Trypanosoma cruzi calreticulin: A possible role in Chagas' disease autoimmunity

Carolina Hager Ribeiro[a], Nandy C. López[a], Galia A. Ramírez[a], Carolina E. Valck[a], María Carmen Molina[a], Lorena Aguilar[a,b], Margarita Rodríguez[a], Ismael Maldonado[a], Ramón Martínez[c], Carlos González[d], Rodrigo Troncoso[e], Sergio Lavandero[b,e], Alexandre R. Gingras[f], Wilhelm Schwaeble[f,**], Arturo Ferreira[a,*]

[a] *Programa Disciplinario de Inmunología, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[b] *Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[c] *Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile*
[d] *Escuela de Medicina Veterinaria, Universidad Andrés Bello, Santiago, Chile*
[e] *Centro FONDAP CEMC, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile*
[f] *Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, United Kingdom*

Keywords:
Chagas' disease
Trypanosoma cruzi
Calreticulin
Autoimmunity

A B S T R A C T

Trypanosoma cruzi (*T. cruzi*) is the causative agent of Chagas' disease, an endemic and chronic illness that affects 18 million people in Latin America. The mechanisms underlying its pathogenesis are controversial. There is a growing body of evidence supporting the view that *T. cruzi* infection elicits severe autoimmune responses in the host, which significantly contribute to the pathogenesis of Chagas' disease, and several recent studies have reported the presence of autoantibodies and effector T lymphocytes against parasite and self antigens in infected patients and experimentally infected animals. *T. cruzi* calreticulin (TcCRT) is a 45 kDa protein, immunogenic in humans, rabbits and mice. It has a high degree of homology with human (HuCRT) and mouse calreticulin (MoCRT), which would explain why an immune response to TcCRT could contribute to autoimmune reactions in Chagas' disease. Anti-TcCRT antibodies generated in A/J mice immunized with recombinant TcCRT (rTcCRT) reacted with rHuCRT and bound to neonatal and adult isogenic cardiomyocytes cultured *in vitro*. Interestingly, histological alterations, such as edema formation and cell infiltrates, which include $CD3^+$ cells, were detected in heart sections from immunized animals. Therefore, in rTcCRT-immunized mice, an autoimmune reaction against host CRT, paralleled by histological cardiac alterations, suggests a role of the parasite molecule in the induction of immunologically mediated heart tissue damage. The data presented here propose that TcCRT participates in the induction of cardiac autoimmunity in Chagas' disease.

1. Introduction

Trypanosoma cruzi (*T. cruzi*) is the hemoflagellate protozoan that causes Chagas' disease (American Trypanosomiasis) (Chagas, 1909), an acute and chronic illness that affects 18 million people in Latin America (Moncayo, 2003). Despite the significant reduction in transmission observed in some countries (Salvatella and Rosa, 2000; Lorca et al., 2001), Chagas' disease is still an important health problem, causing approximately 50,000 deaths every year (Dias et al., 2002; WHO, 2002). Infection with the parasite results in relatively mild acute parasitemia, followed by a long asymptomatic phase. Decades after of the initial infection, one third of infected individuals develop chronic symptoms, such as chagasic cardiomyopathy and gastrointestinal syndromes (Prata, 2001).

Many pathogenic mechanisms have been suggested to explain Chagas' cardiomyopathy, including cellular proliferation in the myocardium (involving fibroblasts and inflammatory cells, mainly T lymphocytes) (Tanowitz et al., 2003), necrosis and apoptosis of cardiac cells (Barcinski and DosReis, 1999), cellular hyperplasia and hypertrophy (Arnaiz et al., 2002), immunity to parasite antigens persisting in the tissue (Gea et al., 1993), and autoimmunity (Leon and Engman, 2001). Even though the presence of parasites in the heart tissue has been considered the primary stimulus for the maintenance of myocardial inflammation and tissue damage (Levin, 1996), the rare detection of parasites in heart lesions raised

* Corresponding author at: Programa Disciplinario de Inmunología, ICBM, Facultad de Medicina, Universidad de Chile, Avda. Independencia 1027, Santiago, Chile. Tel.: +56 2 978 6723; fax: +56 2 978 6979.
** Corresponding author at: Department of Infection, Immunity and Inflammation, Medical Sciences Building, University of Leicester, University Road, Leicester LE1 9HN, UK. Tel.: +44 116 223 1555; fax: +44 116 252 3050.
E-mail addresses: ws5@le.ac.uk (W. Schwaeble), aferreir@med.uchile.cl (A. Ferreira).

the question as to whether *T. cruzi* participates directly in Chagas' cardiomyopathy and proposed the highly debated potential involvement of autoimmunity (Kierszenbaum, 1996). In this sense, the inflammatory response in Chagas' cardiomyopathy would be induced by the parasite, but would also be a consequence of autoimmune cross-reactions with host antigens, mediated by autoreactive B and/or T cells (Oldstone, 1989; Leon and Engman, 2003a,b). In fact, sera from *T. cruzi* infected mice or humans, as well as from mice immunized with parasite proteins, react with heart or skeletal muscle and other self antigens, thus contributing to the pathogenesis of the disease (Laguens et al., 1988; Lozykowski et al., 1991; McCormick and Rowland, 1993; Kierszenbaum, 1999).

Calreticulin (CRT) is a well conserved protein present in cells of all higher organisms, except erythrocytes (Michalak et al., 1999; Johnson et al., 2001). It has been described in a wide range of parasite species, such as *Onchocerca volvulus*, *Schistosoma mansoni*, *Leishmania donovani*, *Plasmodium falciparum*, and *Necator americanus* (reviewed by Ferreira et al., 2004a). We have cloned, sequenced and expressed a *T. cruzi* CRT gene, coding for a 45 kDa protein, TcCRT (Aguillón et al., 1995, 2000a), present on the parasite surface and other cytoplasmic organelles (Ferreira et al., 2004b; Souto-Padrón et al., 2004). TcCRT presents a high degree of identity to CRT from humans and other vertebrates (Ferreira et al., 2004a). As described previously, TcCRT binds to human and mouse C1q, resulting in inhibition of the classical pathway of the complement system (Ferreira et al., 2004b; Aguilar et al., 2005). Recent data from our laboratory show that the TcCRT/C1q interaction favors parasite infectivity (Ribeiro et al., submitted). These findings suggest that TcCRT contributes to early immune evasion strategies and infectivity, allowing the parasite to moderate the innate immune response and to secure its survival in the mammalian host.

