RESUMEN
La enfermedad de Chagas, causada por el parásito protozoario Trypanosoma cruzi, es endémica en América Central y Sudamérica y representa la miocarditis más frecuente a nivel mundial.
El establecimiento de la infección crónica conduce a una patología cardíaca debilitante por la cual mueren más de 50,000 personas cada año. No existe consenso sobre si la causa del daño tisular es ocasionada por el parásito o está exacerbada por una respuesta autoinmune. En ambos escenarios, ha sido sugerido que cruzipaina—la principal cisteína proteasa del T. cruzi—cumple un rol importante en la progresión de la enfermedad.
Cruzipain, miembro de la superfamilia de las papaínas, se expresa como una mezcla compleja de isoformas en los diferentes estados de desarrollo de todas las cepas del parásito. Esta glicoproteína participa en la internalización del T. cruzi en las células mamíferas, lo que ha sido demostrado con inhibidores específicos de la enzima que interfieren en la invasión celular y en la replicación del parásito. Además, cruzipaina genera una fuerte respuesta immune en individuos infectados. Estas características hacen de cruzipain un potencial blanco de drogas terapéuticas.
La presente revisión resume el conocimiento actual sobre el rol de cruzipaina en la patogénesis de la enfermedad, su compromiso en la invasión de células del huésped así como su participación en la activación y evasión de la respuesta inmune en modelos experimentales y en pacientes chagásicos. El avance en esta área de investigación, proveerá nuevas estrategias terapéuticas tendientes a incrementar la respuesta inmunoprotectiva y prevenir la respuesta deletérea producida por el parásito.

PALABRAS CLAVE: Trypanosoma cruzi/ Enfermedad de Chagas/ Cruzipaina/ Patogénesis/ Autoinmunidad/ Cardiomiopatía.

ABSTRACT
The protozoan parasite Trypanosoma cruzi, etiological agent of Chagas disease, is endemic in Central and South America and produces the most common myocarditis worldwide.
Parasite persistence eventually leads to a debilitating heart disease that kills more than 50,000 people every year. There is no consensus as to whether tissue damage is caused entirely by the parasite or is exacerbated by an autoimmune response. In both models of disease progression, cruzipain—the major cysteine protease of T. cruzi—has been suggested to play an important role.
Cruzipain is a member of the papain superfamily, and it is expressed as a complex mixture of isoforms by different strains of the parasite, as well as in all its developmental stages. This parasite glycoprotein plays a role in the process of T. cruzi internalization into mammalian cells, as proved by specific enzyme inhibitors, which interfere with cell invasion and inhibit parasite replication.
In addition, cruzipain not only is essential for parasite survival but also generates a strong immune response in infected individuals. These characteristics point to cruzipain as a potential target for drug therapy and for the generation of immune responses. This review analyses our present knowledge of the role of cruzipain in the disease pathogenesis, its involvement in host cell invasion, immune activation and evasion by T. cruzi in experimental models and human infection. Ongoing studies in this research area may provide novel therapeutic strategies that could enhance the immunoprotective response while preventing the deleterious parasite elicited responses observed during Chagas disease.

KEY WORDS: Trypanosoma cruzi/ Chagas disease/ Cruzipain/ Pathogenesis/ Autoimmunity/ Cardiomyopathy.
INTRODUCTION

The American trypanosome, *Trypanosoma cruzi* is the etiological agent of Chagas disease, which is a common cause of fatal dilated cardiomyopathy. This represents an important public health burden in Latin America, with an estimated 10 million people infected, and approximately 50 million at infection risk. Most patients remain asymptomatic for decades, while a significant fraction of the affected individuals (30%) develop principally heart and gastrointestinal clinical manifestations during chronic Chagas disease. Among the possible mechanisms responsible for the pathogenesis of chronic Chagas disease, autoimmunity has received much experimental support and elicited most controversy during the last 20 years. Nevertheless, there are studies suggesting that parasite persistence in the host tissues is relevant in the pathogenesis, since anti-parasite treatments result in a decrease of disease severity. Taking into account these two hypotheses, Girone and Fresno postulated that *T. cruzi* persistence together with the immune response induced to multiple myocardial antigens may participate in the heart damage. In both speculations, cruzipain (Cz) has been suggested as an important player for the disease progression.

Cruzipain, also known as GP 57/51 or cruzain, belongs to the mammalian papain superfamily but contains, as other cysteine proteases (CPs) from Trypanosomatids, an unusual C-terminal extension. This glycoprotein is synthesized as a zymogen that is activated by cleavage of the N-terminal pro-domain to generate the mature protease. The mature enzyme consists of a catalytic moiety situated at the N-terminal extension that is highly homologous to cathepsin L, and a C-terminal extension showing 36% identity with that described in type 1 CPs from other protozoans (Fig. 1).

