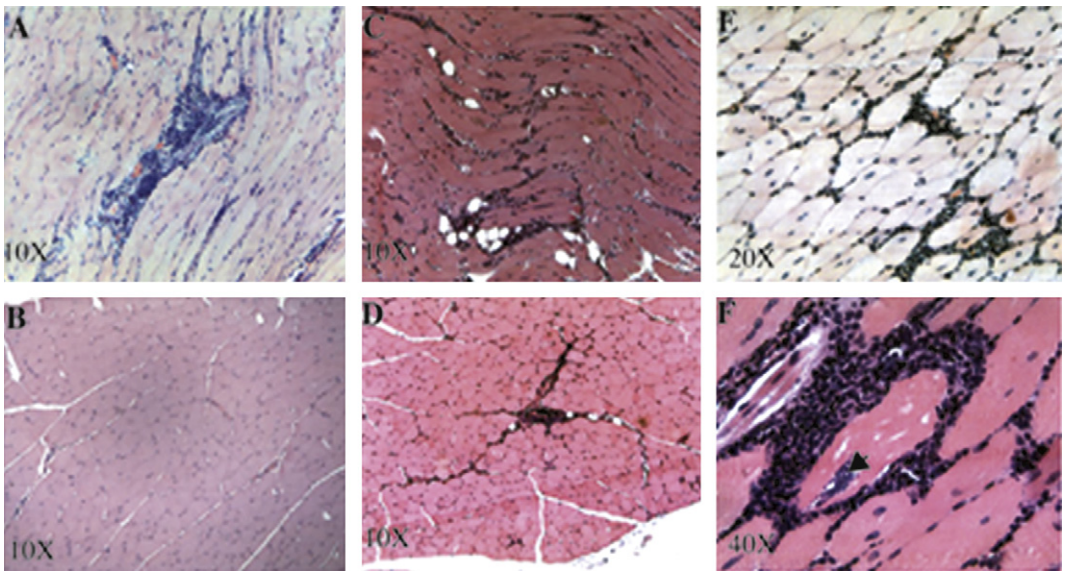

Fig. 3. Representative results of histopathological analysis of the skeletal muscle in genetically immunized mice. Tissues from immunized mice that survived 90 days after infection were analyzed by hematoxylin & eosin staining. (A) Chronically infected mice (not vaccinated); (B) control (not vaccinated, not infected); (C) vaccinated with pELI_Tc1; (D) vaccinated with pELI_Tc2; (E and F) vaccinated with pELI_Tc3 [arrowhead indicates a parasite nest].

3.3. Tertiary screen by immunization and challenge

Immunization of mice with sub-library pELI_Tc2 afforded 50% of protection after *T. cruzi* challenge, with the lowest parasitemia and musculoskeletal tissue damage, including both inflammation and tissue parasitism. Based on these data, sub-library pELI_Tc2 was chosen to be deconvulted into four sub-libraries: pELI_Tc-2A (68 clones), pELI_Tc-2B (68 clones), pELI_Tc-2C (65 clones) and pELI_Tc-2D (63 clones) (see Section 2 for deconvultion details).

The development of anti-*T. cruzi* IgGs was evaluated in vaccinated mice 15 days after the last immunization dose (Fig. 4A). As can be observed, and in agreement with results obtained in the first trial of immunization (Fig. 1A and B), titers triggered by genetic immunization (though specific) are low. The specific total IgG levels were mainly sustained by the specific IgG2a production (Fig. 4B). On the other hand, specific IgG1 was undetectable in vaccinated mice (data not shown).

The level of circulating trypomastigotes and survival were assessed in *T. cruzi*-challenged mice as these parameters are the best read-out of vaccination success in our experimental model. The highest protection was observed in animals immunized with sub-library pELI_Tc-2D, evidenced as 80% of survival of mice and the lowest values of parasitemia (Fig. 5). The protection levels conferred by sub-library pELI_Tc-2D were similar to those of the parent library pELI_Tc2. Of note is that survival rates of mice immunized with sub-library pELI_Tc-2D in both the first and second screenings were 50–55% higher than those recorded for the sham-vaccinated group (0% survival first trial; 25% survival second trial).