CRT has been associated with autoimmunity. For instance, infection with *O. volvulus* results in the exacerbated production of autoantibodies against host CRT, which correlates with autoimmune responses (Lux et al., 1992; Meilof et al., 1993; Eggleton and Llewellyn, 1999; Eggleton and Michalak, 2003). Indeed, the pathogenic feature of anti-CRT antibodies has been extensively described in autoimmune diseases, such as systemic lupus erythematosus (Eggleton et al., 2000), rheumatoid arthritis (Verreck et al., 1995), congenital heart block (Orth et al., 1996), and celiac disease (Tuckova et al., 1997).

TcCRT is highly immunogenic in humans (Aguillón et al., 1997; Marcelain et al., 2000), rabbits (Aguilar et al., 2005), and mice (Aguillón et al., 2000b), suggesting that natural or experimental infection with *T. cruzi* exposes TcCRT to B cells. Accordingly, we decided to investigate whether TcCRT participates in the autoimmune feature of Chagas' disease. We immunized A/J mice with the recombinant parasite protein and analyzed whether the generated anti-rTcCRT antibodies cross-reacted with recombinant human CRT (rHuCRT) and cardiomyocytes. Our findings indicate that rTcCRT-immunized mice produce antibodies that recognize both, rHuCRT and putative native cardiac murine CRT, which correlates with alterations in cardiac histology of immunized mice. The results presented herein strongly support the hypothesis that rTcCRT immunization induces a cross-reactive immune response that associates with the development of cardiac pathology.

2. Materials and methods

2.1. Animals

All experimental procedures, involving animals, were approved by an Institutional Bioethics Committee. Eight to twelve-week-old female A/J mice were maintained under internationally accepted guidelines in our Animal Facility (Faculty of Medicine, University of Chile), including permanent veterinary supervision. All surgical procedures with these animals were performed under general anesthesia with a combination of Ketamin and Xylazine (66 mg/kg and 1.6 mg/kg, respectively).

2.2. Cells

2.2.1. Neonatal mouse cardiomyocyte cultures

Neonatal cardiomyocytes were obtained as described (Wang et al., 1999). For selective enrichment of cardiomyocytes, the isolated cells were first plated on tissue culture dishes for 2 h in order to allow attachment of non-myocytes. The cells in the supernatant were then collected and plated at a density of 2×10^5 on 2% gelatin-treated 12-mm round glass cover slips in wells of tissue culture plates. Cells were kept overnight at 37 °C under 5% CO_2 atmosphere.

2.2.2. Adult mouse cardiomyocyte isolation

Adult cardiomyocytes, from three-months-old mice, were isolated as described (O'Connell et al., 2007), with modifications. Hearts were removed aseptically, leaving 1–2 mm section of the aorta for cannulation purposes. The hearts were immediately placed in a 60-mm dish containing perfusion buffer [Gerard buffer (128 mM NaCl, 4 mM KCl, 0.19 mM NaH_2PO_4, 1.01 mM Na_2HPO_4, 1.39 mM $MgSO_4$, 10 mM HEPES, 5.5 mM glucose, 2 mM piruvic acid, pH 7.4) supplemented with 100 mM 2,3-butanedione monoxide (BDM) (Sigma–Aldrich, USA)], at room temperature (RT). The heart was cannulated on a retrograde perfusion system, firstly using approximately 3 mL Gerard buffer containing 2 mM EGTA for 1 min, followed by perfusion buffer (3 min at 3 mL/min), at 37 °C. A switch to myocyte digestion buffer [perfusion buffer supplemented with 2 mg/mL type II collagenase (Gibco, USA)] was performed for 10–15 min at 3 mL/min. After digestion, the heart was placed in a 60-mm dish containing myocyte digestion buffer, and ventricles were dissociated. The cell suspension was centrifuged (500 rpm for 1 min), and the pellet was resuspended in perfusion buffer. Another centrifugation step followed, and the pelleted cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp., USA), 1000 U/mL penicillin, 50 mg/mL streptomycin, and 100 mM BDM. One milliliter of cell suspension was added to laminin (Invitrogen Corp., USA)-coated (10 μg/mL) 12-mm round glass cover slips (previously treated with HCl to promote laminin attachment to the glass) in wells of tissue culture plates for 1 h at 37 °C under 5% CO_2 atmosphere.

2.3. Recombinant TcCRT and HuCRT

rTcCRT was prepared as previously described (Ferreira et al., 2004b). The human CRT gene (*HuCRT*), coding for residues 18–417, was amplified by PCR using *Taq* DNA polymerase (Promega Corp., USA) from clone phCAR-1 (Stuart et al., 1996). The primers used were: forward (ATAGAATTCCATATGGAGCCTGCCGTCTACTTCA) and reverse (AGAATTCAGATCTTTACAGCTCGTCCTTGGCCTGG) (TAGN, UK). The amplified *HuCRT* DNA was purified and ligated into the EcoRI/NdeI restriction enzyme sites of the pET-15b vector (Novagen, UK). Competent *Escherichia coli* TOP10F′ bacteria were transformed with the plasmid, plated in Luria-Bertani medium, and selected with ampicilin (100 μg/mL). For protein expression, *E. coli* BL21 (DE3) was transformed with the plasmid containing *HuCRT* and grown in the presence of ampicilin (100 μg/mL). Expression constructs were induced in late-log phase with 1 mM IPTG for 3 h. The cells were harvested, sonicated on ice, and centrifuged. The supernatants were filtered and applied to a Ni^{2+} charged iminodiacetic acid-Sepharose column (Hi-Trap Chelating, Amersham Pharmacia Biotech, USA), eluted with an Imidazole gradient, and dialyzed against 20 mM Tris–HCl and 150 mM NaCl, pH 8. The pro-

tein was then digested with thrombin (from human plasma, Roche Diagnostics Corp., USA) to remove the His-Tag. Recombinant HuCRT (rHuCRT) was further purified using anion exchange chromatography (UNO Q-6 Biochromatography column, Bio-Rad, USA). Purity of rHuCRT was assessed by 12% SDS-PAGE.

2.4. Immunizations

Mice were bled from the ventral tail vein and artery to obtain pre-immune sera. One experimental group (10 animals) was immunized four times, at weekly intervals, with 50 μg of rTcCRT. The first three immunizations were subcutaneous (s.c.), while the fourth was intraperitoneal (i.p.). A booster was administered (i.p.) three weeks after the last immunization. The first inoculum contained complete Freund's adjuvant (CFA), while the others the incomplete version (IFA) (both from Sigma–Aldrich, USA). Blood was collected, at weekly intervals, during 80 days. A control group (8 animals) was inoculated with PBS.

