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A systematic review of the *Trypanosoma cruzi* genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy

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Review Article

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Abstract

Chagas disease is a complex tropical pathology caused by the kinetoplastid *Trypanosoma cruzi*. This parasite displays massive genetic diversity and has been classified by international consensus in at least six Discrete Typing Units (DTUs) that are broadly distributed in the American continent. The main clinical manifestation of the disease is the chronic chagasic cardiomyopathy (CCC) that is lethal in the infected individuals. However, one intriguing feature is that only 30–40% of the infected individuals will develop CCC. Some authors have suggested that the immune response, host genetic factors, virulence factors and even the massive genetic heterogeneity of *T. cruzi* are responsible of this clinical pattern. To date, no conclusive data support the reason why a few percentages of the infected individuals will develop CCC. Therefore, we decided to conduct a systematic review analysing the host genetic factors, immune response, cytokine production, virulence factors and the plausible association of the parasite DTUs and CCC. The epidemiological and clinical implications are herein discussed.

Introduction

Chagas disease, also known as American trypanosomiasis, is caused by the parasite *Trypanosoma cruzi*. It is a complex zoonosis that is widely distributed throughout the continent of America. The infection can be acquired by contact with infected triatomine faeces, blood transfusion, laboratory accidents, or by oral and congenital transmission. Chagas disease is a major public-health problem, with approximately 6 million people infected in South America and 25 million at risk of infection, according to the statistics published by the World Health Organization (WHO, 2017). The immigration of infected people from endemic countries has also recently led to Chagas disease becoming an important health issue in other continents, such as Europe, the United States, Australia, Asia and Africa (Rassi *et al.*, 2009; Kuete, 2013; Jackson *et al.*, 2014; Strasen *et al.*, 2014; Angheben *et al.*, 2015). Chagas disease comprises two stages: an acute phase that occurs about 1 week after the initial infection, and a chronic phase that affects about 30–40% of infected patients for which cardiomyopathy is the most frequent and severe clinical manifestation (Rassi *et al.*, 2009; Bern *et al.*, 2011; Dias *et al.*, 2016).

Understanding the epidemiology of Chagas disease, which is considered an anthropozoonosis, is complex. One of the pivotal factors that makes the epidemiological information and the variation in clinical manifestations even more complex is the intraspecific genetic diversity among *T. cruzi* taxon, for which different methods of classification have been developed based on new identification techniques (Zingales *et al.*, 2012; Lima *et al.*, 2015*a*; Brenière *et al.*, 2016). There are seven subtypes within this classification, known as discrete typing units (DTUs), which include *T. cruzi* I (TcI, which includes TcI_{Dom}), *T. cruzi* II (TcII), *T. cruzi* III (TcIII), *T. cruzi* IV (TcIV), *T. cruzi* V (TcV) and *T. cruzi* VI (TcVI), and the recently described genotype associated with anthropogenic bats designated TcBat (Pinto *et al.*, 2012; Guhl et al., 2014; Hernández *et al.*, 2014; Ramírez *et al.*, 2014; Lima *et al.*, 2015*a*, 2015*b*).

Around 30–40% of the infected patients in the symptomatic chronic phase exhibit one or more of the following cardiac clinical conditions: tachycardia, bradyarrhythmias, apical aneurysms, cardiac failure (as a late manifestation with its typical signs and symptoms but a higher mortality rate when caused by Chagas disease), thromboembolic phenomena and sudden death, the latter being the leading cause of death from Chagas cardiac disease, more commonly displayed in the early stages (even in asymptomatic patients and is associated with ventricular fibrillation or tachycardia and less frequently to atrioventricular block or sinus node disease) (Rassi *et al.*, 2010, 2012, 2015). Electrocardiographic manifestations such as premature ventricular beats, ST-T segment changes, abnormal Q waves, voltage drop in the QRS complex, right bundle branch block and left anterior fascicular block are frequent, with the last two symptoms commonly occurring at the same time. Monitoring with a Holter device usually

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reveals ventricular compromise including ventricular dysfunction and tachycardia (Rassi *et al.*, 2015; Sánchez-Montalvá *et al.*, 2016). Chronic chagasic cardiomyopathy (CCC) is considered a multifactorial event in infected patients, with the immune system, host factors and the genetic diversity of the parasite all playing fundamental roles.

Several studies have been conducted over the past 40 years to elucidate the genetic variation of *T. cruzi* across its geographical distribution and the associations with host and vector species. Also, different reviews have been published in the literature showing the plausible effect of *T. cruzi* heterogeneity in the disease outcome and clinical features of patients infected with this parasite (Zingales *et al.*, 2009; Zingales *et al.*, 2012; Messenger *et al.*, 2016; Zingales, 2017). Different studies suggest that this variation may or may not be associated with the different clinical manifestations of Chagas disease. Since 1981, the role of *T. cruzi* genetic diversity on the pathogenicity of Chagas disease has been investigated by Miles *et al.*, with no conclusive answer so far (Miles *et al.*, 1981). Therefore, we conducted a systematic review of cutting-edge research in this field to elucidate the possible associations between *T. cruzi* genetic heterogeneity, host immune response and genetic factors, and the clinical manifestations of CCC considering for the first time, the three players in disease outcome (parasite genetic variability, host genetics and host immune response).

Molecular epidemiology of Chagas disease

The *T. cruzi* parasite comprises a heterogeneous population that displays clonal and/or sexual propagation due to the different cycles of transmission, and the possibility of genetic exchange that can be found in nature and has been previously reported *in vitro* (Gaunt *et al.*, 2003; Sturm *et al.*, 2003; Westenberger *et al.*, 2005; Llewellyn *et al.*, 2009; Ramírez *et al.*, 2012; Ramírez and Llewellyn, 2014; Brenière *et al.*, 2016). *T. cruzi* is genetically diverse and is classified into at least six DTUs known as TcI–TcVI based on different molecular markers and biological features. TcI and TcII are considered as two pure lines with a point of evolutionary separation of approximately 1–3 million years ago. Some authors consider TcIII and TcIV to be the result of hybridization between TcI and TcII, whereas TcV and TcVI are generally accepted to be the result of hybridization between TcII and TcIII (Gaunt *et al.*, 2003; Sturm *et al.*, 2003; Westenberger *et al.*, 2005; Zingales *et al.*, 2012; Ramírez *et al.*, 2014; Brenière *et al.*, 2016; Zingales, 2017). Recently, other authors have proposed divergent evolutionary pathways suggesting three different ancestors (Brenière *et al.*, 2016). Future studies are needed to fully understand the emergence of *T. cruzi* DTUs. Over the last decade, two emergent genotypes have been reported; one associated with anthropogenic bats in Brazil, Colombia, Ecuador and Panama and designated TcBat (Ramírez *et al.*, 2014; Pinto *et al.*, 2015), and a clonal genotype linked to human infections within TcI designated TcI_{Dom} (Llewellyn *et al.*, 2009; Ramírez *et al.*, 2012; Zumaya-Estrada *et al.*, 2012; Sánchez and Ramírez, 2013; Ramírez and Hernández, 2017). However, these emergent genotypes are not as yet considered true DTUs by international consensus.

The DTUs of *T. cruzi* are broadly distributed across the American continent in diverse ecotopes (domestic, peridomestic and sylvatic transmission cycles). Discrimination of the six DTUs has become an important issue in the molecular epidemiology of *T. cruzi*. There are many reports showing algorithms for the molecular characterization of these DTUs based on RAPDs, polymerase chain reaction (PCR)-RFLPs, qPCR, MLST, MLMT and DNA sequencing techniques, but to date there is no gold standard protocol that is accepted internationally for strain typing (Rozas *et al.*, 2007; Duffy *et al.*, 2009; Lewis *et al.*, 2009; Llewellyn *et al.*, 2009; Burgos *et al.*, 2010; Ramírez *et al.*, 2010; Yeo *et al.*, 2011; Higuera *et al.*, 2013; Cura *et al.*, 2015). One of the most frequently used and reliable algorithms for *T. cruzi* typing has been reported by the research of Burgos and colleagues, Higuera and colleagues and Ramírez and colleagues, and has also been applied to the testing of clinical samples (mainly blood samples) (Burgos *et al.*, 2010; Ramírez *et al.*, 2010; Higuera *et al.*, 2013) (Fig. 1).