3.4. Sequence analysis of the protective pool

After the second round of immunization and challenge we retrieved the sequences corresponding to the peptides comprising the protective sub-library pELI_Tc-2D. The nucleotide sequence obtained for each of the 63 clones was conceptually translated to: (1) verify that a His-tagged protein had actually been produced, (2) determine the length of the synthesized peptide and (3) search for similarity of genes of *T. cruzi* (TcruziDB) or other organisms (NR) by BlastP analysis. Clones lacking an open reading frame (ORF) (17) or producing peptides with less than 20 amino acids (10) were discarded. Short peptides were set aside considering that the first 15 amino acids are supplied from the vector background and the next 5 or 12 by the adaptors (1 or 2R, respectively) used to construct the subtractive library. Among the remaining 36 clones, three were repeated three times and three were repeated two times, thus only one of each was considered for further analysis.

We ultimately identified 28 clones that constituted the non-redundant pELI_Tc-2D dataset, which encoded for peptides that ranged between 4.2 kDa and 21 kDa. Only five of these clones were similar to predicted proteins of *T. cruzi* (a putative *trans*-sialidase, a

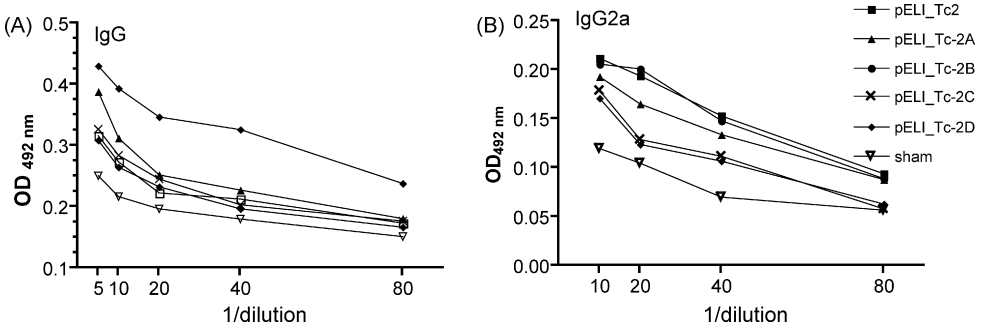

Fig. 4. Humoral immune response following second genetic immunization protocol. Mice were genetically immunized with libraries pELI_Tc-2A, pELI_Tc-2B, pELI_Tc-2C, pELI_Tc-2D, the parental library pELI_Tc2, or empty vectors (sham). Total IgG (A) and IgG2a (B) antibodies production against trypomastigote antigens were evaluated by ELISA on sera from immunized mice obtained 15 days after the last dose.