Different clones and strains of the parasite in all their developmental stages express native Cz as a complex mixture of isoforms. However, Cz is differentially localized to carry out stage-specific functions. In the epimastigotes, Cz is localized to lysosome-like organelles designated as reservosomes, and in this stage shows its highest expression level. In the trypomastigotes, it is localized to the flagellar pocket, and in the intracellular amastigote it is displaced to the cell surface, where it probably functions in the cytoplasm of the host cell. Thus, Cz could be a target for immunity throughout all the phases of *T. cruzi* infection. Furthermore, Cz seems to be important for the survival of the parasite, its growth and cellular differentiation. This enzyme has endopeptidase activity and hydrolyses the Fc moiety of IgG and so, it may be involved in the parasite defense mechanisms against the host immune response. Besides, it was shown that Cz plays a role in the process of internalization of the parasite into mammalian cells and specific enzyme inhibitors have been proved to interfere with cell invasion and inhibit *T. cruzi* intracellular replication.

Cruzipain has a long and highly glycosylated C-terminal extension that is absent from mammalian cathepsins (Fig. 1). Three potential asparagine-glycosylation sites have been described in Cz, two in the enzymatic domain and one in the antigenic domain. Cruzipain is encoded by a high number of genes (up to 130 in the Tul 2 strain) placed in head-to-tail tandems, located on several chromosomes. Mcoply Cz gene family includes polymorphic sequences and the simultaneous expression of several different genes results in the production of a complex mixture of isoforms. The isoforms, which are immunologically cross-reactive with Cz, have a similar apparent molecular mass. Recently, it has been demonstrated that Cz 1 and 2 differ substantially regarding the substrate preference. In this review, we describe the implication of Cz in the interplay between *T. cruzi* and mammalian cells, fociusing mainly on the interactions of this glycoprotein with professional and non-professional phagocytic cells. In addition, we evaluate the role of Cz in immune evasion and disease pathogenesis describing the modulation of immunity in experimental models and human Chagas disease, as well as the relevance of endogenous synthetic CPs inhibitors.
CYSTEIN PROTEASES INHIBITORS

Inhibitors of CPs synthesized by the mammalian host

Cysteine proteases are regulated by reversible natural inhibitors that belong to the cystatin superfamily, among them kininogens, cystatins and stefins. Kininogens are the major physiological inhibitors because of their affinity to CPs and their high plasma concentrations(35). Furthermore, it was demonstrated that Cz has kininogenase activity, since this enzyme is able to release pro-inflammatory kinins from human kinogen. The major kinin released was identified as Lys-bradykinin (kallidin). Moreover, Cz converts plasmatic pre-kallikrein into α-kallikrein, an indirect way of the parasite protease to release bradykinin. Probably, these mechanisms are used by T. cruzi to traverse across capillary vessels to propagate to tissues(23). Thus, Cz mediates invasion of host cells by trypomastigotes through one pathway that involves the triggering of bradykinin B2 receptor (B2 R)(24). In addition, it was reported that heterologous cystatins from chicken, rat, and human bind to and inhibit Cz(25, 26).

Interestingly, it was demonstrated that α2-macroglobulin (α2M) and pregnancy zone protein, two proteinase-inhibiting glycoproteins, inactivate Cz(27). Moreover, increased α2M levels in BALB/c mice were correlated with survival to acute T. cruzi infection(28). Noteworthy, α2M was associated with the surface of the parasite and it was proposed that the inhibitor could stop cell invasion(29).

Endogenous parasite inhibitor of CPs, chagasin

Searching the endogenous inhibitor of CPs, early studies described the presence of the CP inhibitory activity in the extracts of Leishmania(30). More recently, Montero et al.(31) have cloned and expressed chagasin, a T. cruzi protein that was characterized as a novel type of inhibitor of papain-like CPs. Chagasin is an 11-kDa protein of 109 amino acid residues encoded by a single gene, which is expressed at variable levels at the different parasite stages, although at lower concentrations than CPs. The lack of sequence similarity to any other known group of CP inhibitors led to the proposal that chagasin is the representative of a novel family of CP inhibitors, collectively designated Inhibitor of Cysteine Peptidases(32). In epimastigotes, chagasin and Cz co-localize in at least two compartments of the secretory pathways, the Golgi complex and the reservosomes, where proteolytically active Cz is primarily found(13, 33). In addition, transfected T. cruzi epimastigotes expressing four times more chagasin than wild type epimastigotes drastically reduced CP activity, and this was accompanied by reduced capacity to differentiate into trypomastigotes, as well as reduced infectivity in vitro. It was proposed that a fine balance between the expression levels of chagasin and endogenous CPs may ultimately have an impact on parasite differentiation and infectivity(34).

Synthetic CPs inhibitors as potential anti-trypanosomatid drugs

The possibility that inhibitors of CPs could be effective antiparasite agents has been recognized for some time. Considering that Cz is essential for parasite survival, it has been suggested as an attractive drug target.

The use of fluoromethyl ketone and vinyl sulfone derivatized pseudopeptide protease inhibitors has demonstrated efficacy and lack of toxicity in the treatment of acute and chronic experimental T. cruzi infections(31, 33). Vinyl sulfone inhibitors have also provided interesting insights into the processing and subcellular targeting of Cz(15, 35). On the other hand, nonpeptide Cz inhibitors have demonstrated trypanocidal activity in cell cultures(36).