Regarding the geographical distribution of *T. cruzi* DTUs, it is clear that TcI predominates in the terrestrial area from the south of North America to the north of Argentina and Chile, usually due to a sylvatic transmission cycle, but TcI is also a major cause of the disease in countries endemic for trypanosomiasis due to a domestic transmission cycle. By contrast, TcII, TcV and TcVI mainly occur in the southern cone, but this distribution may not be completely accurate since the area includes countries such as Bolivia for which data may be incomplete. TcIII and TcIV are generally characterized by their sylvatic transmission cycles in areas with tropical forest ecosystems (Carrasco *et al.*, 2012; Guhl and Ramírez, 2013; Brenière *et al.*, 2016). This is particularly illuminating given that there are distinct clinical differences between patients presenting with Chagas disease in these geographical regions. Strains appear to differ in terms of pathogenicity and the response to treatment. Both TcI and TcII–VI are associated with cardiac lesions in human infections, but it seems that only TcII, TcV and TcIV are associated with digestive tract lesions (Prata, 2001), despite a report of digestive tract lesions in Colombia caused by TcI (Prata, 2001). In general, however, TcI is considered to be less pathogenic with lower parasitaemias (Burgos *et al.*, 2007) and more chronic cases being asymptomatic compared with Chagas cases caused by TcII, TcV and TcVI in Argentina, Brazil, Chile, Paraguay and Uruguay (Luquetti *et al.*, 2015).

Additionally, some proteomic approaches have been conducted demonstrating differential protein expression among DTUs isolates (Telleria *et al.*, 2010; Díaz *et al.*, 2011). Moreover, there is an observed general partitioning of TcII–TcVI between sylvatic and domestic transmission cycles; with human disease cases associated with TcII, TcV and TcVI and TcIII, TcIV being predominantly sylvatic (Yeo *et al.*, 2005; Zingales *et al.*, 2012; Messenger *et al.*, 2016). However, according to the relationship between the recent TcBat isolates and anthropogenic deaths reported in countries such as Colombia and Brazil, the zoonotic potential of this genotype is being considered (Ramírez *et al.*, 2014). In addition, TcII has also been isolated from the same type of bats from which TcBat originated, and TcII has been shown phylogenetically to be closely related to a common ancestor of the TcI genotype (Lima *et al.*, 2015*b*). Recent advances in next generation sequencing technologies have allowed researchers to obtain three complete *T. cruzi* genomes. The first strain to be fully sequenced was CL Brener (TcVI), which revealed a high degree of repetitive elements along the core genome (El-Sayed *et al.*, 2005). Similarly, the recently sequenced Esmeraldo (TcII) and Sylvio X10 (TcI) genomes revealed the association between repetitive elements and mucin-like proteins that are closely associated with parasite cell invasion and survival, (Franzén *et al.*, 2011) offering new insight into the relationship between *T. cruzi* DTUs and pathogenicity (Andersson, 2011).

Pathophysiology of CCC

Chagas disease is currently considered a multifactorial disease of infectious origin, since several factors such as the mechanisms of action by which the parasite invades, the virulence factors of the parasite and the polymorphic factors of the host come into play, which together determine the severity of the clinical

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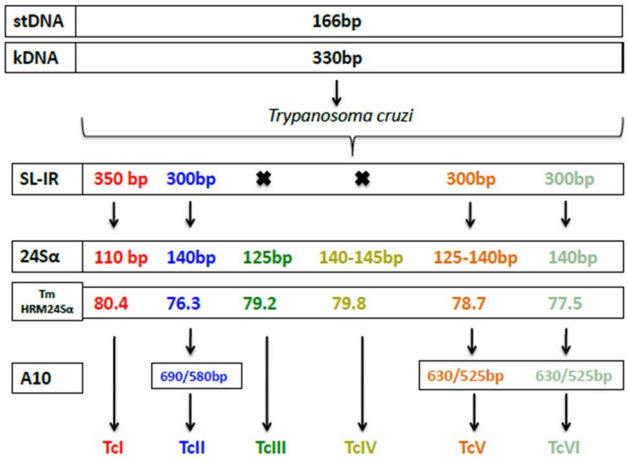

Fig. 1. Algorithm for DTU discrimination of *T. cruzi*.

manifestations of the disease (Prata, 2001). Below, the pathophysiological factors involved in the different presentations of the disease will be discussed, as well as a brief description of the manifestations involved.

The acute phase of *T. cruzi* infection is mediated by both innate and acquired host immunity. Initially, the infection is mediated by phagocytosis with highly virulent metacyclic trypomastigotes being ingested by macrophages. Although most are destroyed within phagocytic vacuoles, some amastigotes escape this immunological action and initiate intracellular replication, causing cell damage and rupture, allowing the parasite to spread through the bloodstream. This leads to the activation of a cascade of cytokines, cationic proteins, complement and transferrin proteins, with *T. cruzi* inducing the expression of interferons and cytokines such as interleukin (IL)-10 and tumour necrosis factor (TNF)-α (Sanjabi *et al.*, 2009; Teixeira *et al.*, 2011). Regarding acquired immunity, certain humoral factors play an important role in control of the infection, among which immunoglobulin G (IgG)2b antibodies are pivotal, which also mediate inflammatory activity during the acute phase. The activation of immune cells, such as CD4+ and CD8+ cells, also plays an important role in acquired immunity (Teixeira *et al.*, 2011).

At the moment the parasite enters the bloodstream, it is directed to the sites with the highest predilection, which in this case are the central nervous system, the cardiac and oesophageal regions, and the colon muscle tissue, as these are sites where the parasite can most readily traverse the vascular endothelium and initiate cellular parasitism (Rassi *et al.*, 2017). The initial process of transmigration through the vascular endothelium prior to transport of the parasite to its target tissues, has been reported by *in vitro* studies in human cells, and is facilitated by chemokines such as bradykinin and chemokine ligand 2 (CCL2). However, the molecules through which the parasite interacts with the endothelium are yet to be determined (Coates *et al.*, 2013).

On reaching and invading its target organs within the host, the parasite undergoes active multiplication during a sustained immunological reaction by the cells that involves CD4+ T lymphocytes, CD8+ and B lymphocytes. This cellular reaction leads to direct induction of anti-trypanosome cytotoxicity, secretion of cytokines and the production of antibodies against the parasite. Inflammatory myocardial lesions of both chronically infected animal and human models are predominantly composed of CD8+ rather than Th1 CD4+ lymphocytes, and increased expression of genes responsible for the production of proinflammatory cytokines and chemokines (especially interferon (IFN)-γ and transforming growth factor (TGF)-β±) has been observed. Other investigators have reported reduced production of regulatory T cells and their cytokines, such as IL-10 and IL-17 (Reis *et al.*, 1993; Sanjabi *et al.*, 2009; Guedes *et al.*, 2012; Cupello *et al.*, 2014). These findings are consistent with an imbalance demonstrated by the upregulation of Th1 cells and the downregulation of Treg cell activity (Fig. 2).

A study using mice susceptible (BALB/c) and non-susceptible (C57BL/6) to *T. cruzi* infection was performed to compare the expression of CD4+ T cells in both the acute and chronic phases of the disease. During the chronic phase, a *T. cruzi*-specific product was amplified by PCR at higher quantities in the surviving BALB/c mice than in the C57BL/6 mice, suggesting that a greater number of parasites were present in the susceptible mice during this phase. Immunological analysis of the chronic phase samples revealed greater expression of Th1 and inflammatory cytokines, such as IFN-γ, TNF and IL-2, which triggered the low but significant activation of CD4+ T cells in the BALB/c mice. However, this cell type was not detected in the infected C57BL/6 mice, in which low levels of proinflammatory cytokines (IFN-γ and IL-2) were detected. Similarly, in BALB/c mice, the production of IL-10 and TGF-β increased, which was not observed in C57BL/6 mice. These results indicated that mice which survive the acute phase and then enter the chronic phase with parasites present in their cardiac tissue trigger a response associated with Th1 cells, but not a Th17 response (Sanoja *et al.*, 2013).

It is also necessary to emphasize the importance of the Toll-like receptors (TLR) since they are responsible for immune recognition of parasites *via* pathogen-associated molecular patterns. *T. cruzi* has chemical structures that stimulate specific TLRs, which subsequently induce the production of nitric oxide and proinflammatory cytokines by monocytic cells. Among them, TLR-2 recognizes trypomastigote-derived glycosylphosphatidylinositol (tGPI) anchored in the mucin-like glycoproteins, TLR-4 recognizes the epimastigote glycoinositolphospholipid (eGIPL) that induces nuclear factor (NF)-κB, TLR-7 recognizes the parasitic RNA and TLR-9 recognizes DNA with abundant oligodeoxynucleotide unmethylated CpG motifs stimulating the cytochemical responses of both macrophages and dendritic cells (de Souza *et al.*, 2010; Rodrigues *et al.*, 2012).

