6

Pathogenesis and Pathology of Chagas' Chronic Myocarditis

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1. Introduction

Chronic chagasic cardiomyopathy (CCC) is the most serious manifestation of the chronic phase of Chagas' disease and constitutes the most common type of chronic myocarditis in the world (Guerri-Guttenberg, et al., 2008, Milei, et al., 1996a, Milei, et al., 2009, Milei, et al., 1992a, Storino, et al., 1992). Chagas' disease, a chronic illness caused by the flagellate parasite *Trypanosoma cruzi* (*T. cruzi*), was first described in 1909 by the Brazilian physician Carlos Chagas (Chagas C, 1909). The insect vectors of the disease are present throughout most of South and Central America, and their zone of distribution extends across the southern United States (Rassi, et al., 2010). It was estimated by year 2000, that in endemic areas 40 million people were considered to be at risk of infection, being 20 million already infected. Every year near 200,000 new cases are expected to happen, and 21,000 deaths per year occur (WHO, 2005).

Although always considered to be confined to Latin America, due to migratory movements from endemic countries to Europe and North America, Chagas' disease is being detected more frequently in developed countries. Europe is estimated to have from 24,001 to 38,708 (lower or upper limit of estimate, respectively) immigrants with *T. cruzi* infection (Guerri-Guttenberg, et al., 2008). In the United States, six autochthonous cases, five transfusion related cases and five transplant related cases have been reported, but migratory movements still remain the main source of Chagas' disease. It has been estimated that around 89,221 to 693,302 infected Latin Americans migrated to the United States in the period 1981 to 2005 (Milei, et al., 2009).

Two phases of the disease can be distinguished: (1) acute phase, with transiently high concentration of parasites in tissue and blood, nonspecific symptoms, and a 5% myocarditis incidence, lasting 4 – 8 weeks; and (2) chronic phase, lasting lifelong. Chronic phase can be presented as indeterminate form, characterized by lack of symptoms and normal ECG and normal radiographic examination of the chest, esophagus and colon. Approximately 60 – 70% of patients remain in this form for the rest of their lives. Only 20-40% of infected individuals, 10-30 years after the original acute infection, will develop cardiac, digestive or mixed form of the disease, characterized by the appearance of megavicera (dilated cardiomyopathy, megaesophagus and/or megacolon). It poses a substantial public health burden due to high morbidity and mortality (Milei, et al., 2009, Rassi, et al., 2000, Rassi, et al., 2010).

CCC is manifested by a chronic, diffuse, progressive fibrosing myocarditis that involves not only the working myocardium but also the atrioventricular (AV) conduction system, autonomic nervous system and microcirculation (Andrade, 1985, Marin-Neto, et al., 2007, Milei, et al., 1991b). This leads to cardiomegaly, cardiac failure, arrhythmias, thromboembolism, and death (Milei, et al., 1991b). Colon and esophagus are also commonly affected by Chagas' disease, being megacolon with constipation and megaesofagus with achalasia also features of the disease (Rassi, et al., 2010).

2. Pathogenesis of Chagas' myocarditis

Milei et al. proposed a combined theory that could explain the pathogenic mechanism in chronic chagasic myocarditis (Milei, et al., 1996a, Storino & Milei, 1994). This theory is based on three ingredients: the parasite, host immune system and fibrosis. These ingredients are proposed as being the primary causative agents of damage on myocardial tissue, conduction system, autonomic ganglia and nerves and microvasculature.

2.1 First ingredient: the parasite

The role of *T. cruzi* in the chronic phase has been previously underestimated due to the fact that its presence was believed to be scarce and unrelated to the inflammatory infiltrate present at this stage. Nowadays, the involvement of the parasite in the chronic phase has been well documented. Using dissimilar methods, different authors demonstrated either the persistence of *T. cruzi* or parasite antigens in mice (Younées-Chennoufi, et al., 1988), the parasite DNA sequence amplified by the polymerase chain reaction (PCR) (Jones, et al., 1993, Schijman, et al., 2004), *T. cruzi* antigens from inflammatory lesions in human chagasic cardiomyopathy (Higuchi, Brito et al. 1993), or the immunohistochemical finding of the parasite in endomyocardial biopsies with PCR confirmation (Añez, Carrasco et al. 1999). This would suggest a direct role for the parasite in the perpetuation of myocardial inflammation. In other words, the antigen stimulation would persist throughout the chronic stage, even though the parasites are not morphologically detectable by light microscopy (Andrade 1992).

The role of parasitemia is more controversial. High parasitemia correlated with severity of disease in one report (Basquiera, et al., 2003), but showed no association in another (Castro C., et al., 2005).

Interestingly, it has been observed that immunosuppression reactivates rather than ameliorates the disease, as seen in patients receiving immunosuppressive therapy to prevent transplant rejection and in AIDS patients. Accordingly, many experimental models where strains of genetically manipulated mice lacking various immune functions showed increased susceptibility to develop the disease (Tarleton & Zhang, 1999).

2.1.1 Life cycle of *Trypanosoma cruzi*

When a reduviid bug feeds from an infected mammal, it takes up circulating trypomastigotes, which reach then the bug's gut. There, they differentiate to amastigotes, which proliferate and start to differentiate into epimastigotes. In this process, when amastigote is still sphere-shaped but has developed its flagellum, some authors call this stage spheromastigotes. Then, it elongates its cell body and flagellum, taking the classical epimastigote shape. At this stage, the parasite undergoes metacyclogenesis, differentiating in metacyclic trypomastigotes, the infective form for mammals. When the bug feeds again,

it excretes trypomastigotes with feces, which in turn reach blood torrent through bug's wound. Trypomastigotes can infect a wide variety of host cells, within them it differentiate into amastigotes and proliferate. Then, they can differentiate into trypomastigotes again, reach circulation and infect new cells. If an uninfected bug feeds from the animal in the moment of parasitemia, cycle starts again (Tyler & Engman, 2001).

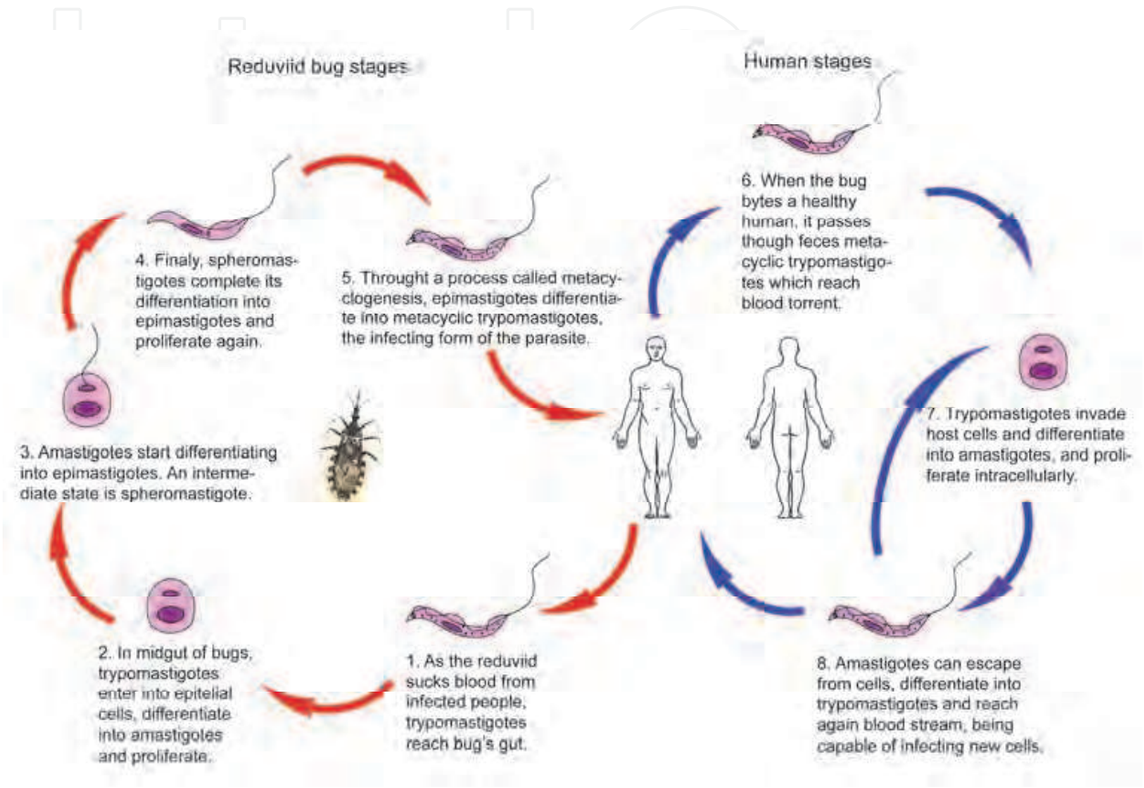

Fig. 1. Life cycle of *Trypanosoma cruzi*.

