

Redirection of the Immune Response to the Functional Catalytic Domain of the Cystein Proteinase Cruzipain Improves Protective Immunity against *Trypanosoma cruzi* Infection

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q3 **Despite the strong immune responses elicited after natural infection with *Trypanosoma cruzi* or vaccination against it, parasite survival suggests that these responses are insufficient or inherently inadequate. *T. cruzi* contains a major cystein proteinase, cruzipain, which has a catalytic N-terminal domain and a C-terminal extension. Immunizations that employed recombinant cruzipain or its N- and C-terminal domains allowed evaluation of the ability of cruzipain to circumvent responses against the catalytic domain. This phenomenon is not a property of the parasite but of cruzipain itself, because recombinant cruzipain triggers a response similar to that of cruzipain during natural or experimental infection. cruzipain is not the only antigen with a highly immunogenic region of unknown function that somehow protects an essential domain for parasite survival. However, our studies show that this can be reverted by using the N-terminal domain as a tailored immunogen able to redirect host responses to provide enhanced protection.**

Several studies of Chagas disease vaccines have identified antigens, immunization routes, adjuvants, and immune mechanisms that are protective against *Trypanosoma cruzi* infection [1, 2]. However, the immunity

granted by all the vaccination regimens tested thus far was unable to prevent infection. Despite strong immune responses triggered by most of these vaccination approaches, parasite survival suggests that, similarly to what happens in natural infection, the elicited immune response is either insufficient or inherently inadequate to confer protection. Thus, a better understanding of host-parasite relationship and parasite escape mechanisms, as well as a deeper knowledge of the immune reactions triggered during natural infections, are needed to design more effective vaccines.

T. cruzi contains a major lysosomal cystein proteinase called cruzipain. This enzyme exhibits properties that make it an attractive antigen for vaccine development [3–11]. Cruzipain presents a catalytic domain with high homology to enzymes belonging to the papain family and a C-terminal domain that, in contrast to other enzymes of this type, is retained in the mature protein [12–15]. Although the role of the C-terminal domain

Received 25 September 2009; accepted 7 January 2010; electronically published XX May 2010.

Potential conflicts of interest: none reported.

Financial support: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), University of Buenos Aires and Agencia Nacional de Investigaciones Científicas y Técnicas (grant PICT 608 to ELM), Argentina. ELM is also supported by the Fogarty International Center (FIRCA; grant 5R03TW007972–02), USA. SIC was in part supported by a fellowship from the German Academic Exchange Service (DAAD).

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The Journal of Infectious Diseases 2010;201(12):000–000

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0022-1899/2010/20112-00XX\$15.00

DOI: 10.1093/infdis/jiq287

is not well understood, it may enhance the catalytic efficiency of cruzipain [16], and it is also the major immunogenic part of cruzipain in naturally infected humans [17, 18].

We herein demonstrated that the presence of the C-terminal domain allows cruzipain to circumvent the immune response to the catalytic N-terminal domain. This phenomenon is not a property of the parasite but of the molecule itself, because inoculation of the full-length recombinant cruzipain is able to produce a similar effect to the specific immune response against cruzipain in natural or experimental infection. Once the C-terminal domain is not present, the immune system is able to mount a stronger and more efficient response that confers improved protection by greatly reducing the parasite load after challenge and by diminishing tissue damage.

MATERIALS AND METHODS

Full-length N- and C-terminal cruzipain. Genomic DNA from *T. cruzi* epimastigotes was used as a template for polymerase chain reaction amplification. The amplified 644 base pair (bp) (215 residues, from the N-terminal) and 392 bp (130 residues, from the C-terminal) fragments were digested and ligated into pET-23a. The recombinant plasmids were transformed into BL21-D3 strain for expression. Proteins were purified using a Ni²⁺-NTA sepharose. Properly folded proteins were obtained by dialysis. Purity assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was >98%. Native cruzipain was obtained from epimastigotes [9, 19], and recombinant cruzipain was produced as described elsewhere [10]. The folded proteins were separated by electrophoresis, blotted, and incubated with either horseradish peroxidase-conjugated Ni²⁺-NTA or a polyclonal antibody against cruzipain. Endotoxin was removed by polymyxin B-agarose. Endotoxins in the proteins were <10 U/mg.

