Immunological and non-immunological effects of cytokines and chemokines in the pathogenesis of chronic Chagas disease cardiomyopathy

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The pathogenesis of Chagas disease cardiomyopathy (CCC) is not well understood. Since studies show that myocarditis is more frequent during the advanced stages of the disease, and the prognosis of CCC is worse than that of other dilated cardiomyopathies of non-inflammatory aetiology, which suggest that the inflammatory infiltrate plays a major role in myocardial damage. In the last decade, increasing evidence has shown that inflammatory cytokines and chemokines play a role in the generation of the inflammatory infiltrate and tissue damage. CCC patients have an increased peripheral production of the inflammatory Th1 cytokines IFN-γ and TNF-α when compared to patients with the asymptomatic/indeterminate form. Moreover, Th1-T cells are the main producers of IFN-γ and TNF-α and are frequently found in CCC myocardial inflammatory infiltrate. Over the past several years, our group has collected evidence that shows several cytokines and chemokines produced in the CCC myocardium may also have a non-immunological pathogenic effect via modulation of gene and protein expression in cardiomyocytes and other myocardial cell types. Furthermore, genetic polymorphisms of cytokine, chemokine and innate immune response genes have been associated with disease progression. We will review the molecular and immunological mechanisms of myocardial damage in human CCC in light of recent findings.

Key words: Chagas disease cardiomyopathy - cytokines - chemokines - gene polymorphism - energy metabolism

The most important clinical consequence of chronic Chagas disease is chronic Chagas disease cardiomyopathy (CCC), an inflammatory cardiomyopathy that develops in up to 30% of infected individuals. A significant proportion of those patients subsequently develop dilated cardiomyopathy with a fatal outcome. Heart failure of Chagasic aetiology has a worse prognosis and 50% lower survival rate than cardiomyopathies of non-inflammatory aetiology, like ischemic and idiopathic dilated cardiomyopathy (Mady et al. 1994, Bocchi 1994, Bestetti & Muccillo 1997). Vector transmission is under control in several regions and countries in Latin America; nevertheless, hundreds of thousands of patients already have CCC, not to mention numerous other individuals that currently have the indeterminate phase and still have the potential to develop cardiac symptoms. Although currently used of trypanocidal drugs are effective in the treatment of acute infection or recent infection in children from endemic areas, its efficacy in halting the progression of cardiac lesions has not been established (Marin-Neto et al. 2008). Available treatment for heart failure of CCC is the same supportive therapy used for other non-inflammatory aetiologies of heart failure. Our limited understanding of the pathogenesis of CCC may be the underlying reason for the lower survival of CCC patients. Thus, the need to find adequate treatment for CCC patients underscores the importance of studying CCC pathogenesis.

The pathogenesis of CCC is still matter of intense debate. The susceptibility factors that lead to 30% of individuals to develop CCC after *Trypanosoma cruzi* infection remain unknown. However, there are three main pathogenetic mechanisms to explain CCC development: cardiac dysautonomy, disorders of the microvascular circulation and inflammatory/immunological tissue damage. Since the majority of evidence indicates that the inflammatory infiltrate is a significant effector of heart tissue damage, we will review the effects of cytokines and chemokines in the pathogenesis of CCC. A recent review regarding other pathogenic mechanisms can be found in Marin-Neto et al. (2007).

Pathogenesis: mononuclear inflammatory infiltrate

The most striking histopathological feature in cardiac lesions of CCC patients is the presence of a diffuse myocarditis - albeit with focal aspects - with intense myocardial remodelling, fibrosis, cardiomyocyte hypertrophy and damage (Pereira-Barretto et al. 1986, Higuchi et al. 1987). In CCC, the inflammatory infiltrate is com-