2.5. ELISA

Nunc Maxisorb polystyrene plates (Fisher Scientific, USA) were coated with 100 μL/well of rTcCRT or rHuCRT (3 μg/mL) diluted in carbonate buffer, followed by overnight incubation at 4 °C. Control wells received buffer alone. Nonspecific binding sites were blocked (2 h at 37 °C) with 0.5% soybean proteins (SBP) in PBS (PBS-SBP) (Aguillón et al., 1992). Each step was followed by washes with PBS/0.05% Tween-20 (PBS/Tw). Pre-immune and immune sera, diluted in PBS-SBP, were added in triplicate wells, followed by incubation for 90 min at 37 °C. A monoclonal antibody (mAb) to TcCRT (E2G7) (Ferreira et al., 2004b) and a mouse anti-mouse CRT mAb (BD Biosciences, USA) were used as positive controls for rTcCRT and rHuCRT, respectively. Affinity purified horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma–Aldrich, USA) was added. Enzyme activities were assessed by addition of 2-2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) with hydrogen peroxide. Optical density was read at 405 nm.

2.6. Western blots

Standard procedures for SDS-PAGE and Western blots were used (Mesri et al., 1990). rTcCRT or rHuCRT (3 μg/track) were loaded onto 12% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in PBS. After several washes with PBS/Tw, sera from rTcCRT-immunized or control mice were incubated with recombinant proteins-sensitized membranes for 90 min at RT. E2G7 and anti-mouse CRT mAbs were used as positive controls. As a secondary antibody, alkaline phosphatase (AP)-conjugated goat anti-mouse polyclonal IgG (Dako, USA) was used. The reactive bands were visualized using a Nitro blue tetrazolium/Phosphate 5-bromo-4-cloride-indol (NBT/BCIP) solution.

2.7. Histopathology

Immunized and control mice were sacrificed 50 days after the last rTcCRT immunization or saline inoculation, respectively. Their hearts were removed, rinsed with PBS and fixed in 10% buffered formalin for 24 h. Fixed hearts were embedded in paraffin, and three sections (4 μm) were taken from each heart, including both atria, atrial–ventricular intersection, and both ventricles. Sections were stained with hematoxylin–eosin and submitted to a blind light microscopy evaluation. A total of 5 microscopic fields (mf) per section were screened to assess for evidence of histological alterations, such as vascular and muscle fiber degenerative changes, as well as inflammation. According to the extent of damage, a standard score system was assigned for each histological finding: normal (−); mild (+) (equal or less than 10% of heart involvement); moderate (++) (10–50% involvement); severe (+++) (more than 50% involvement) (Smith and Allen, 1991; Sun and Tarleton, 1993).

2.8. Immunohistochemistry

Immunostaining of heart tissue sections was performed for the detection of $CD3^+$ lymphocytes. Serial sections from the same areas analyzed for histological changes were plated on silanized glass slides, deparaffinized, and rehydrated. Antigen retrieval was performed with 20 mg/mL Proteinase K (Dako), and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min at RT. Nonspecific binding was blocked with 1% BSA in PBS for 20 min at RT. The sections were then incubated with the primary antibody [polyclonal rabbit anti-human CD3 antibody (Dako, USA) or rabbit normal serum (isotype control)] in 1% BSA, overnight at 4 °C. A secondary step was performed using biotinylated goat anti-rabbit IgG (Sigma–Aldrich, USA) in 1% BSA, for 60 min at RT. Incubation with streptavidin-HRP-conjugate (Dako, USA), in 1% BSA for 60 min, was then performed. As a peroxidase substrate, 3,3-diaminobenzidine tetrahydrochloride (DAB Chromogen) (Dako, USA) was used. Each step was followed by extensive slide washes with PBS. The sections were counter-stained with Harris' haematoxylin (Linsan Laboratories, Chile) and mounted with aqueous medium (Merck, Germany). The specificity of the reaction was tested by omission of the primary antibody.

2.9. Immunofluorescence

Neonatal and adult cardiomyocytes, cultured on glass cover slips, were washed three times with cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 for 5 min at RT. Nonspecific binding sites were blocked with PBS-BSA for 90 min at RT. Neonatal and adult cardiomyocytes were then incubated overnight at 4 °C with one of the following primary antibodies, diluted in the blocking buffer: anti-mouse CRT mAb, polyclonal rabbit anti-human muscarinic acetylcholine receptor M2 IgG (mAChR M2) (Santa Cruz Biotechnology, USA) (for cardiomyocyte staining), E2G7, pre-immune and immune sera (obtained after 5 doses of rTcCRT). Secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG, both labeled with FITC) (Sigma–Aldrich, USA) were then incubated for 1 h at 4 °C. Each step was followed by extensive washes with PBS. The specificity of the reaction was tested by omission of the primary antibody. The cover slips were placed on slides with fluorescent mounting medium (Dako, USA). Confocal images were captured with a Zeiss LSM 5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 image capture and analysis software and a Plan-Apochromat 63x/1.4 oil DIC.

2.10. Statistical analysis

ELISA results were subjected to analysis of variance (ANOVA) using GraphPad Prism software (version 5.0), followed by post hoc Bonferroni's analysis for multiple comparisons. p values of <0.05 were regarded as statistically significant.

3. Results

3.1. TcCRT immunization induces cross-reactive antibodies to HuCRT

Sera from rTcCRT-immunized mice, obtained after 5 inoculations with the parasite protein, contained antibodies that