2.1.2 Genetic variability of *Trypanosoma cruzi* and its relation to its pathogenesis

The genetics of *T. cruzi* caught the attention of researchers in late 80' and early 90'. First studies on variability were performed analyzing electrophoretic variants on cellular enzymes. The groups resulting were called zymodemes and were named Z1, Z2, Z3. Only Z2 was associated with domestic transmission cycle.

The development of PCR based techniques, allowed the study of new variant regions and the characterization of multiple variants of a great number of genes. All these variants showed significant correlation with each other, suggesting the existence of two subtypes of *T. cruzi* based on these data (Macedo, et al., 2004). Moreover, *T. cruzi II* which is clearly linked to human pathology, being *T. cruzi I* mainly related to infection of wild sylvatic mammals. Even, applying LSSP-PCR to the study of the variable region of kinetoplast minicircle from *T. cruzi* provided evidence of a differential tissue distribution of genetically diverse *T. cruzi* populations in chronic Chagas' disease, suggesting that the genetic variability of the parasite is one of the determining factors of the clinical form of the disease (Vago, et al., 2000).

2.1.3 Cell host invasion and intracellular survival by *Trypanosoma cruzi*

Once *T. cruzi* reaches blood torrent, it invades a great variety of cells in the host. When parasiting non phagocytic cells, *T. cruzi* uses some surface glycoproteins to attach to cell: gp82, gp30 and gp35/50. All three glycoproteins are known to induce calcium mobilization from intracellular reservoirs. Gp82 is linked to the phospholipase C (PLC) and inositol 1,4,5 – triphosphate (IP3). Gp 35/50 is associated to increasing intracellular levels of cyclic AMP. On the other side, cruzipain, a protein known to be secreted by *T. cruzi*, acts on kininogen and produces bradykinin, which binds to its receptor, further increasing intracellular calcium. Increased intracellular calcium produces modifications in cytoskeleton that lead to parasite endocytosis (Yoshida & Cortez, 2008).

In the parasitoforous vacuole, mainly by the action of gp85/TS a glycoprotein with trans-sialidase action, and TcTox, a protease, the parasite degrades the membrane of the vacuole, escapes from it and proliferates within the cell (Alves & Colli, 2007).

2.1.4 Molecular mimicry

The induction of autoimmunity by similarities between *T. cruzi* and host epitopes has been long proposed as a mechanism that leads to tissue damage in the chronic phase of the disease. Both humoral and cellular autoimmune responses have been described, but we will discuss them in more detail in the section of immune system. The real importance of molecular mimicry in the pathogenesis of chagasic myocarditis is still a matter of debate (Girones, et al., 2005).

Although it seems that in some cases this mechanism triggers autoimmunity, in many others, autoimmunity seems to be an epiphenomenon of cellular destruction, with exposition of intracellular epitopes not normally exposed to the immune system. This, in turn may activate autoreactive lymphocytes leading to the appearance of autoantibodies that are not the cause of damage, rather a consequence (Girones, et al., 2005).

The most important cross reacting epitopes of *T. cruzi* and the correspondent epitopes in humans are listed in table 1, as well as the kind of immune response they elicit.

2.2 Second ingredient: host immune system

When the three ingredients theory was first proposed (Storino and Milei 1994, Milei, et al. 1996), second ingredients were mainly T lymphocytes and macrophages. In the subsequent years some evidence grew about the participation of humoral immune system through autoantibodies in the pathogenesis. As a consequence, the whole immune system of the host is now considered as the second ingredient.

As described earlier, mononuclear cells persist in the chronic stage of the disease, contributing to the inflammation through its products of secretion or through its own cytotoxicity (suppressor T cells) and cytolytic action (macrophages) (Storino & Milei, 1994).

As previously stated, molecular mimicry may be the main explanation of autoimmunity, triggering both cellular and humoral autoreactivity (Girones, et al., 2005). Figure 2 summarizes the most important immune events in CCC pathogenesis.

2.2.1 Innate immunity

In recent years innate immunity came to the attention of researchers of Chagas' disease pathogenesis. The role of NK cells has been particularly studied in early and late indeterminate forms of the disease and in CCC patients. In early indeterminate patients,

Parasite antigen	Human Antigen	Immune reaction
B13	Cardiac myosin heavy chain	Autoantibodies Autoreactive T cells
R13 (ribosomal protein)	Ribosomal protein β_1-adrenergic receptor M_2-muscarinic receptor 38-kDa heart antigen	Autoantibodies
Ribosomal protein PO	β_1-adrenergic receptor	Autoantibodies
FL-160	47-kDA neuron protein	Autoantibodies
Shed acute-phase antigen (SAPA)	Cha antigen	Autoreactive T cells
TENU2845/36 kDa	Cha antigen	Autoantibodies
Calcireticulin	Calcireticulin	Autoantibodies Autoreactive T cells
Galactosyl-cerebrosides	Galactosyl-cerebrosides	Autoantibodies
Unknown	Neurons, liver, kidney, testis	Autoantibodies
Sulphated glycolipids	Neurons	Autoantibodies
150-kDa protein	Smooth and striated muscle	Autoantibodies
Cruzipain	Cardiac myosin heavy chain M_2-muscarinic receptor	Autoantibodies
Microsomal fraction	Heart and skeletal muscle	Autoantibodies
Cytoskeleton	95-kDa myosin tail	Autoantibodies
SRA	Skeletal muscle Ca^{2+} dependent SRA	Autoantibodies
MAP	MAP (brain)	Autoantibodies
Soluble extract	Myelin basic protein	Autoantibodies Autoreactive T cells
55-kDa membrane protein	28-kDa Lymphocyte membrane protein	Autoantibodies

Table 1. Examples of cross-reacting epitopes (Girones, et al., 2005, Marin-Neto, et al., 2007).

compared to non infected people, increased values of pre-natural killer (NK)-cells ($CD3^-$ $CD16^+$ $CD56^-$), and higher values of proinflammatory monocytes ($CD14^+$ $CD16^+$ $HLA\text{-}DR^{++}$) were found. The higher values of activated B lymphocytes ($CD19^+$ $CD23^+$) contrasted with impaired T cell activation, indicated by lower values of $CD4^+$ $CD38^+$ and $CD4^+$ $HLA\text{-}DR^+$ lymphocytes, a lower frequency of $CD8^+$ $CD38^+$ and $CD8^+$ $HLA\text{-}DR^+$ cells; a decreased frequency of $CD4^+$ $CD25^{HIGH}$ regulatory T cells was also observed. All these data suggest a rather proinflammatory profile (Vitelli-Avelar, et al., 2006). This profile may be useful to limit parasitemia and confine infection to tissues. In fact, it has been demonstrated that NK cells are important in defense against the spread of parasitic infection (Brener & Gazzinelli, 1997), and are an important source of INF-γ, a key cytokine to activate macrophages and help with parasite clearance (Camargo, et al., 1997).

In late indeterminate form, $CD3^-CD16^+CD56^+$ and $CD3^-CD16^+CD56^{DIM}$ NK cells are increased but are in normal range in CCC patients, suggesting a protective role for them (Vitelli-Avelar, et al., 2005). NK cells showing $CD56^{DIM}$ may play a role in the down

modulation of cytotoxic deleterious T $CD8^+$ response reported in CCC patients (Sathler-Avelar, et al., 2009).
Monocytes display different cytokine profile. In indeterminate patients they produce more IL-10 (Gomes, et al., 2003) while in CCC patients they produce more TNF-α (Vitelli-Avelar, et al., 2008), leading to a proinflammatory profile that could be responsible for chronic myocarditis.
Toll-like receptors (TLR) are also implied in the response to acute infection with *T. cruzi*. TLR-2 has been shown to recognize GPI surface molecules from the parasite. In vitro and in vivo studies have demonstrated that macrophages stimulated with GPIs through TLR-2/CD14 receptors produce NO, TNF-α and IL-12 (Campos & Gazzinelli, 2004).