Other factors related to cardiac involvement are neurological or microvascular alterations, immune-mediated tissue injury and parasite-dependent damage. These play a secondary role in the development of cardiac lesions and complications. However, some authors consider these critical factors in the persistence of parasites and in the inflammatory reaction and the initiation and progression of chronic myocarditis (Tarleton and Zhang, 1999, Marin-Neto *et al.*, 2015). Immune-mediated tissue injury is caused by polymorphonuclear leucocyte infiltration and the production of deleterious cytokines, mechanisms that are probably triggered by the persistence of the parasite in the tissue. Autoimmunity generated by molecular mimicry with parasite antigens and the resulting polyclonal activation have been reported; however, validation of autoimmunity is difficult and therefore remains a controversial issue (Tarleton, 1991, 2003). Evidence for an additional potential autoimmune mechanism came from the detection of mitochondrial DNA from the parasite in the genome of chickens in a model in which infection was induced in the egg phase (Teixeira *et al.*, 2011).

Metabolomic studies have revealed that the proteasome is the key protease in the generation of peptides for the presentation of antigens through MHC-I. In one study, it was shown that the biosynthesis of the immunoproteasome subunits Bl1, B2i and B5i, as well as PA28B, TAP1 and MCH-I (in macrophages *via* the SAPK/JNK signalling pathway) were downregulated in HeLa cells by *T. cruzi*, although the last three were not degraded by the parasite (Camargo *et al.*, 2014). By contrast, the parasite does not affect

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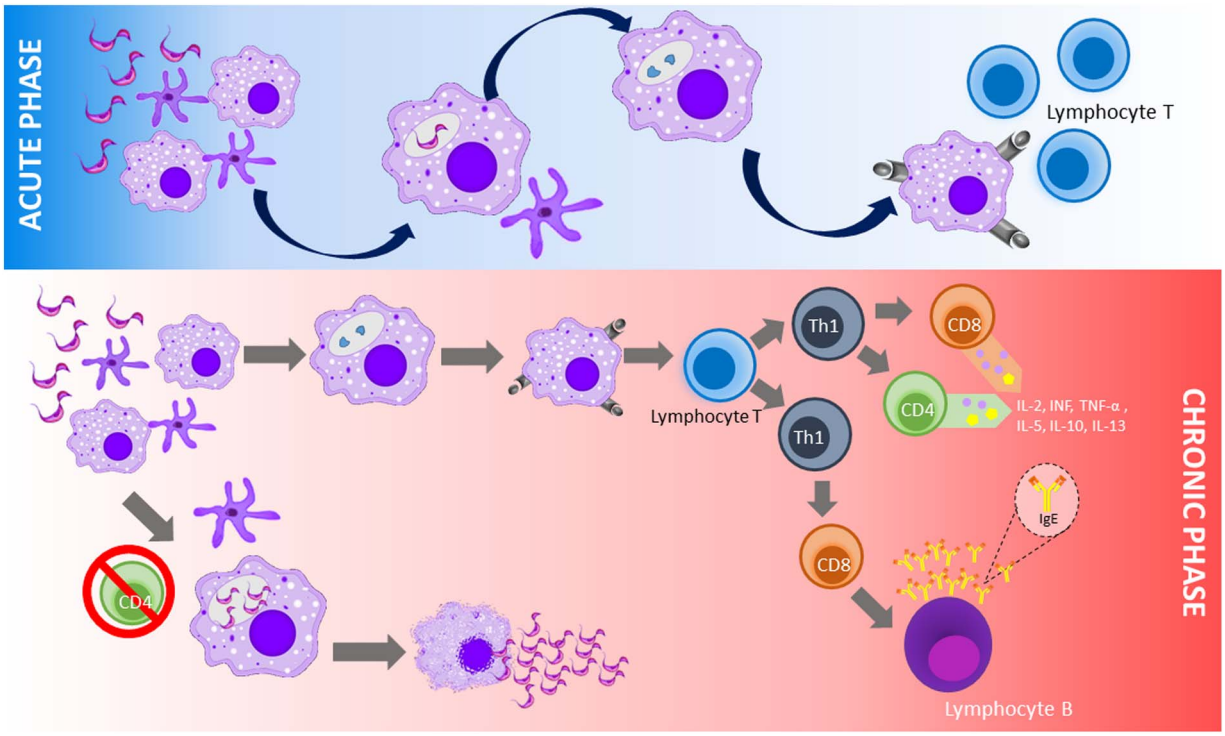

Fig. 2. Cytokine profile during *T. cruzi* infection. The acute phase shows the immune system response and the chronic phase shows the activation of cytokines.

the transcription or expression of proteins of the constitutive subunits of the proteasome. This provides evidence that this protozoan is able to modulate infection through post-transcriptional mechanisms that affect the translation of host proteins, as well as limiting their recognition by CD8+ cells, thus favouring parasite evasion from the immune response (Camargo *et al.*, 2014; Tarleton, 2015).

Thus, when the protozoan comes into contact with neurons and glial cells, it carries out the process of cell invasion by binding to receptor tyrosine kinase A and C (TrkA and TrkC), which are normally activated by neurotrophin (NT) nerve growth factor and NT-3, respectively, and whose activation is essential for maintenance of the nervous system (Chuenkova and Pereira, 2009; Aridgides *et al.*, 2013). Recognition of these receptors occurs through parasite-derived neuropathic factor (PDNF), a GPI-linked neuraminidase/trans-sialidase (TS), whose interaction, as with other protein kinases (Akt), is independent of sialic acid (Chuenkova and Pereira, 2011). Subsequently, these same receptors were identified in cardiomyocytes (Meloni *et al.*, 2010; Aridgides *et al.*, 2013), where it was shown that these cells are also invaded *via* a mechanism that involves PDNF binding to TrkC receptor primarily, or TrkA receptor, which offers some protection against oxidative stress and parasite invasion (Aridgides *et al.*, 2013).

Parasite invasion induces multiple responses in the heart. Studies have shown that Cox2 mRNA and protein expression is induced in murine models during parasitic loading and myocarditis. This upregulation is also associated with the induction of cardiac dysfunction markers such as endothelin-1 (ET-1) and atrial natriuretic peptide (ANP), the first considered important in signalling pathways that allow Ca activation of ERK1/2 leading to cyclin-D1 expression and inflammation-related gene expression. In addition, Cox2 is induced *via* the Ca/calcineurin/NFAT pathway allowing overproduction of eicosanoids (thromboxane A2 (TXA2) and prostaglandins E2 and F2a) (Petkova *et al.*, 2000; Salomone *et al.*, 2001; Hassan *et al.*, 2006; Corral *et al.*, 2013), and TXA2 is induced exacerbating cardiomyocyte apoptosis, facilitating cytokine biosynthesis by monocytes, and leading to endothelial and platelet activation, aggregation and degranulation (Corral *et al.*, 2013).

Virulence factors of *T. cruzi*

T. cruzi possesses a wide variety of virulence factors that allow it to reproduce and effectively evade host defences and generate damage or morbidity in the affected individual (Brown *et al.*, 2012), these virulence factors are described below.

Many studies have focused on finding and characterizing the molecules involved in the process of infection by *T. cruzi*, but little is known about the molecules involved in cellular invasion by extracellular amastigotes. Recently, a group of amastine proteins was reported, for which there are four subfamilies (alpha, beta, delta and gamma), of which the beta and delta proteins are found in *T. cruzi* (Jackson, 2010). These proteins are located on the amastigote cell surface and in strain G were demonstrated to increase the rate of differentiation towards metacyclic trypomastigotes. Studies with recombinant proteins both *in vitro* and *in vivo* demonstrated that these proteins also participated in the adhesion to and invasion of host cells by the amastigotes, and intracellular survival of the parasites by maintaining the pH inside parasitic vacuoles potentially *via* a mechanism that involves proton and ion trafficking through the membrane (Cruz *et al.*, 2012).