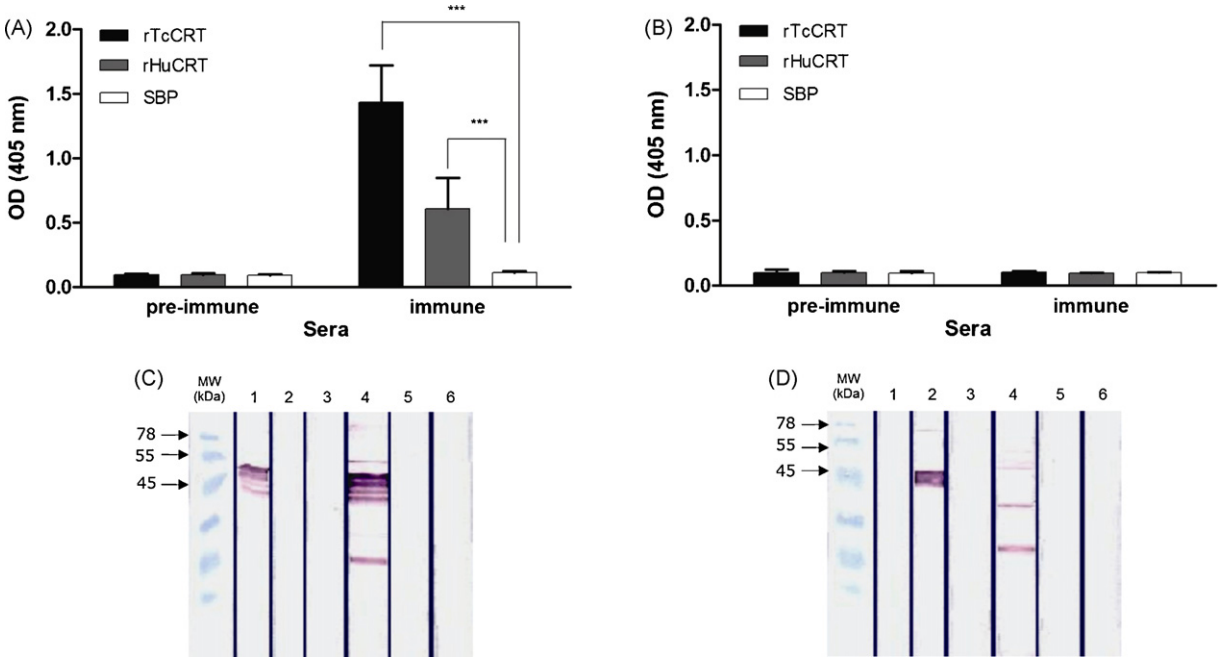

Fig. 1. Immunization with rTcCRT induces anti-rTcCRT antibodies that cross-react with rHuCRT. A/J mice were inoculated with rTcCRT (A) or with PBS (B). For ELISA, plates were coated with rTcCRT or rHuCRT in carbonate buffer. After blocking with PBS/soybean proteins (SBP), pre-immune and immune sera (obtained after the last doses of rTcCRT or PBS) were added. Reactivity was detected by a HRP-conjugated anti-mouse IgG. Mean values ± SD obtained from 10 immunized and 8 control mice, in triplicate wells, are shown. OD values for rTcCRT and rHuCRT were compared to SBP values. Statistical analyses were performed with two-way ANOVA. Sera reactivity with rTcCRT, rHuCRT and SBP is shown in black, gray and white, respectively. $^{***}p < 0.001$. In WB assays, rTcCRT (C) and rHuCRT (D) were incubated with pre-immune (lane 3) and immune (lane 4) sera from an immunized mouse, as well as with pre-immune (lane 5) and PBS-inoculated (lane 6) sera from a control mouse. Lane 1: E2G7 mAb; lane 2, anti-human CRT mAb. Reactivity was detected by AP-conjugated anti-mouse IgG.

specifically bound to immobilized rTcCRT in ELISA ($p < 0.001$) (Fig. 1A). Interestingly, rTcCRT-immune sera also recognized rHuCRT ($p < 0.001$), as compared to pre-immune sera (Fig. 1A) and sera from control mice (Fig. 1B).

Our data with WB assays confirmed these results. As expected, a strong band of 45 kDa was observed when rTcCRT-immune sera were incubated with rTcCRT on nitrocellulose membranes, as seen in Fig. 1C, lane 4 [a representative result comparable to that observed between the parasite protein and the E2G7 mAb (Fig. 1C, lane 1)]. Additionally, immune sera also recognized a band of equivalent molecular weight in rHuCRT sensitized membranes (Fig. 1D, lane 4). A similar reaction was observed with a commercial mAb against HuCRT (Fig. 1D, lane 2). Such bands were not detected by pre-immune sera (Fig. 1C and D, lanes 3) or by pre-immune and control sera (Fig. 1C and D, lanes 5 and 6, respectively). It is apparent that both, the human and parasite recombinant molecules, are relatively susceptible to proteolysis. It is also evident that the monoclonal and polyclonal antibodies used recognize different electrophoretic patterns, as a consequence of their intrinsic specificities. These results indicate that immunization with *T. cruzi* CRT generates specific anti-rTcCRT antibodies that cross-react with mammalian recombinant CRT.

3.2. Anti-TcCRT antibodies react with murine cardiomyocytes in vitro

We observed that neonatal and adult cardiomyocytes, obtained from A/J mice, were recognized by isogenic murine rTcCRT-immune sera by indirect immunofluorescence (IIF), as shown in Fig. 2A and B, respectively. This recognition is consistent with the fact that a 45–50 kDa band is recognized by the same anti-sera in cardiomyocyte extracts (data not shown). A pre-immune serum showed very low unspecific reactivity with neonatal and adult cells (Fig. 2C and D, respectively), while the anti-rTcCRT mAb, anti-mouse CRT mAb, and anti-mAChR M2 IgG also reacted with both cell types (data not shown). No specific reaction was detected when the primary antibody was omitted (Fig. 2E and F). These results suggest that polyclonal antibodies against parasite CRT, obtained from rTcCRT-immunized mice, cross-react and bind putatively to native mouse CRT on cardiomyocytes.

3.3. TcCRT immunization induces heart histological alterations

We then investigated whether rTcCRT immunization induces autoimmune lesions of myocardial tissue. Mice were sacrificed 50 days after the last booster immunization with rTcCRT or PBS (as controls). Fig. 3 is representative of 15 heart sections from each of 6 immunized and 7 control mice analyzed. Mild mononuclear perivascular and interstitial heart infiltrate foci were observed only in rTcCRT-treated mice (Fig. 3A), while no infiltrates were detected in the hearts of control animals (Fig. 3B). In addition, muscular degenerative changes, such as eosinophilia and fiber fragmentation, were apparent only in rTcCRT-immunized animals (Table 1).

T. cruzi CRT immunization induced the migration of inflammatory $CD3^+$ T cells to the cardiac tissue (Fig. 4A), as compared to control animals (Fig. 4B). Our results suggest that rTcCRT immunization leads to autoimmune lesions and structural damage of the heart, which is accompanied by infiltration of the myocardial tissue with $CD3^+$ T cells.