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posed of macrophages (50%), B cells and T cells (10%) and very few NK cells (Milei et al. 1992, Higuchi et al. 1993). Also, there is a 2:1 predominance of $CD8^+$ over CD4+ T cells, with increased numbers of granzyme-positive cells (Higuchi et al. 1993, Reis et al. 1993b) and restricted heterogeneity of T cell receptor variable alpha chain transcripts (Cunha-Neto et al. 1994), further indicating an antigen-driven inflammatory infiltrate. Several clinicopathological data suggest that the infiltrate plays a major role in the development and progression of the disease: (i) the mononuclear infiltrate is associated with local cardiomyocyte destruction and fibrosis, (ii) CCC presents a shorter survival and worse prognosis than cardiomyopathies of non-inflammatory aetiology, (iii) the frequency of myocarditis in endomyocardial biopsies correlates with the severity of the functional heart damage, being low among asymptomatic individuals with the indeterminate form, intermediate among patients with ECG abnormalities and very frequent (93%) among CCC patients with dilated cardiomyopathy (Higuchi et al. 1987) and (iv) we found a positive correlation between the cellularity of the infiltrate and degree of ventricular dilation (unpublished observations) among hamsters chronically infected with *T. cruzi*. $CD8^+$ T cells recognizing *T. cruzi* antigens, such as cruzipain and FL-160, are also found in the CCC inflammatory infiltrate (Fonseca et al. 2005). Although it is difficult to locate *T. cruzi* nests in the myocardium of immunocompetent CCC patients, the presence of *T. cruzi* antigens and DNA has already been demonstrated (Higuchi et al. 1993, Jones et al. 1993) and an association between the persistence of parasite DNA and intense myocarditis has been reported (Benvenuti et al. 2008). On the other hand, $CD4^+$ T cells can cross-react recognizing cardiac myosin the most abundant heart protein and *T. cruzi* protein B13 have also been recovered from the myocardium of CCC patients (Cunha-Neto et al. 1996, Abel et al. 1997, 2005, Iwai et al. 2005). Whatever the antigenic stimulus may be, it is long-lasting and maintains the local inflammatory infiltrate. Besides the observed tissue damage, histiocytes and endothelial cells display increased expression of HLA class I and class II molecules, ICAM and E-selectin, while cardiomyocytes express increased levels of HLA class I, which are markers normally found on cells in response to local production of inflammatory cytokines (Higuchi et al. 1993, Reis et al. 1993a).

Cytokines and chemokines in the acute phase of *T. cruzi* infection

Data from animal models show that inflammatory cytokines play a central role in acute *T. cruzi* infection. Shortly after the acute infection starts, *T. cruzi* components - including its DNA and membrane glycoconjugates - trigger innate immunity via Toll-like receptors 2, 4 and 9 in macrophages and dendritic cells (Bafica et al. 2006). Upon activation, such cells secrete pro-inflammatory cytokines and chemokines, upregulate expression of co-stimulatory receptors and increase endocytosis and intracellular killing of parasites through release of reactive oxygen and nitrogen species. Pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18, IL-27, and TNF-α are promptly released, and further activate other inflammatory cells (Michailowsky et al. 2001, Bilate & Cunha-Neto 2008). Macrophages and dendritic cells that have endocytosed the parasite subsequently elicit a strong T cell and antibody response against *T. cruzi*. Interferon-γ (IFN-γ) - producing *T.cruzi* - specific T cells are thus generated (Bilate & Cunha-Neto 2008), and migrate together with other blood leukocytes to sites of *T. cruzi*-induced inflammation in response to chemokines such as CCL2, CCL3, CCL4, CCL5 and CXCL10, and participate in the immune response against the parasite (Teixeira et al. 2002). This inflammatory T cell and antibody response leads to control - but not complete elimination - of tissue and blood parasitism. On the other hand, the blockade of CCR5 with Met-RANTES significantly decreased the intensity of cardiac inflammatory infiltrate, suggesting that lymphocyte migration to the myocardium during acute infection is dependent on CCR5 ligands (Marino et al. 2004). The Syrian hamster model of *T. cruzi* infection reproduces the range of different outcomes of human Chagas disease (Ramirez et al. 1994, Bilate et al. 2003). During acute *T. cruzi* infection, hamsters displaying high cardiac parasitism also showed increased expression of TNF-α, IFN-γ, IL-10 and CCL3 mRNA, as well as acute phase signs such as weight loss, vomiting and diarrhoea while animals with low cardiac parasitism displayed a modest increase in cytokine/chemokine mRNA and no acute phase signs (Bilate & Cunha-Neto 2008). Cardiac parasitism was apparently related to the increased expression of cytokines and chemokines, as well as to lower levels of α-crystalin B chain (CRYAB) and increased expression of desmin and other structural proteins as suggested by proteomic analysis (Bilate & Cunha-Neto 2008). On the other hand, animals with low parasitism displayed increased levels of CRYAB and unchanged levels of structural proteins. These results are in line with the idea that CRYAB isoforms may have a protective effect against cytoskeleton disruption and it has been reported that it exerts an inhibitory action on inflammatory cytokine production, although it cannot be excluded that the intense parasitism limits CRYAB expression in animals with acute phase signs and high parasitism. Acutely infected children have shown increased expression inflammatory cytokines, such as circulating IL-6 and TNF-α (Moretti et al. 2002) and have also displayed increased production of IFN-γ by mononuclear cells (Samudio et al. 1998).

