**This is a postprint of an article published in
Cazorla, S.I., Frank, F.M., Becker, P.D., Corral, R.S., Guzmán, C.A.,
Malchiodi, E.L.**

**Prime-boost immunization with cruzipain co-administered with MALP-2
triggers a protective immune response able to decrease parasite burden and
tissue injury in an experimental Trypanosoma cruzi infection model
(2008) Vaccine, 26 (16), pp. 1999-2009.**

Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental *Trypanosoma cruzi* infection model

Silvia I. Cazorla^{a,b,c}, Fernanda M. Frank^{a,b}, Pablo D. Becker^c, Ricardo S. Corral^d, Carlos A. Guzmán^{c*} and Emilio L. Malchiodi^{a,b*}

^a Cátedra de Inmunología and Instituto de Estudios de la Inmunidad Humoral (IDEHU), CONICET-UBA, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

^b Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Argentina.

^c Department of Vaccinology, Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124 Braunschweig, Germany.

^d Servicio de Parasitología-Chagas, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina.

Running title: Prime-boost with Cz-MALP-2 protects against *T. cruzi* infection

Keywords: Chagas disease, *Trypanosoma cruzi*, MALP-2

* Corresponding authors. Tel.: +54 114 9648259 (E.L. Malchiodi) and +49 531 6181558 (C.A. Guzmán).

E-mail address: emalchio@ffyb.uba.ar (E.L. Malchiodi) and carlos.guzman@helmholtz-hzi.de (C.A. Guzmán)

Abbreviations: Cz, cruzipain; i.n., intranasal; i.d., intradermal; MALP-2, macrophage activating lipopeptide-2; CpG-ODN, oligodeoxynucleotides containing CpG motifs; CK, creatine kinase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase

Abstract

Cruzipain (Cz), a key *Trypanosoma cruzi* enzyme, is a main candidate antigen for vaccines against Chagas' disease. We evaluated a vaccination protocol based on intradermal priming with recombinant Cz and intranasal boosting with rCz co-administered with a derivative of the TLR2/6 agonist MALP-2. Vaccination triggered strong systemic and mucosal antibody responses, and a vigorous cell-mediated immunity characterized by lymphoproliferation, DTH reactivity and IFN- γ production. The immune responses protected against a lethal trypomastigote challenge and, upon sub-lethal infection, immunized mice showed reduction of tissue damage and normal enzymatic markers of muscle injury. This prime-boost regimen appears promising for further development, since warranted survival, provided efficient control of parasite load and restricted inflammatory myopathy.

1. Introduction

Chagas' disease, or American trypanosomiasis, caused by the protozoan parasite *Trypanosoma cruzi* has a widespread distribution in America. An estimated 16 to 18 million persons are infected, and close to 100 million people are at risk of infection [1]. Transmission to humans occurs primarily through blood-sucking reduviid bugs, which deposit infective feces on the skin when they are feeding [2]. The disease is characterized by an initial acute phase during which trypomastigotes multiply in the blood, followed by an indeterminate phase with very low parasitemia and no apparent pathology. Susceptible hosts then enter a chronic phase with increasing tissue damage, mostly in cardiac and skeletal muscle tissues but sometimes also in the liver, spleen, colon, or esophagus, which progressively leads to cardiac failure and death [3-5]. Parasite replication is hampered by both innate and acquired immune responses. Both CD4⁺ and CD8⁺ T cells are involved in *T. cruzi* systemic immunity, since mice lacking functional cells of either type have increased susceptibility to *T. cruzi* [6-10]. CD4⁺ Th1 responses (characterized by IFN- γ production) are associated with *T. cruzi* resistance, whereas CD4⁺ Th2 responses (characterized by IL-4 production) are associated with *T. cruzi* susceptibility [11-13]. In addition to the induction of protective effector functions, such as microbicidal NO [14], CD4⁺ Th1 cells serve as important helper cells enhancing both CD8⁺ T-cell and antibody responses. CD8⁺ T-cells are important for recognition of parasite antigens presented on the surfaces of infected cells. On the other hand, lytic serum antibodies can be protective against the extracellular life stages of *T. cruzi* [15-17] and secretory immunoglobulin A (sIgA) responses may be relevant for protection against mucosal transmission from the reduviid vector [18].

There is still considerable debate about the mechanisms involved in Chagas disease's pathology. Nevertheless, sensitive examination techniques confirmed that parasites persist in tissues and that there is a correlation between this persistence and tissue damage [10,19,20]. These results strongly encourage development of vaccines and new trypanocidal drugs directed to control *T. cruzi* infection in order to prevent or arrest Chagas pathogenesis.

Vaccines represent an outstanding success story in modern medicine and have had a dramatic effect on morbidity and mortality worldwide. Most of currently available vaccines are delivered through parenteral routes. However, mucosal vaccination offers several benefits, including higher acceptance by the public, easiness of administration, no risk of cross-contamination by infected needles, and more importantly, the induction of mucosal as well as systemic immunity, which might facilitate to block the agent at a very early stage of the infection process. Another attractive option for vaccine delivery is transcutaneous immunization. The skin, especially its epidermal layer, is an accessible and competent immune environment populated with Langerhans cells, which are efficient

and potent antigen-presenting cells. Induction of protective immunity may be accomplished by targeting antigens to the skin through intradermal (i.d.) injection or immunostimulant patches. The i.d. presentation of antigens to the immune system is able to raise a macrophage-dependent T-lymphocyte response via specific epidermal cells, which finally stimulates both T helper and cytolytic activities. Cross-presentation involves the uptake and processing of exogenous antigens within the major histocompatibility complex (MHC) class I pathway. This process primarily performed by CD8⁺ dendritic cells is likely to be important for the generation of cytotoxic T cell immunity [21].