Immunizations and challenge. Experiments that used animals were approved by the Review Board of Ethics of the Instituto de Estudios de la Inmunidad Humoral (IDEHU) and conducted in accordance with the guidelines established by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

Inbred female 6–8-week old C3H/HeN (H-2^K haplotype) mice were vaccinated and boosted 15 days later with 10 μg of the corresponding antigens coadministered with 100 μg of CpG-ODN [9–11] by bilateral injection in the quadriceps as follows: GI: PBS plus CpG-ODN; GII: N-terminal domain plus CpG-ODN; GIII: C-terminal domain plus CpG-ODN; and GIV: recombinant cruzipain plus CpG-ODN. Two weeks after the last vaccine administration, half of the mice were killed by cervical dislocation, and their spleens were removed aseptically, whereas the remaining animals were challenged with 50 blood-stream trypomastigotes (RA strain).

Delayed-type hypersensitivity reactions. The delayed-type

hypersensitivity (DTH) test was performed 13 days after the last vaccine administration by intradermal challenge with 5 μg of recombinant cruzipain, N- or C-terminal domain. The thickness of both hind footpads was measured before injection and 48 h after the injection of the antigens.

Peptide synthesis. We synthesized 3 peptide sequences on the basis of their ranks in the Proped, Rankpep, and MAPP databases: pep2, SGHTVGATI (residues 226–234); pep3, SEQLDHGVLLV (279–289); and pep4, KEEASSAVV (328–336). A nonrelated peptide was used as control.

In vitro CD8⁺ T cell-mediated cytotoxicity assay. Spleen cells from immunized mice were isolated 15 days after the last boost with recombinant cruzipain or the N-terminal domain, pooled, and restimulated in vitro with 10 μg/mL recombinant cruzipain for 5 days. Then, splenocytes were depleted of CD4⁺ T cells by incubation with the supernatant of an anti-CD4 T cell hybridoma (clone GK1.5), followed by complement lysis. Peritoneal cells were removed from naive mice and pulsed with 5 μmol/L peptides. The cells were then labeled with 1 μmol/L of carboxyfluorescein succinimidyl ester (CFSE). Effector and target cells were mixed at a 20:1 ratio and incubated for 4 h, and 5 μg/mL propidium iodide was added to labeled lysed cells. Fluorescence-activated cell sorting, or FACS, analysis was performed using a Partec flow cytometer. Results are expressed as a lysis ratio using the following formula:

$$\frac{\%CFSE^{+}PI_{pept}^{+}/\%CFSE_{pept}^{+}}{\%CFSE^{+}PI_{none}^{+}/\%CFSE_{none}^{+}}$$

Cell invasion assay. In vitro *T. cruzi* infection was performed with the nonphagocytic Vero cell line. Irradiated cells were infected with trypomastigotes at a parasite-cell ratio 10:1. Before the infection experiments, trypomastigotes were incubated by triplicate for 10 min at room temperature with diluted (1:50) serum from mice belonging to each immunization group. Controls included uninfected Vero cells (0% infection control) and cells infected with trypomastigotes (100% infection control). After 2 h of coculture, plates were washed to remove unbound parasites, and 1 μCi of [³H]-thymidine was added. The counts per minute (cpm) were measured 48 h later, and infection percentages were calculated as follows:

$$\frac{\text{Each well (cpm)} - 0\% \text{ control well (cpm)}}{100\% \text{ control well (cpm)}} \times 100.$$

Complement-mediated killing of parasites. Trypomastigotes were incubated by triplicate with serum collected from immunized and challenged mice. Incubation was performed in 200 μL of normal donor fresh serum. Then, the number of living parasites was counted by using a Neubauer chamber.

Measurement of muscle damage. Muscle injury was evaluated at 100 days after infection by determining the serum

levels of the myopathy-linked enzyme markers creatine kinase and lactate dehydrogenase (LDH). The histological features of heart and quadriceps from vaccinated and infected mice were also investigated (100 days after infection). A blind histological test was performed, and tissue observations were scored on the basis of previous studies [20].

Statistics. Statistical analyses were performed with Prisma software (version 3.0; Prisma Software), using 1-way analysis of variance and a Bonferroni posttest. The long rank test was used for survival curves. $P < 0.05$ was considered significant.

RESULTS

Characterization of recombinant proteins. Cruzipain and its N- and C-terminal domains were expressed in *Escherichia coli*, purified with Ni^{2+} -NTA, and refolded by dialysis. The purified proteins, with sizes of 48–60, 34–32, and 26–24 kDa, corresponding to cruzipain and the N- and C-terminal domains, respectively, were recognized in Western blot by both horseradish peroxidase-conjugated Ni^{2+} -NTA and polyclonal antibodies against n-cruzipain (Figure 1A). Determination of enzyme activity in SDS-PAGE gels containing a copolymerized gelatin substrate [21] demonstrated that the recombinant full-length cruzipain is an active enzyme, whereas the domains are not active (Figure 1B). These results indicated that (1) it is possible to independently express both the N- and C-terminal domains in prokaryotic cells, (2) the domains have an independent folding, and (3) the enzymatic activity is only preserved in the recombinant full-length protein, because it is lost in the N- and C-terminal domains.