4. Discussion

Several lines of evidence indicate that, as occurs in autoimmune diseases, parasite infections induce cross-reacting immune responses against host CRT, with subsequent damages to the host

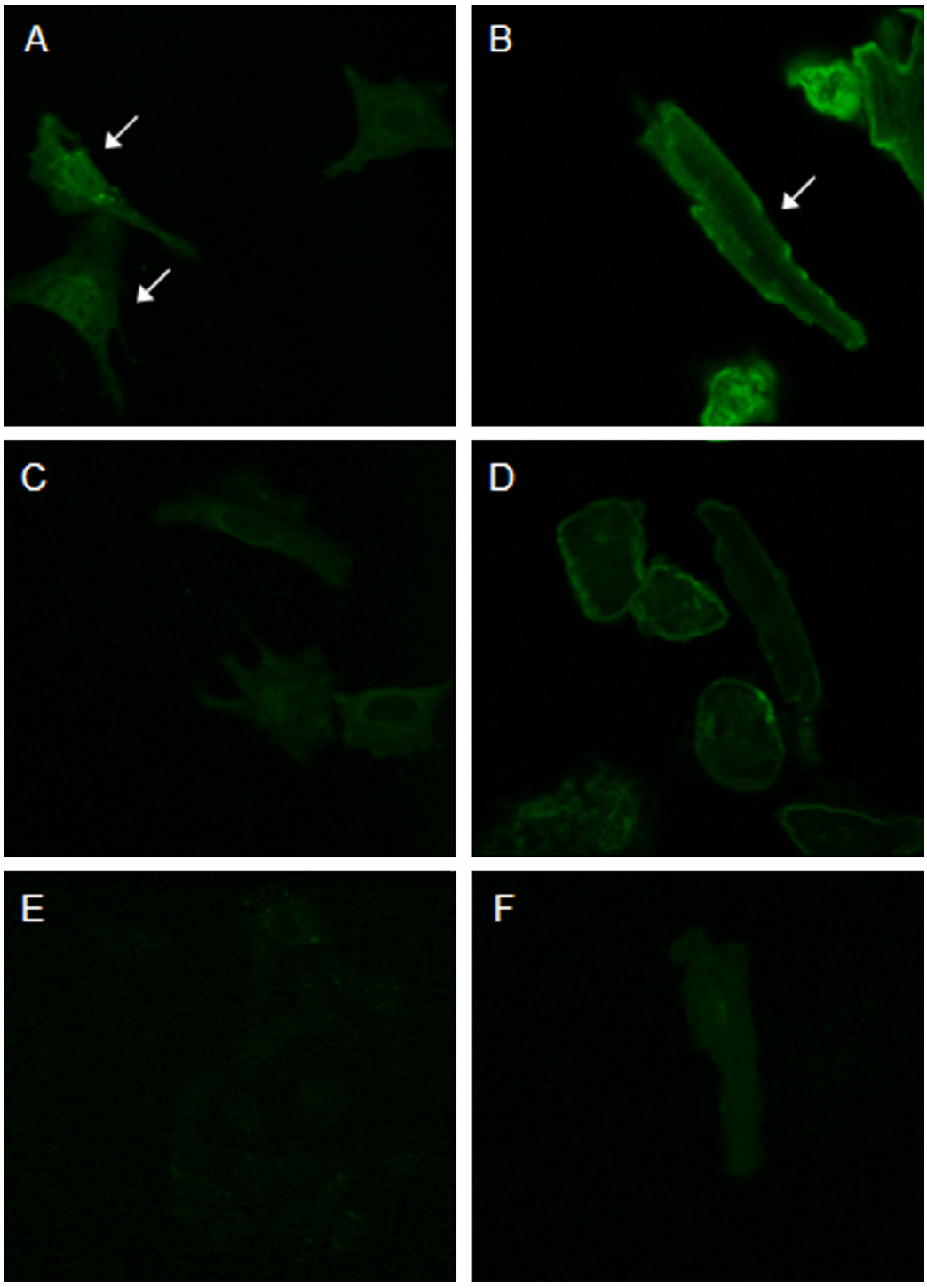

Fig. 2. Murine anti-rTcCRT antibodies react with neonatal and adult mouse cardiomyocytes. Cells were obtained from normal A/J mice, as described in Section 2. For immunofluorescence, neonatal and adult cardiomyocytes were incubated with serum from an rTcCRT-immunized isogenic mouse ((A and B) respectively), as well as with pre-immune serum from the same animal ((C and D) respectively). Neonatal and adult cells were also incubated in the absence of primary antibodies ((E and F) respectively). FITC-conjugated anti-mouse IgG was used as secondary antibody. Reactive myocytes (arrows) were visualized under a light microscope.

(Eggleton and Llewellyn, 1999). We have previously demonstrated that *T. cruzi*, the parasite causing Chagas' disease, expresses TcCRT, which is immunogenic in humans, rabbits and mice (Ferreira et al., 2004a). Our results strongly suggest a role for TcCRT in the autoimmune pathology of Chagas' disease. Thus, mice immunized with the parasite molecule generate cross-reactive antibodies against mammalian CRT, which correlates with histological alterations of the heart tissue.

While the autoimmune contribution to the pathogenesis of Chagas' disease is still a matter of controversy (as reviewed by Kierszenbaum, 2005), a growing body of evidence suggests that parasite-mediated molecular mimicry, bystander activation or epitope spreading, essentially contributes to tissue damage (Leon and Engman, 2001). It is generally accepted that a persistent and chronic inflammatory process caused by autoimmune responses may result in chronic chagasic cardiomyopathy leading to heart fail-

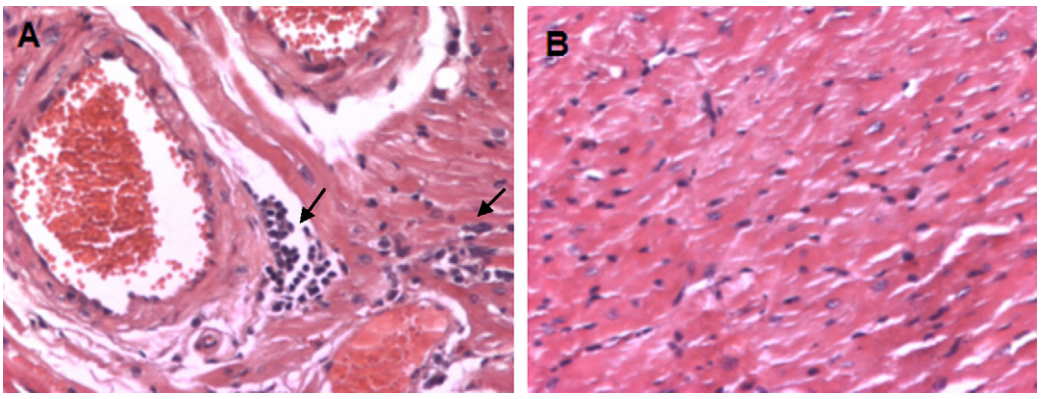

Fig. 3. The cardiac tissue from rTcCRT-immunized mice is histologically abnormal. Representative heart sections from an immunized (A) and a control (B) mouse, obtained 50 days after the 5th doses of rTcCRT or PBS inoculation, respectively, are shown. Results represent 15 histological sections from each of 6 immunized and 7 control mice. Arrows indicate mononuclear perivascular and interstitial heart infiltrate foci. Magnification, ×200.