Cytokines and chemokines in the chronic phase of *T. cruzi* infection

Inflammatory cytokines are produced during the chronic phase of Chagas disease. Mononuclear cells increase their cytokine production, leading to increased plasma levels of TNF-α and IFN-γ, and are even detected in infected individuals with indeterminate forms of Chagas disease (Ribeirão et al. 2000, Abel et al. 2001, Ferreira et al. 2003, Talvani et al. 2004), which is probably in response to parasite persistent. The subset of patients that develop Chagas cardiomyopathy displays an array of immunological alterations consistent with an exacerbated Th1 immune response. CCC patients

display increased circulating levels of TNF-α and CCL2 compared to individuals with the indeterminate form of Chagas disease, or those with ECG abnormalities alone but with no ventricular dysfunction (Ferreira et al. 2003, Talvani et al. 2004). Additionally, CCC patients show increased numbers of IFN-γ-producing $CCR5^+$ $CXCR3^+$ $CD4^+$ and $CD8^+$ T cells, with reduced numbers of IL-10-producing and $FoxP3^+$ regulatory T cells (Abel et al. 2001, Gomes et al. 2003, 2005, Araujo et al. 2007) as compared to patients with the indeterminate form of Chagas disease.

The exacerbated Th1 response observed in the peripheral blood is reflected by the nature of the inflammatory infiltrate found in the myocardium of Chagas cardiomyopathy patients (Cunha-Neto et al. 2005). Mononuclear cells infiltrating CCC heart tissue express IFN-γ, TNF-α and IL-6, with lower levels of IL-2, IL-4 and IL-10 (Reis et al. 1993b, 1997, Abel et al. 2001). IL-7 and IL-15, which are cytokines that promote cell survival, expression in CCC heart tissue, is also found to be increased and may be the underlying reason for the predominance of $CD8^+$ T cells, which express increased levels of IL-15Rα and γ_c chain receptor (Fonseca et al. 2007). Gene expression analysis of inflammatory mediators using real time-PCR allowed us to define the profile of inflammatory mediators in the CCC myocardium. We observed significantly increased expression of chemokine receptors CCR5, CXCR3 and CCR7 and their ligands, as well as IL-18 in the myocardium CCC patients, in comparison with samples from heart donors and non-inflammatory cardiomyopathy patients (Cunha-Neto et al. 2005 and our unpublished observations). For the first time, we observed a correlation between myocardial expression of a chemokine (in this case, a Th1 chemoattractant) and myocarditis intensity, thus corroborating our hypothesis that such chemokines contribute to the migration and accumulation of inflammatory cells in the myocardium CCC patients. We were able to detect mononuclear cells that express CXCR3, CCR5, CXCL9 and CCL5 in the myocardium of CCC patients using confocal immunofluorescence assays (unpublished observations). These results are consistent with the hypothesis that circulating $CCR5^+$ $CXCR3^+$ $CCR7^+$ Th1 inflammatory cells previously activated in the periphery via encounter with *T. cruzi* (Gomes et al. 2005), interact with CCR7 ligands in high endothelial venules in inflamed myocardium - where they may interact with soluble ligands for CCR5 and CXCR3 receptors (our unpublished data) and migrate into tissue. Local IL-18 production may push these cells into producing increased levels of IFN-γ in a positive feedback loop. Also, IFN-γ-dependent chemokines, such as CCL5, CXCL9 and CXCL10, may increase the chemotactic signal and cause migration of more $CCR5^+$ $CXCR3^+$ T lymphocytes to the myocardium. On the other hand, genes expressed by regulatory T cells (TGF-β and Foxp3) or Th2 cells (IL-4 and IL-13) had reduced or undetectable expression in CCC heart tissue, suggesting that the intense Th1 inflammatory response in the myocardium of CCC patients occurs in the absence of other regulatory mechanisms.