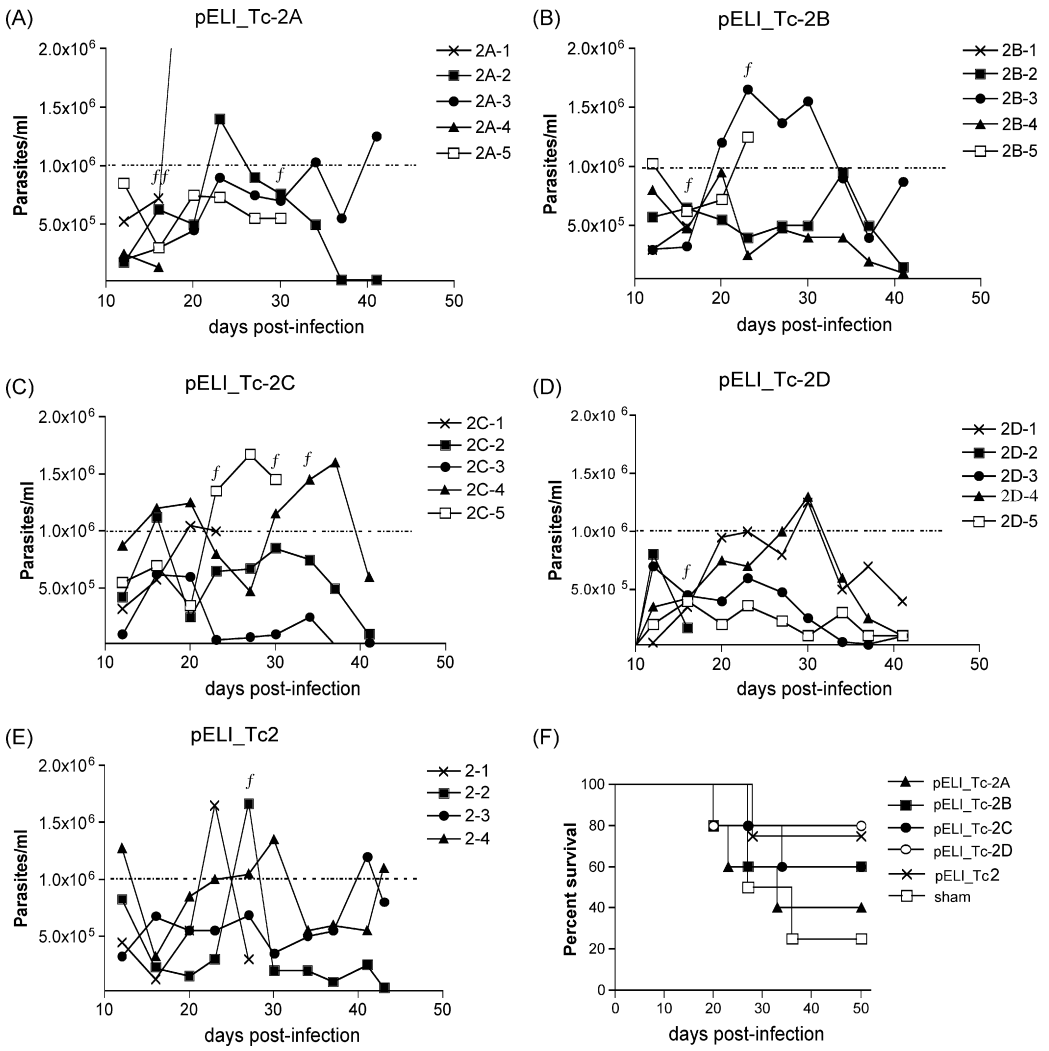

Fig. 5. *T. cruzi* challenge following second genetic immunization protocol: parasitemia and survival. Mice were immunized with plasmid DNA from each sub-library by the intramuscular route. Twenty days after the last immunization mice were challenged with 500 trypomastigotes. Individual parasitemias from mice immunized with pELI_Tc-2A (A; $n = 5$), pELI_Tc-2B (B; $n = 5$), pELI_Tc-2C (C; $n = 5$) and pELI_Tc-2D (D; $n = 5$) and whole pELI_Tc2 (E; $n = 4$) and survival (F) of vaccinated mice are shown. *f* indicates animal death.

mucin-associated surface protein (MASP), a lisosomal/endosomal protein and two hypothetical proteins) (Table 2). Most of the pELI_Tc-2D clones (17/28; 60.71%) matched with ORFs longer than 100 amino acids deposited at the *T. cruzi* database but were not significantly similar to any annotated *T. cruzi* protein. This finding indicates both that the expressed peptides were actually *T. cruzi* coding sequences and that – as previously reported [34] – the *T. cruzi* genome is sub-annotated. The complete sequence of the ORFs that matched with the pELI_Tc-2D clones were retrieved and used as bait to search the NR database at NCBI. Only 8 out of 17 produced significant matches and, among these, most "best hits" were with *T. cruzi* proteins and only two with proteins of known function of other organisms. It is worth mentioning that six pELI_Tc-2D clones corresponded to inserts cloned in antisense orientation or wrong frame, as they only give significant alignments when tested by BlastX (DNA vs. protein database) but not by BlastP (protein vs. protein database) against *T. cruzi*. A complete list including the sequences of the pELI_Tc-2D peptides as well as their detailed matches against *T. cruzi* and NR databases is provided as additional material (Table S1).

3.5. *In silico analysis of the identified sequences*

T cell-mediated immunity, especially immunity mediated by CD8+ CTL, has been demonstrated to play a crucial role in the control of the infection of humans and mice with *T. cruzi* [35]. Antigen presentation to CD8+ T cells is mediated by major histocompatibility complexes (MHC) class I and it is generally accepted that only peptides that bind to MHC with an affinity above a threshold [typically a value of 500 nM] can act as T cell epitopes [36]. Being aware that, within a target molecule, mouse T cell epitopes will certainly differ from human T cell epitopes we screened for the presence of both human and mouse CTL epitopes in all *T. cruzi* proteins and ORFs retrieved from the protective non-redundant ELI_Tc-2D dataset (cloned in the sense orientation; $n = 22$). We restricted our analysis to the HLA-A2 human allele owing to its high frequency of expression in individuals living in Chagas' disease endemic areas of Latin America [37] and to the mouse MHC class I gene product H-2K^k present in the mouse strain used in this work. Results indicated that 21 out 22 proteins and ORFs presented one or more peptides that bind with strong or intermediate affinities (i.e. IC50 <500 nM) to human MHC I, in context HLA-A2. The same predictor ran for mouse H2-k^k showed that 17/22 putative novel vaccine candidates have CTL epitopes. Besides, for HLA-A2, 18 ORFs (out of the 21 with predicted CTL peptides) have – in total – 52 peptides that bind with affinities lower than 50 mM to MHC class I; 18 peptides with these strong binding affinities were found for H2-k^k. The whole information about CTL epitopes and MHC binding affinities are provided as Supplementary material (Table S2).