Antibody response. Groups of mice were inoculated with recombinant cruzipain or its domains, combined with CpG-ODN. Two weeks after the last vaccine administration, the specific IgG antibody response was measured by coating enzyme-linked immunosorbent assay (ELISA) plates with either C-terminal or N-terminal fragments. Antibodies in mice immunized with the full-length recombinant cruzipain were directed to the C-terminal domain, with only negligible antibody reactivity against the N-terminal portion (Figure 2A). To compare the level of antibodies elicited by immunization with the recombinant cruzipain and the domains, the full-length protein was immobilized. The highest titer was observed in mice vaccinated with the C-terminal domain and a slightly lower titer was observed in mice immunized with full-length recombinant cruzipain. In contrast, mice immunized with the N-terminal domain presented a low antibody titer against recombinant cruzipain (Figure 2B). In all the immunized groups, a predominant IgG2a response with low IgG1 titers (Figure 2C) was observed. This isotype profile is consistent with the well-known ability of CpG-ODN to drive immunity toward a T-helper 1 (Th1) response pattern.

Overall, these results suggest that the major cysteine protease

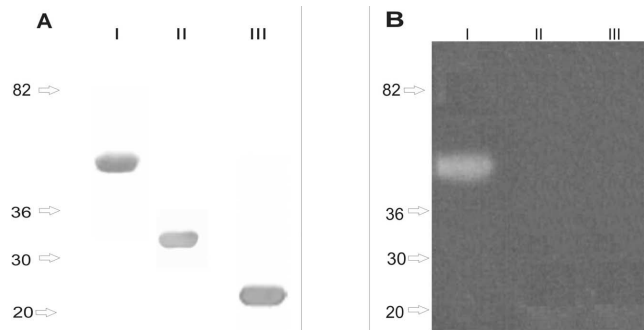


Figure 1. A, Immunoblots of recombinant cruzipain (I), its N-terminal domain (II), and its C-terminal domain (III). B, Corresponding results obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis containing 0.2 % gelatin.

of *T. cruzi* displays immunodominant B-cell determinants at the C-terminal domain but not at the N-terminal domain, where the essential catalytic activity for parasite survival is located.

Cellular response. To investigate whether immunization with recombinant cruzipain elicited cellular immunity against the different domains, we performed skin tests on vaccinated mice. Hind footpad thickness was measured before and 48 h after the intradermal injection of 5 μg of the N- or C-terminal domains of cruzipain. Mice injected with the C-terminal domain displayed higher reactivity than those observed in the N-terminal domain-injected group (Figure 3A). When full-length recombinant cruzipain was inoculated, it evoked a significant DTH response in mice immunized with full-length recombinant cruzipain. Surprisingly, in contrast to the antibody response, we found a strong DTH response in mice immunized with the N-terminal domain but not in those vaccinated with the C-terminal domain, whose results were undistinguishable from control animals (Figure 3B).

The ability of cruzipain and its domains to promote an antigen-specific cellular response was also determined *ex vivo* by lymphocyte proliferation assays using recombinant cruzipain for restimulation. Similarly to results obtained *in vivo*, we observed only a vigorous lymphoproliferation in splenocytes from recombinant cruzipain and N-terminal domain-immunized mice (Figure 3C). Immunization with the C-terminal domain proved to be unable to activate antigen-specific lymphocytes. Splenocytes from mice vaccinated with the N-terminal domain, C-terminal domain, or whole recombinant cruzipain produced significantly enhanced levels of interferon γ (IFN- γ) (Figure 3D) and interleukin 10 (IL-10) (Figure 3E) with respect to control mice (phosphate-buffered saline [PBS]). However, no significant differences in the expression of IL-10 or IFN- γ were observed between animals receiving the C-terminal domain, N-terminal domain, or full-length recombinant cruzipain as immunogen.