One of the most important virulence factors in *T. cruzi* is the TS, which allow for sialylation of *T. cruzi* glycoconjugate membranes at terminal positions. There are two isoforms of TS identified in *T. cruzi*, one of which is enzymatically active (aTS) and the other is inactive (iTS), but still retains the ability to bind to sugar substrates. These proteins are secreted into the bloodstream, where they are systemically distributed and induce modifications in host cells by sialylation (Freire-de-Lima *et al.*, 2015). After intracellular replication of the parasites and transformation into trypomastigotes, a large amount of TS is released into the cytoplasm of the parasitized cell, which is then lysed leading to an increase in the concentration of systemic TS that cannot be

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efficiently combated by antibodies. The amount of enzyme secreted correlates with the virulence of the strain and high levels of TS are associated with a wide variety of abnormalities during the early stages of infection, including thymocyte depletion (thymocyte apoptosis), the absence of germinal centres in secondary organs, thrombocytopenia and erythropenia (Freire-de-Lima *et al*., 2015; San Francisco *et al*., 2017) (Fig. 3).

TS also inhibit lymphocyte proliferation *via* IL-2 signalling, and are associated with the ability of the parasite to reduce IL-2Ra expression and IL-2 production by T cells. In addition, they can manipulate the maturation of CD4+ cells inducing non-protective Th2 genotypes in naive T cells while downregulating Th1 cells by IL-10 induction/expression during antigenic presentation by host cell antigens (ACP) to favour the survival and infection of the parasite. Additionally, CD8+ T cells from infected animals have been found to be highly sialylated, which reduces their ability to infiltrate tissues. This provides a dual benefit to the parasite, since it favours its intracellular replication while preventing extensive tissue damage of the infected tissue (Tarleton, 2015; San Francisco *et al*., 2017).

Similarly, the parasite is covered by a group of mucin glycoconjugates, which are classified depending on whether they are present in the vector (TcMUC) or in the host (TcSMUG). These mucin glycoconjugates offer protection against the vector or host defences, thereby assuring cellular invasion (Osorio *et al*., 2012). Surface antigens also exist that are related to these glycoconjugates, such as the mucin-like gp35/50 surface antigen that compromises cellular invasion and the trypomastigote small surface antigen (TSSA) of the trypomastigote. Genetic analysis of the TSSA revealed a strong relationship with TcMUC and variations among the sequences of the six DTUs of *T. cruzi* (TcI–TcVI). However, unlike TcMUC, TSSA is a hypoglycosylated molecule that participates in the infectivity of the trypomastigote by acting as an adhesion molecule between the host and parasite and playing a role in amastigogenesis. There are two variants of TSSA, one of which shows adhesive properties (TSSA-CL), and the other is non-adhesive (TSSA-Sy). TSSA is a potential target as a diagnostic and therapeutic effectiveness agent (Yoshida, 2006; Balouz *et al*., 2017; Cámara *et al*., 2017).

The *T. cruzi* parasite has mechanisms for the evasion of reactive species of oxygen and hydrogen (e.g. O_2^- and H_2O_2), produced mainly by macrophages, that generate direct oxidative damage. These defence mechanisms are mainly coordinated by five different types of peroxidase (TcGPXI, TcGPXII, TcCPX, TcMPX and TcAPX), the first two conferring protection against exogenous hydroperoxides, and TcCPX, TcMPX, and small chains of organic hydroperoxides and TcAPX conferring protection against H_2O_2. *T. cruzi* also possesses an iron superoxide dismutase (Fe-SOD) that prevents damage by O_2^- from mitochondrial, cytosolic or glycosomal sources (Osorio *et al*., 2012; Malvezi *et al*., 2014).

T. cruzi possesses other molecules that contribute to the evasion of host immune responses, such as those that inhibit the coupling of complement molecules. Complement regulatory proteins (CRPs) expressed only in trypomastigotes, inhibit the classical and alternative pathways by binding to C3b and C4b. For example, trypomastigote decay accelerating factor (T-DAF) interferes with the coupling of C3 in both pathways, and its metacyclic trypomastigote form is able to induce blood cells to produce microvesicles that bind to the surface of the parasite and stabilize it to C3. Similarly, the complement C2 receptor inhibitor trispanning protein (CRIT) inhibits complement activation *via* lectin, while calreticulin (TcCRT) captures C1 molecules and uses them as host cell recognition molecules to the benefit of the parasite (Osorio *et al*., 2012; Henrique *et al*., 2016).

Other molecules such as calpain, a calcium-dependent lysosomal peptide cysteine, present in the endosome–lysosome system of the epimastigotes, on the epimastigote cell surface and in the amastigote–trypomastigote transitional forms, is secreted from the flagellar pocket and intervenes in the processes of cytoskeleton remodelling, proliferation, differentiation and regulation of cellular calcium. Calpain operates through the cleavage of the high molecular weight kininogen protein, which stimulates the release of calcium through inositol triphosphate, among other molecules (de Souza *et al*., 2010; Branquinha *et al*., 2013). Oligopeptidase B, a serine endopeptidase cytosolic, secreted by trypomastigotes, has also been implicated in the induction of calcium release during invasion of the parasite (Burleigh *et al*., 1997; Burleigh and Woolsey, 2002; de Souza *et al*., 2010).

Finally, alteration in the metabolism of phospholipids has recently been reported as another possible mechanism by which parasites interact with the host. For example, molecules such as phospholipase A1 (PLA1) have been shown to participate in parasite–cell interactions prior to cell invasion through the generation of lipids that act as secondary messengers and co-activate kinase C (Belaunzaran *et al*., 2013). The expression of phosphatidylinositol phospholipase C (TcPI-PLC) correlates with the decrease in phosphatidylinositol-4,5-bisphosphate (PIP2) and the increase in its product inositol-1,4,5-triphosphate in the cell host demonstrating that overexpression of TcPI-PLC can inhibit the progression of trypomastigotes to amastigotes (Okura *et al*., 2005; Osorio *et al*., 2012).

The main limitations in the identification of these virulence factors is that there is no clue if these are DTU-dependent. Most of the studies include merely the Y strain (typed as TcII). Therefore, future studies should establish if these virulence factors are deviated by the massive genetic diversity among *T. cruzi* taxon.

Host factors

Polymorphisms among certain host factors, such as those that affect the expression of cytokines involved in the immune response, can play an important role in the course of a disease by attenuating the defensive capacity of the host against an invading pathogen. The relationship between CCC and cytokine expression (e.g. for IL-1, IL-10, IL-12, IL-17 and IL-18) has been studied, along with preliminary studies to analyse the relationship between this disease and other polymorphisms among host immune factors (Leon *et al*., 2016).

For IL-1, a proinflammatory cytokine that has been implicated in the mediation of both acute and chronic manifestations of CCC disease, has been reported to offer protection in terms of the haplotypes IL-1A, IL-1B and IL-1RN (Flórez *et al*., 2006). For IL-10, polymorphisms that lead to reduced expression, such as IL-1082G/A, are associated with the development of CCC. For IL-17, a proinflammatory cytokine produced by CD4+ T cells, some studies have shown that lower expression of this cytokine correlates with cardiac manifestations, whereas associations between certain polymorphisms (rs2275913, rs763780) have been related to the severity of left ventricular systolic function in patients with CCC (Costa *et al*., 2009; Magalhães *et al*., 2013; Reis *et al*., 2017). As for IL-18, reduced expression of this cytokine has been associated with the early stage response to CCC (rs360719*C) by permitting the activation of transcription factor OCT-1; however, certain polymorphisms (rs5744258, rs360722) showed no statistically significant relationship with susceptibility to disease, and other studies found that rs2043055 was associated with the modulation of Chagas disease severity (Esper *et al*., 2014; Nogueira *et al*., 2015). Further studies are therefore required to investigate the relationships between host polymorphisms and CCC disease.