Table 1
Histopathological analysis of the cardiac muscle of rTcCRT-immunized and control A/J mice.

Group	Cross-reactivity with rHuCRT	Degenerative changes		Inflammation[a]	
		Eosinophilia	Fragmentation	Lymphocytes	Macrophages[b]
Immunized ($n = 10$)	10	+	+	+	+
Control ($n = 8$)	ND	–	–	–	–

ND: not detected.

[a] Scores for histological alterations were determined as described in Section 2: normal (–), mild (+).

[b] Results were derived from mean values of five different mf/section, three sections/heart, and six to seven mice/determination.

ure. The inflammatory environment (necessary for the stimulation and expansion of autoreactive cells), and the presence of antibodies directed against cardiac proteins, such as calreticulin, is seen to be a pre-requisite for the development of the terminal phase of Chagas' disease (Cunha-Neto et al., 2006).

For this study, the A/J murine strain, susceptible to experimental autoimmunity, was chosen. This strain develops myocarditis in response to coxsackievirus infection or immunization with cardiac myosin in Freund's adjuvant (Leon and Engman, 2001). This strain also develops strong parasite-specific immunity and autoimmunity following *T. cruzi* infection (Leon and Engman, 2001). We found that immunization of A/J mice with TcCRT in Freund's adjuvant induced specific humoral immunity (Fig. 1). In addition, the anti-TcCRT polyclonal antibodies generated also reacted with human CRT (Fig. 1).

These results are in agreement with earlier reports showing that mice immunized with parasite proteins develop cross-reactive antibodies against host proteins. For example, immunization with a parasite ribosomal P protein elicits antibodies that cross-react with host M2 muscarinic receptor (Mahler et al., 2004), host ribosomal protein (Lopez Bergami et al., 1997) and β1 adrenergic receptor (Lopez Bergami et al., 2001). Moreover, immunization of mice with cruzipain induces antibodies reactive to mouse cardiac and skeletal muscle myosin, which was associated with heart conduction abnormalities (Giordanengo et al., 2000a), inflammatory infiltrates, and other histological changes in skeletal muscle of immunized animals (Giordanengo et al., 2000b).

In the present study, in addition to the observed reactivity between polyclonal anti-TcCRT antibodies and putative CRT derived from a cardiac cell extract, we demonstrated that primary cultured cardiomyocytes, isolated from normal neonatal and adult A/J mice, are recognized by anti-TcCRT polyclonal antibodies (Fig. 2), suggesting that self cardiac proteins, most likely CRT, are detected by these antibodies.

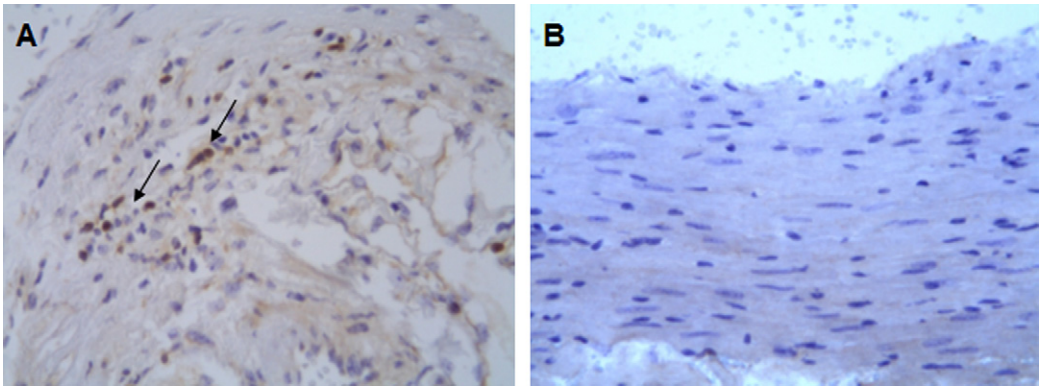

Fig. 4. $CD3^+$ cell infiltrates are present in the cardiac tissue of rTcCRT-immunized A/J mice. Representative paraffin-embedded heart sections, obtained from an immunized (A) and a control (B) mouse, and processed for immunohistochemistry and stained with anti-human CD3 antibody (diluted at 1/75), are shown. Biotinylated anti-rabbit IgG was used as secondary antibody. Slices were analyzed with a 600× objective. The arrows indicate $CD3^+$ mononuclear cells.

IgG2a, the main isotype involved in the production of autoantibodies in the chronic phase of *T. cruzi* experimental infection (Spinella et al., 1992), can lead to complement fixation, inflammation and subsequent pathology (Giordanengo et al., 2000a). Immunization with TcCRT elicits specific IgG1 and IgG2a isotypes (Ribeiro et al., submitted). Examination of cardiac tissues from rTcCRT-immunized mice revealed the presence of mild mononuclear interstitial infiltrates (Fig. 3), including $CD3^+$ cells (Fig. 4). Additionally, muscular degenerative changes, such as eosinophilia and fiber fragmentation, were also observed in the hearts of immunized animals (Table 1). Therefore, our results are consistent with a pathogenic role of the immune response to TcCRT. This immune response may promote tissue damage, with release of parasite and self antigens. Induction of cross-reactive responses against self antigens may then occur, thus generating a self-perpetuating pathogenic cycle, with subsequent chronic damage to the host.

In synthesis, we present here the first demonstration that immunization with recombinant *T. cruzi* calreticulin can induce autoimmune pathology of the heart by induction of autoimmune humoral and cellular responses similar to those observed in advanced stages of human Chagas' disease.

Acknowledgements

This research was sponsored by the following Chilean Public Research Grants: Bicentennial ACT 29 and R-07, both from CONICYT; FONDAP 1501006 and MECESUP-Red UCH 0115. We are grateful to Ruth Mora, Juana Orellana, Nancy Fabres, Miguel Sepúlveda, and Fidel Albornoz for their excellent expert technical assistance.

References

Aguilar, L., Ramírez, G., Valck, C., Molina, M.C., Rojas, A., Schwaeble, W., Ferreira, V., Ferreira, A., 2005. F(ab′)2 antibody fragments against *Trypanosoma cruzi* calreticulin inhibit its interaction with the first component of human complement. Biol. Res. 38, 187–195.

Aguillón, J.C., Ramos, R., Chiong, M., Vallejos, P., Ferreira, A., 1992. Soybean proteins: alternative blocking agents for immunoassays using nitrocellulose or plastic solid phases. Biol. Res. 25, 79–83.