A possible limitation of these synthetic compounds would be the emergence of parasite populations developing resistance to CP inhibitors. In this sense, Yong et al reported that a phenotypically stable T. cruzi cell line displayed increased resistance to an irreversible CP inhibitor decreasing the availability of Cz, the most sensitive target(37). Thus, there is an urgent need to develop an improved Chagas therapy due to the toxicity of existing drugs. However, the major pharmaceutical companies have little interest in the small profit and high cost of the development of new drugs for the treatment of Chagas disease.

ROLE OF CRUZIPAIN IN IMMUNE EVASION AND DISEASE PATHOGENESIS IN EXPERIMENTAL MODELS

A range of immune system evasion mechanisms operate during the infection by T. cruzi and contribute to both the chronicity and the pathologic changes associated with it. This parasite evades its destruction by macrophages by escaping from phagolysosomes and invading non-phagocytic cells as well as by modulating the pattern of secreted cytokines at the transcriptional level.

Effects of cruzipain on host phagocytic cell activation

Trypanosoma cruzi infects mammalian cells by a process of endocytosis following an initial step of parasite host-cell recognition(28, 38). Recently, it was reported that trypomastigotes exploit an alternate actin-independent invasion pathway that involves formation of associated host cell plasma membrane-derived vacuoles, enriched in the lipid products of class I PI-3-kinases(39, 40). Different parasite molecules not only located on its surface but
also liberated to the extracellular milieu may participate in this process\(^\text{41}\). Cz has been involved in macrophage infection during the first step of parasite-cell recognition. Furthermore, the F(ab)\(_2\) portion of a monoclonal anti-Cz antibody significantly inhibited the ingestion of parasites by macrophages\(^\text{13}\). The inhibition of its enzymatic activity prevented growth and differentiation of \(T. cruzi\) in cultures\(^\text{17, 42}\) as well as rescued mice from a lethal infection\(^\text{33}\).

As discussed before, the activation of the pro-inflammatory kinin cascade by Cz up-regulated the infectivity of \(T. cruzi\)\(^\text{24, 43}\). In the same way, Aliberti et al. tested the possibility that activation of the kinin cascade system may likewise modulate dendritic cell (DC) function\(^\text{44}\). Thus, they demonstrated that kinins mobilize DC to produce IL-12 through activation of the bradykinin B\(_2\) R and induce an IL-12 response. This is tightly regulated by both angiotensin-converting enzyme -a kinin-degrading peptidase- and by endogenous IL-10. These data indicated that kinin peptides can serve as danger signals that trigger DC to produce IL-12\(^\text{44}\). Thus, it is possible that during \(T. cruzi\) infection, enzymatically active Cz can liberate bioactive kinins and subsequently, activate host cells through bradykinin receptors, stimulating endocytic uptake of the pathogen. Rather than unilaterally enhancing parasite infectivity, the liberated kinins activate innate immunity by stimulating DC maturation via the bradikinin B\(_2\) R\(^\text{45}\). However, it would be interesting to test whether Cz devoid of enzymatic activity directly stimulates DC, an issue unknown at present.

Macrophages have a major role in innate immunity and participate as effector cells in adaptive immune responses. The classical metabolic pathway in which macrophages stimulated by Th1 cytokines possess cytotoxic and antimicrobial effector functions is based on their ability to produce nitric oxide (NO). The production of NO and L-citrulline from the substrate L-arginine is catalyzed by the enzyme inducible nitric oxide synthase (iNOS). In contrast, the alternative pathway of macrophages is favored by Th2 cytokines resulting in an anti-inflammatory process with an enhanced phagocytic capacity and diminished killing functions\(^\text{46, 47}\). This later metabolic pathway is catalyzed by the enzyme arginase, which acts on L-arginine producing urea and L-ornithine. Activation of these metabolic pathways after the interaction between Cz and macrophages has not been investigated. Our group was the first in demonstrating that spleen macrophages were alternatively activated in BALB/c mice immunized with Cz – devoid of protease activity -. Associated with the enhanced arginase activity, these mice developed a type II cytokine profile and showed an increase in CD11b+GR1+ spleen immature myeloid cells\(^\text{48}\). In this sense, it is well known that microbial antigens together with Th1 and Th2 cytokines influence the heterogeneity and state of macrophage activation. In turn, the alternative macrophage activation is able to induce ornithine decarboxilase activity, an enzyme involved in the synthesis of polyamines, which are essentials for intracellular parasite replication\(^\text{49}\).

Studies performed recently in our laboratory demonstrated that Cz immunization of C57BL/6 (B6) mice induced a predominantly type I cytokine profile with high levels of IFN-\(\gamma\)\(^\text{49}\). B6 spleen adherent cells, which are a macrophage enriched cell population, were classically activated by Cz producing high levels of NO and pro-inflammatory cytokines (Guiñazú et al. unpublished results). Moreover, these cells showed an increase in iNOS mRNA and protein expression. Accordingly, these cells were able to control the parasite replication \textit{in vitro} (Guiñazú et al. unpublished results). These apparently contrasting results may account for the different cytokine profile induced in the two mouse strains. Supporting our results, Planelles et al. demonstrated that spleen macrophages from \(T. cruzi\) infected BALB/c mice secreted lower concentration of nitrates than B6 mouse cells, indicating that impaired function of myeloid cells in susceptible mice could be a contributing factor to infection persistence\(^\text{50}\). These and other studies indicate that the host genetic background is another important determinant influencing the nature of the immune response and the outcome of the infection.