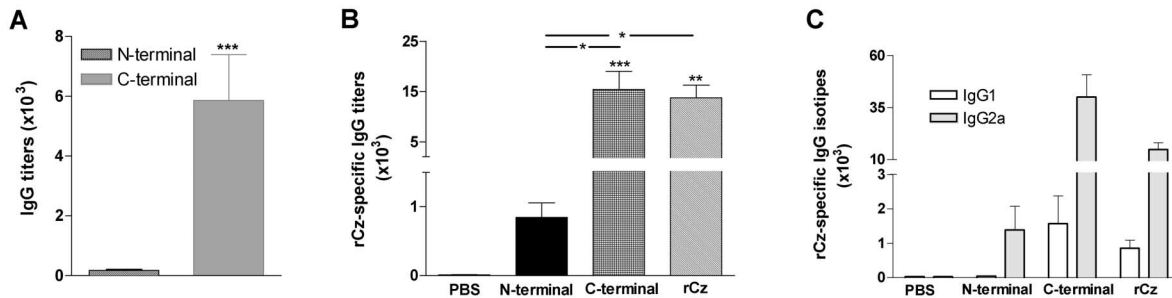


Figure 2. Antibody response in mice immunized with full-length recombinant cruzipain (rCz) or its domains: results for serum samples assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of (A) N-terminal and C-terminal specific antibodies, (B) recombinant cruzipain-specific antibodies, or (C) recombinant cruzipain-specific immunoglobulin G (IgG) isotypes. The results are representative of 3 independent experiments ($n = 6$). Stars at the top of a bar indicate comparison with the phosphate-buffered saline (PBS) group: * $P < .05$, ** $P < .01$, and *** $P < .001$.

Considering the critical role that CD8⁺ T cells play in parasite clearance [22–25], we also evaluated cytotoxic responses stimulated in N-terminal domain-immunized mice using macrophages pulsed with cruzipain peptides. The highest rate of specific lysis was observed when phagocytes loaded with the KEEASSAVV peptide were used as targets (Figure 3F). These findings enabled us to identify an MHC-I-restricted cytotoxic T cell epitope within the N-terminal domain of cruzipain.

Protection against infection. Next, *in vitro* studies were performed to assess the biological activity of the antibodies stimulated after vaccination. When trypomastigotes were incubated with serum from mice immunized with the C-terminal domain, *in vitro* infection rates were similar to those recorded for parasites treated with naive mice serum. In contrast, *T. cruzi* invasiveness was blocked in the presence of antibodies from recombinant cruzipain-vaccinated mice. This reduction was even more dramatic when parasites were incubated with serum from N-terminal domain-immunized mice (Figure 4A). Because this group displayed significantly (15 times) lower ELISA titers than those in C-terminal- and recombinant cruzipain-immunized groups (Figure 2B), antibody-mediated blocking of cell invasion seems to be dependent on the domain against which the antibody response is directed, rather than on the much enhanced antibody levels.

To determine whether the domains of cruzipain were able to induce protective immunity against *T. cruzi* infection, 2 weeks after immunization mice were sublethally challenged with trypomastigotes. A comparison of parasite loads at their peak (10 days after infection) in the 4 groups of mice shows that N-terminal domain immunization is able to strongly reduce the level of circulating parasites (Figure 4B). Conversely, mice immunized with the C-terminal domain did not show blood trypomastigote levels that were significantly different from those in the PBS control mice. Moreover, when the area under the parasite-load curves were analyzed (Figure 4C), control mice and recombinant cruzipain-immunized mice presented 300% and 100% more parasites than did N-terminal

domain-immunized mice, respectively, whereas the C-terminal domain conferred less protection, with 200% more parasites than were found in N-terminal domain-immunized mice.

We also analyzed whether antibodies in vaccinated and infected mice were able to increase the complement-mediated killing of *T. cruzi* at the time of subpatent parasite loads (100 days after infection). A significant reduction in the number of parasites became evident when treatment was accomplished with antibodies from mice vaccinated with the N-terminal domain, whereas the outcome after incubation with serum from mice vaccinated with full-length recombinant cruzipain or its C-terminal domain did not differ from the results obtained using serum from nonimmunized mice (Figure 4D).

Protection against tissue damage. We then investigated whether vaccinations were also effective in limiting tissue injury. Serum levels of the cardiomyopathy-associated enzymes creatine kinase and LDH were measured in infected mice at 100 days after infection. N-terminal-immunized and *T. cruzi*-challenged mice exhibited a markedly decreased concentration of enzymes compared with that observed in nonvaccinated infected control mice (Figure 5A), and comparable levels to those found in normal nonimmunized, uninfected mice. In contrast, the levels in C-terminal-immunized mice were indistinguishable from those in PBS control-infected mice. We were not able to identify structural abnormalities in the hearts from vaccinated or control animals. However, the skeletal muscle from mice inoculated with PBS showed a severe inflammation after the *T. cruzi* challenge, which was mainly characterized by lymphocytes with enlargement of the interstitium. Noteworthy, skeletal tissue sections from recombinant cruzipain- or C-terminal-immunized mice showed moderate lesions, thereby contrasting with the mild inflammation in N-terminal immunized mice (Figure 5B). Overall, these results indicate that immunization with the enzymatic domain of cruzipain elicits a protective immune response capable of reducing the muscle damage caused by *T. cruzi* infection.