2.2.2 Cellular adaptative immunity

The role of immune cells in the pathogenesis of Chagas' heart disease has been the dominant hypothesis for many years. The paucity of parasite cells in the inflamed myocardium and the presence throughout the evolution of the disease of macrophages and lymphocytes in patched infiltrates lead to this hypothesis. As early as in 1929, Magariños Torres, observing those infiltrates postulated an "allergic" mechanism for CCC. Further, Mazza and Jörg followed this thought and supported the "allergic" theory (Storino & Milei, 1994).
The study of circulating lymphocytes in peripheral blood of chagasic patients showed an increase in the percentages and actual numbers of double-positive cells of the phenotype CD3+/HLA-DR+, as well as decrease in the percentage of CD45RA+/CD4+ and CD45RA+/CD8+ T cells, indicating greater numbers of activated T cells circulating. Consistent parallel increases were seen also in the B lymphocyte subset which stained double-positive for CD19/CD5 (Dutra W. O., et al., 1994). These results were similar for both indeterminate and CCC patients. Moreover, activated T cells lacking the co-stimulatory molecule CD28 are increased in chagasic patients (Menezes, et al., 2004) and express high levels of HLA-DR molecules (Dutra, et al., 2000). Some interesting differences were demonstrated between indeterminate and CCC patients. $CD28^-$ T cells in indeterminate patients showed expression of CTLA-4, which recognizes the same ligands as CD28, but instead of inducing cell activation it causes down modulation of T cells. On the contrary, T cells in CCC patients do not up-regulate CTLA-4 (Souza P. E. A., et al., 2007). It is particularly interesting that CD8+CD28- cells are increased in CCC patients compared to indeterminate patients, and that these cells do not require co-stimulation to exert their cytotoxic functions. More strikingly, CD4+CD28- cells behave differently in indeterminate and CCC patients. In the formers, they are closely related to IL-10 levels, while in CCC patients they correlate with INF-γ levels (Menezes, et al., 2004).
Another interesting difference has been found in cellular response between indeterminate and CCC patients. CD4+ cells from CCC patients had an increased expression of Vβ5+-TCR, not found in indeterminate patients. When CCC patients mononuclear cells from peripheral blood were cultured in the presence of trypomastigotes antigens, a selective expansion of CD4+ Vβ5+ cells was obtained; while when cultured in the presence of epimastigotes antigens, an expansion of CD8+ Vβ5+ cells was also noted. These findings could not be repeated in indeterminate patients. Trypomastigote stimulation led to the expansion of CD4+ Vβ17+ in indeterminate patients, not seen in CCC patients. This suggests that CCC patients and indeterminate patients respond to different antigen repertoires (Costa, et al., 2000).

Monocytes from indeterminate patients, when infected *in vitro* with *T. cruzi*, express low levels of HLA-DR and high levels of CD80, a ligand for CTLA-4 (Souza P. E., et al., 2004). The interaction of these monocytes with CTLA-4$^+$ T cells leads to the expression of IL-10, a cytokine known to down-modulate inflammatory responses (Gomes, et al., 2003). This is not observed in CCC patients. CD28$^-$ T cells, not expressing CTLA-4, express TNF-α and INF-γ (Menezes, et al., 2004).

In the same direction, CD4$^-$CD8$^-$ γδ T cells are found to be increased in indeterminate patients compared with CCC ones. These cells are also linked to the production of IL-10 and a down modulator effect on inflammation (Villani, et al., 2010).

Cells infiltrating myocardium have also been studied. As demonstrated with immunostaining of endomyocardial biopsies by our group, leukocytes infiltrating myocardium in Chagas' disease were approximately 50% macrophages, and 50% lymphocytes, mainly T lymphocytes (Milei, et al., 1992b). Further immunohistochemical characterization of these cells with CD45R for lymphocytes, CD20 and lambda and kappa light chains for B lymphocytes, CD45R0 for T lymphocytes and CD68 for macrophages, confirmed these findings (Milei, et al., 1996a).

Autoreactive T cells have caught the attention of many investigators. In experimental models, CD4$^+$ T cells from infected mice showed a proliferative response to the exposition to human cardiac myosin heavy chain and to *T. cruzi* B13 protein. They also arrested the beating of fetal heart cells and, more importantly, induced myocarditis in immunized mice and promoted rejection of transplanted normal hearts in the absence of *T. cruzi* (Ribeiro-Dos-Santos, et al., 2001). Also, it has been described that T cells infiltrating the myocardium of chagasic patients cross react with human cardiac myosin heavy chain and to *T. cruzi* B13 protein and express high levels of INF-γ and low levels of IL-4, switching to a Th1 profile (Cunha-Neto Edecio & Kalilf, 2001).

In recent years, Treg cells have come to attention in relation to Chagas' disease pathogenesis. These cells are characterized by the expression of CD4, CD25 and FOXP3 (Ziegler & Buckner, 2009). Treg cells are increased in indeterminate patients compared to CCC, which correlates negatively with levels of activated CD8$^+$ (Vitelli-Avelar, et al., 2005).

A second subset of T CD4+ cells, recently described, the Th17 cells, resulted important in Chagas' disease pathogenesis. These cells, mainly linked to autoimmune pathology, are characterized by the expression of CD4+, RORγt, and secrete IL-17 (Di Jin, et al., 2008). They were increased in a murine model of acute myocarditis induced by *T. cruzi* infection, as well as by immunization with heat-killed *T. cruzi* antigens (Bonney, et al., 2011). Both cell subsets seem to be related, as they require TGF-β to differentiate. In the presence of proinflammatory cytokines, differentiation to Th17 cell prevails and a pro-autoimmune profile develops (Ziegler & Buckner, 2009).

An additional mechanism is the bystander activation. This is the activation of autoreactive lymphocytes by antigen presenting cells in a proinflammatory environment (Fujinami, et al., 2006). This kind of autoreactive T cells activation has been described in Chagas'disease (Fedoseyeva, et al., 1999).

2.2.3 Humoral adaptative immunity

The importance of humoral immunity in controlling *T. cruzi* acute infection has been clearly established. Mice lacking B lymphocytes rapidly succumb to infection (Kumar & Tarleton, 1998). But the fact that attracted most attention is the production of autoantibodies.

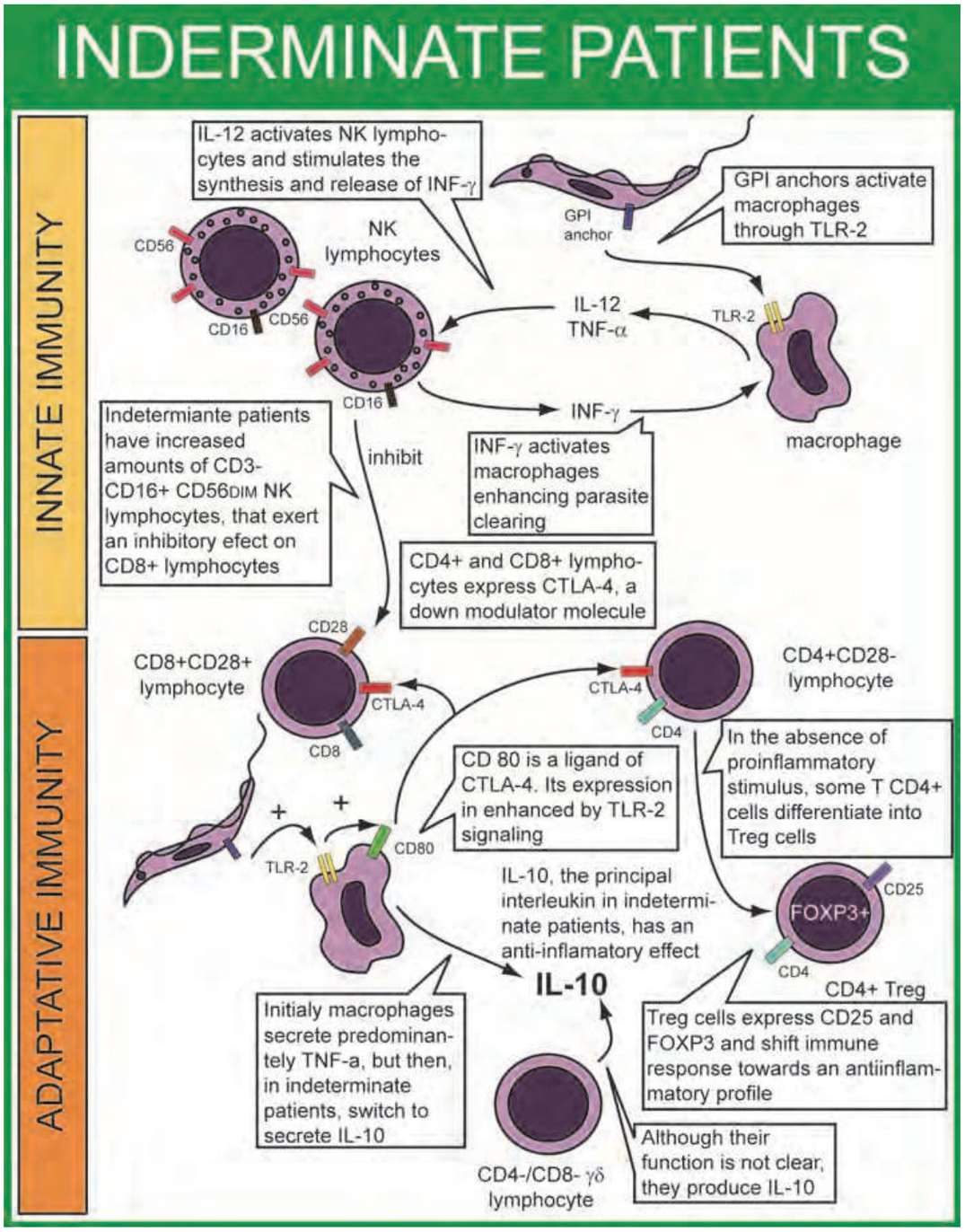

Fig. 2A. The immune pathogenesis of Chagas disease in indeterminate patients. The presence on numerous down regulating mechanisms shift the response towards an anti-inflammatory profile.