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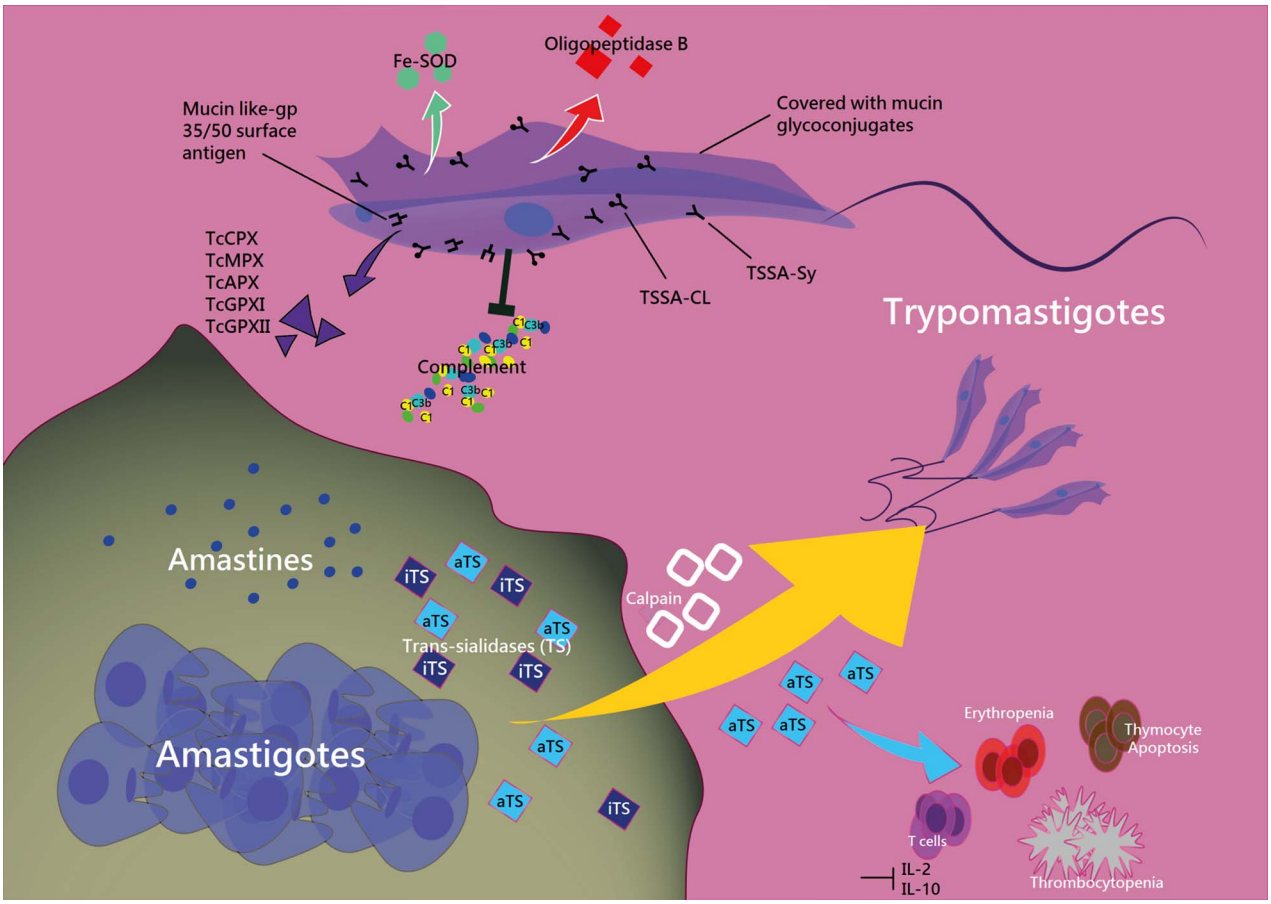

Fig. 3. Virulence factors and immunological response elicited by *T. cruzi* infection.

Similarly, polymorphisms among chemokines have been implicated in the pathology of CCC disease. Chemokines play an important role in the recruitment of inflammatory cells to heart tissue in studies of mice with both acute and chronic myocarditis. Variations in CCL2, a cytokine that participates in the elimination of parasites through NO-dependent pathways, have been associated with the development of CCC. Similarly, polymorphisms in CCR5, which is essential in the control of acute infections and in the maintenance of inflammation that causes tissue damage, have been related to CCC disease severity (CCR5 rs1799988) and other polymorphisms are more frequently associated with asymptomatic patients (CCR5 59029A/G). Furthermore, the pleomorphism rs10336 in CXCL9 [105–110] correlated with the diminution of the intensity of myocarditis. By contrast, variations in CCL4, CCL5, CCL17, CCL19 and CXCR3 showed no association with CCC (Fernández-Mestre *et al.*, 2004; Nogueira *et al.*, 2012).

Variations in HLA supertypes (such as HLA-A1, A2 and A24, which are present in CD8+ T cells where most of their epitopes are restricted to the HLA-A allele) have also been found to relate to disease pathology (Lasso *et al.*, 2016), with polymorphisms in HLA I and HLA II being reported to be associated with CCC (Deghaide *et al.*, 1998). Of the HLA I genes, it was shown that variations in HLAC*03 have a strong association with CCC compared with asymptomatic patients. Similarly, DPB1*0401 presents a higher frequency in affected patients compared with asymptomatic patients, with asymptomatic patients showing a higher frequency of DPB1*0101 (Colorado *et al.*, 2000; Layrisse *et al.*, 2000). As for the HLA II genes, a comparison was made between alleles DRB1 and DQB1 and polymorphisms DRB1*14 and DQB1*0303 were found to be protective against the chronic phase of the disease. In addition, the DRB1*01, DRB1*08 and DQB1*0501 polymorphisms showed a higher frequency in patients with arrhythmia and congestive heart failure, whereas the DRB1*1501 polymorphism presented a lower frequency in such patients. These findings suggested that the HLA II genes may be associated with the development of chronic infection and cardiac tissue damage. Similarly, polymorphisms in HLA-DQ1 are considered to confer susceptibility, whereas HLA-DQ7 confers protection against the development of CCC disease (Deghaide *et al.*, 1998; Cunha-Neto and Chevillard, 2014).

Finally, for TNF, the TNFA-308 polymorphism and variations in the microsatellites of TNF-α from patients with end-stage CCC correlated with significantly shorter survival times compared with individuals who did not possess these variations (Drigo *et al.*, 2006). Furthermore, variants in the promoter region of the IKBL gene (IKBL-62A/T, IKBL-262A/G) were associated with susceptibility to CCC (Ramasawmy *et al.*, 2008). It has been recently suggested that the parasympathetic nervous system and the endocrine system may play important roles in the clinical manifestations associated with CCC (Roggero *et al.*, 2016; Savino, 2017). Further investigations are now needed on large study populations to determine the frequency of certain polymorphisms among individuals and the roles of host factor gene variations on the susceptibility and severity of CCC disease pathology (Prata, 2001).

Cytokine profile during *T. cruzi* infection

The expression of cytokines and their kinetics are key factors in the development and progression of disease. At present, the pathophysiological mechanisms of Chagas' disease have not

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been completely elucidated; however, two hypotheses have been established. The first relates to the early stages of infection where an appropriate inflammatory response is beneficial but a dysregulated response allows for tissue damage. In the acute phase of infection, it has been proposed that the immune response of the host may be associated with aggressive autoimmunity. The second hypothesis is based on an autoreactive process in response to the lack of adequate immune modulation between excessive proinflammatory cytokines and the loss of anti-inflammatory cytokines that plays an important role in the progression of human Chagas disease from asymptomatic to severe forms (cardiomyopathy and megavisceral syndromes) and is associated with molecular mimicry (Nagib *et al*., 2007; Pissetti *et al*., 2011; Longhi *et al*., 2014).

During the acute phase of the disease, it has been suggested that the immune response employs three mechanisms to counteract the infection. The first is the detection and destruction of the parasite *via* macrophages and dendritic cells. The second involves the activation of dendritic cells and macrophages that initiate the presentation and activation of specific antigens to elicit immune responses. The third mechanism is the detection of infection by non-hematopoietic cells, which play a major role in protecting against the invasion of *T. cruzi* (Poveda *et al*., 2014).

In the chronic phase, antigens that are produced by the autoimmune response are mediated by T cells, as an important step in the progression of the disease. Several studies in children have found T cells to be predominant proinflammatory and cellular monocyte regulators. CD8+ T cells play an important role in combatting intracellular pathogens. This is due to the fact that this cell type is able to recognize infected cells, when such a function is eliminated or inhibited, there is no parasitic control in the early phase of the disease leading to the exacerbation of infection and the onset of chronic disease (Sanmarco *et al*., 2016).

Immune mechanisms that operate to control the parasite prior to intracellular infection are controlled by the CD4+ and CD8 cellular responses, which inhibit the replication of *T. cruzi* as demonstrated *in vitro*. These cells and the protective functions of IL-2, IFN-γ and TNF-α produced by Th1 cells, have been shown to be associated with heart disease. IL-5, IL-10 and IL-13 regulate the inflammatory humoral response and the stimulation of IgE, eosinophils and mastocytes (Teixeira *et al*., 2011).