Given the major role of Th1/inflammatory infiltrate in CCC heart, we tried to dampen the inflammatory response in an attempt to reduce the heart damage. Using the hamster model of dilated cardiomyopathy of human CCC, researchers found that blocking TNF-α with the soluble receptor Etanercept paradoxically worsened cardiac function (Bilate et al. 2007) in the absence of increased parasitism, direct drug toxicity, or increased myocarditis. These results suggest that there is a beneficial role for residual TNF-α signalling in Chagas disease cardiomyopathy and also suggests that TNF-α antagonism during the chronic phase of the *T. cruzi* infection worsens experimental cardiomyopathy, providing a cautionary note for cytokine-blocking intervention in human Chagas disease.

Non-inflammatory effects of cytokines and mediators on the myocardium

Our group and others have found significant evidence of non-inflammatory cytokine and chemokine effects on cardiomyocytes and other myocardial cell types, in addition to the inflammatory effects of cytokines and chemokines. Significant IFN-γ signalling was observed in the myocardium of CCC patients, including genes that are not ordinarily expressed by inflammatory cells. In vitro experiments have shown that IFN-γ alone or in combination with CCL2 may induce profound changes in the cardiomyocyte gene expression program, including induction of atrial natriuretic factor and the hypertrophic gene expression program (Cunha-Neto et al. 2005). IL-18 and CCR7 ligands are upregulated in CCC myocardium and can induce cardiomyocyte hypertrophy and molecules involved in the fibrotic process (Riol-Blanco et al. 2005, Sakai et al. 2006, Reddy et al. 2008). Transgenic mice overexpressing CCL2, TNF-α or IFN-γ in the myocardium develop myocardial hypertrophy and ventricular dilation (Kolattukudy et al. 1998, Kubota et al. 2000, Reifenberg et al. 2007).

Inflammatory cytokines may also affect myocardial energy metabolism. Several heart disorders, especially those with ventricular dysfunction, are associated with reduced energy metabolism, especially when mitochondrial fatty acid or other energy metabolism enzymes are involved (Johnston et al. 1991, Carvajal & Moreno-Sanchez 2003). Treatment of cardiomyocytes with IFN-γ inhibited oxidative metabolism and ATP production (Wang et al. 1996). Additionally, IFN-γ treatment reduces gene and protein expression of creatine kinase, which is responsible for translocation of mitochondrial ATP to the sarcoplasm in cultured human skeletal muscle cells (Kalovidouris et al. 1993). We observed that the myocardium of CCC patients displayed reduced expression of some key energy metabolism enzymes, including isoforms of creatine kinases, Krebs cycle enzymes and members of the ATP synthase complex, in comparison with the myocardium of patients from non-inflammatory cardiomyopathies and heart donors (unpublished observations), which could be partly due to IFN-γ/inflammatory cytokine signalling. Using cDNA microarrays, our group also observed increased expression of genes

encoding a number of proteins involved in oxidative phosphorylation and lipid catabolism in CCC myocardium as compared to idiopathic dilated cardiomyopathy or donor myocardium (Cunha-Neto et al. 2005). The increased expression of such genes could be a compensatory mechanism for cytoplasmic ATP depletion, as observed in mice genetically deficient for creatine kinases (Heddi et al. 1999, de Groof et al. 2001). cDNA microarray experiments in mice experimentally infected with *T. cruzi* showed changes in oxidative phosphorylation and depressed energy metabolism (Garg et al. 2003) and respiratory chain complexes with reduced ATP-generating capacity (Vyatkina et al. 2004). Thus, both IFN-γ and *T. cruzi* infection can depress energy metabolism, thus reducing myocardial ATP generation, which has potential consequences for myocardial contractility, electric conduction and rhythm.

We can thus hypothesise that, apart from the direct inflammatory damage, non-immunological effects of several mediators locally produced in the myocardium, such as IFN-γ, TNF-α, IL-18, CCL2 and CCL21, may play a significant pathogenic role in CCC, by modulating gene and protein expression of cardiomyocytes and fibrocytes in pathways essential for the development of CCC, such as hypertrophy, fibrosis and energy metabolism. It is clear that T cell migration to the myocardium and non-immunological effects of chemokines and other mediators are prime candidates for intervention in Chagas disease. Understanding the importance of these pathways in the pathogenesis may be instrumental for the development of more adequate therapy for chronic CCC.