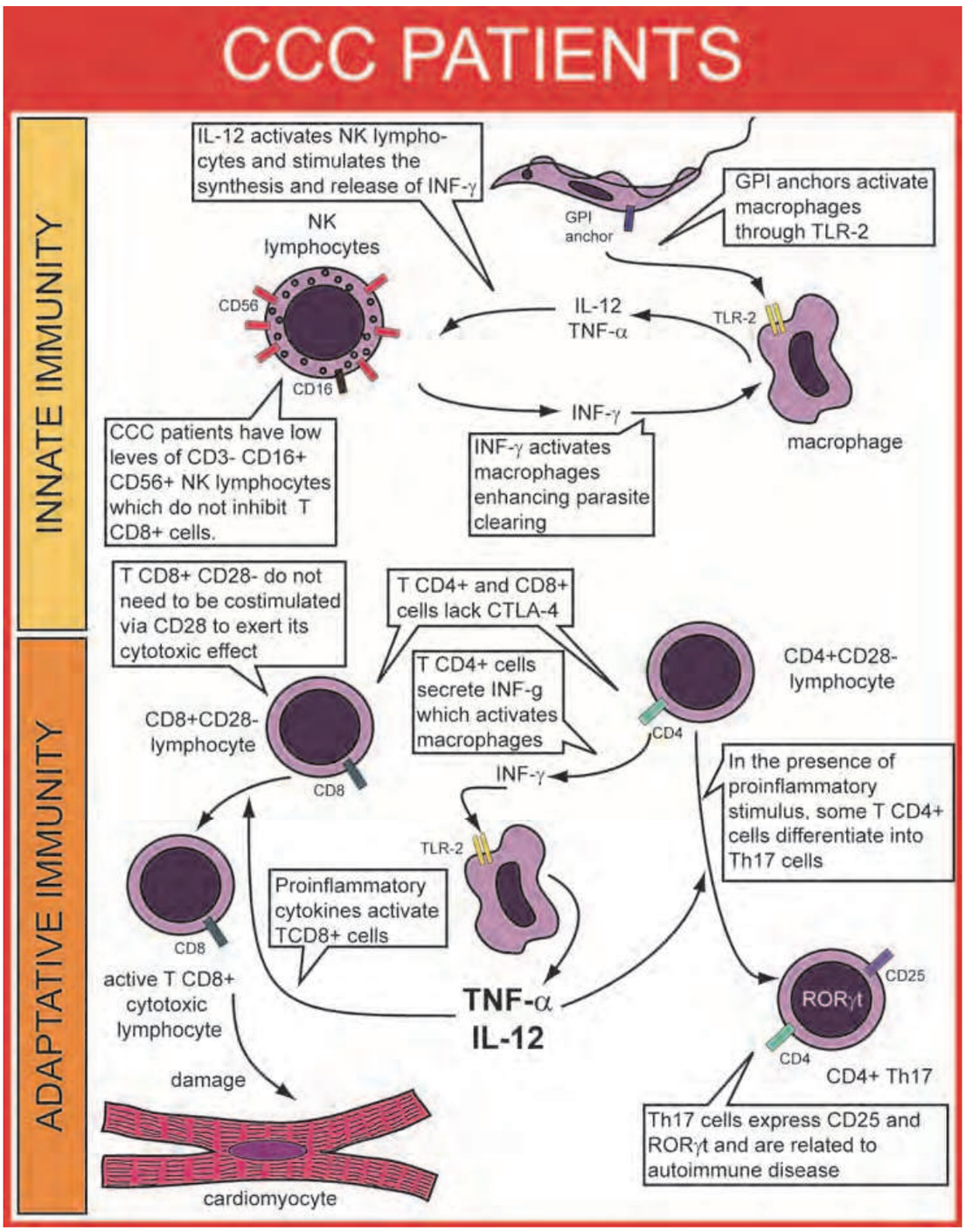

Fig. 2B. The immune pathogenesis of Chagas disease in CCC patients. Cells evolve towards a proinflammatory profile, with development of autoimmunity.

The first autoantibody to be described was one that reacted to endocardium, blood vessels and interstitium of skeletal muscle (EVI) (Cossio, et al., 1974), but was the same group of investigators who recognized the heterophil nature of the antibody and realized that had no pathogenic role (Khoury, et al., 1983).

Another autoantibody, studied by our group, was anti-laminin antibody (Sanchez, Milei et al. 1993, (Milei, et al., 1993). These antibodies were shown to react against *T. cruzi* amastigotes and trypomastigotes and human laminin (Szarfman, et al., 1982) and deposition of this antibody in marked thickened basement membranes of myocytes, endothelial cells, and vascular smooth muscle cells was shown by us with light microscopy, electron microscopy and immunohistochemical techniques in endomyocardial biopsies of chagasic patients (Sanchez, et al., 1993) but then we found that only 50% of patients had the antibody on their sera and no correlation with disease severity could be established (Milei, et al., 1993).

Anti-myosin antibodies were postulated by some authors to be generated through molecular mimicry with two *T. cruzi* antigens: B13 protein (Gruber & Zingales, 1993) and cruzipain (Giordanengo Laura, et al., 2000a, Giordanengo Laura, et al., 2000b). Although cruzipain antibodies mainly react to skeletal muscle myosin, they can cause conduction disturbances when transferred to uninfected mice and, when transferred to pregnant animals, they caused conduction disturbances in pups (Giordanengo Laura, et al., 2000b). On the other hand, immunossuppresed mice did not mount any humoral response when immunized with myosin but still develop myocarditis (Neu, et al., 1990). This fact made some authors doubt on the molecular mimicry hypothesis and rather consider antibodies to myosin a consequence of myocyte damage (Kierszenbaum, 2003).

Antibodies that react with muscarinic receptors were intensely studied. In early 1990's IgG from chagasic patients was observed to bind to muscarinic M2 receptors and activate them (Sterin-Borda L, et al., 1991). These anti-muscarinic antibodies were found to increase intracellular cGMP and decrease cAMP (Goin J., et al., 1997) and were positively related to the presence of dysautonomia (Goin J. C., et al., 1994). These antibodies also caused accumulation of inositol phosphate and nitric oxide synthase stimulation, with a negative inotropic effect on myocardium (Sterin-Borda Leonor, et al., 1997). As mentioned before, anti-muscarinic autoantibodies are positively related to the presence of dysautonomia (Goin J. C., et al., 1994), the presence of achalasia in chagasic patients (Goin J. C., et al., 1999), sinus node dysfunction (Altschuller, et al., 2007), but are not related with the degree of myocardial dysfunction (Altschuller, et al., 2007, Talvani Andre, et al., 2006), nor with the presence of brain lesions (Py, et al., 2009). In fact, patients with cardiomyopathy and left ventricular dysfunction but without autonomic dysfunction show low levels of anti-muscarinic antibodies (Sterin-Borda Leonor & Borda, 2000).

Antibodies against β_1-adrenergic receptors were also deeply studied. Described in early 1980's (Borda E., et al., 1984) these antibodies increased cAMP in mouse atrial fibers, increasing the release of PGE_2 and TXB_2 causing diminished contractility (Gorelik, et al., 1990). Increased cAMP activates PKA and then increases the intracellular calcium concentration. This causes in turn inhibition of the Na^+/K^+-ATPase and stimulates Ca^{2+}-ATPase activity leading to intracellular depletion of K^+ and further increase in Ca^{2+}. These alteration alter contractility and electric impulse generation and conduction (Borda E. S. & Sterin-Borda, 1996). Antiadrenergic autoantibodies titers could not be related to the severity of left ventricular dysfunction (Talvani Andre, et al., 2006) and patients with overt cardiomyopathy but without autonomic dysfunction show low levels of these antibodies (Sterin-Borda Leonor & Borda, 2000). Antibodies against β_2-adrenergic receptors have also been described but are mainly related to megacolon (Wallukat, et al., 2010).