A previous study detected a switch between the anti-inflammatory cytokines IL-13, IL-5 and IL-10 and proinflammatory cytokines IL-2, IL-6, IL-9 and IL-12 in 109 seropositive patients and 21 seronegative controls, who were classified into two groups, CARD (heart disease) and NON-CARD (no heart disease), and a clear lack of immune modulation was reported. By measuring the mean fluorescence intensity of IL-12, IFN-γ, IL-1, IL-6 and IL-9 for discriminant analysis of principal components (DAPC), a cluster was observed for the CARD patients that was not present with the NON-CARD patients, potentially indicating that some NON-CARD patients may be predisposed to developing cardiomyopathy or mega-viscera syndrome explaining why the cytokine levels are not homogeneous for this group and suggesting that these cytokines could be used in combination as progression markers (Poveda *et al*., 2014).

Role of cytokines in the genetic variability of *T. cruzi* DTUs

Previous studies to analyse the association between *T. cruzi* genetic variability and the different clinical manifestations of Chagas disease found an association between TcI and cardiomyopathy, and between TcII, TcV and TcVI and mega-visceral syndrome. We used sera from CCC patients infected with different DTUs (20 TcI, 20 TcII and 15 mixed TcI + TcII) to observe whether the genetic variability of the parasite was associated with the pathogenesis of Chagas disease. A proinflammatory profile was observed for all groups as expected, and this profile was similar to that identified for the cardiac group. However, higher levels of cytokines were found in the TcII and mixed groups compared with the TcI group, where the levels did not exceed 50%. By contrast, we found higher levels of the cytokines IL-6 for TcI, IL-1 for TcII and IL-22 for the mixed TcI/TcII group (Rassi *et al*., 2009). This is interesting since it is known that IL-6 is secreted by T cells and macrophages, IL-1 by macrophages and lymphocytes, and IL-22 by dendritic cells and T cells (Rojas. *et al*., 2017) (Table 1).

A recent study showed that the immune profile relating to the cardiac inflammatory response involved the expression of TNF, IL-2, IL-10 and IFN-γ in human cardiac cell infiltrate samples, which may suggest that these factors play an important role in the variable susceptibility to the chronic phase of the disease. Other studies demonstrated the presence of IL-2, IL-4 and IL-6 in infected cardiac tissue. Even in patients with ventricular dysfunction, IL-10, IFN-γ, IL-6, TNF and IL-1 have been reported to increase plasma levels. This suggests that there is a relationship between the secretory response of T cells in the immunological profile of infected heart tissue (Vicco *et al*., 2013; Rodríguez *et al*., 2014). However, a study by Vicco and colleagues in Wistar mice showed that the administration of diluted phosphorus allowed for some modulation of inflammation in the cardiac tissue *via* IFN-γ and TNF-α (Ferreira *et al*., 2017).

In contrast to previous studies that showed that patients with less aggressive forms of cardiomyopathy produced higher levels of IL-17 (Guedes *et al*., 2012), we hypothesize that DTU-specific recognition by the immune system (antibodies, B cells or T cells), could lead to the differential responses observed. Our results suggest that patients with more severe cardiomyopathy would be those with TcI, followed by those with a mixed infection, and finally those infected with TcII. This is in accordance with a descriptive analysis performed by our group where we detected that patients infected with the TcI DTU displayed more cardiac alterations than those infected with TcII (Ramírez *et al*., 2010).

Finally using IFN-γ, IL-12, IL-22 and IL-10 for DAPC analysis, we found clusters for patients infected with TcI and mixed TcI + TcII, suggesting that there is a likely association between the genetic variability of *T. cruzi* (TcI, TcII and mixed TcI/TcII) and the levels of some cytokines. The sympathetic nervous system is thought to play an important role in the survival of *T. cruzi* infection based on studies in mice (C57B1/6) given the possible participation of mechanisms that upregulate the production of proinflammatory cytokines, as well as cellular immune responses and the restriction of parasitic proliferation (Roggero *et al*., 2016).

T. cruzi heterogeneity and CCC

The classification of *T. cruzi* parasites is important in defining the biological, clinical and pathological characteristics associated with specific populations of *T. cruzi* (Mantilla *et al*., 2010; Dias *et al*., 2016). Despite intensive research into the molecular epidemiology of *T. cruzi*, few studies have investigated the association between the genetic heterogeneity of *T. cruzi* and the clinical outcomes of Chagas disease. The DTUs of *T. cruzi* involved in an infection can alter the humoral response affecting the pathogenesis of the disease (Santi-Rocca *et al*., 2017). For this reason, different authors have proposed the use of lineage-specific serology markers to detect the serological profile of an infection according to each DTU. These assays have clearly demonstrated that the immune response elicited by each DTU may be different and highlights the need for further research in this field (Zingales *et al*., 2009). TSSA antigen and B-cell epitopes have been investigated for this purpose of developing specific serology markers but

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Table 1. Association between the *T. cruzi* DTUs and the related clinical form and immune response

DTU	Related clinical form	Immune response	References
TcI	Cardiomyopathy (less pathogenic with lower parasitaemias with more chronic cases)	TNF, IL-2, IL-4, IL-6, IL-10 and IFN-γ in human cardiac cell	Burgos *et al*., 2007; Vicco *et al*., 2013; Rodríguez *et al*., 2014; Poveda *et al*., 2014
TcII	Mega-visceral syndrome and less cases of cardiomyopathy	Higher levels of IL-1β, IL-2, IL-4, IL-6, IL-5, IL-17a, IL-18, IL-13. It has many virulence factors as enzyme and receptors to avoid host immune response	Prata 2001; Rassi *et al*. 2009; Zingales *et al*., 2012; Poveda *et al*., 2014
TcIII	Cardiomyopathy and mega-visceral syndrome, less human cases (sylvatic transmission)	It is considered rare in human infection, needs more investigation about immune response profile	Zingales *et al*., 2012; Poveda *et al*., 2014
TcIV	Cardiomyopathy and mega-visceral syndrome, less human cases (sylvatic transmission)	In *in vitro* antigenic stimulation in monkeys showed higher levels of TNF and INF-γ, and minor IL-10 production	Prata, 2001, Vitelli-Avellar *et al*., 2017
TcV	Mega-visceral syndrome and cardiomyopathy	In less aggressive forms of cardiomyopathy produced higher levels of IL-17	Prata, 2001; Guedes *et al*., 2012
TcVI	Mega-visceral syndrome and cardiomyopathy	In less aggressive forms of cardiomyopathy produced higher levels of IL-17	Zingales *et al*., 2012; Guedes *et al*., 2012
TcBat	Anthropogenic deaths reported in countries such as Colombia and Brazil, the zoonotic potential of this genotype is being considered	This DTU needs more investigation, including recent cases and the immune response	Ramírez *et al*., 2014

experiments were hindered by the lack of specificity of the *in vitro* assays (Bhattacharyya *et al*., 2010, 2014) (Fig. 4).

Several studies have provided evidence of the existence of a relationship between *T. cruzi* DTUs and clinical manifestations. TcI has been reported to be the most abundant DTU across the American continent, being detected among a wide range of reservoirs and triatomines, in which its presence is derived predominantly from sylvatic rather than domestic transmission cycles. The infection of humans by this DTU is concentrated in the north of South America reaching the centre of America, and is mainly associated with chagasic cardiomyopathy, predominantly in the domestic cycle (Zingales *et al*., 2009; Leiby *et al*., 2017). It has been reported that this particular DTU plays an important role in severe forms of chagasic heart disease (Guhl, 2013; Guhl and Ramírez, 2013). In a study carried out in Argentina of 239 patients with a diagnosis of severe myocarditis, TcI was detected in 4.2% of the patients from either blood samples, biopsies or the organs from those who underwent a transplant (Burgos *et al*., 2010; Guhl, 2013). In Colombia, similar studies showed that TcI caused more cardiac alterations that TcII across a large cohort of chronic symptomatic patients (Ramírez *et al*., 2010).

Studies on TcII have shown that this DTU of *T. cruzi* prevails in the central and southern regions of America, and is related to transmission of the domestic cycle. TcII has been associated with the clinical manifestations of moderate chagasic cardiomyopathy, concomitant with mega syndromes such as megacolon and megaesophagus, also associated with blood donors (Zingales *et al*., 2009; Guhl and Ramírez, 2013; Leiby *et al*., 2017). In addition, Bisio and coworkers confirmed that TcII was present in two patients with cardiac manifestations. However, these estimations are not absolute and patients across the continent with CCC can be infected by TcII (Burgos *et al*., 2007).