Considering that through mucosa or skin lesion *T. cruzi* finds its way to invade host cells, the elicitation of an effective immune response in those sites is highly desirable, as it may clearly contribute to control the infection at portal of entry. Several studies have focused on characterization of parasite antigens which may be used as vaccine candidates. In this regard, the major cysteine proteinase of *T. cruzi*, named cruzipain (Cz), has shown promise because it induced significant protection in mouse experimental models [22,23]. In the present study, we evaluated whether intranasal (i.n.) and i.d. administration of recombinant Cz (rCz) combined with different synthetic adjuvants, namely oligodeoxynucleotides containing CpG motifs (CpG-ODN) and a derivative of a macrophage-activating lipopeptide from *Mycoplasma fermentans* (MALP-2), is able to confer protection against experimental *T. cruzi* infection. CpG-ODN are powerful enhancers of Th1 immune responses [23-25] through the activation of Toll-like receptor-9 (TLR-9)-dependent cascades [26], whereas MALP-2 acts as a TLR-2/6 agonist [27,28] improving humoral and cell-mediated immunity. To further modulate and broaden the effective responses elicited, a prime-boost regimen including i.d. and mucosal rCz-based immunizations was also assessed.

2. Materials and methods

2.1. Parasites

T. cruzi epimastigotes (RA strain), were grown in biphasic medium as previously described [29]. Parasites were harvested during exponential growth phase by centrifugation at 5000 x g for 15 min and washed three times with 0.1 M phosphate buffered saline, pH 7.2 (PBS). Bloodstream trypomastigotes of the same parasite strain were isolated from acutely infected mice at the peak of parasitemia.

2.2. Cruzipain cloning and expression

DNA from epimastigotes of *T. cruzi* (RA strain) was purified using the Genomic DNA extraction kit (QIAgen, Valencia, CA). Isolated DNA was used as a template for PCR amplification using a forward primer 5'-CATGGGATCCGCGCCCGCGGCAGTGGATTG-3', with the *Bam*H I site underlined, and a reverse primer 5'-CGCGCGCTCGAGGCGGCGATGACGGC-3', with an *Xho* I site underlined. PCR was performed using Platinum Pfx (Invitrogen, Carlsbad, CA) DNA polymerase with an annealing temperature of 55°C. The amplified 1037 bp fragment (encompassing bp 367 to 1404 of the sequence; accession number AF265226) was ligated into a prokaryotic expression plasmid (pET-23a) and used for transforming *Escherichia coli* DH5 host cells. After confirmation of the sequence by DNA sequencing, the recombinant plasmid was transformed into BL21-D3 strain host cells for expression.

Recombinant Cz (rCz) was obtained by inducing bacterial cultures with 1 mM isopropyl-L-thiol-β-D-galactoside (IPTG) for 4 h. Cells were harvested, centrifuged, and resuspended in lysis buffer containing 100 mM NaH₂PO₄, 10 mM TrisHCl, 8 M urea, 1 mM PMSF, 1 μM E-64, pH 8.0. The cells were stirred at room temperature for 60 min and then centrifuged at 10,000 x g for 20 min to pellet cellular debris.

rCz was purified under denaturing conditions by affinity chromatography using a Ni²⁺-NTA sepharose matrix. After several washes in lysis buffer pH 6.3, the protein was eluted at pH 5.5. Properly folded rCz was obtained by extensive dialysis against PBS and stored at -70°C until use. Purity assessed by SDS-PAGE was >98%. Endotoxin was removed by a column of polymyxin B agarose (Sigma, St Louis, MO). Endotoxin levels in the final protein preparations were <10 EU/mg, as determined using a *Limulus* amoebocyte lysate analysis kit (Whittaker Bioproducts, Walkersville, MD). Protein concentration was determined by Bradford (Bio-Rad, Hercules, CA), using bovine serum albumin (Sigma) as standard. A 10 μg dose of rCz was chosen for experimental immunization.

2.3. Adjuvants

CpG-ODN 1826 was used. The sequence is: 5'-TCCATGACGTTCCTGACGTT-3', with the immunostimulatory motifs underlined for clarity. CpG-ODN was synthesized under GMP conditions with a nuclease-resistant phosphorothioate backbone by Oligos Etc (Wilsonville, OR). We employed 100 µg of CpG-ODN 1826 for immunization protocol.

A pegylated derivative of the macrophage activating lipopeptide from *M. fermentans* (MALP-2) was synthesized according to established protocols [30], and 0.5 µg/dose of the active moiety was employed where indicated.

Endotoxin was undetectable (<10 EU/mg) in both adjuvant preparations, as determined by the *Limulus* test.

2.4. Immunizations and challenge

All studies were carried out in inbred female 6-8 week-old C3H/HeN mice breed at the University of Buenos Aires, using 18 animals in each group which were maintained under standard conditions. Mice were immunized with the following formulations: PBS (GI) as control; rCz-CpG-ODN i.d. on days 0, 10, 20 and 30 (GII); rCz i.d. on days 0, 10, 20, 30 (GIII); rCz i.d. on days 0 and 10, plus rCz-MALP-2 i.n. on days 20 and 30 (GIV); and rCz-MALP-2 i.n. on days 0, 10, 20, 30 (GV). Blood was collected at different times post-immunization and the sera were analyzed for the presence of specific antibodies. Two weeks after the last immunization, six mice per group were killed by cervical dislocation to analyze the acquired immune responses elicited, whereas the remaining animals were challenged intraperitoneally with bloodstream trypomastigotes. Two different experimental infection models were used in this study: (i) acute and lethal, in mice receiving 5×10^3 parasites, and (ii) sub-lethal, in mice inoculated with 50 trypomastigote forms. Parasitemia was monitored by counting peripheral parasites every 2 days in 5 µl of blood diluted 1/5 in lysis buffer (0.75% NH₄Cl, 0.2% Tris, pH 7.2) by direct microscopy examination in a Neubauer chamber. Mortality was recorded daily.