Autoantibody	Hypothetic pathogenic role	Reference
Anti-Cerebroside	Probably related to neurologial symptoms	(Avila & Rojas, 1990)
Anti-Gal	Apparently protective	(Gazzinelli, 1991)
Anti-Brain Microtubules	Unknown	(Kerner, et al., 1991)
Anti-Ribosome	Unknown	(Levitus, et al., 1991, Skeiky, et al., 1992)
Anti- UsnRNPs	Unkwnown	(Bach-Elias, et al., 1998)
Anti-Sulfatides	May cause myocarditis and induce arrhythmias	(Garcia, et al., 1998)
Anti-Galectin-1	Increased in CCC patients	(Giordanengo L., et al., 2001)
Anti-Cha R3	Specific of CCC	(Girones, et al., 2001a)
Anti-Desmoglein-1	Related to Penphigus foliaceum	(Diaz, et al., 2004)
Anticardiolipin	Unknown	(Pereira De Godoy, et al., 2005)
Anti- TrkA, TrkB and TrkC	Prevents apoptosis of neurons and helps cellular invasion	(Lu, et al., 2010)
Anti-MBP	Related to gastrointestinal form	(Oliveira E. C., et al., 2009)

Table 2. Less studied autoantibodies in Chagas' disease.

Antibodies against AV node and sinus auricular node tissues have been studied as markers of CCC. When compared in chronic chagasic cardiopathy patients, non-chagasic cardiopathy patients, indeterminate chagasic subjects and healthy blood donors as controls, they more frequently found in chronic chagasic cardiopathy, but not enough to be good markers for chagasic cardiopathy group. Besides, no clear association with complex rhythm or conduction alterations was found (Arce-Fonseca, et al., 2005).

Many other autoantibodies have been described (table 2) but are not so widely studied and their role in pathogenesis of chagasic myocarditis is not clear.

2.2.4 Genetic factors

Human leukocyte antigens (HLA) have shown some relation to the development of CCC. HLA-B40 and Cw3 combination was protective for CCC (Llop, et al., 1991), as resulted DRB1*14, DQB1*0303 (Fernandez-Mestre, et al., 1998), HLA-DQB1*06 (Deghaide, et al., 1998) and HLA-A68 (Cruz-Robles, et al., 2004). On the other hand, HLA-C*03 (Layrisse, et al., 2000), DRB1*1503 (Garcia Borras, et al., 2009), DRB1*01, DRB1*08, DQB1*0501 (Fernandez-Mestre, et al., 1998) and HLA-DR16 alelles (Cruz-Robles, et al., 2004) were positively related to the development of CCC.

A number of other genes related to immune system have been studied in order to determine their relation to a predisposition to develop CCC. In table 3 we list those positively related to the appearance of CCC (Cunha-Neto E., et al., 2009).

Gene	**Polymorphism**
CCL2/MCPI	- 2518
CCR5	+ 53029
TNF-α	- 308
LTA	+ 80, + 252
BAT-1	- 22, - 348
NFkBIL-1	- 62, - 262
IL-1B	- 31, + 3954, + 5810
IL-10	- 1082
IL-12B	+ 1188
MAL/TRIAP	S180L

Table 3. Genetic polymorphisms related to CCC. Adapted from (Cunha-Neto E., et al., 2009).

2.2.5 The cytokines and chemokines

Although proinflammatory cytokines seem to be necessary for controlling parasitemia during acute phase of the disease (Cunha-Neto E., et al., 2009), CCC patients display a rather proinflammatory cytokine while indeterminate patients display a down modulator one. CCC patients had higher levels of TNF-α and CCL2 than indeterminate patients (Ferreira, et al., 2003, Talvani A., et al., 2004). Infiltrating macrophages from CCC patients expressed INF-γ, TNF-α and IL-6 but showed low levels of IL-2, IL-4 and IL-10 (Abel, et al., 2001, Reis D. D., et al., 1993, Reis M. M., et al., 1997). Also CCR5, CXCR3 and CCR7 and their ligands were increased in hearts of CCC patients, as well as monocytes expressing CXCR3, CCR5, CXCL9 and CCL5 (Cunha-Neto E., et al., 2009). It has been shown that INF-γ and CCL2 induceed myocytes to secrete arial natriuretic factor and caused hypertrophy (Cunha-Neto E., et al., 2005), and IL-18 and CCR7 ligands, which are increased in CCC, caused cardiomyocyte hypertrophy and fibrosis (Reddy, et al., 2008, Riol-Blanco, et al., 2005, Sakai, et al., 2006).

2.3 The third ingredient: fibrosis

Fibrosis is one of the most striking characteristics of CCC. In our patients with CCC in endomyocardial biopsies, fibrosis had replaced between 8.2 and 49% of contractile myocardium, with only one patient having less than 10% (Milei, et al., 1992b). While in autopsies, fibrosis was more extensive reaching in the conduction system than in the contracting myocardium (51.5 ± 18% vs 43.4 ± 8%, $p < 0.05$) (Milei, et al., 1996a).

The deposition of laminin in extracellular and basement membranes has been implicated in the pathogenesis of inflammatory process, as laminin is able to bind proinflammatory citokines (Savino, et al., 2007). The inflammatory infiltrate in CCC was related to the production of citokines such as INF-γ, TNF-α, IL-18, CCL2 and CCL21, that may have modulator actions on fibrotic process (Cunha-Neto E., et al., 2009).

3. A combined theory that could explain the pathogenic mechanism in chronic chagasic myocarditis

With the perpetuation of inflammation, necrosis and scarring fibrosis, damage to all histological components of myocardium occurs. Damage to contracting myocardial fibers

determines contractile failure as well as electrophysiological disturbances. Conduction system, nervous autonomic system and microvasculature are also damaged and as a consequence they cause further damage to contractile myocardium and produce electrical instability. Figure 3 illustrates with a flow chart the interactive network of different elements in the pathogenesis of CCC.

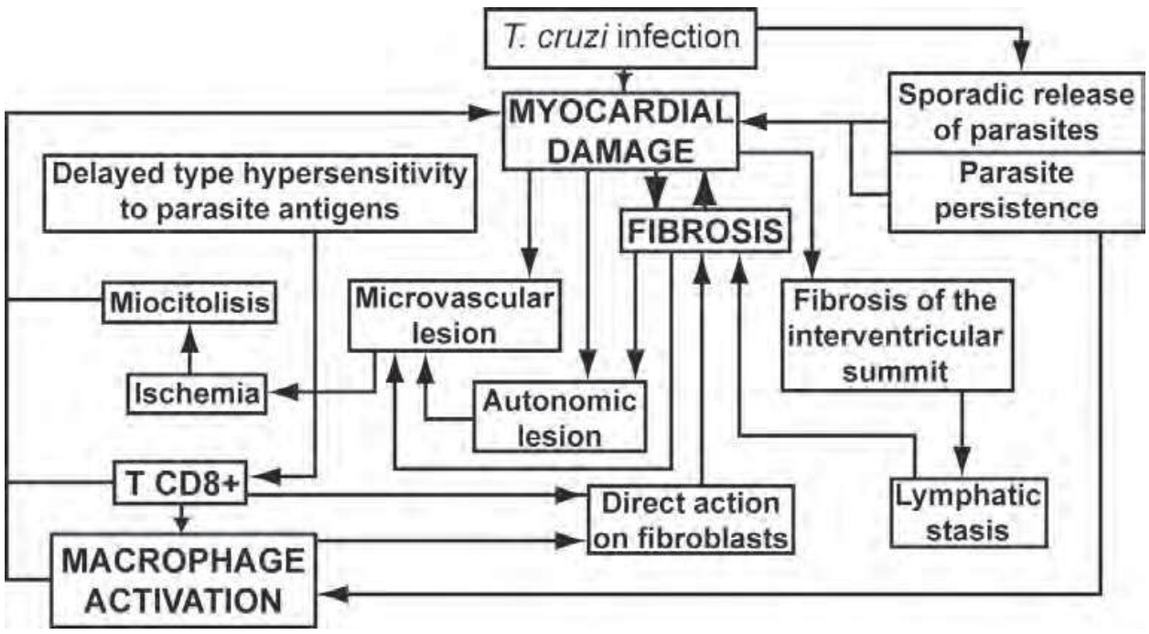

Fig. 3. Schematic representation of the integrated theory of multiple factors that determine myocardial damage in CCC.

4. Pathophysiological consequences of organ damage

4.1 Dysautonomia

As early as 1922 Carlos Chagas noted that the chronotropic response to atropine was altered in chagasic patients (Chagas C. & Vilella, 1922), but it was not until late 1950's that Köberle published his works showing impressive neuronal depopulation in microscopic sections obtained from the intercaval atrial strip in chagasic patients using a standardized technique of cardiac intramural neuronal counting developed by himself (Köberle, 1956a, 1956b). These findings led to the "neurogenic hypothesis" (Köberle, 1959), which explained all megas in Chagas' disease as a consequence of neuronal depletion.