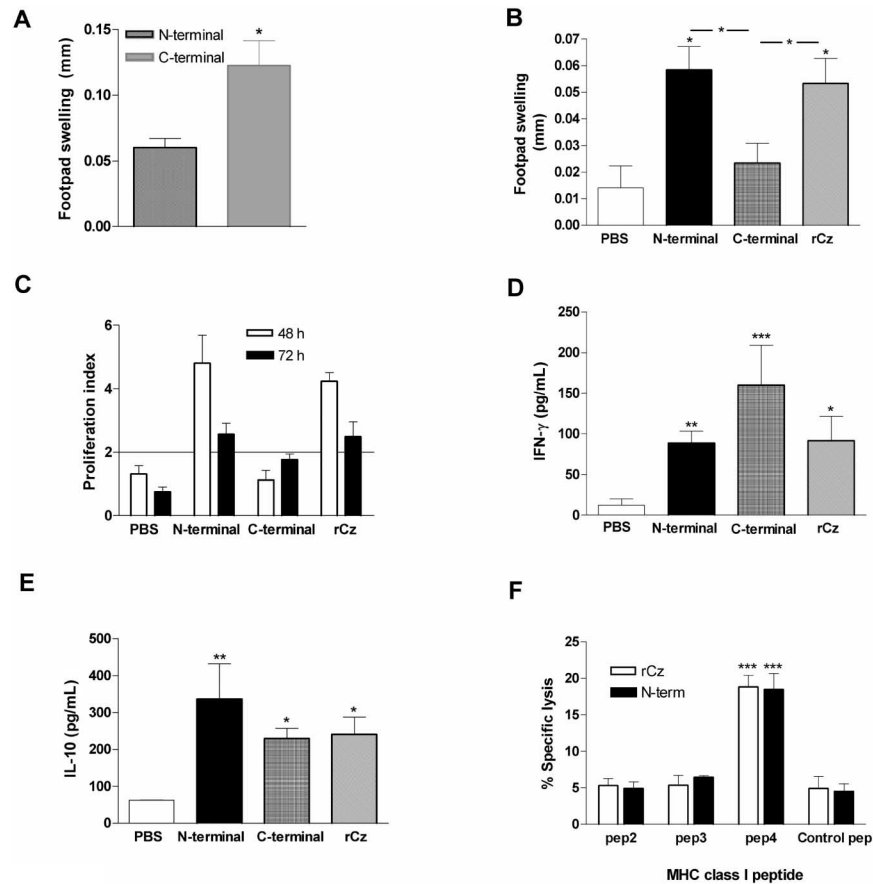


Figure 3. Cellular immune response in mice immunized with full-length recombinant cruzipain (rCz) or its domains. Delayed-type hypersensitivity tests in animals immunized with full-length recombinant cruzipain or its domains 13 days after the last vaccine dose. Footpad thickness was measured before and 48 h after the inoculation of 5 μ g of N- or C-terminal domains (A) or recombinant cruzipain (B). C, Proliferative response of spleen cells from immunized mice stimulated with 10 μ g/mL of recombinant cruzipain. Interferon γ (IFN- γ) (D) and interleukin 10 (IL-10) (E), secreted by splenocytes from mice immunized with full-length recombinant cruzipain or its domains in the presence of recombinant cruzipain (10 μ g/mL). F, In vitro CD8⁺ T cell-mediated cytotoxicity. The results are representative of 3 independent experiments ($n = 6$). The line at the top of the bars indicates comparison between the 2 groups. * $P < .05$, ** $P < .01$, and *** $P < .001$.

DISCUSSION

By analyzing the antibody and cellular immune responses elicited by immunization with full-length recombinant cruzipain or its N- and C-terminal domains, we were able to dissect the ability of a key parasite enzyme to direct immune responses. Thus, we found that cruzipain promotes the strongest antibody and cellular immune responses against its C-terminal domain, of unknown function, somehow protecting its catalytic N-terminal domain.