In endemic areas of the Amazon region of Brazil, and in eastern Colombia and Venezuela, TcIV is considered to be the predominant DTU responsible for most of the acute diseases caused by this DTU (Carrasco *et al*., 2012; Guhl and Ramírez, 2013; Segovia *et al*., 2013; Monteiro *et al*., 2013*a*; 2013*b*; Margioto Teston *et al*., 2017). TcIV has also been strongly incriminated with lethal cases relating to oral transmission in Colombia and Brazil (Monteiro *et al*., 2013*a*; 2013*b*; Ramírez *et al*., 2013; Dario *et al*., 2016; Hernández *et al*., 2016). As for TcV and TcVI, comparative genetic studies proposed that these DTUs are TcII and TcIII hybrids that correlate with chagasic cardiomyopathy and megavisceral syndrome in the southern cone of the American continent (Zingales *et al*., 2009; Guhl and Ramírez, 2013). In a study from Argentina, TcV or TcII/V/VI were found to be the most prevalent DTUs among 226 of 239 patients studied (89.9%). Whereas, TcV was present in 90.9% of the TcII/V/VI group samples studied that correlated with Chagas' moderate chronic heart disease in the study by Burgos and colleagues (Burgos *et al*., 2010).

Another study revealed that in the southern region of Latin America (in particular Argentina), blood samples and cultures isolated from patients with CCC were predominately of the TcII, TcV and TcVI *T. cruzi* DTUs. To date, in this geographic location, TcI has not been considered the dominant DTU in heart disease (Cura *et al*., 2012). However, in terms of clinical associations, descriptive molecular epidemiology studies linked TcI with severe forms of myocarditis in cardiac samples from CCC patients in Argentina and no specific clinical manifestations related to *T. cruzi* DTUs in Bolivian CCC patients showing the pleomorphism of *T. cruzi* (Moncayo and Yanine, 2006; Ramírez *et al*., 2009; Burgos *et al*., 2010; del Puerto *et al*., 2010). The direct detection of *T. cruzi* DTUs in the blood of CCC patients was established by amplification of the 24Sα rDNA divergent domain and the mitochondrial housekeeping genes (Mantilla *et al*., 2010). In this study, molecular characterization of *T. cruzi* DTUs showed that most of the patients were infected with TcI and some were infected with TcII (9.9%). Recently, a new approach for *T. cruzi* DTU detection in CCC patients has been developed that showed that TcI was the predominant DTU and TcII was also detected, furthermore, the genetic characteristics of the TcII parasites found in Colombia were similar to those of the TcII parasites found in Bolivia and Chile (Mantilla *et al*., 2010). Regarding the genetic variability of the parasite, prognosis markers based on mitochondrial genes are being developed, since specific mutations in these genes can trigger complications in the chronic phase of the disease in asymptomatic patients (Carranza *et al*., 2009).

Despite the genetic variability, it is important to consider the presence of *T. cruzi* clones that have been detected in different tissues. Several studies have demonstrated specific histotropism of *T. cruzi* in mice showing differences in the pathological, immunological and clinical features that the parasite can elicit in the host (Carareto *et al*., 2008; Ramírez *et al*., 2010; Cruz *et al*., 2016; Leon *et al*., 2017). Moreover, some authors have shown

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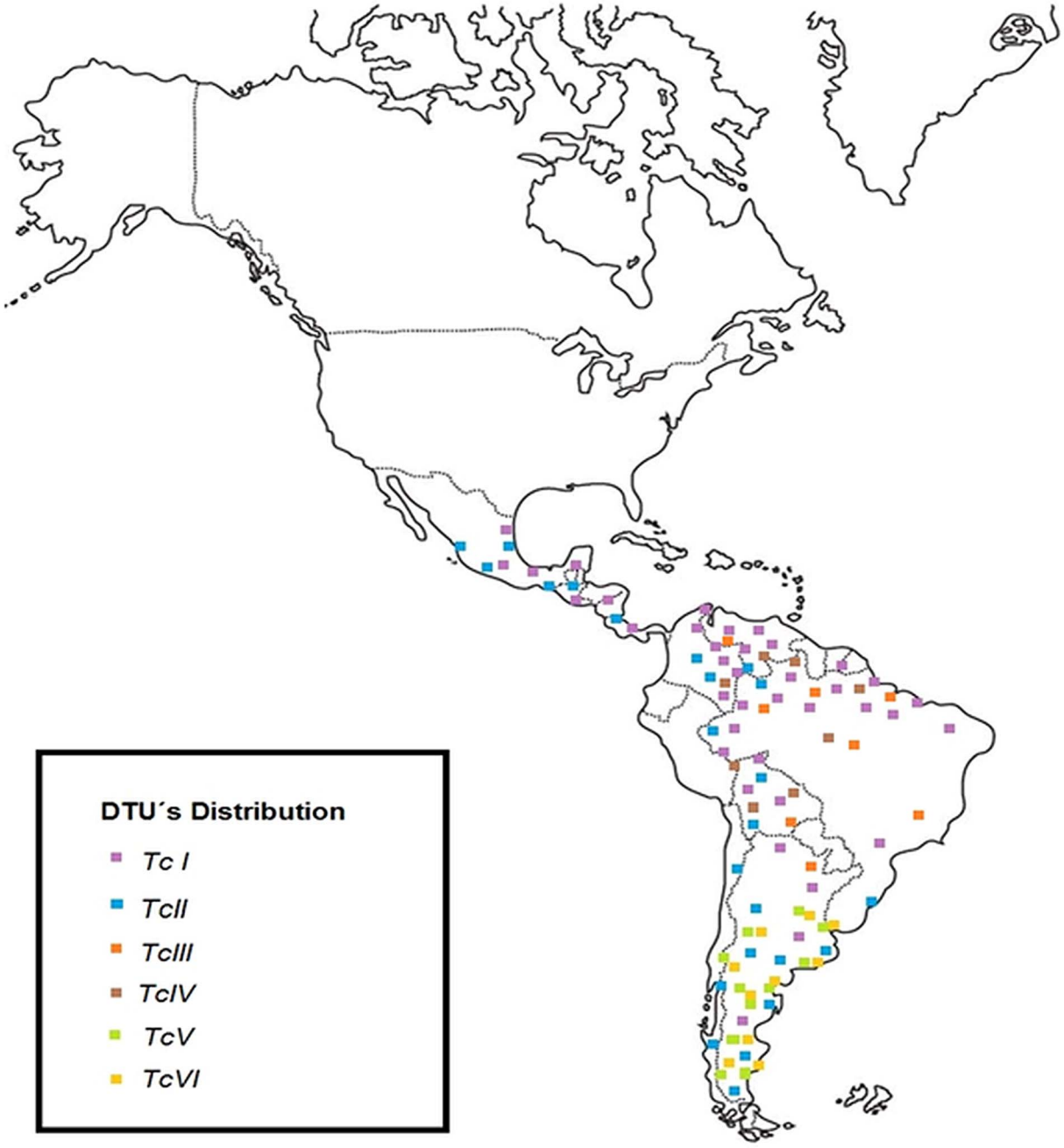

Fig. 4. Overview of the geographical distribution of *T. cruzi* DTUs associated with CCC across the American continent.

that the *T. cruzi* population in a patient's bloodstream may differ from the parasite population that causes tissue damage (Vago *et al.*, 2000; Macedo *et al.*, 2004). Differences were found in *T. cruzi* populations in the bloodstreams of patients with CCC and of chagasic patients without cardiomyopathy (Venegas *et al.*, 2009). Microsatellite analyses have shown multiclonality in samples from the heart and bloodstream of infected patients demonstrating that specific populations of *T. cruzi* may determine the disease outcome (Burgos *et al.*, 2007; Valadares *et al.*, 2008). Finally, studies from clones obtained from hemoculture from one patient (chronic symptoms) showed divergent multilocus genotypes, reinforcing this hypothesis (Ramírez *et al.*, 2012, 2013). One group proposed a model of clonal histiotropism to explain how a composite of clones may be related to disease outcomes (Macedo and Segatto, 2010). Recent reports from Colombia corroborate this premise since they describe cardiac biopsies infected with sylvatic genotypes and TcI circulating in the bloodstream of patients (Ramírez *et al.*, 2010; Zafra *et al.*, 2011). The case of mixed infections appears to be the rule rather than the exception in chagasic patients. It has been proposed that such mixed infections play a major role in Chagas pathogenicity, with some paradox traits: in animal models, infection by a 'slow' genotype and by a 'fast' genotype is faster than the faster genotype alone. This has been recently corroborated by Llewellyn *et al.* in 2015 where they explored *T. cruzi* infection multiclonality in the context of age, sex and clinical profile among a cohort of chronic patients, as well as paired congenital cases from Cochabamba, Bolivia and Goias, Brazil using amplicon deep sequencing technology (Llewellyn *et al.*, 2015). Their conclusions showed that no specific association was found between the number and diversity of parasite genotypes in each patient with their age, sex or disease status. Also, they were able to detect the transmission of multiple parasite genotypes between mother and foetus. This clearly opens the window about the need to explore

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in deep the case of mixed infections across a well-characterized cohort of chagasic patients and demonstrate the pivotal role of multiclonality in a plausible severity of disease outcome (Llewellyn *et al.*, 2015).