2.5. Antibody determination

Serum Cz-specific antibody titers were determined by ELISA, as previously described [22]. The amount of Cz-specific IgA present in bronchial lavages was determined as previously described [27]. To compensate for variations in the efficiency of recovery of secretory antibodies among animals, the Cz-specific IgA in each sample was normalized with the total amount of IgA present in the

lavage. Total IgA was detected by ELISA using plates coated with anti-IgA antibodies (Sigma) and purified IgA (Sigma) for the generation of the standard curve.

2.6 Delayed-type hypersensitivity (DTH) reactions

The DTH test was performed 13 days after the last immunization by i.d. challenge with 5 µg of rCz in the left footpads. The thickness of hind footpads was measured before and 48 h after the injection of the antigen with a Digital Indicator (Schwyz). Results are expressed as the difference in thickness of footpads after and before the inoculation

2.7. Proliferation assays and cytokine quantification

Spleen cells from controls and immunized mice were aseptically removed and cell suspensions were prepared. Cell proliferation was assayed as previously reported [22]. The production of cytokines in spleen cell supernatants was determined by capture ELISA (R&D System, Minneapolis, MN).

To determine the number of IFN- γ -secreting CD8⁺ T cells, splenocytes were negatively enriched by using anti CD4 beads (DynaL Biotech, Hamburg, Germany). Then, a murine IFN- γ ELISPOT kit (BD Biosciences, San Jose, CA) was used according to manufacturer's instructions. Colored spots were counted with a C.T.L. ELISPOT reader and analyzed using the ImmunoSpot image analyzer software v3.2. These results are expressed as Stimulation Index, which represents the relationship between the number of spots per well in presence of antigen and the number of spots per well with medium alone.

2.8. Measurement of muscle damage

Muscle injury was evaluated through the determination of a panel of myopathy-linked enzyme markers. Serum levels of creatine kinase (CK), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured at 100 days post infection (dpi). The assays were done by ultraviolet spectrophotometry following the specifications of the manufacturer (Wiener Lab, Buenos Aires, Argentina).

The histological features of heart and skeletal (quadriceps) muscles from vaccinated and infected mice (100 dpi) were also investigated. The removed organs were rinsed with PBS, fixed for 24 h in 10% buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and

examined by light microscopy. A blind histological test was done, analyzing ten microscopic fields in ten sections of each organ.

2. 9. Statistics

Statistical analyses were carried out with the Prisma 3.0 Software (GraphPad, San Diego, California) using one-way analysis of variance (ANOVA) for proliferation, cytokine, antibody and enzyme assays, and parasitemia data. Long rank test was used for survival curves. All the comparisons were referred to control group immunized with PBS (GI), except when indicated. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Cloning, characterization and expression of Cz

The genomic DNA from RA strain epimastigotes was extracted, a segment encompassing base pairs 367 to 1404 of the gene coding for Cz was PCR amplified and cloned in the pET23a vector. Nucleotide sequencing showed more than 98% homology with previously reported Cz gene sequences. It is important to mention that Cz is a *T. cruzi* glycoprotein encoded by members of the cruzipain gene family, which has been shown to consist of up to 130 genes [31]. Affinity chromatography with a Ni^{2+} -NTA agarose resin led to the purification of proteins with sizes of 50-60 kDa from pET23a-Cz transformed *E. coli* BL21-D3 strain (**Fig. 1A**). Although the predicted molecular weight of Cz from amino acid sequence is 36.4 kDa, it has been reported that both native Cz [32] and recombinant Cz [23] can migrate in SDS-PAGE with masses larger than those predicted by their amino acid sequences. The purified protein was recognized in Western blot by both Ni^{2+} -NTA horseradish conjugate (Ni^{2+} -NTA-HRP, QIAgen), which detects 6xHis-tagged proteins, and by polyclonal antibodies against native Cz (**Fig. 1B**). Amino acid sequencing of the eight N-terminal residues confirmed the identity of the recombinant protein with Cz.

3.2. Cz immunization elicits strong antibody responses

We assessed the stimulation of humoral immune responses by i.d. and/or i.n. administration of rCz in mice immunized with: PBS i.d. as control group (GI); [rCz-CpG-ODN] i.d. (GII); rCz i.d. (GIII); rCz i.d. prime + [rCz-MALP-2] i.n. boost (GIV); and [rCz-MALP-2] i.n. (GV). As shown in

Fig 2A, ELISA analysis of serum specimens showed that all i.d. immunized mice displayed enhanced Cz-specific IgG antibody reactivity respect to PBS-receiving controls. The highest titers were observed in GII ($6.82 \pm 0.8 \times 10^6$), followed by GIII ($4.7 \pm 0.4 \times 10^5$) and GIV ($1.3 \pm 0.3 \times 10^5$). Conversely, mice vaccinated just by i.n. route (GV) did not display significant specific IgG titers. We then evaluated whether the different vaccination regimes were able to stimulate anti-Cz mucosal immunity by determining IgA in bronchial lavages (**Fig. 2B**). Increased levels of Cz-specific sIgA could only be demonstrated in samples from i.n. immunized mice. Remarkably, we observed a stronger response of Cz-specific sIgA in mice primed by i.d. route with rCz and boosted by i.n. route with rCz-MALP-2 (GIV), as compared to mice intranasally immunized with rCz-MALP-2 (GV). This difference could be explained considering that the B cells intradermally primed with rCz switched from IgG producers, as shown in **Fig. 2A** GIII, to IgA producers, when mice were boosted intranasally with rCz-MALP-2.