Table 2
BlastP matches of non-redundant pELI_Tc-2D clones to protein databases at www.TcruziDB.org and NCBI.

Description	Number	Percentage
Non-redundant pELI_Tc-2D dataset	28	100
T. cruzi annotated proteins	5	17.86
pELI_Tc-2D _A11: hypothetical protein;		
Tc00.1047053511675.3		
Comments: protein with signal peptide and transmembrane domain		
pELI_Tc-2D _G2: hypothetical protein, conserved;		
Tc00.1047053507003.70,		
Comments: orthologs in *T. brucei* and several *Leishmania* sp.		
pELI_Tc-2D _A12: lysosomal/endosomal membrane protein;		
Tc00.1047053510825.30		
Comments: characterized in *T. brucei* [p67]		
pELI_Tc-2D _B8: mucin-associated surface protein (MASP);		
Tc00.1047053510047.60		
Comments: *T. cruzi*-specific gene family		
pELI_Tc-2D _F2: *trans*-sialidase (TS), putative;		
Tc00.1047053509629.10		
Comments: other members of TS family assayed as vaccines		
Not annotated ORFs [*T. cruzi* DB]	17[a]	60.71
Detail of matches against NR database[a]		
(i) *T. cruzi* proteins	6	
(ii) Proteins from other organisms	2	
(iii) Non-significant matches	9	
Antisense cloned	6	21.43

All 28 pELI_Tc-2D clones in the non-redundant dataset were conceptually translated and compared to "protein and ORFs higher than 100 amino acid" in the database hosted at www.tcruziDB.org [28].

[a] These 17 ORFs (with the best hit to each pELI_Tc-2D clone) were used to search the NR database at NCBI by BlastP.

4. Discussion

Up to date, there is no available vaccine for the treatment or prevention of Chagas' disease, one of the major health problems in Latin America. Although sustained fumigation has reduced the insect-mediated transmission of *T. cruzi* in some endemic areas, the fight for disease eradication must be carried out from several angles and, certainly, the gold standard for the control of the human infection is to find an effective vaccine. Classical methods for vaccine discovery have focused on subunit vaccines. A limitation of this approach is that, in most cases, antigens are selected based on their availability, the previous knowledge of the gene, or because they elicit a strong (not necessarily protective) immune response on the infected host. This has been the case for *T. cruzi* vaccine development (except for two recently published works that tested *in silico* identified antigens [5,38]) and, as a consequence, none of the antigens currently under experimentation is 100% protective nor avoids the infection. Therefore, there is a mandatory need to identify additional vaccine candidates to foster the development of an effective vaccine.

In the present work we identified a new pool of *T. cruzi* gene fragments, which improved survival of mice after challenge with parasites following genetic vaccination with sequential fractions of a stage-specific expression library. Our hypothesis was that genes preferentially expressed in the trypomastigote bloodstream represent potential targets for immunization, since this parasite stage mediates tissue dissemination and cell invasion in the vertebrate host. Other approaches to identify protective antigens (i.e. reverse vaccinology) rely on the *a priori* selection of target genes to express and test them as vaccines, even though the best algorithm or set of algorithms for the prediction of a good vaccine candidate remains to be found [39,40]. We decided to screen a trypomastigote-enriched cDNA library by genetic immunization in order to allow the host immune system to select the best protective antigens. For this, we took advantage of having a model of murine experimental infection where the read-out of the immunization trial is parasitemia and mortality. The summary of our research is depicted in Table 3: we started from a subtractive library containing ~5000 expressed sequence tags and after one round of library screening to select for peptide-producing clones followed by two rounds of animal trials by genetic immunization and pathogen challenge, we identified a group of 28 peptides composing a protective pool, thus reducing the complexity of the original pELI_TcT-E library by more than 96%.

The *T. cruzi* diploid genome is estimated to be ~110 Megabases (Mb) but only 22,500 protein-coding genes had been annotated in 60.4 Mb; 12,000 genes compose the haploid genome of the parasite [41]. The mean length of coding sequences is 1500 bases, which indicates that approximately 25.5 Mb of the genome are intergenic and therefore non-coding regions. These facts have direct implications in the method we used to construct the expression library for genetic immunization. First, and as mentioned before, an ELI approach based on cDNA instead of genomic DNA would be more convenient, as it significantly diminishes the probability of cloning non-coding regions [22]. Second, we used an epimastigote-subtracted trypomastigote cDNA library (TcT-E) since subtractive libraries not only suppress sequences with identical abundance and subtract between two mRNA populations but also normalize cDNA levels [24]. This may be useful to increase the representation in the ELI_TcT-E library some trypomastigote-specific but relatively poorly expressed proteins that are essential for parasite survival. The method of subtractive library construction also implies that genes are split by a restriction enzyme to achieve better subtraction efficiency. It has been reported that short peptides might have a greater opportunity to be presented in the context of MHC giving a superior immunostimulatory capacity to that of complete genes [20]. This is desired when dealing with pathogens with intracellu-

Table 3
Sequential deconvultion of the original ELI_TcT-E library for the identification of vaccine candidates.