Aguillón, J.C., Bustos, C., Vallejos, P., Hermosilla, T., Morello, A., Repetto, Y., Hellman, U., Orn, A., Ferreira, A., 1995. Purification and preliminary sequencing of Tc-45, an immunodominant *Trypanosoma cruzi* antigen: absence of homology with cruzipain, cruzain, and a 46-kilodalton protein. Am. J. Trop. Med. Hyg. 53, 211–215.

Aguillón, J.C., Harris, R., Molina, M.C., Colombo, A., Cortés, C., Hermosilla, T., Carreno, P., Orn, A., Ferreira, A., 1997. Recognition of an immunogenetically selected *Trypanosoma cruzi* antigen by seropositive chagasic human sera. Acta Trop. 63, 159–166.

Aguillón, J.C., Ferreira, L., Pérez, C., Colombo, A., Molina, M.C., Wallace, A., Solari, A., Carvallo, P., Galindo, M., Galanti, N., Orn, A., Billetta, R., Ferreira, A., 2000a. Tc45, a dimorphic *Trypanosoma cruzi* immunogen with variable chromosomal localization, is calreticulin. Am. J. Trop. Med. Hyg. 63, 306–312.

Aguillón, J.C., Hermosilla, T., Molina, M.C., Morello, A., Repetto, Y., Orn, A., Ferreira, A., 2000b. *Trypanosoma cruzi*: H2 complex and genetic background influence on the humoral immune response against epimastigotes. Int. J. Parasitol. 30, 981–984.

Arnaiz, M.R., Fichera, L.E., Postan, M., 2002. Cardiac myocyte hypertrophy and proliferating cell nuclear antigen expression in Wistar rats infected with *Trypanosoma cruzi*. J. Parasitol. 88, 919–925.

Barcinski, M.A., DosReis, G.A., 1999. Apoptosis in parasites and parasite-induced apoptosis in the host immune system: a new approach to parasitic diseases. Braz. J. Med. Biol. Res. 32, 395–401.

Chagas, C., 1909. Nova tripanozomiase humana: Estudos sobre a morfolojia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen., n. sp., ajente etiolojico de nova entidade morbida do homem. Mem. Inst. Oswaldo Cruz. 1, 159–218.

Cunha-Neto, E., Bilate, A.M., Hyland, K.V., Fonseca, S.G., Kalil, J., Engman, D.M., 2006. Induction of cardiac autoimmunity in Chagas heart disease: a case for molecular mimicry. Autoimmunity 39, 41–54.

Dias, J.C., Silveira, A.C., Schofield, C.J., 2002. The impact of Chagas disease control in Latin America: a review. Mem. Inst. Oswaldo Cruz. 97, 603–612.

Eggleton, P., Llewellyn, D.H., 1999. Pathophysiological roles of calreticulin in autoimmune disease. Scand. J. Immunol. 49, 466–473.

Eggleton, P., Ward, F.J., Johnson, S., Khamashta, M.A., Hughes, G.R., Hajela, V.A., Michalak, M., Corbett, E.F., Staines, N.A., Reid, K.B., 2000. Fine specificity of autoantibodies to calreticulin: epitope mapping and characterization. Clin. Exp. Immunol. 120, 384–391.

Eggleton, P., Michalak, M., 2003. Introduction to calreticulin. In: Calreticulin, 2nd ed. Kluwer Academic Plenum Publishers, NY, pp. 1–8.

Ferreira, V., Molina, M.C., Valck, C., Rojas, A., Aguilar, L., Ramírez, G., Schwaeble, W., Ferreira, A., 2004a. Role of calreticulin from parasites in its interaction with vertebrate hosts. Mol. Immunol. 40, 1279–1291.

Ferreira, V., Valck, C., Sanchez, G., Gingras, A., Tzima, S., Molina, M.C., Sim, R., Schwaeble, W., Ferreira, A., 2004b. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*. J. Immunol. 172, 3042–3050.

Gea, S., Ordoñez, P., Cerban, F., Iosa, D., Chizzolini, C., Vottero-Cima, E., 1993. Chagas' disease cardioneuropathy: association of anti-*Trypanosoma cruzi* and anti-sciatic nerve antibodies. Am. J. Trop. Med. Hyg. 49, 581–588.

Giordanengo, L., Maldonado, C., Rivarola, H.W., Iosa, D., Girones, N., Fresno, M., Gea, S., 2000a. Induction of antibodies reactive to cardiac myosin and development of heart alterations in cruzipain-immunized mice and their offspring. Eur. J. Immunol. 30, 3181–3189.

Giordanengo, L., Fretes, R., Diaz, H., Cano, R., Bacile, A., Vottero-Cima, E., Gea, S., 2000b. Cruzipain induces autoimmune response against skeletal muscle and tissue damage in mice. Muscle Nerve 23, 1407–1413.

Johnson, S., Michalak, M., Opas, M., Eggleton, P., 2001. The ins and outs of calreticulin: from the ER lumen to the extracellular space. Trends Cell Biol. 11, 122–129.

Kierszenbaum, F., 1996. Chronic chagasic tissue lesions in the absence of *Trypanosoma cruzi*: a proposed mechanism. Parasitol. Today 12, 414–415.

Kierszenbaum, F., 1999. Chagas' disease and the autoimmunity hypothesis. Clin. Microbiol. Rev. 12, 210–223.

Kierszenbaum, F., 2005. Where do we stand on the autoimmunity hypothesis of Chagas disease? Trends Parasitol. 21, 513–516.

Laguens, R., Meckert, P., Chambo, J., 1988. Antiheart antibody-dependent cytotoxicity in the sera from mice chronically infected with *Trypanosoma cruzi*. Infect. Immun. 56, 993–997.

Leon, J.S., Engman, D.M., 2001. Autoimmunity in Chagas heart disease. Intern. J. Parasitol. 31, 555–561.

Leon, J.S., Engman, D.M., 2003a. The significance of autoimmunity in the pathogenesis of Chagas heart disease. Front. Biosci. 8, 315–322.

Leon, J.S., Engman, D.M., 2003b. The contribution of autoimmunity to Chagas heart disease. In: Tyler, K.M., Miles, M.A. (Eds.), World Class Parasites: American Trypanosomiasis. Kluwer Academic Publishers, Boston/Dordrecht/London, pp. 97–106.

Levin, M.J., 1996. In chronic Chagas heart disease, don't forget the parasite. Parasitol. Today 12, 415–416.

Lopez Bergami, P., Cabeza Meckert, P., Kaplan, D., Levitus, G., Elias, F., Quintana, F., Van Regenmortel, M., Laguens, R., Levin, M.J., 1997. Immunization with recombinant *Trypanosoma cruzi* ribosomal P2beta protein induces changes in the electrocardiogram of immunized mice. FEMS Immunol. Med. Microbiol. 18, 75–85.