Although many other authors claimed to have confirmed this finding (Mott & Hagstrom, 1965, Oliveira J. S., 1985), other authors called to attention about the criteria used to diagnose neuronal depletion because of the great variability in the number of neurons in autonomic ganglia (Rossi L., et al., 1994) and they also remarked that the only right criterion to establish neuronal depletion is the presence of proliferation of satellite cells, with the formation of Terplan's nodules, a characteristic lesion described as proliferating satellite cells which replace degenerating neurons, forming nodular structures. These lesions, once considered patognomonic, can be found in other cardiomyopathies (Rossi L., et al., 1994). The same author could not confirm the loss of neurons or denervation in CCC (Rossi L., 1988). Finally, it was demonstrated that, using Terplan's nodules as diagnostic criterion,

CCC patients with heart failure had more neuronal depletion than patients with dilated cardiomyopathy of other causes (Oliveira J. S., 1985). In our experience the neuroganglionic involvement was variable in autopsies of chagasic hearts (Milei, et al., 1991b).
According to pioneer neurogenic hypothesis (Köberle, 1959), early and irreversible damage to the parasympathetic system during acute phase of the disease causes a cathecolaminergic cardiomyopathy, but this point of view has been debated and evidence is contradictory. Functional test performed in CCC patients demonstrated impaired parasympathetic heart rate regulation (metaraminol, phenylephrine and atropine intravenous injections, facial immersion, Valsalva maneuver, head-up and head-down tilt tests, respiratory sinus arrhythmia, handgrip, graded dynamic exercise, and spectral analysis of Holter recordings) (Amorim, et al., 1968, Amorim, et al., 1973, Gallo, et al., 1975, Guzzetti, et al., 1991, Junqueira Junior, et al., 1985, Manço, et al., 1969, Marin-Neto, et al., 1975, Sousa, et al., 1987). However, a careful analysis of these data showed that many patients had normal autonomic function and most patients had heart failure, that could explain autonomic dysfunction *per se* (Davila, et al., 1998).
On the other hand, the study of indeterminate patients has shown conflicting results. While some authors could demonstrate impaired autonomic function (Molina, et al., 2006, Vasconcelos & Junqueira, 2009) others could demonstrate that autonomic function was normal in patients without myocardial damage and that abnormalities in autonomic dysfunction was proportional to heart dysfunction, leading these authors to propose that these abnormalities arise as a compensating mechanism for the progressive left ventricular dilatation (Davila, et al., 1991, Davila Spinetti, et al., 1999). These findings led to a new "neurogenic theory", which considers autonomic dysfunction as secondary to ventricular dilatation and hemodynamic alterations, but once installed, acts synergistically with parasitism and inflammation to cause further myocardial damage (Davila, et al., 2004).

4.2 Microvascular damage

Microcirculation abnormalities in CCC have been firstly pointed out by Jorg as an angiographic anarchy due to capillary loss (Jörg, 1974) and furtherly demonstrated in experimental models as well as in clinical practice (Rossi M. A., et al., 2010).
Many investigators have found abnormal myocardial perfusion using isonitrile-99m-technetium (Castro R., et al., 1988) and thallium-201 (Hagar & Rahimtoola, 1991, Marin-Neto, et al., 1992) scintigraphy in chagasic patients with normal epicardial coronary arteries. Furthermore, the progression of left ventricular systolic dysfunction is associated with both, the presence of reversible perfusion defects and the increase in perfusion defects at rest (Hiss, et al., 2009, Schwartz & Wexler, 2009). Anatomopathological studies in humans also provided evidence of microvascular damage in CCC. In late 1950's first reports showing collapse of arterioles and intimal proliferation (Torres, 1960) caught the attention of investigators. Also, microthrombi have been described (Rossi M. A., et al., 1984). As said, in endomyocardial biopsies thickening of capillary basement membranes was also found (Milei, et al., 1992b).
Additional evidence of microvascular damage was obtained from experimental models. Vascular constriction, microaneurysms, dilatation and proliferation of microvessels has been demonstrated (Factor & Sonnenblick, 1982, Morris, et al., 1989, Tanowitz H. B., et al., 1996, Tanowitz Herbert B., et al., 1992b).
Many factors have been advocated in the genesis of these lesions. First, the parasite itself. It was shown that *T. cruzi* produces a neuraminidase that removes sialic acid from de surface

of endothelial cells. This results in thrombin binding and platelet aggregation (Libby, et al., 1986). *T. cruzi* also produces tromboxane A_2 (TXA_2), specially during amastigote state (Ashton, et al., 2007), also favouring platelet aggregation and vascular spasm. Direct parasitism of endothelial cells by *T. cruzi* has also been demonstrated, and this causes the activation of the NF-κB pathway increasing the expression af adhesion molecules (Huang, et al., 1999a), and secreting proinflammatory citokines (Tanowitz Herbert B., et al., 1992a) and iNOS (Huang, et al., 1999b).

Endothelin-1 (ET-1) is another proposed pathogenic element. Elevated levels of mRNA for preproendothelin-1, endothelin converting enzyme and endothelin-1 were observed in the infected myocardium (Petkova Stefka B., et al., 2000), and elevated levels of ET-1 have been found in CCC patients (Salomone, et al., 2001). Mitogen-activated protein kinases and the transcription factor activator-protein-1 regulate the expression of endothelin-1, and both are shown to be increased in myocardium, interstitial cells and vascular and endocardial endothelial cells (Petkova S. B., et al., 2001). Besides, treatment with phosphoramidon, an inhibitor of endothelin converting enzyme, decreases heart size and severity of lesions in an experimental model of Chagas' disease (Jelicks, et al., 2002).

Inflammation also produces dysfunction of endothelial cells. Macrophages secrete TXA_2 and platelet activating factor (PAF) act on endothelium causing vasoconstriction (Rossi M. A. & Carobrez, 1985). Endothelial cells infected *in vitro* with *T. cruzi* lose their antithrombotic properties in response to interleukin 1 β (IL-1β) (Bevilacqua, et al., 1984, Nachman, et al., 1986).

It is remarkable that, although the data presented, endothelial function seems to be normal in CCC patients without heart failure, as measured by increases in blood flow in response to acetylcholine and sodium nitroprusside (Consolim-Colombo, et al., 2004). Further, in our concept, microvascular damage found in CCC, seems to be secondary to fibrosis and distortion of myocardial fiber arrangement by necrosis and chronic infiltrates, but as once established, may contribute to the perpetuation of myocardial damage.

5. Pathology

Pathological findings are described mostly according to our own findings.

5.1 Macroscopic features

The most striking characteristic of CCC is enlargement of heart with variable degrees of dilatation of chambers (Andrade, 1985) (Figure 4A). In autopsy series, hearts were overweighted (Andrade, 1985, Baroldi, et al., 1997, Bestetti, et al., 1993, Lopes, et al., 1981) compared with indeterminate chagasic patients and non-chagasic subjects. Marked cardiomegalies reached up to 500 grams. Right ventricle (RV) and atrium (RA) were generally more compromised than left chambers, being RV the most dilated in one paper (Laranja, et al., 1956) but RA was in other (Andrade, 1985).

A second remarkable feature is the thinning of the left ventricular apical wall, resulting in apical aneurysm, a very characteristic lesion in CCC (Figure 4B) (Moia, et al., 1955).

Other lesions described are flattening of the papillary muscles and a marked subendocardial sclerosis, parietal and/or aneurismal thrombosis and fibrotic plaques in pericardium (Milei, et al., 1996a, Milei, et al., 1991b, Storino & Milei, 1994).

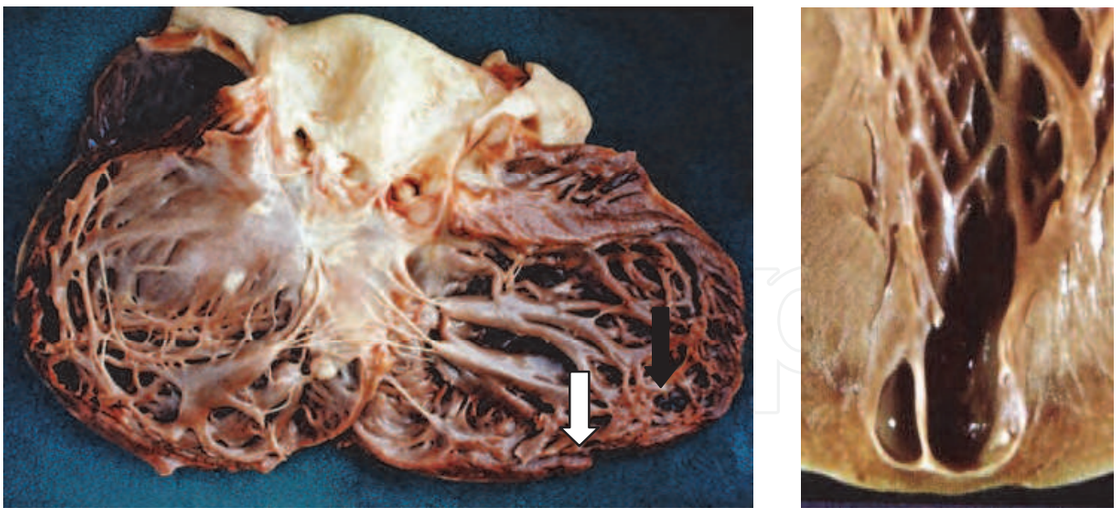

Fig. 4. A. High grade heart dilatation. Thining of the apical wall of the left ventricle (white arrow) and cavitary thrombus (black arrow). B. Characteristic apical aneurysm. A from Milei, et al., 1996b, B from Milei, et al., 2008.