Protective library	Number of clones (% of reduction)
ELI_TcT-E	~5000
pELI_TcT-E (peptide-producing clones)	772 (100)
pELI_Tc2 (first deconvultion)	264 (65.8)
pELI_Tc-2D (second deconvultion)	63 (91.8)
pELI_Tc-2D non-redundant dataset (sequencing)	28 (96.37)

lar stages primarily controlled by cellular immune responses as is the case of *T. cruzi*.

The genetic immunization protocol used in this work was able not only to improve survival but also to reduce tissue parasitism and inflammation of skeletal muscle and to induce cellular immune response to native *T. cruzi* antigens. Several evidences have indicated that activation of CD4+ and CD8+ T cells are required for protection in infection caused by *T. cruzi*. T cells contribute to control of parasitemia and mortality and thus to the elimination of parasite by secretion of Th1 type cytokines such as IFNγ, TNFα or IL-12 [35,42,43]. More recently, through *in vivo* depletion studies, Araujo et al. [4] and Chou et al. [44] have found that treatment with anti-CD8, but not with anti-CD4 antibodies, completely reversed the protective immunity elicited by immunization, thus reinforcing the role assigned to CD8+ T cells in control of *T. cruzi* infection. However, regarding the cytokine production by splenocytes, we could not associate a Th1 profile with a more protective response. Actually, a biased Th1 profile was found both in the group of mice that showed maximum and minimum survival rates (sub-library pELI_Tc2 and complete library pELI_TcT-E in the first immunization and challenge protocol). These results confirm that a correlation between the immune response elicited by vaccination and disease control remains still uncertain as immune response against *T. cruzi* relies on multiple cellular and humoral mechanisms that were not directly addressed in the present work [35,45]. Nevertheless, in our experimental model, cross protection between *T. cruzi* strains was demonstrated. In fact, animals immunized with plasmids containing sequences from the reference CL Brener strain (*T. cruzi* lineage I/II) were partially protected when challenged with the highly virulent RA strain (*T. cruzi* lineage II). Cross-strain protection is a desirable characteristic for the design of a vaccine, as the goal is to protect against a broad spectra of parasite isolates that circulate in large endemic areas, as it is the case of *T. cruzi*. Moreover, recent studies focused on immune response and immunotherapy against *T. cruzi* infection support the idea that immunodominance of the few known *T. cruzi* antigens, might be a strong escape mechanism of the parasite [46,47]. Perhaps, vaccination should also focus on subdominant or cryptic epitopes in order to break this immunodominance.

The humoral immunological parameters indicate that immunization with pCINot vectors carrying *T. cruzi* inserts induced parasite-specific production of IgG, although titers were very low. The absence of a strong anti-*T. cruzi* antibody production in immunized mice was not unexpected since the vaccine formulation employed here (DNA inoculated by the intramuscular route), is not the most suitable to induce a humoral response [31]. Indeed, we privileged the immunization protocol used as the cellular response has been proposed as the most critical mechanism of control for *T. cruzi* infection, as discussed above. Besides, the dilution of the antigens that occurs by immunization with a large number of clones containing a mixture of antigens also does not favor the assembly of a strong anti-*T. cruzi* humoral response. Particularly in the mouse model of Chagas disease, protection against a lethal *T. cruzi* challenge after genetic vaccination in the absence of significant specific IgG antibodies has been previously reported [8,10]. Conversely, other *T. cruzi* antigens like *trans*-sialidase are good inducers of specific IgG when delivered in genetic vaccines but fail to induce protective immunity, suggesting that a strong IgG response is not always a good correlate of protection [9].