3.3. Efficient cellular immune responses are stimulated by the prime-boost vaccination protocol

In order to investigate *in vivo* whether inoculation of Cz elicited cellular immunity, we performed skin tests on vaccinated mice to evaluate the delayed-type hypersensitivity developed by the immunization protocols. Only animals primed intradermally with rCz and boosted with rCz-MALP-2 by i.n. route (GIV) displayed significant DTH response ($p < 0.01$, **Fig 3A**). Additionally, lymphoproliferative assays were carried out *in vitro* to evaluate and compare the efficacy of the different vaccination protocols in inducing cell-mediated immune response. An increased cell proliferation was observed in mice vaccinated according to the prime-boost vaccination protocol (GIV), with respect to the control groups ($p < 0.01$, **Fig 3B**). Spleen cells from control mice did not proliferate in response to rCz, but responded to ConA mitogenic stimulus, as expected (data not shown).

3.4. The rCz-based prime-boost immunization protocol induces a Th1-biased response

To evaluate the effect of the vaccination strategy on the elicited T helper response, we analyzed the predominance of the IgG1 or IgG2a subclass -representative of Th2- and Th1-type immunity, respectively- within the pool of rCz-specific serum antibodies (**Fig. 4**). As previously reported by our group when native Cz was employed [22], mice immunized with rCz by i.d. route (GIII) predominantly raised an IgG1 response with lower amounts of IgG2a (IgG1: $1.4 \pm 0.3 \times 10^4$; IgG2a: $0.2 \pm 0.8 \times 10^4$). Conversely, immunization with rCz-CpG-ODN by i.d. route (GII) predominantly

raised an IgG2a dominant response (IgG1: $1.1 \pm 0.3 \times 10^4$; IgG2a: $5.9 \pm 1.4 \times 10^4$), whereas GIV presented a mixture of both isotypes with IgG2a twice as much as IgG1 (IgG1: $1.1 \pm 0.4 \times 10^4$; IgG2a: $2.6 \pm 1.5 \times 10^4$, **Fig 4**). In correspondence to what observed for total IgG (**Fig. 2**), administration of rCz-MALP-2 by i.n. route (GV) promoted negligible IgG1 and IgG2a responses (**Fig. 4**).

Cytokine profiles of rCz-vaccinated mice were also determined. To this end, we quantified by capture ELISA the concentration of IFN- γ , IL-4 and IL-10 in supernatant fluids of spleen cells (mainly CD4⁺ derived) re-stimulated in vitro with rCz. Splenocytes from mice in GII and GIV produced significantly enhanced levels of IFN- γ (812 ± 230 and 198 ± 42 pg/ml, respectively) and IL-10 (1383 ± 246 and 1027 ± 59 pg/ml, respectively; **Fig 5**), while GIII and GV mice could not be distinguished from control mice. In none of the groups, spleen cell-derived IL-4 was detected after in vitro re-stimulation with rCz. As expected, ConA induced the secretion of cytokines in all groups (data not shown).

CD8⁺ T cells seem to play a critical role in the control of *T. cruzi* infection. Accordingly, we measured the abundance of rCz-specific IFN- γ secreting CD8⁺ T cells in splenocytes depleted of CD4⁺ lymphocytes by ELISPOT assay. Except from GV immunized with rCz i.n., we found a significant increase in the frequency of antigen-specific IFN- γ -releasing cells in specimens collected from all groups with the higher number in mice from GIV ($p < 0.001$; **Fig 6**).

3.5. The prime/boost immunization protocol protects mice against *T. cruzi* infection

To determine whether the different strategies could induce immunoprotection against *T. cruzi*, 15 days after the last immunization mice were challenged with bloodstream trypomastigotes and individual parasitemia levels were assessed every other day.

When we inoculated mice with a lethal dose of live parasites, a significant ($p < 0.05$) reduction in the level of circulating trypomastigotes was recorded for GIV throughout the acute phase of infection and particularly at the peak of parasitemia ($4.9 \pm 0.9 \times 10^5$ parasites/ml in GIV vs. $1.9 \pm 0.4 \times 10^6$ parasites/ml in controls, at 14 dpi; **Fig 7A**). Most importantly, in mice immunized with the prime-boost regimen (GIV) there was an important delay in the mean time to death, with a 60% survival rate at the end of experiment (100 dpi), compared to the remaining groups that died at 14-27 dpi (**Fig 7B**).