5.2 Histological features

Microscopically, myocardial lesions consisted of a chronic inflammatory process with fibrotic scars and extensive mononuclear infiltrates. Such infiltrates were more prominent in the working myocardium and in the specialized cells of the left branch of the His bundle than in the AV node and in the right hisian branch, showing a microfocal disposition (Figure 5A). The percentage of fibrosis was variable and ranged between 8.2 to 49% (Milei, et al., 1996a, Milei, et al., 1992b) (Figure 5B).

Extensive myocytolysis and spotty contraction band necrosis were observed. Cell hypertrophy in the apparently preserved myocytes was revealed by hypertrophic bizarre nuclei. Dilated lymphatic channels widespread in the ventricular septum and in the AV node, His bundle, and in the root of the right and left bundles branches were observed. In the case of apical aneurysm of the left ventricle, dilated lymphatic were distributed subepicardically (Milei, et al., 1996a).

The serial sectioning of the conducting system showed prominent lesions. Sino-atrial node presented mononuclear infiltrates, necrosis of specialized fibers, and intense fibrosis (Milei, et al., 1991b). In the remaining specialized system lesions consisted of mild to moderate diffuse fibrosis of the AV node and of the penetrating and branching portions of the His bundle, complete destruction of the proximal segments of the right and left bundles branch by varying degrees of replacement by dense collagen tissue (Figure 5A). The remaining specialized fibers presented atrophy and mild fatty infiltration and were surrounded in most cases by infiltrates consisting mainly of lymphocytes and macrophages. The subendocardial Purkinje fibers were usually damaged by chronic inflammation and fibrosis (Milei, et al., 1991b) (Figure 5B). These vast fibrosis in the conduction system (Figure 5C) showed severe conduction alterations in electrocardiograms, although curiously in one revision, there were needed sophisticated electrophysiological studies to demonstrate electrical abnormalities in these patients (Andrade, et al., 1988)

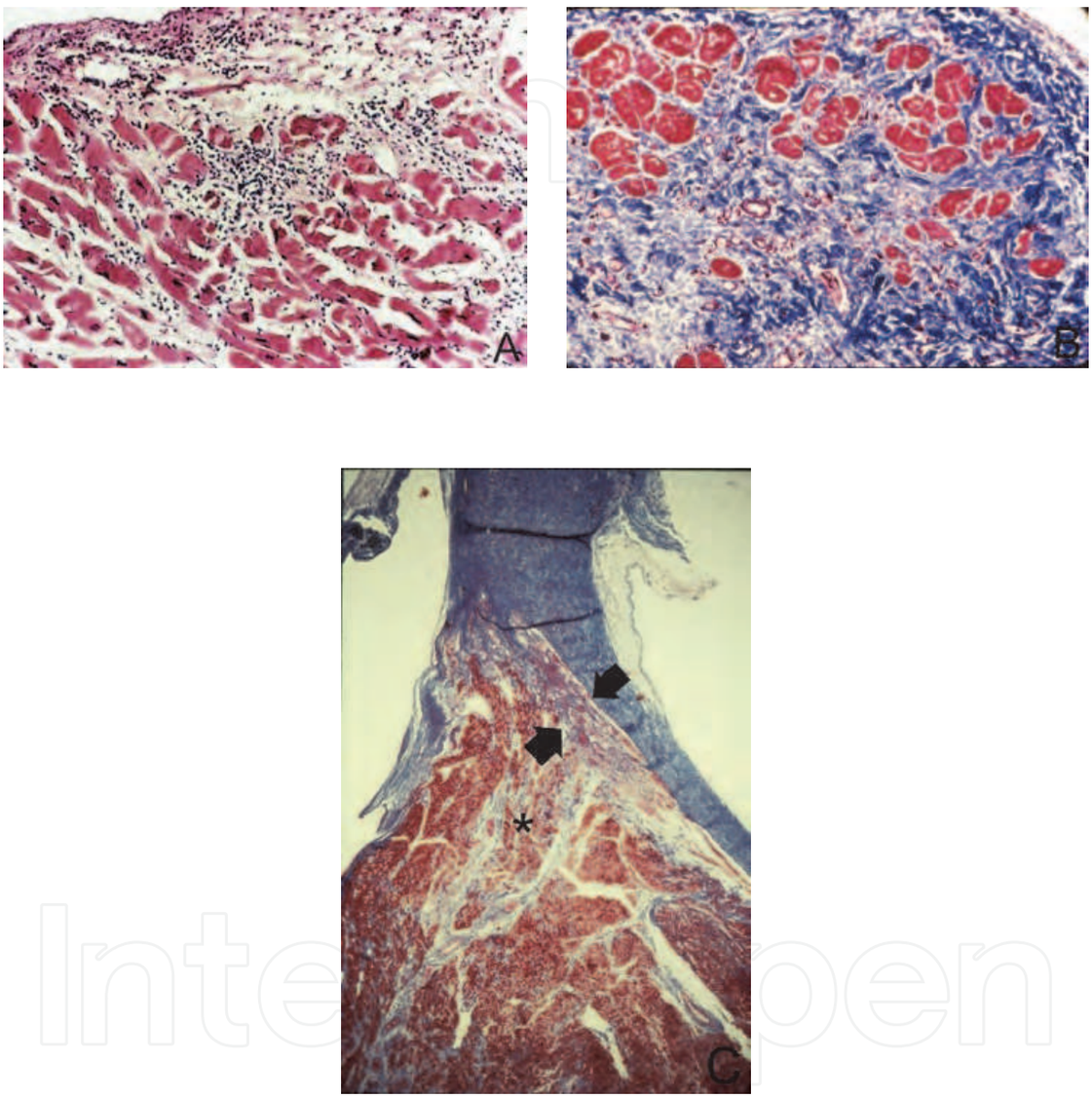

Fig. 5. A. Extensive mononuclear infiltrates, myocytes loss, and subendocardial fibrosis. Hematoxylin and eosin stain, X25. B. Atrophic myocardial fibres (red) separated by thick bands of fibrous tissue (blue). Mallory trichrome, X 25. C. Bifurcating His bundle showing severe fibrosis at the left branch (between arrows). The right branch (asterisk) is intramyocardial and surrounded by connective tissue. Mallory trichrome, X25. A and C from Milei, 1996a. B from Milei, 2008.

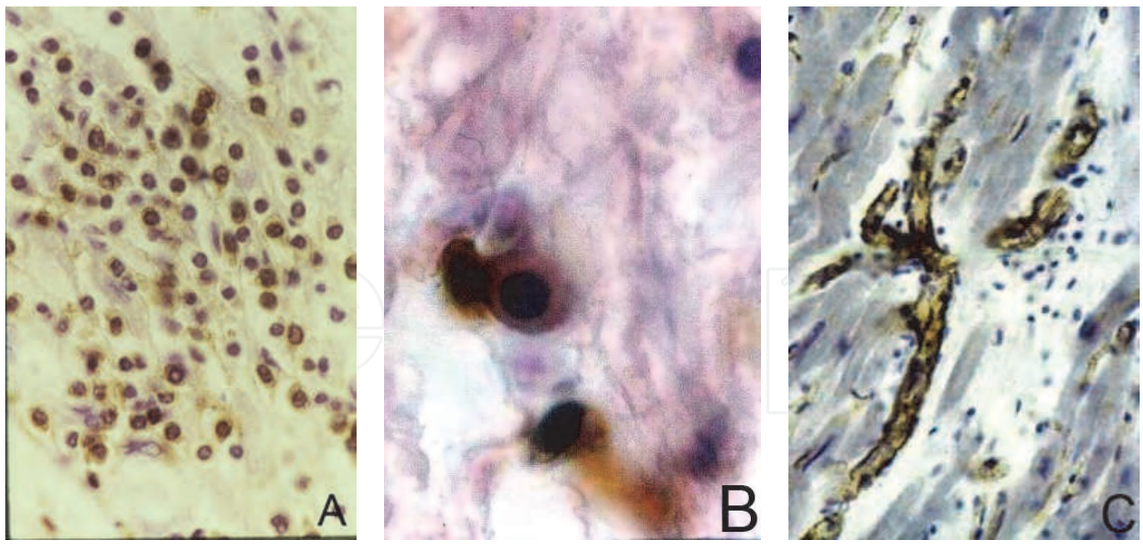

Fig. 6. A. Detail of the left bundle of His is shown. Immunostaining for T lymphocyte. Positive cells express CD45R0 antigen (brown); specialized myocardial cells have almost disappeared. Extensive mononuclear infiltrate, the majority of them being T lymphocytes. X20. B. Double immunostaining for the simultaneous demonstration of T lymphocytes (CD45RO) and macrophages (CD68). T lymphocytes (brown) in close contact with a macrophage (pinky cytoplasm). X1000. C. Immunostaining to show endothelial cells. Capillaries and small vessels are clearly showed by the expression of CD31. Vessels are midly or moderately disorted because of the surrounding fibrosis. X100. From Milei, 1996a.