The *T. cruzi* genome annotation has assigned a putative function only to 50.8% of the predicted *T. cruzi* coding genes on the basis of significant similarity to previously characterized proteins or functional domains [41]. Among these, 18% represent repetitive sequences of large gene families of surface proteins like MASP, TS, mucins and gp63. In retrospect, it is predictable, therefore, that (1) most of the clones that we identified in the protective pool pELI_Tc-2D are *T. cruzi* CDSs not-annotated as genes and that (2) among clones similar to protein-coding genes, two are of unknown function (hypothetical), two belong to large surface protein families (TS, MASP) and only one is a gene of inferred function because of its similarity to a previously characterized protein (lysosomal/endosomal membrane protein p67). This gene, besides having a motif of laminin-A common to membrane-associated intracellular proteases, is an ortholog to the p67 protein of the closely related kynetoplastid *Trypanosoma brucei* (Tbp67), whose function has been recently characterized [48,49]. Tbp67 is a lysosomal glycoprotein essential not only to preserve the morphology of lysosomes but also for the normal growth of the mammalian bloodstream but not the procyclic insect-stage of African trypanosomes [48]. The ablation of Tbp67 expression confers resistance to human serum trypanosome lytic factor (TLF), indicating this protein as the target for the TLF-mediated killing [48]. The role of this protein in *T. cruzi* biological processes remains to be elucidated but what we learned from the *T. brucei* ortholog increases its potential as a vaccine candidate.

On the other hand, 60.71% (17) of the clones in the protective pool encompass non-annotated CDSs, with nine of them lacking sequence homologs in any of the protein databases searched (Table 2). This scenario was not unexpected taking into account the incompleteness – due to its highly repetitive nature – of the *T. cruzi* genome assembly and annotation [34]. The absence of identity of the nine clones described above might suggest that, at least some of them, encode for *T. cruzi*-specific proteins, and therefore could be relevant as new candidates for vaccine development. Likewise, since no function has been assigned to most of the genes with identity to clones of the pELI_Tc-2D protective pool, these promising target antigens would certainly not have been identified by other methods for vaccine discovery.

In summary, we present here the results of the screening that led to the identification of novel putative vaccine candidates for Chagas' disease. The challenge remains to understand the biological functions of the products of these genes in order to add these molecular targets to rationally design multi-component formulations (DNA or peptide-based vaccines) as they could increase protection against the extracellular stage of the parasite. Additional studies are also necessary to enhance the individual *in vivo* expression of the protective gene fragments in order to improve the efficacy of vaccination. One interesting approach is to give a prime immunization with plasmid DNA (in the presence or not of adjuvant) and a second and third boost with recombinant proteins in order to achieve both good cellular and humoral immune responses.

Another forthcoming issue should be to demonstrate the immunogenicity of these vaccine candidates in human *in vitro* models or by using humanized transgenic mice. To preliminary address this matter we performed an *in silico* approach to potentially identify relevant vaccine candidate CTL epitopes for humans among proteins and ORF derived from the sequences of the clones from the protective pool. Algorithms showed that 21 out 22 proteins and ORFs retrived from the non-redundant protective pool dataset, presented peptides that bind with strong or intermediate affinities to the most frequent HLA allele present among inhabitants of *T. cruzi* endemic areas.

Our idea is that the novel vaccine targets identified in the present study will complement but not necessarily replace the previously identified antigens in view of developing multi-component vaccines. This type of vaccines seems the most useful against a protozoan with multiple developmental stages and a great capacity for immune evasion as is *T. cruzi*.

Acknowledgements

We thank DJ Comerci and JE Ugalde for their kind gift of pCI vectors and F Peri and E Gimenez for their valuable technical assistance.

This work was supported by grants of UNSAM (SC 06/52 to VT) and ANPCyT, Argentina (PICT 05-11002 to VT, PICT 01-10888 to DS, and PICT 05-14241 to SMGC). VT, SMGC, MP and DS are members of the Research Career of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. CDAS is a post-doctoral fellow of CONICET.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2008.12.056.