On the other hand, Stempin et al. demonstrated that Cz is able to trigger the secretion of IL-10 and TGF\(\beta\) by J774 cells, as well as spleen and peritoneal macrophages from normal BALB/c mice. These cells cultured with Cz displayed also an enhancedarginase activity, and developed higher number of intracellular parasites compared to unstimulated cells\(^\text{51}\). This group also analyzed the intracellular signaling pathways triggered by this parasite antigen employing J774 macrophages. They found that arginase induction by Cz was mediated by tyrosine kinase, protein kinase A and p38 MAPK signaling\(^\text{52}\). This mechanism would contribute to parasite persistence and growth within infected cells, and may influence the pathogenesis of Chagas disease, favoring the onset of the chronic infection. Alternatively, during the late phase of acute \(T. cruzi\) infection, the activation of macrophage arginase may occur as a modulator mechanism to counteract the possible overproduction of harmful inflammatory mediators, such as NO.

It has been suggested that Toll like Receptor (TLR) signaling pathways in the innate immune response to \(T. cruzi\), and particularly TLR2, may play an important role by regulating the initial inflammatory response\(^\text{53}\). Thus, our
group demonstrated that Cz in vitro up-regulated TLR2 surface expression in immune B6 spleen total and F4/80+ cells (Guiñazú et al. unpublished results). Therefore it is likely that Cz, by increasing TLR2 expression, could modulate functional responses to the T. cruzi ligands that bind to TLR2. In this sense, Campos et al. have postulated that another parasite antigen, the T. cruzi glycosylphosphatidylinositol anchor, may directly initiate IL-12, TNF-α and NO production through activation of TLR2, thus promoting host resistance during early infection[54, 55].

Implications of cruzipain-non professional phagocytic cell interaction

To promote the penetration of non-phagocytic cells, infective trypomastigotes exploit an arsenal of heterogenous surface glycoproteins, secreted proteases and signaling agonists to actively manipulate multiple host cell signaling pathways[50]. In this regard, in vitro models of infection have been very useful for studying the molecular and cellular basis of host cell response after T. cruzi infection.

In parallel to the mechanism described above, kinin peptides and the cognate bradikinin B2 and B1 R were identified as members of a Cz-driven activation pathway involved in T. cruzi signaling and invasion of non-professional phagocytic cells like endothelial cells and cardiomyocytes[54, 43]. More recently, the same group reported a Cz-mediated invasion route that is not blocked by a kinin receptor antagonist. The activation of this pathway requires Cz-mediated processing of a trypomastigote molecule associated with parasite-shed membranes[41].

Similar to other intracellular protozoan parasites, T. cruzi invades and resides inside different types of cells, avoiding direct destruction by the immune system. The infected cell, however, has still the capacity to counteract the invasive pathogen by initiating its own death by apoptosis. This potent defense mechanism of the host cell puts strong selective pressure on the parasites, which have in turn evolved strategies to modulate the apoptotic program of the host cell to their favor[57]. In this way, it has been reported that the transialidase of T. cruzi rescues target cells like PC12 and Schwann cells from apoptotic death caused by serum deprivation[58, 59]. The authors demonstrated that both T. cruzi and transialidase activate PI3K/Akt protein kinase signaling in the Schwann cell, which is utilized as a survival pathway by a variety of cell types[60].

The first report showing the survival effect of T. cruzi infection on cardiomyocytes was made by our group[61]. We found that T. cruzi infection protected isolated cardiac cells against apoptosis induced by growth factor deprivation, increasing the expression of the antiapoptotic factor Bcl-2. This protection was further increased by the pre-treatment with Cz, devoid of enzymatic activity[61].

As discussed above, it is widely known that resistance to T. cruzi infection is associated with the capacity of lymphocytes to generate IFN-γ, which in turn activates macrophages to produce NO. In cardiomyocytes, although the IFN-γ induced trypanocidal action has been unquestionably demonstrated, some authors found that IFN-γ exerted a parasiticidal action without significant NO production[62]. In contrast, Machado’s group reported that iNOS-derived NO generated by IFN-γ is the final responsible for the trypanocidal effect[63]. In this context, we demonstrated that IFN-γ plus LPS treatment of cardiomyocytes drastically increased NO production[61]. On the other hand, in our experimental model, Cz treatment stimulates the alternative L-arginine metabolic pathways mediated by arginase. Interestingly, we also found an increased arginase activity and expression of isoform II, which is involved in the antiapoptotic effect of cardiomyocytes exerted by Cz.