In another experimental setting with groups of mice immunized as previously, a sub-lethal model of murine *T. cruzi* infection was established after vaccination. When we analyzed the area under the parasitemia curve in all immunization groups, we observed an important decrease in the

number of circulating parasites (2 to 4 times lower comparing with control group), with a significant ($p < 0.01$) reduction at the peak of parasitemia on day 23 (**Fig 8**). Thus, we further investigated whether the immunization regimes capable of reducing parasitemia were additionally effective in limiting tissue injury, particularly cardiac muscle damage, a pathognomonic feature of chronic Chagas' heart disease. Accordingly to the known limited parasitism in murine heart upon infection with the RA strain, we were unable to identify by microscopic examination of stained tissue sections any abnormality in the myocardial tissues from either rCz-immunized or control groups. We then analyzed a more sensitive marker of muscle damage, by measuring serum levels of the cardiomyopathy-associated enzymes (i.e., CK, LDH and AST) in infected mice at 100 dpi. Immunized and challenged mice exhibited a marked decrease of circulating enzymes compared with the levels observed in non-immunized infected controls. Although this reduction was observed in all groups, it was strikingly remarkable in mice subjected to the rCz prime-boost protocol, where a 6-7 fold-decrease respect to controls was observed (**Fig 9**). It is noteworthy that mice immunized following that regimen presented serum enzyme levels similar to basal values recorded in untreated mice (28.67 ± 10.41 , 1087 ± 240 and 8.6 ± 4.6 for CPK, LDH and AST, respectively). These results were in agreement with the histopathologic analysis of skeletal muscles, in which mice that received rCz prime-boost protocol exhibited negligible myopathy, in opposition to the inflammatory foci and fiber muscle infarction observed in the quadriceps from control animals and mice in the remaining vaccinated groups (**Table 1**).

4. Discussion

There is an increasing need for novel vaccines able to stimulate efficient and long-lasting responses for the prevention of infectious diseases. In the case of Chagas' disease, chemotherapeutic agents have limited effectiveness against chronic *T. cruzi* infection, and no prophylactic vaccines are currently available. Studies in experimental models have delineated the effector mechanisms that are essential to provide resistance to *T. cruzi* infection. Several well-defined vaccine candidate antigens are known to elicit partially protective immunity against this protozoan parasite. These include Cz, the major cysteine proteinase of *T. cruzi* [22]. This enzyme is expressed in all strains and developmental forms of the parasite (epimastigote, amastigote and trypomastigote), it is accumulated in lysosomes, is secreted and it is also present at surface level, being able to hydrolyse IgG [33]. Moreover, specific enzyme inhibitors interfere with cell invasion and *T. cruzi* replication [34]. In vitro, it has been shown that Cz is capable of modulating the immune response towards a type-2 profile by increasing IL-10 and TGF- β secretion, while simultaneously decreasing the production of

IL-12 [35]. In vivo it has been shown that the administration of IL12 and anti-IL4 is able to modulate the profile induced by Cz from predominantly a Th2/3 to a Th1, and this switch leads to control the infection [23]. Nevertheless, the administration of interleukins or antibodies against them is not a useful approach for the development of human vaccines. We previously demonstrated that native Cz co-administered with ODN-CpG, was able to confer protection against a lethal challenge with trypanomastigotes [22]; but purification of native Cz is too laborious and costly, requires complex manufacturing, and the resulting product is considered unstable and too-expensive for vaccine development. Here, we were able to overcome this problem by the highly efficient expression and purification of rCz (13.5 mg per liter of bacterial culture).

In parallel with the attempts to identify vaccine candidates, further efforts are being focused on the identification of efficient antigen delivery systems, adjuvants and vaccination regimens to enhance the protective responses to defined immunogens. In the present study, we investigated whether the immunoprotective response previously reported by us using native Cz could be achieved with rCz. We also evaluated if the elicited responses can be modulated and broadened by the use of different adjuvants and inoculation routes. Finally, we assessed a protocol based on i.d. priming with rCz and boosting with protein co-administered intranasally with the TLR2/6 agonist MALP-2. This vaccination regimen is known to stimulate both Th and CTL activities, as a result of the i.d. priming, and humoral and mucosal responses, on account of the i.n. boost [36].

The characterization of the immune responses obtained upon Cz-based vaccination demonstrated that i.d. immunization with rCz or rCz + CpG-ODN, but not rCz co-administered intranasally with MALP-2, clearly induced a strong Cz-specific serum IgG response. Nevertheless, mice receiving the i.d. prime and i.n. boost protocol still showed significant IgG titers at systemic level and, more importantly, also a strong mucosal response in terms of sIgA, which was not seen after i.d. immunization. The IgG isotype titers were also dependent on the adjuvant and inoculation route. Immunization with rCz by i.d. route resulted in high IgG1 titers, and low amounts of IgG2a, whereas the inverse situation was seen in mice immunized with rCz-CpG by i.d. route. The i.n. boost with rCz-MALP after i.d. inoculation of rCz was able to increase the levels of IgG2a over IgG1, suggesting a switch from a Th2- to a Th1-like profile that was confirmed by the IFN- γ levels.

Elicitation of strong systemic and mucosal anti-*T. cruzi* immunity, as that evidenced for the rCz prime-boost immunization protocol, represents one of the main goals pursued by the majority of experimental vaccine approaches against this human pathogen. The prime-boost vaccination regimen utilized in our experimental model was capable of raising a robust cell-mediated immunity, characterized by vigorous lymphoproliferation and DTH reactivity, as well as enhanced production of IFN- γ , with increased number of IFN- γ -producing T cells (likely from the CD8⁺ subpopulation),

counterbalanced by a significant release of IL-10. A similar Th1-associated cytokine pattern was observed for mice receiving rCz plus CpG-ODN. However, although an early onset of the IFN- γ -predominant Th1 response is critical for the resistance against *T. cruzi* infection, its IL-10-mediated down-regulation [37,38] is also of great relevance to avoid inflammatory disorders. Most importantly, upon an otherwise lethal challenge with bloodstream trypomastigotes, only mice subjected to prime-boost immunization presented a marked reduction in the level of circulating parasites and survived the infection. This result indicates that a Cz-based vaccine administered following a prime-boost protocol is able to confer immunoprotection against murine *T. cruzi* infection.