Immunization with full-length recombinant cruzipain elicits a strong antibody response against the C-terminus, whereas a negligible amount of antibodies are directed against the N-terminal domain. Immunization with each domain suggests that the N-terminus displays less B-cell epitopes, because a 15-fold lower antibody response was observed, as compared with serum samples from mice vaccinated with the C-terminus. Owing to the use of a recombinant protein or the immunization

protocols, the N-terminus can be ruled an artifact, because it has been reported that most serum samples from naturally infected chagasic patients react against the C-terminus of cruzipain [17], and additional boosting of mice did not significantly enhance the ratio of N-terminal antibodies to C-terminal antibodies (results not shown). The fact that the major cysteine protease of *T. cruzi* displays immunodominant B-cell determinants restricted to the C-terminal region could be interpreted as a parasite immune escape mechanism. This would avoid antibody production against the catalytic N-terminal domain, whose activity is essential for parasite survival in the host [3]. This hypothesis is reinforced by the results showing that serum samples from N-terminal-immunized mice were able to dramatically reduce the infection of Vero cells, when compared with serum samples from C-terminal or recombinant cruzipain-vaccinated animals. This is even more significant, considering that the antibody titers against the N-terminal domain

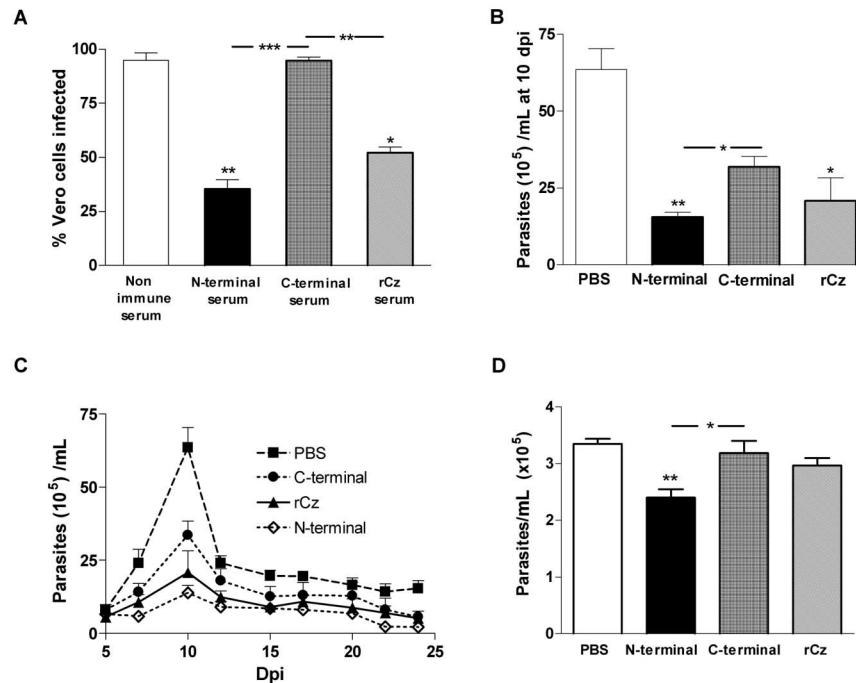


Figure 4. Results for the infection of Vero cells and mice with trypomastigotes. *A*, In vitro infection of Vero cells with trypomastigotes. Parasites were incubated with serum from mice previously immunized with phosphate-buffered saline (PBS), N-terminal, C-terminal or full-length recombinant cruzipain (rCz) before the infection of irradiated Vero cells. *B*, Parasite loads during the acute phase of *T. cruzi* infection in immunized mice. Animals ($n = 6$) were challenged 15 days after the last vaccine administration with 50 trypomastigotes (RA strain) administered by the intraperitoneal route. *C*, Number of parasites at the peak of parasite load (10 days after infection). *D*, Trypomastigote lyses by antibodies from immunized and infected mice mediated by complement. Parasites were incubated with serum from mice immunized with PBS, N-terminal, C-terminal, or full-length recombinant cruzipain. The results are representative of 3 independent experiments. The star at the top of a bar indicates comparison with the control group. The line at the top of bars indicates comparison between the 2 groups. * $P < .05$, ** $P < .01$, and *** $P < .001$.

were at least 15-fold lower than anti-recombinant cruzipain or anti-C-terminal domain titers. Consequently, these phenomena could be interpreted as a combination of a reduced number of B-cell epitopes within the N-terminal domain and/or its masking by the presence of the C-terminal domain. Alternatively, it could also be possible that the N-terminal domain has lost conformational B-cell epitopes due to an improper folding, because only linear epitopes are present. These epitopes might be subdominant during natural parasite infection but could retain high neutralizing activity, as already observed for other pathogens [26–28].