Lopez Bergami, P., Scaglione, J., Levin, J.M., 2001. Antibodies against the carboxyl-terminal end of the *Trypanosoma cruzi* ribosomal P proteins are pathogenic. FASEB J. 15, 2602–2612.

Lorca, M., García, A., Bahamonde, M.I., Fritz, A., Tassara, R., 2001. Serological certification of the interruption of the vectorial transmission of Chagas disease in Chile. Rev. Med. Chile 129, 264–269.

Lozykowski, M.G., McCormick, T.S., Rowland, E.C., 1991. Coincidence of tissue antibody and cardiac pathology in mice infected with *Trypanosoma cruzi*. Trans. R. Soc. Trop. Med. Hyg. 85, 225–226.

Lux, F.A., McCauliffe, D.P., Büttner, D.W., Lucius, R., Capra, J.D., Sontheimer, R.D., Lieu, T.S., 1992. Serological cross-reactivity between a human Ro/SS-A autoantigen (calreticulin) and the lambda Ral-1 antigen of *Onchocerca volvulus*. J. Clin. Invest. 89, 1945–1951.

Mahler, E., Hoebeke, J., Levin, M.J., 2004. Structural and functional complexity of the humoral response against the *Trypanosoma cruzi* ribosomal P2 beta protein in patients with chronic Chagas' heart disease. Clin. Exp. Immunol. 136, 527–534.

Marcelain, K., Colombo, A., Molina, M.C., Ferreira, L., Lorca, M., Aguillón, J.C., Ferreira, A., 2000. Development of an immunoenzymatic assay for the detection of human antibodies against *Trypanosoma cruzi* calreticulin, an immunodominant antigen. Acta Trop. 75, 291–300.

McCormick, T.S., Rowland, E.C., 1993. *Trypanosoma cruzi*: recognition of a 43 kDa muscle glycoprotein by autoantibodies present during murine infection. Exp. Parasitol. 77, 273–281.

Meilof, J.F., Van der Lelij, A., Rokeach, L.A., Hoch, S.O., Smeenk, R.J., 1993. Autoimmunity and filariasis: autoantibodies against cytoplasmic cellular proteins in sera of patients with onchocerciasis. J. Immunol. 151, 5800–5809.

Mesri, E., Levitus, G., Hontebeyrie-Joskowicz, M., Dighiero, G., Van Regenmortel, M.H.V., Levin, M.J., 1990. Major *Trypanosoma cruzi* determinant in Chagas' heart disease shares homology with the systemic lupus erythematosus ribosomal P protein epitope. J. Clin. Microbiol. 28, 1219–1224.

Michalak, M., Corbett, E.F., Mesaeli, N., Nakamura, K., Opas, M., 1999. Calreticulin: one protein, one gene, many functions. Biochem. J. 344, 281–292.

Moncayo, A., 2003. Chagas disease: current epidemiological trends after the interruption of vectorial and transfusional transmission in the Southern Cone countries. Mem. Inst. Oswaldo Cruz. 98, 577–591.

O'Connell, T.D., Rodrigo, M.C., Simpson, P.C., 2007. Isolation and culture of adult mouse cardiac myocytes. Methods Mol. Biol. 357, 271–296.

Oldstone, M.B.A., 1989. Molecular mimicry as a mechanism for the cause and as a probe uncovering etiologic agent(s) of autoimmune disease. Curr. Top. Microbiol. Immunol. 145, 128–135.

Orth, T., Dorner, T., Meyer Zum Buschenfelde, K.H., Mayet, W.J., 1996. Complete congenital heart block is associated with increased autoantibody titers against calreticulin. Eur. J. Clin. Invest. 26, 205–215.

Prata, A., 2001. Clinical and epidemiological aspects of Chagas disease. Lancet Infect. Dis. 1, 92–100.

Salvatella, R., Rosa, R., 2000. La interrupción en Uruguay de la transmisión vectorial de *T. cruzi*, agente de la enfermedad de Chagas, por control de *T. infestans*. Rev. Pat. Trop. 29, 213–231.

Smith, S.C., Allen, P.M., 1991. Myosin-induced acute myocarditis is a T cell mediated disease. J. Immunol. 147, 2141–2147.

Souto-Padrón, T., Labriola, C.A., de Souza, W., 2004. Immunocytochemical localisation of calreticulin in *Trypanosoma cruzi*. Histochem. Cell. Biol. 122, 563–569.

Spinella, S., Liegeard, P., Hontebeyrie-Joskowicz, M., 1992. *Trypanosoma cruzi*: predominance of IgG2a in nonspecific humoral response during experimental Chagas' disease. Exp. Parasitol. 74, 46–56.

Stuart, G.R., Lynch, N.J., Lu, J., Geick, A., Moffatt, B.E., Sim, R.B., Schwaeble, W.J., 1996. Localisation of the C1q binding site within C1q receptor/calreticulin. FEBS Lett. 397, 245–249.

Sun, J., Tarleton, R.L., 1993. Predominance of CD8+ T lymphocytes in the inflammatory lesions of mice with acute *Trypanosoma cruzi* infection. Am. J. Trop. Med. Hyg. 48, 161–169.

Tanowitz, H.B., Factor, S.M., Shirani, J., Ilercil, A., Wittner, M., Scharfstein, J., Kirchhoff, L.V., 2003. Recent developments in the pathology of Chagas disease with emphasis on the cardiovascular system. In: Tyler, K.M., Miles, M.A. (Eds.), World Class Parasites: American Trypanosomiasis. Kluwer Academic Publishers, Boston/Dordrecht/London, pp. 82–96.

Tuckova, L., Karska, K., Walters, J.R., Michalak, M., Rossmann, P., Krupickova, S., Verdu, E.F., Saalman, R., Hanson, L.A., Tlaskalova-Hogenova, H., 1997. Anti-gliadin antibodies in patients with celiac disease cross-react with enterocytes and human calreticulin. Clin. Immunol. Immunopathol. 85, 289–296.

Verreck, F.A., Elferink, D., Vermeulen, C.J., Amons, R., Breedveld, F., de Vries, R.R., Koning, F., 1995. DR4Dw4/DR53 molecules contain a peptide from the autoantigen calreticulin. Tissue Antigens 45, 270–275.

Wang, G.W., Schuschke, D.A., Kang, Y.J., 1999. Metallothionein-overexpressing neonatal mouse cardiomyocytes are resistant to H_2O_2 toxicity. Am. J. Physiol. 276, 167–175.

WHO 2002. WHO Expert Committee Control of Chagas disease, 2002. World Health Organization Technical Report Series. 905:109.