Our recent studies have demonstrated that there are at least two signal transduction pathways responsible for the antiapoptotic properties of Cz, the PI3K-Akt and MEK 1/ ERK[64]. Interestingly, these mediators were also triggered in cardiomyocytes exposed to T. cruzi trypomastigotes. Noteworthy, live parasites activated stronger and earlier signals than Cz, indicating that other parasite molecules may be involved. Additionally, using a pharmacological inhibition approach, we further demonstrated that the ERK1/2 signaling, but not the PI3k/Akt pathway, was involved in Cz-mediated up-regulation of Bcl-2. Finally, both signaling pathways interfered with the caspase cascade by downregulating activation of caspase 3, the central executioner of the apoptotic program. In summary, altogether our findings show how a parasite molecule, which is released to the cellular milieu, can exploit the cardiomyocyte machinery to favour parasite replication and drive the signaling pathways to maintain a comfortable niche (Fig. 2).

Increasing evidences show that the myocardium possesses a functionally intact innate immune system. In this context, we additionally found that enzymatically inactive Cz strongly up-regulates the protein and mRNA expression of TLR2 but not TLR4. In turn, the transcription factor NF-κB is also activated by Cz treatment and it is involved in the survival effect (Aoki MP, unpublished results).

In our study, protease activity of Cz was inhibited excluding the action of the enzymatic activity. The elucidation of the enzymatic effect of Cz on the survival rate will be subject of future investigations. In this regard, it is important to stress that Schwann cell survival promotion exerted by transialidase was independent of its enzymatic activity[58, 60].
In agreement with our results the group of Hashimoto confirmed the anti-apoptotic effect of *T. cruzi* (65). They found that the cellular FLICE inhibitory protein, an anti-apoptotic factor, is accumulated in *T. cruzi*-infected mouse heart muscle cells. More recently, Petersen et al., also demonstrated the ability of this parasite to counteract cardiomyocyte apoptosis induced by TNF-α or growth factor deprivation, which is mediated by the activation of host NF-κB transcription factor (66). But in contrast to our findings, the authors found that the survival response could not be reproduced by soluble factors released by the parasite. In fact, treatment of an embryonic cardiac myoblast cell line with the conditioned media induced a decrease in the apoptotic rate but it was not significantly different to controls. It is not surprising that a transformed myoblast could not respond in the identical way than end-differentiated cardiac cells. In this regard, *T. cruzi* provides one of the most striking examples of how a parasite acts in a cell-type specific manner (57, 67, 68).

On the other hand, it was also reported that apoptosis of cardiomyocytes occurs during *T. cruzi* infection in *in vivo* and *in vitro* models (69). It is likely that the apparent discrepancy between results could be due to a different parasite and mouse strain used.

The data discussed herein clearly illustrate that *T. cruzi* benefits itself in several ways from manipulating the apoptotic pathways of host cells, favoring its own survival in the parasitized host.

**Modulation of the immune response to cruzipain in animal models**

Cystein proteases may be instrumental in modulating the host immune response to favor parasite survival and proliferation, nevertheless they are themselves immunogenic. Thus, our group reported for the first time that BALB/c mice immunization with enzymatically inactive Cz plus complete Freund adjuvant (CFA) was able to induce high levels of specific IgG1 and IgE antibodies and a lymphoproliferative response. Immunization with this parasite glycoprotein significantly enhanced the survival percentage of mice challenged with trypomastigote from the Tulahuen strain (70). Moreover, we also demonstrated that the immunization of BALB/c provoked extramedullary
hemopoiesis since immune mice develop a transient splenomegaly, as consequence of an increased production of hemopoietic progenitor cells. The expansion of these cell populations could result from the production of hemopoietically active cytokines such as GM-CSF(71). In fact, high levels of this cytokine were detected in supernatants of immune splenocytes stimulated with Cz. These results were consistent with the findings obtained through flow cytometry, which revealed an increased number of Mac-1(+), Gr-1(+) and CD19(+) splenocytes. These findings were associated with high levels of IL-4, IL-5 and IL-10 and low levels of IFN-γ and IL-12 after splenocyte stimulation with Cz, suggesting a preferential activation of Th2 type cytokines(80).

It is widely known that resistance to T. cruzi infection is associated with a Th1 pattern of cytokine production, whereas susceptibility to this infection is related with a Th2 cytokine profile(79). In the context of our current findings, we propose that Cz would play a key role during T. cruzi infection leading to a Th2 cytokine profile, favoring the permanence of parasite in the host. As mentioned above, Th1 and Th2 cytokines are able to enhance the classical and alternative activation of macrophages, respectively. Thus, during the acute phase of infection, this mechanism could promote survival and replication of the parasite(78).

In contrast with latter results, in a similar experimental model, our group also demonstrated that the immunization of B6 mice with Cz induced spleen cells to produce high levels of IFN-γ and low levels of IL-4, compatible with a Th1 profile. Although B6 and BALB/c mice are prone to elicit Th1 or Th2 cytokines, respectively, in this work we also demonstrated that the immunization of BALB/c with T. cruzi transialidase plus CFA elicited high levels of INF-γ. Thus, the cytokine profile is not only correlated with the murine genetic background, but also with the nature of the antigen, which is an important factor in determining the outcome of the immune response induced upon immunization (Fig. 3).