Similarly, the DTH response was poorly restimulated with the N-terminal domain in mice immunized with recombinant cruzipain, whereas most of the response was evoked by the C-terminal domain. However, the DTH response and the proliferation index were much higher when the N-terminal domain was used for immunization. A potential explanation is that, somehow, the cellular immune response can be down-regulated by full-length cruzipain to avoid the reaction against the N-terminal domain. This hypothesis is supported by the fact that recombinant cruzipain is the only molecule analyzed with enzymatic activity. However, there are T cell epitopes in the N-

terminal domain that are, indeed, able to stimulate a stronger reaction than the C-terminus when used as a single domain in immunization. These results are in agreement with those describing that peptides deriving from the C-terminal domain failed to elicit a proliferative response on cruzipain specific T cell lines from chronic patients, whereas a peptide from the N-terminus elicited a strong T cell response [18]. In C3H mice, we identified an MHC-I-restricted cytotoxic T cell epitope in the N-terminus, which is able to stimulate specific CD8⁺ T cells. However, we cannot exclude that NK cells in the CD4⁺ T cell-depleted population might have some contribution to the observed effect.

Strikingly, we found that immunization with the N-terminal domain did not raise a differential cytokine pattern. The fact that the 2 individual domains as well as the full-length recombinant cruzipain were able to induce both IFN- γ and IL-10 is consistent with a Th1 or regulator T cell mixed cytokine profile. This is surprising in view of the successful protection attained. In this sense, although a Th1 response is required for *T. cruzi* control, it is reasonable to assume a mixed Th1 or regulator T cell profile could promote protection without significant bystander tissue damage [29–31]. In fact, mice vaccinated with