In our studies in endomyocardial biopsies, infiltrates were approximately 50% lymphocytes and 50% macrophages. Almost 80% of lymphocytic population were T lymphocytes, being only 20% B lymphocytes. Eosinophils were scarce in infiltrates reaching 5%, and were associated with areas of necrotic myocardium. Mast cells also were scarce or absent in specialized and in contracting myocardium. (Milei, et al., 1996a, Milei, et al., 1992b)
Histological study of aneurisms showed a thinned wall 2-4 mm, with sclerotic plaques of thickened endocardium of up to 92% of total tissue and extensive mononuclear chronic inflammatory infiltrates and widespread fibrosis in myocardium. Myocytes were organized in thin bands or atrophic units surrounded by fibrotic tissue (Figure 5B) (Milei, et al., 1991a).
Autonomic ganglia showed above described Terplan's nodules, with satellite cell proliferation replacing degenerated autonomic neurons. As stated, these lesions, once considered patognomonic, can be found in other cardiomyopathies (Rossi L., et al., 1994).

5.3 Immunohistochemical findings

Immunophenotyping of infiltrates allowed a better characterization of the cells participating in the inflammatory infiltrates, mainly macrophages ($CD68^+$) and lymphocytes ($CD45R^+$). In our works 26.5% percent of them were T lymphocytes ($CD45R^+$, $CD45R0^{+)}$ and 10.5% were B lymphocytes ($CD20^+$, light chains kappa and/or lambda$^+$) (Figure 6A). Thirty percent of the infiltrate was composed of macrophages ($CD68^+$). The remaining infiltrate was composed of mononuclear cells resembling macrophages and CLA-negative mononuclear cells. Contacts between CD68 positive cells and T lymphocytes were frequently found

(Figure 6B). CD31 antibodies clearly pointed out normal endothelial cells, in either normal or damaged vessels (Figure 6C) (Milei, et al., 1996a).

5.4 Ultrastructural features

Myocardial fibers showed nuclear enlargement, nuclear membrane invaginations, lipofuscin deposits, myofibrils derangements and loss, swelling, mitochondrial atrophy, dilatation of sarcotubular system, and interstitial fibrosis (Carrasco, et al., 1982, Palacios-Prü, et al., 1982). These findings have been confirmed by our group in endomyocardial biopsies (Ferrans, et al., 1988, Milei, et al., 1992b). Platelet thrombi can be demonstrated within capillaries (Figure 7B).

Other striking alteration in these specimens was the thickening of the basement membranes of cardiac myocytes (Figures 7A, 7C), endothelium Figure 7C) and vascular smooth muscle up to 20 times their normal thickness of 500 Å (Ferrans, et al., 1988). The thickened basement membranes appeared structurally homogeneous, without being multilayered or subdivided into a lamina rara and a lamina densa. They were of relatively low electron density, had a finely fibrillar appearance at high magnification and measured up to 1 μm in thickness. Using gold-conjugated antibodies, we could demonstrate the presence of laminin in the thickened basal membranes of myocytes and endothelium (Sanchez, et al., 1993).

Regarding the ultrastructure of aneurysms resected from chagasic patients we observed, hypertrophy of myocytes, with swelling, partial or complete loss of myofibrils, swelling of mitochondria, disruption of mitochondrial cristae, lipofuscin granules, and intact sarcolemmas . Basement membranes were thickened, as previously described (Milei, et al., 1991a)

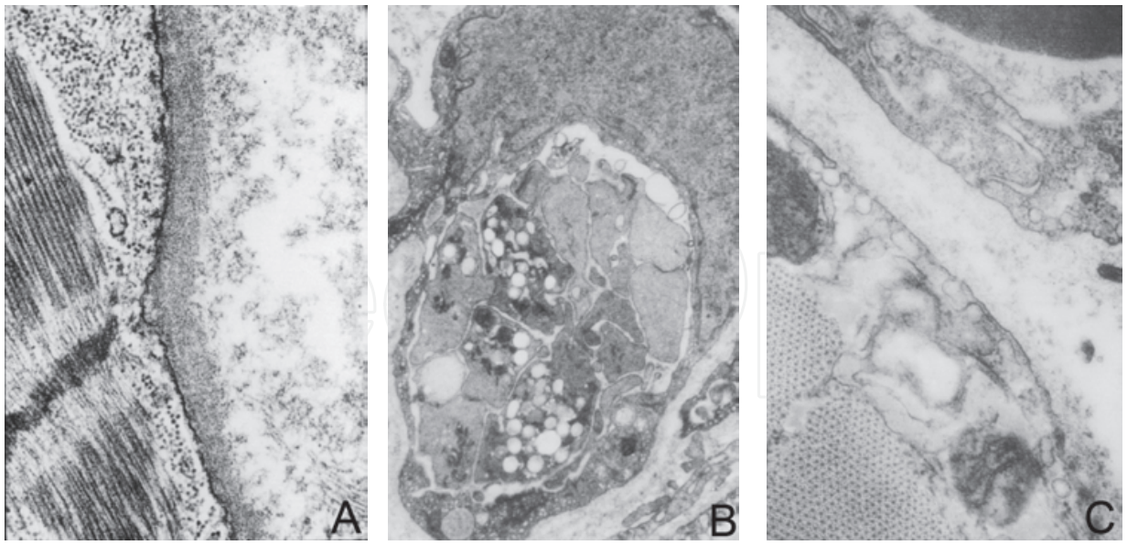

Fig. 7. A. Myocardial fibre with thickened basement membrane. B. Platelet thrombus within a capillary. C. Thickened basement membranes in a myocardial fibre and a capillary. From Milei, et al., 2008.

6. Conclusions

As shown across the sections of this chapter, the numerous hypothesis about pathogenic pathways of CCC have supporting data and pitfalls. Finally all proposals interact with each other, giving us the idea that none of these theories explains the very complex development of CCC by itself. Rather, it seems more feasible that all these hypothesis conform a network of damaging elements, and that all ingredients cause and/or enhances each other. The triggering factor is obviously the interaction between parasite and host's immune system. Cell parasitism, the inflammatory process and consequent necrosis and fibrosis cause damage to contracting myocardium, autonomic system, conduction system and microcirculation. Autonomic damage causes impaired regulation of microvasculature and further alterations in blood flow. Ischemia causes more myocardial damage. Necrosis exposes intracellular epitopes and causes autoantibodies production with more necrosis, fibrosis and so on. It seems that, if adequate down modulator immune mechanisms work properly, this vicious circle stops and patients do not develop cardiomyopathy, rather they remain in the indeterminate form lifelong.

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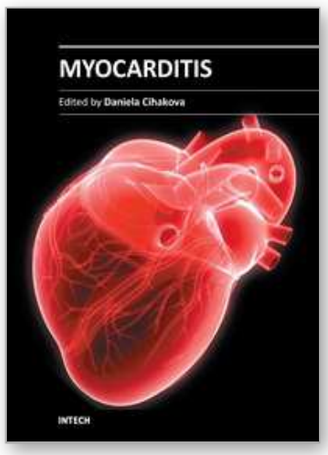

Myocarditis

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Myocarditis, the inflammation of the heart muscle, could be in some cases serious and potentially fatal disease. This book is a comprehensive compilation of studies from leading international experts on various aspects of myocarditis. The first section of the book provides a clinical perspective on the disease. It contains comprehensive reviews of the causes of myocarditis, its classification, diagnosis, and treatment. It also includes reviews of Perimyocarditis; Chagasâ€™ chronic myocarditis, and myocarditis in HIV-positive patients. The second section of the book focuses on the pathogenesis of myocarditis, discussing pathways and mechanisms activated during viral infection and host immune response during myocarditis. The third, and final, section discusses new findings in the pathogenesis that may lead to new directions for clinical diagnosis, including use of new biomarkers, and new treatments of myocarditis.

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