Figura 3. Immune response induced by Cz in BALB/c and B6 mice. Cz immunization activates T (TL) and B (BL) lymphocytes able to recognize Cz in both mouse strains. In BALB/c, these cells also recognize myosin and produce autoantibodies that may contribute to the onset of heart abnormalities(82). Activated TL from BALB/c secrete type II cytokines, which favor the alternative macrophage activation(46), while B6 TL show a type I cytokine profile, which contributes to classic macrophage activation(46).---

Considering the importance of Th1 mechanisms in the resistance against this intracellular parasite, Frank et al. analyzed the ability of Cz plus another adjuvant, CpG-ODN, as immunogen in C3H/HeN mice. In the study, this group demonstrated the induction of high titers of specific antibody, mostly of the IgG2a isotype, and a strong proliferation of spleen cells secreting IL-2 and IFN-γ. Immunized mice also displayed lower parasitemia than unimmunized ones and all animals survived to acute infection with T. cruzi RA strain(74).

The apparent conflicting results presumably reflect the complexity of the immune response generated to Cz and how the response may be determined by the particular immunization conditions (via, adjuvant, antigen dose) but also by host genetic background or parasite strain.

On the other hand, in a murine model of chronic T. cruzi infection Shnapp et al. demonstrated that Cz is a target of both B-cell and CD4+ T cell immunity and that at least some of the epitopes responsible for the Cz immunogenicity are preserved in the recombinant protein(79). These data demonstrated that this parasite glycoprotein expressed by the invading parasites is immunogenic in mice during T. cruzi infection. Furthermore, DNA immunization with a Cz expression vector induced a T cell response capable of lysing T. cruzi-infected cells. Thus, the authors suggested that Cz is a promising candidate for use in the development of a T. cruzi vaccine. This group also reported that Cz-specific CD4+ Th1 cells can induce macrophages to produce NO and inhibit parasite replication in vitro. They also demonstrated that specific Th1 cells induced by a recombinant Cz vaccine can protect mice against virulent T. cruzi mucosal and systemic challenges. However, adoptive transfer of these cells alone did not protect BALB/c mice, suggesting that additional immune mechanisms are important for Cz-specific immunity(76).

In a murine model of acute T. cruzi infection, our group found that B6 mice are able to better control the parasitemia although they have higher mortality than BALB/c mice.
These results were associated with a strong pro-inflammatory cytokine profile in B6 compared with BALB/c when splenocytes from each mouse strain were stimulated with Cz (Carrera-Silva AE, unpublished results). Thus, a highly specific parasite immune response, capable to keep parasite in check, might also be deleterious for the host.

One attractive point to be elucidated was whether the immune response developed to Cz could be pathogenic. Although there is a considerable controversy about whether the clinical signs of Chagas disease are exacerbated by autoimmunity, there is no doubt that the anti-self is present during chronic T. cruzi infection. Sera from chagasic patients as well as T. cruzi infected mice react to heart and skeletal muscle, as well as to some other antigens(3,4). One question to be elucidated is whether the presence of parasites is essential to induce cardiac injury or whether the immune response induced by the parasite or by antigens may promote heart damage. Parasite fractions, T. cruzi ribosomal proteins and peptides derived from them have been implicated in such responses(77-79).

With the purpose to know whether Cz was able to induce an immunopathogenic response and tissue injury, our group studied the effect of the immunization with enzymatically inactive Cz plus CFA in BALB/c mice. In this model, IgG antibodies binding to a 210-kDa molecule from a syngeneic skeletal muscle extract were detected. The absorption of immune sera with purified myosin completely eliminated this reactivity, confirming that the protein identified was really myosin. In addition, spleen cells from immunized mice proliferated in response to skeletal muscle extract enriched in myosin and to purified myosin. Moreover, an increase in plasma creatine kinase activity accompanied of electromyography abnormalities during chronic T. cruzi infection. Sera from chagasic patients as well as T. cruzi infected mice react to heart and skeletal muscle, as well as to some other antigens(3,4). One question to be elucidated is whether the presence of parasites is essential to induce cardiac injury or whether the immune response induced by the parasite or by antigens may promote heart damage. Parasite fractions, T. cruzi ribosomal proteins and peptides derived from them have been implicated in such responses(77-79).

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Because protease activity was inhibited in the Cz preparation, a direct action of this enzyme in induction of tissue damage was excluded in our experimental model. These results were the first experimental evidences showing that the immune response induced by Cz could be involved in the pathogenesis of experimental Chagas disease. In another report, we also demonstrated that Cz immunization of BALB/c was able to induce antibodies that bind to a 223-kDa antigen from a mouse heart extract. This protein was identified as the mouse cardiac myosin heavy chain by sequencing analysis. Moreover, the autoimmune response was associated to heart conduction disturbances associated with cardiomyocyte severe ultrastructural alterations. Another important finding of this study was the demonstration of heart conduction abnormalities in the offspring of mothers immunized with Cz. It is likely that autoantibodies generated by immunization are capable of passing from the mothers to the fetus via the placenta, and trigger an inflammatory process associated with disorders in the conduction system(82).