References

[1] Stuart K, Brun R, Croft S, Fairlamb A, Gurtler RE, McKerrow J, et al. Kinetoplastids: related protozoan pathogens, different diseases. J Clin Invest 2008;118(4):1301–10.
[2] Garg N, Bhatia V. Current status and future prospects for a vaccine against American trypanosomiasis. Expert Rev Vacc 2005;4(6):867–80.
[3] Cazorla SI, Becker PD, Frank FM, Ebensen T, Sartori MJ, Corral RS, et al. Oral vaccination with *Salmonella enterica* as a cruzipain-DNA delivery system confers protective immunity against *Trypanosoma cruzi*. Infect Immun 2008;76(1):324–33.
[4] Araujo AF, de Alencar BC, Vasconcelos JR, Hiyane MI, Marinho CR, Penido ML, et al. CD8+-T-cell-dependent control of *Trypanosoma cruzi* infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect Immun 2005;73(9):6017–25.
[5] Bhatia V, Garg NJ. Previously unrecognized vaccine candidates control *Trypanosoma cruzi* infection and immunopathology in mice. Clin Vaccine Immunol 2008;15(8):1158–64.
[6] Fontanella GH, De Vusser K, Laroy W, Daurelio L, Nocito AL, Revelli S, et al. Immunization with an engineered mutant trans-sialidase highly protects mice from experimental *Trypanosoma cruzi* infection: a vaccine candidate. Vaccine 2008;26(19):2322–34.
[7] Fralish BH, Tarleton RL. Genetic immunization with LYT1 or a pool of trans-sialidase genes protects mice from lethal *Trypanosoma cruzi* infection. Vaccine 2003;21(21–22):3070–80.
[8] Garg N, Tarleton RL. Genetic immunization elicits antigen-specific protective immune responses and decreases disease severity in *Trypanosoma cruzi* infection. Infect Immun 2002;70(10):5547–55.
[9] Vasconcelos JR, Hiyane MI, Marinho CR, Claser C, Machado AM, Gazzinelli RT, et al. Protective immunity against *Trypanosoma cruzi* infection in a highly susceptible mouse strain after vaccination with genes encoding the amastigote surface protein-2 and trans-sialidase. Hum Gene Ther 2004;15(9):878–86.
[10] 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(11):5073–81.
[11] Martin DL, Weatherly DB, Laucella SA, Cabinian MA, Crim MT, Sullivan S, et al. CD8+ T-cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. PLoS Pathog 2006;2(8):e77.
[12] Doolan DL, Aguiar JC, Weiss WR, Sette A, Felgner PL, Regis DP, et al. Utilization of genomic sequence information to develop malaria vaccines. J Exp Biol 2003;206(Pt 21):3789–802.
[13] Doolan DL, Southwood S, Freilich DA, Sidney J, Graber NL, Shatney L, et al. Identification of Plasmodium falciparum antigens by antigenic analysis of genomic and proteomic data. Proc Natl Acad Sci U S A 2003;100(17):9952–7.
[14] Barry MA, Howell DP, Andersson HA, Chen JL, Singh RA. Expression library immunization to discover and improve vaccine antigens. Immunol Rev 2004;199:68–83.
[15] Barry MA, Lai WC, Johnston SA. Protection against mycoplasma infection using expression-library immunization. Nature 1995;377(6550):632–5.
[16] Ivey FD, Magee DM, Woitaske MD, Johnston SA, Cox RA. Identification of a protective antigen of *Coccidioides immitis* by expression library immunization. Vaccine 2003;21(27–30):4359–67.
[17] McNeilly CL, Beagley KW, Moore RJ, Haring V, Timms P, Hafner LM. Expression library immunization confers partial protection against *Chlamydia muridarum* genital infection. Vaccine 2007;25(14):2643–55.
[18] Melby PC, Ogden GB, Flores HA, Zhao W, Geldmacher C, Biediger NM, et al. Identification of vaccine candidates for experimental visceral leishmaniasis by immunization with sequential fractions of a cDNA expression library. Infect Immun 2000;68(10):5595–602.
[19] Piedrafita D, Xu D, Hunter D, Harrison RA, Liew FY. Protective immune responses induced by vaccination with an expression genomic library of *Leishmania major*. J Immunol 1999;163(3):1467–72.
[20] Stemke-Hale K, Kaltenboeck B, DeGraves FJ, Sykes KF, Huang J, Bu CH, et al. Screening the whole genome of a pathogen in vivo for individual protective antigens. Vaccine 2005;23(23):3016–25.
[21] Yero D, Pajon R, Perez Y, Farinas M, Cobas K, Diaz D, et al. Identification by genomic immunization of a pool of DNA vaccine candidates that confer protective immunity in mice against *Neisseria meningitidis* serogroup B. Vaccine 2007;25(28):5175–88.
[22] Talaat AM, Stemke-Hale K. Expression library immunization: a road map for discovery of vaccines against infectious diseases. Infect Immun 2005;73(11):7089–98.