With the aim of being more close to what happens in humans, where most patients overcome the acute phase of infection, we decided to further evaluate Cz-dependent resistance to a sub-lethal inoculum of trypomastigotes. Mice vaccinated with rCz alone or combined with diverse adjuvants, by either i.d. or i.n. route, displayed humoral and cellular specific immune responses efficient enough to achieve reduction of parasitemia levels. Moreover, the triggered immune responses were capable of restricting the generation of tissue pathology at the chronic stage of infection. Since *T. cruzi* RA strain is known to produce limited cardiac parasitism in chronic murine infection [39], we analyzed more sensitive and broader markers of muscle damage, by measuring the serum levels of the myopathy-associated enzymes CK, LDH and AST. Our rationale for choosing these enzymes was their abundant distribution in heart and muscles, and their increased serum activity in chronically infected hosts showing inflammatory damage [40]. We found significantly reduced enzyme activity in mice vaccinated using distinct immunizing strategies respect to non-vaccinated and infected animals. Remarkably, mice under the prime-boost regimen presented values as low as those detected in naive mice under basal conditions. Accordingly, rCz-vaccinated animals from this group presented no inflammatory infiltrates in their striated muscles. Encouraging results have also been recently reported by our group using *Salmonella*-mediated delivery of a Cz-based DNA vaccine [41]. However, it would be difficult to implement such approach in the field. The existence of pre-existing immunity to the carrier in the Chagas' disease endemic area, which could in turn affect the overall efficacy of the intervention, may represent a roadblock for mass implementation of a prophylactic vaccine. On the other hand, the potential risk of integration of DNA vaccines should be carefully evaluated in terms of cost-benefit, particularly for an intervention aimed at the pediatric population. Therefore, a simple and robust approach, such as the one described here, which is based on the use of a recombinant protein and a synthetic adjuvant with a well-defined molecular target appeals as easier to implement and more promising than DNA vaccines.

Taken together, our findings indicate that prime-boost immunization with rCz favors the elimination of blood and tissue parasites, consequently precluding Chagas pathogenesis. Given the parasites' ability to evade immune detection and survive long-term in an immunocompetent host, it is unlikely that anti-*T. cruzi* vaccines would be effective in preventing infection or in providing sterilizing immunity. It is, however, important that the Cz-based vaccination following a prime-boost protocol was capable of eliciting immune responses able to keep the parasite burden below a threshold. This could in turn be effective in eliminating the parasite-mediated immune activation and tissue injury, thereby arresting the progression of *T. cruzi* infection to Chagas' disease. On the other hand, the presence of limited number of parasites might act as a booster during primary infection, thereby increasing the overall effectiveness of the vaccination strategy (i.e., immune responses against vaccine components and additional antigens might be triggered under controlled conditions). We consider that our evaluation of the Cz route delivery, immunization regimens, and the role of adjuvants in eliciting a maximal protective immunity in mice contributes to provide an impetus for future developments which could be exploited to protect infants from endemic areas, and may also alleviate or prevent the pathogenic responses of chronic Chagas' disease.

Acknowledgments

Financial support was received from: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); University of Buenos Aires and Agencia Nacional de Investigaciones Científicas y Técnicas (PICT 12217), Argentina (ELM). SIC was in part supported by a fellowship from the German Academic Exchange Service (DAAD).

References

- [1] Tropical disease research. In: Twelfth Programme Report of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease (TDR). Geneva, Switzerland: World Health Organization; 1999.
- [2] Moncayo A. Progress towards interruption of transmission of Chagas disease. Mem Inst Oswaldo Cruz 1999;94 Suppl 1:401-4.
- [3] Andrade ZA. Pathogenesis of Chagas' disease. Res Immunol 1991;142(2):126-9.
- [4] Bestetti RB, Muccillo G. Clinical course of Chagas' heart disease: a comparison with dilated cardiomyopathy. Int J Cardiol 1997;60(2):187-93.
- [5] Rassi A Jr, Rassi A, Little WC. Chagas' heart disease. Clin Cardiol 2000;23(12):883-9.

- [6] Araujo FG. Development of resistance to *Trypanosoma cruzi* in mice depends on a viable population of L3T4⁺ (CD4⁺) T lymphocytes. *Infect Immun* 1989;57(7):2246-8.
- [7] Tarleton RL. Depletion of CD8⁺ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. *J Immunol* 1990;144(2):717-24.
- [8] Tarleton RL, Sun J, Zhang L, Postan M. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infect Immun* 1994;62(5):1820-9.
- [9] Rottenberg ME, Riarte A, Sporrang L, Altchek J, Petray P, Ruiz AM, et al. Outcome of infection with different strains of *Trypanosoma cruzi* in mice lacking CD4 and/or CD8. *Immunol Lett* 1995;45(1-2):53-60.
- [10] Tarleton RL, Zhang L, Downs MO. "Autoimmune rejection" of neonatal heart transplants in experimental Chagas disease is a parasite-specific response to infected host tissue. *Proc Natl Acad Sci USA* 1997;94(8):3932-7.
- [11] Hoft DF, Lynch RG, Kirchhoff LV. Kinetic analysis of antigen-specific immune responses in resistant and susceptible mice during infection with *Trypanosoma cruzi*. *J Immunol* 1993;151(12):7038-47.
- [12] Humphrey JS, McCormick TS, Rowland EC. Parasite antigen-induced IFN- γ and IL-4 production by cells from pathopermissive and pathoresistant strains of mice infected with *Trypanosoma cruzi*. *J Parasitol* 1997;83(3):533-6.
- [13] Kumar S, Tarleton RL. Antigen-specific Th1 but not Th2 cells provide protection from lethal *Trypanosoma cruzi* infection in mice. *J Immunol* 2001;166(7):4596-603.
- [14] Munoz-Fernandez MA, Fernandez MA, Fresno M. Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-alpha and IFN-gamma through a nitric oxide-dependent mechanism. *Immunol Lett* 1992;33(1):35-40.
- [15] Kierszenbaum F, Howard JG. Mechanisms of resistance against experimental *Trypanosoma cruzi* infection: the importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. *J Immunol* 1976;116(5):1208-11.
- [16] Lima-Martins MV, Sanchez GA, Krettli AU, Brener Z. Antibody-dependent cell cytotoxicity against *Trypanosoma cruzi* is only mediated by protective antibodies. *Parasite Immunol* 1985;7(4):367-76.
- [17] 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* 2000;68(9):4986-91.