Molecular epidemiology studies of *T. cruzi* have attempted to establish the effects of different DTUs in the clinical progression of Chagas disease. Several studies have shown the effect of genetic variability on the host immune response (Ramírez *et al.*, 2009). However, a study carried out in the Chilean population, in which the DTUs TcI, TcII, TcV and TcVI were present and TcV predominated, sought to establish a relationship between the parasitic burden, the DTUs and the clinical manifestations of the disease, but found no correlation between the DTUs and the cardiac manifestations of the disease (Apt *et al.*, 2015). It had previously been established that the cardiopathologies in southern cone countries were caused by TcII, TcV and TcVI, but it has recently been demonstrated that TcI can also play an important role specifically in the severe cardiopathologies related to Chagas disease. Studies of cardiac biopsies from Argentinean patients revealed that patients with severe myocarditis were infected with TcI, whereas those with moderate or absent myocarditis were infected with TcII, TcV or TcVI (Burgos *et al.*, 2010). Furthermore, among patients with CCC, the TcIDOM genotype was most commonly found in the bloodstream, whereas sylvatic-like TcI parasites were most commonly found in cardiac biopsies. These results were consistent with reports from patients in Colombia, where the least and most prevalent TcI genotypes in adult patients with CCC were sylvatic-like TcI parasites and TcIDOM, respectively (Ramírez *et al.*, 2010; Hernández *et al.*, 2016). These results suggest potential histotropism by TcI genotypes and the epidemiological importance of this DTU in the southern American countries, where cardiopathologies were previously thought to be caused primarily by TcII, TcV and TcVI.

The main problem in establishing the real picture of *T. cruzi* heterogeneity in Chagas disease patients is the low parasitic load in the chronic phase of the disease. Furthermore, a temporal variation pattern has been detected whereby the *T. cruzi* population may change at 10-day intervals (Sánchez and Ramírez, 2013). Therefore, it is imperative to improve the current methodologies for strain typing. Recently, with the rise of next generation sequencing technologies, researchers have been able to deploy multilocus sequence typing (MLST) schemes to infer the genetic divergence of this parasite (Lauthier *et al.*, 2012; Messenger *et al.*, 2012). These methodologies have been applied to specific clinical phenotypes such as oral Chagas disease, which is an eminent public-health problem in those areas were vectorial transmission has been interrupted. In Colombia, six outbreaks of oral Chagas disease have been reported and strains isolated from these outbreaks were analysed by MLST schemes. The results showed a predominance of TcI in the cases with a foreseen infection of TcIV, suggesting the unlikely relatedness of sylvatic strains with the oral outbreaks (Hernández *et al.*, 2016).

T. cruzi I has shown a relevant genetic heterogeneity and some authors have subdivided it into at least two near-clades (domestic and sylvatic TcI) (Ramírez and Hernández, 2017). However, other studies showed that only domestic TcI is a robust genotype across the American continent which clearly reflects that TcI isolates are highly variable (Zumaya-Estrada *et al.*, 2012). Recent genomic approaches by whole genome sequencing and multi-SNP typing will most probably uncover lesser genetic subdivisions within it. This intra-DTU diversity is remarkable in the light of plausible histiotropism where has been revealed that domestic TcI circulates in the bloodstream and sylvatic TcI shows tropism for heart tissue (Burgos *et al.*, 2010). Such micro-molecular epidemiology tends to become routine in bacteria and has started in parasites (Wong *et al.*, 2015). Such studies show that upper evolutionary units such as *T. cruzi* I are to broad units of analysis for refined epidemiological studies. Future studies must consider intra-DTU genetic variation and plausible disease outcome.

The analysis of specific haplotypes incriminated the TcI sylvatic-like strains in the oral cases of Chagas disease highlighting the need to improve current epidemiological surveillance systems in endemic areas to detect the invasion of sylvatic-like genotypes in domestic cycles of transmission (Ramírez *et al.*, 2013). This type of analysis was also conducted in the largest urban oral outbreak of Chagas disease ever reported in Caracas, Venezuela, where the authors were able to track the source of infection attributing it to TcI sylvatic-like strains (Segovia *et al.*, 2013). This scenario proposes the relevance of the typing schemes to track the phylodynamics of *T. cruzi* and also indicates that some TcI DTUs are likely more susceptible to causing oral infections. However, some authors have proposed that TcI and TcII express different glycoproteins that facilitate their survival in the gastric mucosa leading to infection *via* the oral route (Yoshida *et al.*, 2011; Sánchez and Ramírez, 2013). This area therefore requires further study.

Studies that encompass molecular epidemiology and molecular biology techniques, such as PCR, allow us to establish the relationship between different DTUs of *T. cruzi* and the clinical manifestations of the disease (including Chagas' cardiomyopathy) in various populations. The findings of such studies indicate that certain genotypes of this parasite exert damage at the cellular level and therefore are not the same as those detected in the blood that cause parasitaemia and infection during the acute phase of the disease. In addition, the various serotypes of trypanosomes that cause non-heart-related pathologies and those that cause Chagas cardiomyopathy may differ, meaning that organisms belonging to a specific genotype may predominate in particular clinical manifestations. This suggests that specific genotypes may determine the clinical course of the disease (Guhl and Ramírez, 2013).

One interesting and important study was conducted by Santi-Rocca *et al.* in 2017 using syngeneic mice infected acutely or chronically with six DTUs, 66 parameters were analysed, including parasite tropism, organ and immune responses (local and systemic) and clinical presentations of CCC. The authors of that study found that the parasite genetic background consistently impacts most of these parameters, but they remain highly variable impeding reliable one-dimensional association with phases, strains and damage, but the use of multi-dimensional statistics overcame this extreme intra-group variability and revealed some pathophysiological patterns that accurately allow defining (i) the infection phase, (ii) the infecting parasite strains and (iii) organ damage type and intensity (Santi-Rocca *et al.*, 2017). This study was very important towards the understanding of the association between *T. cruzi* genetic diversity, host genetics and disease outcome. However, the results are not merely conclusive and research must focus on this topic.

However, the existence of studies showing the non-existence of a relationship between the clinical manifestations of Chagas disease and the genetic heterogeneity of the parasite must be considered. According to one study, no lineage was found to have a significant association with any clinical manifestation in particular, nor exclusively with patients who presented with the chronic phase of the disease (del Puerto *et al.*, 2010).

Conclusions

This paper enhances our understanding of CCC as a multifactorial disease and highlights the heterogeneity of *T. cruzi* and how this heterogeneity relates to the pathology of disease. By collating the data from epidemiological studies, it becomes possible

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to gain an overview of which DTUs are predominant in particular geographic locations and which are associated with particular pathologies. These findings are useful in many respects including the development of control strategies to prevent the spread of the disease, for example, by preventing the migration of infected patients to non-endemic regions.

In addition, the clinical course of CCC disease depends on the immunological status of the host, as all aspects of innate immunity, as well as cytokine induction, are required to respond to *T. cruzi* infection. The pathology of disease is also affected by the response of the host to the virulence factors of the parasite. Processes such as pathogen recognition and parasite internalization into host cells all play a part in determining the pathophysiology of the disease. Despite intensive efforts of the scientific community, the studies to date are not conclusive regarding the true drivers of CCC. It is a complex interaction between the parasite and the host and for us is very speculative to propose responses based on the current data. We believe that the advent of new technologies such as QTL analysis using human and parasite genomes and also the cytokinome efforts would contribute to the future understanding of the CCC drivers. The new PacBio *T. cruzi* genome assembly and the high level of human genome annotation would be pivotal to solve this enigmatic question.

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