Interestingly, immunization with Cz also triggered IgG antibodies, which were able to interact with the cardiac M2 muscarinic acetylcholine receptor (mAChR) on heart cells, leading to activation of signaling pathways downstream of this receptor. It was postulated that chronic fixation of IgG and activation of M2 mAChR could induce myocardial dysfunction and injury, leading to intracellular myosin exposure with induction of anti-myosin antibodies(83). These events might perpetuate myosin antibodies and lead to the symptoms commonly observed in chronic cardiac Chagas disease(84). Collectively, we postulated that inflammatory signals triggered by Cz plus CFA in an appropriate MHC context on accessory cells could activate autoreactive T and B cells and so, lead to the breakdown of the self-tolerance. However, the pathogenesis of Chagas disease is multifactorial and other mechanisms and parasite molecules may also be involved in tissue damage.

Taking into account that the pathogenesis of this disease has been closely related to the host genetic background we decided to study whether cardiac autoimmunity and injury could be induced in B6 mice immunized with enzymatically inactive Cz. The findings revealed a lack of heart autoreactive response against myosin and myosin- or Cz-derived peptides. Conversely, autoreactive splenocytes present in immune BALB/c proliferated against cardiac myosin extract and homologous peptides derived from Cz (P291-302) and myosin (P7-18). Reinforcing our findings, Leon and colleagues demonstrated that B6 mice failed to develop cardiac autoimmunity after T. cruzi infection as well as after myosin immunization(85).

Interestingly, in the described model, the study of the B cell compartment from BALB/c and B6 immunized with Cz revealed important differences between strains. B cells from immune BALB/c presented higher activation and proliferation than those from B6 mice in cultures stimulated with specific antigen or LPS (Pellegrini AV, unpublished results).

Thus, we provide compelling evidence supporting the importance of the genetic background of the mouse strain in the outcome of the immune response to Cz and heart damage(86).
IMMUNE RESPONSE TO CRUZIPAIN IN HUMAN CHAGAS DISEASE

Humoral immune response in patients with chronic Chagas disease

Human infection with *T. cruzi* is usually accompanied by humoral and cellular immune responses to a major antigen *Cz* (GP57/51). In fact, antibodies against this parasite glycoprotein were detected in most sera from chronic Chagas disease patients (CDP) being proposed as an antigen of potential interest to use as a diagnostic reagent. It is noteworthy that immunoreactivity was shown not only for the intact *Cz* but also for 25-35 kDa fragments (some of them corresponding to the central domain and some to the C-terminal domain)\(^{(86)}\). In this sense, the 25 kDa self-proteolysis fragment, corresponding to the C-terminal domain of *Cz*, earlier named GP25, has been proposed as a diagnostic antigen\(^{(87)}\). In another study, sera from chronic CDP affected with cardiomyopathy or with the asymptomatic form, showed similar reactivity against GP57/51 by Western blot and this antigen complex was recognized by 100% of sera from CDP\(^{(88)}\).

In addition, antibodies against three recombinant fragments of *Cz*, expressed in bacteria, were detected in sera from chronic CDP. Most antibodies directed against this glycoprotein were found to react with the C-terminal extension, thus suggesting the presence of immunodominant B-cell epitopes within this protein domain. Immunoprecipitation with these antibodies did not impair enzyme activity, suggesting that *Cz* consists of an enzymatic domain and a non-enzymatic immunodominant domain, which corresponds to the C-terminal extension\(^{(89)}\).

On the other hand, Duschak et al reported that the reactivity of anti-*T. cruzi* microsomal fraction monoclonal antibodies (MoAb 5A9B11), which also recognize epitopes on *Cz*, was completely inhibited by sera of severe cardiomyopathy patients, while only a partial inhibition was found with sera from CDP with mild disease\(^{(80)}\). Moreover, when the overall anti-*Cz* antibody response was evaluated, 70% of the patients with severe disease showed anti-*Cz* titers higher than 1/800. The authors proposed that antibodies against different *Cz* epitopes appeared to be related with the severity of heart Chagas disease. Similar results have been reported assaying the ability of sera from CDP with different degrees of cardiac dysfunction to block the immune recognition of MoAb 5A9B11\(^{(90)}\).

In addition, our group reported that antibodies to *T. cruzi* acidic antigens, among them anti-*Cz* antibodies, were able to bind *T. cruzi* epimastigote surface and human heart tissue epitopes, suggesting the presence of cross-reactive epitopes. These results were demonstrated assaying sera from symptomatic and asymptomatic CDP absorbed with fixed parasite or human heart tissue extract\(^{(92)}\).