[23] Tekiel V, Oliveira GC, Correa-Oliveira R, Sanchez D, Gonzalez-Cappa SM. Chagas' disease: TCRBV9 over-representation and sequence oligoclonality in the fine specificity of T lymphocytes in target tissues of damage. Acta Trop 2005;94(1):15–24.
[24] Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 1996;93(12):6025–30.
[25] Moore RJ, Lenghaus C, Sheedy SA, Doran TJ. Improved vectors for expression library immunization—application to *Mycoplasma hyopneumoniae* infection in pigs. Vaccine 2002;20(1–2):115–20.
[26] Sambrook J, Russell DW. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2001.
[27] Aguero F, Abdellah KB, Tekiel V, Sanchez DO, Gonzalez A. Generation and analysis of expressed sequence tags from *Trypanosoma cruzi* trypomastigote and amastigote cDNA libraries. Mol Biochem Parasitol 2004;136(2):221–5.
[28] Aguero F, Zheng W, Weatherly DB, Mendes P, Kissinger JC. TcruziDB: an integrated, post-genomics community resource for *Trypanosoma cruzi*. Nucleic Acids Res 2006;34(Database issue):D428–31.
[29] Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. Nucl Acids Res 2008;36(Web Server issue):W509–12.
[30] Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, et al. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci 2003;12:1007–17.
[31] Sasaki S, Takeshita F, Xin KQ, Ishii N, Okuda K. Adjuvant formulations and delivery systems for DNA vaccines. Methods 2003;31(3):243–54.
[32] Widdison S, Schreuder LJ, Villarreal-Ramos B, Howard CJ, Watson M, Coffey TJ. Cytokine expression profiles of bovine lymph nodes: effects of *Mycobacterium bovis* infection and bacille Calmette-Guerin vaccination. Clin Exp Immunol 2006;144(2):281–9.
[33] Stober CB, Lange UG, Roberts MT, Alcami A, Blackwell JM. IL-10 from regulatory T cells determines vaccine efficacy in murine *Leishmania major* infection. J Immunol 2005;175(4):2517–24.
[34] Beucher M, Norris KA. Sequence diversity of the *Trypanosoma cruzi* complement regulatory protein family. Infect Immun 2008;76(2):750–8.
[35] Martin D, Tarleton R. Generation, specificity, and function of CD8+ T cells in *Trypanosoma cruzi* infection. Immunol Rev 2004;201:304–17.
[36] Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM, et al. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J Immunol 1994;153:5586–92.
[37] Santiago-Delpin EA. Transplantation in Latin America. Transplant Proc 1991;23(2):1855–60.
[38] Silveira EL, Claser C, Haolla FA, Zanella LG, Rodrigues MM. Novel protective antigens expressed by *Trypanosoma cruzi* amastigotes provide immunity to mice highly susceptible to Chagas' disease. Clin Vaccine Immunol 2008;15(8):1292–300.
[39] Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarselli M, et al. Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science 2005;309(5731):148–50.
[40] Bhatia V, Sinha M, Luxon B, Garg N. Utility of the *Trypanosoma cruzi* sequence database for identification of potential vaccine candidates by in silico and in vitro screening. Infect Immun 2004;72(11):6245–54.
[41] El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 2005;309(5733):409–15.
[42] 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(1):13–22.
[43] Abrahamsohn IA, Coffman RL. *Trypanosoma cruzi*: IL-10, TNF, IFN-gamma, and IL-12 regulate innate and acquired immunity to infection. Exp Parasitol 1996;84(2):231–44.
[44] Chou B, Hisaeda H, Shen J, Duan X, Imai T, Tu L, et al. Critical contribution of immunoproteasomes in the induction of protective immunity against *Trypanosoma cruzi* in mice vaccinated with a plasmid encoding a CTL epitope fused to green fluorescence protein. Microbes Infect 2008;10(3):241–50.
[45] Tarleton RL. Immune system recognition of *Trypanosoma cruzi*. Curr Opin Immunol 2007;19(4):430–4.
[46] Rodrigues MM, de Alencar BC, Claser C, Tzelepis F. Immunodominance: a new hypothesis to explain parasite escape and host/parasite equilibrium leading to the chronic phase of Chagas' disease? Braz J Med Biol Res, in press.
[47] Tzelepis F, de Alencar BC, Penido ML, Claser C, Machado AV, Bruna-Romero O, et al. Infection with *Trypanosoma cruzi* restricts the repertoire of parasite-specific CD8+ T cells leading to immunodominance. J Immunol 2008;180(3):1737–48.
[48] Peck RF, Shiflett AM, Schwartz KJ, McCann A, Hajduk SL, Bangs JD. The LAMP-like protein p67 plays an essential role in the lysosome of African trypanosomes. Mol Microbiol 2008;68(4):933–46.
[49] Kelley RJ, Alexander DL, Cowan C, Balber AE, Bangs JD. Molecular cloning of p67, a lysosomal membrane glycoprotein from *Trypanosoma brucei*. Mol Biochem Parasitol 1999;98(1):17–28.