Genetics and differential evolution to CCC

Mechanisms underlying differential progression to CCC are still incompletely understood. Familial aggregation of CCC has been described (Zicker et al. 1990), suggesting that there might be a genetic component to disease susceptibility. This is also supported by the fact that only one third of *T. cruzi*-infected individuals develop CCC. Given the importance of inflammatory mechanisms for CCC pathogenesis, genetic susceptibility to CCC may result from functionally relevant genetic polymorphisms that lead to variations in the intensity of the innate or acquired immune response and in inflammatory cytokines and chemokines involved in the pathogenesis of the disease. During the past few years, our group has shown associations between SNPs in genes such as CCL2, BAT1 (an inhibitor of inflammatory cytokines), Lymphotoxin-α, NFKBIL1 (potential inhibitor of NFKB) and MAL/TIRAP (an adaptor protein involved in the TLR2 and TLR4 signalling pathway) with CCC when compared with the indeterminate group (Ramasawmy et al. 2006a, b, 2008, 2009). Furthermore, we have shown that severe CCC patients carrying the

TABLE

Host genetic polymorphisms studied in Chagas disease

Gene	Polymorphism	Susceptibility/association	Reference
HLA	several, class I and II	contradictory	Deghaide et al. 1998, Fernandez-Mestre et al. (1998), Colorado et al. (2000), Fae et al. (2000), Layrisse et al. (2000), Nieto et al. (2000), Cruz-Robles et al. (2004)
β Cardiac myosin heavy chain	(CATT)n	negative	Fae et al. (2000)
CCL2/MCP1	-2518	CCC	Ramasawmy et al. (2006a)
CCR5	+53029	CCC	Calzada et al. (2001a)
NRAMP1	5'(GT)n, -236 C-->T, D543N, 3'UTR deletion	negative	Calzada et al. (2001b)
NOS2	(CCTTT)n	negative	Calzada et al. (2002)
TNF-α	-308, -238, TNFa	negative	Beraun et al. 1998, Drigo et al. (2007)
TNF-α	-308	CCC	Rodriguez-Perez et al. (2005)
TNF-α	-308, TNFa	progression to death/ heart transplant	Drigo et al. (2006)
LTA	+80, +252	CCC	Ramasawmy et al. (2007)
BAT-1	-22, 348	CCC	Ramasawmy et al. (2006b)
NFkBIL-1	-62, -262	CCC	Ramasawmy et al. (2008)
IL1B	-31, +3954, +5810	CCC	Flórez et al. (2006)
IL-10	-1082	CCC	Costa et al. (2009)
IL-12B	+1188	CCC	Zafra et al. (2007)
TLR1, 2, 4, 5, 9		negative	Ramasawmy et al. (2009),
MAL/TIRAP	S180L	CCC	Ramasawmy et al. (2009),
ACE	I/D	negative	Cuoco et al. 2005, Pascuzzo-Lima et al. (2009)

high TNF-α expresser genotype have shorter survival (Drigo et al. 2006). The Table shows all host genetic polymorphisms in Chagas disease published to date. The search for associations between candidate genes with the development of CCC has been focused on genes involved in immune response and inflammation. The association of SNPs in genes such as IL1B, TNF, LTA, IL10, IL12B, MAL/TIRAP, CCL2 and CCR5 with the progression to CCC is in accordance to what is expected, especially when the importance of migration of innate immune cells, inflammatory cytokines and chemokine-dependent migration in the pathogenesis of CCC is considered. As in with every multi-faceted disease, the input of each gene involved in the development of CCC is expected to be very small (1-10% of the susceptibility). However, identification of key genes and potent genetic combinations coupled with environmental factors may lead to the identification of *T. cruzi*-infected individuals that will progress to CCC. SNPs in other genes such as those involved in cardiovascular diseases (heart failure and ischemic heart disease) also need to be screened in *T. cruzi*-infected individuals. The first candidate cardiovascular polymorphism (ACE I/D polymorphism) showed no association with progression to CCC. The pace of discovery has been slow; in the 12 years since the first publication, in 1998, 19 loci were studied, and only 10 showed any association with disease progression. Better success in identification of genetic markers in Chagas disease will only come when the sample size exceeds the ones previously used in the past CCC studies. Large sample size (on the order of 1000's for each clinical group) will allow us to use high-throughput SNP detection to define a pattern of genetic susceptibility in the development of CCC, which may thus draw new modalities for prognosis and appropriate treatment.

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