- [18] Hoft DF, Farrar PL, Kratz-Owens K, Shaffer D. Gastric invasion by *Trypanosoma cruzi* and induction of protective mucosal immune responses. *Infect Immun* 1996;64(9):3800-10.
- [19] Brandariz S., Schijman A, Vigliano C, Arteman P, Viotti R, Beldjord C, et al. 1995. Detection of parasite DNA in Chagas' heart disease. *Lancet* 1995;346(8986):1370-1.
- [20] Tarleton RL, Zhang L. Chagas disease etiology: autoimmunity or parasite persistence? *Parasitol Today* 1999;15(3):94-9.
- [21] Schnorrer P, Behrens GM, Wilson NS, Pooley JL, Smith CM, El-Sukkari D, et al. The dominant role of CD8⁺ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc Natl Acad Sci USA* 2006;103(28):10729-34.
- [22] Frank FM, Petray PB, Cazorla SI, Munoz MC, Corral RS, Malchiodi EL. Use of a purified *Trypanosoma cruzi* antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypomastigotes. *Vaccine* 2003;22(1):77-86.
- [23] Schnapp AR, Eickhoff CS, Sizemore D, Curtiss R3rd, Hoft D.F. Cruzipain induces both mucosal and systemic protection against *Trypanosoma cruzi* in mice. *Infect Immun* 2002;70(9):5065-74.
- [24] Krieg, AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995;374(6522):546-9.
- [25] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 1997;186(10):1623-31.
- [26] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000;408(6813):740-5.
- [27] Rharbaoui F, Drabner B, Borsutzky S, Winckler U, Morr M, Ensoli B, Muhlradt PF, Guzman CA. The *Mycoplasma*-derived lipopeptide MALP-2 is a potent mucosal adjuvant. *Eur J Immunol* 2002;32(10):2857-65.
- [28] Borsutzky S, Kretschmer K, Becker PD, Muhlradt PF, Kirschning CJ, Weiss S, Guzman CA. The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. *J Immunol* 2005;174(10):6308-13.
- [29] Chiari E, Camargo EP. Culturing and cloning of *Trypanosoma cruzi*. In: Morel CM, editor. *Genes and antigens of parasites*. Rio de Janeiro, Fundação Oswaldo Cruz, 1984: 23-6.
- [30] Mührladt PF, Kieß M, Meyer H, Süßmuth R, Jung G. Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from *Mycoplasma fermentans* acting at picomolar concentration, *J Exp Med* 1997;185(11):1951-58.
- [31] Campetella O, Henriksson J, Aslund L, Frasc AC, Pettersson U, Cazzulo JJ. The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is encoded by multiple polymorphic

- tandemly organized genes located on different chromosomes. *Mol Biochem Parasitol* 1992;50(2):225-34.
- [32] Martinez J, Cazzulo JJ. Anomalous electrophoretic behaviour of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* in relation to its apparent molecular mass. *FEMS Microbiol Lett* 1992;74(2-3):225-9.
 - [33] Berasain P, Carmona C, Frangione B, Cazzulo JJ, Goni F. Specific cleavage sites on human IgG subclasses by cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*. *Mol Biochem Parasitol* 2003;130(1):23-9.
 - [34] Cazzulo JJ, Stoka V, Turk V. The major cysteine proteinase of *Trypanosoma cruzi*: a valid target for chemotherapy of Chagas disease. *Curr Pharm Des* 2001;7(12):1143-56.
 - [35] Stempin C, Giordanengo L, Gea S, Cerban F. Alternative activation and increase of *Trypanosoma cruzi* survival in murine macrophages stimulated by cruzipain, a parasite antigen. *J Leukoc Biol* 2002;72(4):727-34.
 - [36] Borsutzky S, Ebensen T, Link C, Becker PD, Fiorelli V, Cafaro A, Ensoli B, Guzman CA. Efficient systemic and mucosal responses against the HIV-1 Tat protein by prime/boost vaccination using the lipopeptide MALP-2 as adjuvant. *Vaccine* 2006;24(12):2049-56.
 - [37] Dennis VA, Jefferson A, Singh SR, Ganapamo F, Philipp MT. Interleukin-10 anti-inflammatory response to *Borrelia burgdorferi*, the agent of Lyme disease: a possible role for suppressors of cytokine signaling 1 and 3. *Infect Immun* 2006;74(10):5780-9.
 - [38] Rajasingh J, Bord E, Luedemann C, Asai J, Hamada H, Thorne T, et al. IL-10-induced TNF- α mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression. *FASEB J* 2006;20(12):2112-4.
 - [39] Tekiel VS, Mirkin GA, Gonzalez Cappa SM. Chagas' disease: reactivity against homologous tissues induced by different strains of *Trypanosoma cruzi*. *Parasitology* 1997;115(Pt 5):495-502.
 - [40] de Souza AP, Olivieri BP, de Castro SL, Araújo-Jorge TC. Enzymatic markers of heart lesion in mice infected with *Trypanosoma cruzi* and submitted to benznidazole chemotherapy. *Parasitol Res* 2000;86(10):800-8.
 - [41] Cazorla SI, Frank FM, Becker P, Sartori MJ, Corral RS, Malchiodi EL, Guzman CA. Oral vaccination with *Salmonella* as cruzipain-DNA delivery system confers protective immunity against *T. cruzi*. *Infect Immun* 2008;76(1):324-33.