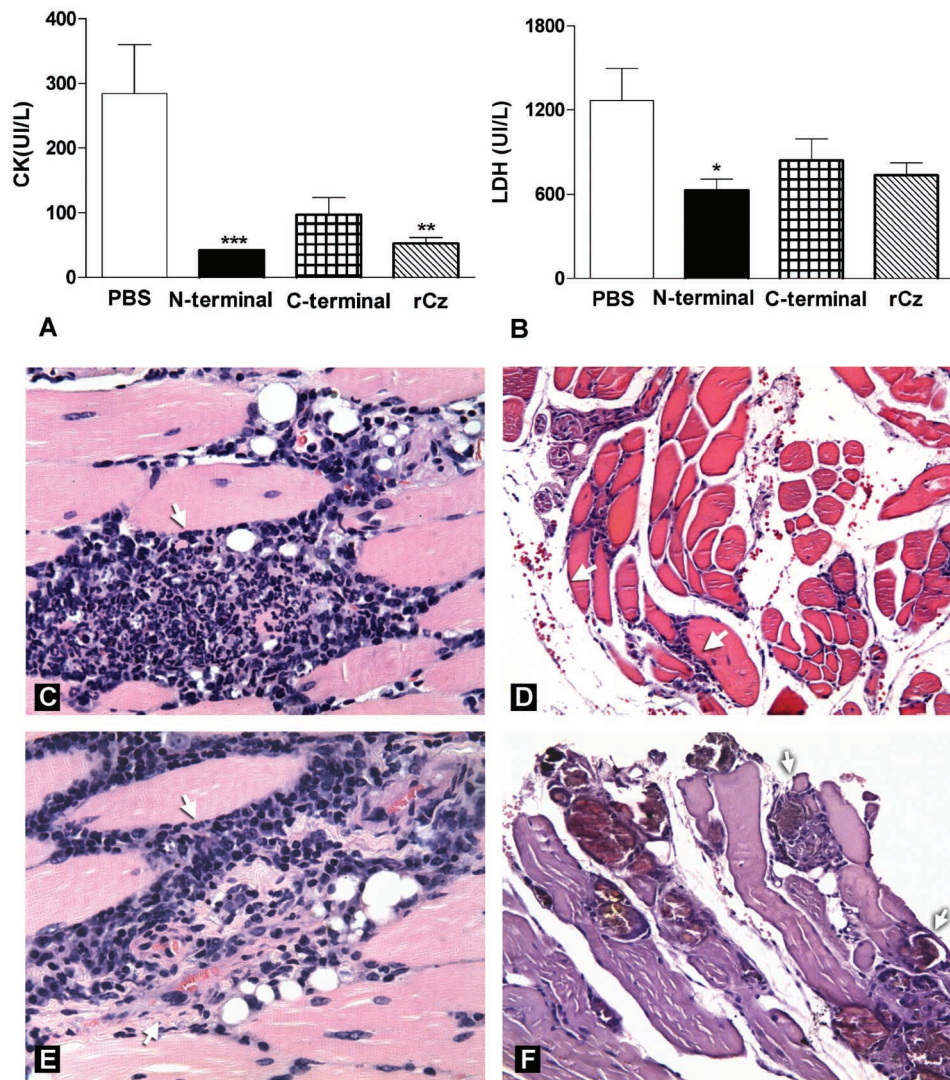


Figure 5. Tissue damage after challenge. Serum levels of creatine kinase (A) and lactate dehydrogenase (B) in immunized mice after challenge. The bars represent the group mean ($n = 6$) \pm standard error of mean. The results are representative of 3 independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$. C, Micrographs of skeletal muscle from mice inoculated with phosphate-buffered saline (PBS) and challenged shows severe inflammation, mainly characterized by lymphocytes with enlargement of the interstitium (arrow) (magnification, $\times 40$). D, The micrograph shows moderate inflammation (lower arrow) with edema (upper arrow) of the peripheral tissue (magnification, $\times 10$) in mice immunized with recombinant cruzipain. E, Mice immunized with the C-terminal domain presented moderate inflammation (upper arrow) and foci of collagen deposition (lower arrow) (magnification, $\times 40$). F, Mild inflammation (isolated inflammatory cells) with foci of calcifications (shaded arrows) was observed in N-terminal immunized mice (magnification, $\times 20$).

cruzipain N-terminal domain showed less tissue damage than mice vaccinated with the C-terminal domain. Additionally, the induction of IL-10 might have contributed to parasite clearance by favoring production of *T. cruzi*-neutralizing antibodies [32] and antibody-dependent cellular cytotoxicity [33], as well as by promoting CD14-mediated phagocytosis [34] and/or NK cell activity [35]. Thus, the better outcome could be attributed to the ability of the N-terminal domain to concurrently promote parasite-specific cytotoxic CD8⁺ T cells and antibody-mediated neutralization, together with IL-10-associated anti-inflammatory mechanisms [36, 37].

We also addressed the question of whether the immunization with the domains of cruzipain were able to protect against infection with trypomastigotes. A prominent finding of this study has been that, although immunization with the N-terminal domain elicited low cruzipain-specific antibody titers, these antibodies significantly reduced trypomastigote infection of mammalian cells, perhaps because of effective complement fixation. Inhibition was even more effective than that observed with antibodies generated after immunization with full-length recombinant cruzipain. These improved antibody effector functions, together with the secretion of IFN- γ and CD8⁺ T cell

activation, allowed N-terminal-immunized mice to control *T. cruzi* infection, resulting in significantly lower parasite loads throughout the acute phase. By contrast, immunization with the C-terminal domain generated high cruzipain-specific antibody titers that were unable to significantly reduce the number of circulating parasites with respect to control mice. Clearly, the higher antibody titer against the C-terminal domain was neither able to mediate an effective complement fixation nor to neutralize cell invasion. These results suggest that the ability of antigens to trigger vigorous immune responses against *T. cruzi* is not necessarily associated with the ability to induce protection. A strong immune response with high antibody titers, T cells and cytokine release does not mean better protection. In fact, natural *T. cruzi* infection elicits most of these immune reactions and parasites still survive for years within their hosts.

We demonstrated that full-length cruzipain in some immunization protocols (but not in other studies) induces protection against *T. cruzi* [9–11]. Here, we assessed whether the ability of the N-terminal domain to elicit a differential immune response could have a stronger impact on immunoprotection against a sublethal challenge with trypomastigotes. By comparing the parasite loads of the immunized mice, we showed that the N-terminal domain is able to confer better protection by diminishing blood parasites count at the peak of parasite loads and during the first 25 days after infection. Moreover, immunization with the N-terminal domain is able to limit the tissue injury caused by the parasite after infection when compared with that of the C-terminal domain and, most importantly, full-length cruzipain-vaccinated mice, as demonstrated by the levels of cardiomyopathy-associated enzymes and histological analysis of the affected tissues. The reduced damage observed after N-terminal domain immunization could also be related to the lack of enzymatic activity displayed by this domain or its inability to promote bystander inflammation.

The presence of additional domains or regions of unknown function as the 1 presented by cruzipain was described for other *T. cruzi* antigens. For example, the immunomodulatory portion of the *T. cruzi* Tc52 virulent factor may reside, at least in part, in a conserved sequence within its C-terminal domain, which contributes to immune dysfunction by modulating T cell and macrophage activation [38]. Similarly, DNA vaccination with ASP-2 protects ~65% of highly susceptible A/Sn mice against *T. cruzi* infection [39]. However, immunization with residues 261–500 of ASP-2 induced 100% protection [40]. In addition, the *T. cruzi* trans-sialidase uses an alternative mechanism to distract the immune response by including, on the C-terminus of the molecule, an antigenic domain (SAPA), which is made up of 12 tandemly repeated residues. These B-cell epitopes act as a diversion for the immune system to concentrate the antibody response against it, thereby preserving the enzymatic

activity of the N-terminal domain that induces an early and strong antibody response [41–43]. However, our studies showed that these mechanisms can be reverted by using optimized immunogens able to redirect host responses to provide enhanced protection.

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