Cellular immune response and partial cruzipain epitope mapping

In human T cell response studies, highly purified GP57/51 demonstrated its capacity to promote specific proliferation of peripheral blood mononuclear cells (PBMC) *in vitro*, suggesting that the immunity to this particular glycoprotein may be an important component of human immune responses against *T. cruzi*\(^{(88)}\). In this study, a comparative human T-cell responses between CDP affected with cardiomyopathy or with the asymptomatic form revealed that both groups actively responded to GP57/51, although the reactivity did not reveal pronounced differences between the different groups\(^{(88)}\). In this regard, as was mentioned above, complexes formed by *Cz* and α2M were efficiently internalized by human monocytes resulting in enhanced presentation of *Cz* peptides to CD4+ T cells from CDP\(^{(89)}\).

In another study, the T cell response against *Cz* was analyzed with cells from a panel of chronic CDP with cardiomyopathy. PBMC proliferative response to native (n)- or recombinant (r)-*Cz* was of varied intensity, possibly reflecting differences in MHC restriction or influences determined by T cell subset selection. The authors noted that the enzymatic activity of n-*Cz* was not required for proliferative responses\(^{(93)}\). Furthermore, the fact that anti-*Cz* T cell lines generated with n-*Cz* or r-*Cz* were reciprocally reactive indicated that these molecule forms share epitopes. However, the proliferation response was stronger using n-*Cz*. The analysis of cytokine production suggested that Th1-like subsets dominate the patient’s responses and that IFN-γ was induced on stimulation with either n-*Cz* or r-*Cz*. In contrast, IL-4 was present in very small concentrations or was undetectable.

To define T cell epitopes of *Cz*, the authors used a panel of 11 synthetic peptides spanning portions of the central (catalytic) domain and C-terminal extension. Surprisingly, the peptides deriving from the C-terminal domain failed to elicit responses. The screening of peptides from the catalytic domain revealed that one 33mer peptide (P214) was able to induce a strong proliferation on T cell lines originated specifically to n-*Cz* from three patients. This result suggested that the peptide might contain immunodominant epitopes. Interestingly, P214 has significant sequence homologies with lysosomal enzymes found in human tissues, such as cathepsin L and S. Noteworthy, P214 aminoacid sequence shares some homology with
cardiac myosin (12mer) derived peptides that were immunogenic in the BALB/c experimental model immunized with Cz. Fine epitope mapping, combined with MHC restriction analysis, should determine whether anti-Cz T cell responses have the potential to cross-react with their tissue homologues and which are the pathologic implications during chronic Chagas infection.

Recently, in order to investigate the recognition of Cz epitopes by CD8+ T cells from CDP, Fonseca et al. selected a panel of 12 synthetic peptides from this protein, which were predicted to bind to HLA-A2 after an in vitro assay. The HLA-A2 allele was selected given its high frequency in the Brazilian population. Since Cz is secreted from the parasite it is likely of being presented by the MHC class I pathway. Thus, IFN-γ ELISPOT assays indicated that several HLA-A*0201-restricted Cz epitopes were frequently recognized by PBMC from HLA-A2+ CDP, suggesting that infection induces a significant antigen specific peripheral CD8+ T cell expansion.

Notably, CD8+ T cells from the inflammatory infiltrate in the heart lesion from a single CDP with cardiomyopathy also recognized some of these epitopes: a tetrameric HLA-A*0201 complex built with the Cz 60-68 peptide, which is frequently recognized in the periphery, also bound to CD8+ T cells from a heart-infiltrating T cell line. The authors postulated that the observed heterogeneity of inter-individual responses may be due to differences in T cell recognition repertoire and/or host genetic background; alternatively, they could be related to sequence polymorphism in some of the tested Cz epitopes. Experiments involving analysis of heart infiltrating T cell lines from patients may elucidate whether CD8+ T cells play a pathogenic or protective role in chronic CDP.

CONCLUDING REMARKS

Nearly 100 years since its discovery by the Brazilian physician Carlos Chagas, the protozoan parasite *Trypanosoma cruzi* remains the most common cause of myocarditis worldwide. During these years, substantial progress has been made in the comprehension of the multiple roles of CPs in infection and invasion by the pathogenic *T. cruzi*. These cumulative findings show us that CPs are attractive potential targets for the treatment of infection because they are essential during the development of Chagas disease pathogenesis.

Cruzipain, the major CP of *T. cruzi*, plays a role at multiple points of the host immune response as illustrated by autoimmune phenomena and immune evasion elicited in experimental models as well as in chagasic patients. According to the experimental evidence, the results described herein show that Cz, a single parasite antigen, is able to drive the behavior of the target cell to favor parasite survival and dissemination within the host. Altogether the different activated mechanisms could lead to the establishment of the long-term myocardial infection. Nevertheless, it is important to stress that the manipulation of host functions is extremely complex, and this became apparent with the comparative experiments employing different mouse strains, adjuvants or means of immunization. Thus, the final host response will result from the integrated information where the host genetic background and the parasite strain will be determinant.

From a therapeutic point of view, there is an urgent need to develop an improved Chagas therapy due to the toxicity of existing drugs and emerging drug resistance. Cz, a factor of virulence and pathogenicity in this parasite disease, emerges as an important target to develop selective inhibitors. Thus, good science, and unfailing financial support can remove this killing disease from the list of the most neglected »poor people’s disease».

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DISCLOSURES

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