Legends

Fig. 1. Cloning and expression of cruzipain. (A) SDS-PAGE of rCz purified under denaturing conditions by affinity chromatography using Ni²⁺-NTA agarose. Lane 1, supernatant of bacterial lysate; lane 2, eluted rCz. (B) Immunoblots of refolded rCz incubated with either Ni-NTA-HRP (lane 1) or a murine polyclonal serum anti-nCz (lane 2).

Fig. 2. Specific antibody responses in Cz-vaccinated C3H mice. Mice were immunized with PBS as control (GI); [rCz-CpG] i.d.(GII); rCz i.d.(GIII); rCz i.d. prime + [rCz-MALP-2] i.n. boost (GIV); or rCz-MALP-2 i.n (GV). Two weeks after the last immunization, sera were assayed by ELISA for the presence of Cz-specific IgG, and bronchial lavages were analyzed by ELISA for Cz-specific secretory IgA. (A) Antigen-specific serum IgG titers. (B) Antigen-specific sIgA titers. Results are expressed as Cz-specific sIgA titers per µg of total IgA. The results presented are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 3. Cellular immune responses in vaccinated mice. (A) Delayed-type hypersensitivity tests in immunized animals on day 13 after the last immunizing dose. The thickness of footpads was measured before and 48 h after the inoculation of 5 µg of rCz. Results are expressed as the differences between the values of measurements performed after and before inoculation. (B) Proliferative response of spleen cells from immunized mice after 4 days of *in vitro* restimulation with rCz. Results are expressed as the difference between cpm values obtained from stimulated and non stimulated cultures. Groups are as in Fig. 2. Each bar represents the group mean $\pm$ SEM. The results are representative of three independent experiments. ** $p < 0.01$.

Fig. 4. Analysis of Cz-specific IgG1 and IgG2a isotypes in vaccinated animals. Mice were immunized by different routes with rCz, alone or co-administered with different adjuvants, as indicated in Fig. 2. Two weeks after the last immunization, blood samples were collected and sera were assayed by ELISA for the presence of different subclasses of Cz-specific IgG antibodies. Each bar represents the group mean (n=6) $\pm$ SEM. The presented results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 5. Cytokine responses of spleen cells from Cz-immunized mice. Spleen cells from immunized mice were harvested 2 weeks after the last immunization and cultured by duplicate in the presence of rCz (10 µg/ml). Supernatants were collected 48 h later and assayed in duplicate by capture ELISA for (A) IFN- γ and (B) IL-10. Groups are as in Fig. 2. ** $p < 0.001$ and *** $p < 0.001$.

Fig. 6. Detection of IFN- γ -secreting CD8⁺ T cells. Spleen CD8⁺ T cells-enriched preparation recovered from Cz-immunized mice were incubated for 16 h in the presence of rCz, and the number of IFN- γ -producing cells was determined by ELISPOT. Results (mean $\pm$ SEM of triplicate wells) expressed as stimulation index, represent the ratio between the number of spots/well in the presence of antigen and the number of spots/well in nonstimulated cultures. Groups are as in Fig. 2. Three independent experiments were performed. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 7. Parasitemia levels and survival curve in Cz-immunized and *T. cruzi*-challenged mice. Animals were challenged with 5×10^3 trypomastigotes of the *T. cruzi* RA strain 15 days after the boost. Groups are as in Fig. 2. **(A)** Parasitemia was monitored by counting the number of parasites in 5 μ l of fresh blood collected from tail vein every 2 or 3 days. **(B)** Mortality was recorded every day. The results are representative of three independent experiments.

Fig. 8. Parasitemia levels in Cz-immunized mice sublethally infected with *T. cruzi*. Animals were challenged with 50 trypomastigotes of the *T. cruzi* RA strain 15 days after the boost. Parasitemia was determined as indicated in the Methods section. Groups are as in Fig. 2. The results are representative of three independent experiments.

Fig. 9. Serum levels of myopathy-linked enzyme markers from immunized mice after challenge. Blood was collected 100 dpi from mice immunized and infected with 50 trypomastigotes and assays were performed to determine the levels of CK **(A)**, LDH **(B)** and AST **(C)** by ultraviolet spectrophotometry. The bars represent the average of 6 determinations, and the SEM is indicated by vertical lines. Groups are as in Fig. 2. The results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

TABLE 1. Score of skeletal muscle damage from vaccinated mice after a sublethal *T. cruzi* challenge.

Groups	Inflammatory foci	Fiber muscle infarction
PBS	++++	+++
rCz-CpG i.d.	++	+
rCz i.d.	+++	+
rCz i.d. + rCz-MALP i.n.	+	-
rCz-MALP i.n.	+++	++

Groups are as in Fig. 2. Tissue observations were arbitrarily scored on a gradual scale from (-) to (++++), representing normal and maximal injury, respectively.

A

B

Parasites/ml Blood ($\times 10^5$)

Days postinfection