Current Understanding of Immunity to *Trypanosoma cruzi* Infection and Pathogenesis of Chagas Disease

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Abstract

Chagas disease caused by *Trypanosoma cruzi* remains an important neglected tropical disease and a cause of significant morbidity and mortality. No longer confined to endemic areas of Latin America, it is now found in non-endemic areas due to immigration. The parasite may persist in any tissue, but in recent years there has been increased recognition of adipose tissue both as an early target of infection and a reservoir of chronic infection. The major complications of this disease are cardiomyopathy and megasyndromes involving the gastrointestinal tract. The pathogenesis of Chagas disease is complex and multifactorial involving many interactive pathways. The significance of innate immunity, including the contributions of cytokines, chemokines, reactive oxygen species, and oxidative stress, has been emphasized. The role of the components of the eicosanoid pathway such as thromboxane A_2 and the lipoxins has been demonstrated to have profound effects as both pro-and anti-inflammatory factors. Additionally, we discuss the vasoconstrictive actions of thromboxane A_2 and endothelin-1n Chagas disease. Human immunity to *T. cruzi* infection and its role in pathogen control and disease progression have not been fully investigated. However, recently, it was demonstrated that a reduction in the anti-inflammatory cytokine IL-10 was associated with clinically significant chronic chagasic cardiomyopathy.

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Introduction

The genus *Trypanosoma* encompasses important diseases of humankind. Thus, *Trypanosoma brucei gambiense* and *T.b. rhodesiens*e cause human African trypanosomiasis (sleeping sickness), and *Trypanosoma cruzi* is the cause of American trypanosomiasis, also known as Chagas disease. The outcome of Chagas disease in humans is related both to the virulence of the parasite strain as well as the host response to infection. Interestingly, paleo-parasitological studies demonstrating the presence of *T. cruzi* in tissues of 9,000-year-old mummies from coastal northern regions of Chile have pointed to the possibility that human Chagas disease was present in South America before its "discovery" in 1909 [1,2]. The year 2009 marked the centennial of the discovery of Chagas disease, and, in recognition of this event, many journals published reviews on various aspects of this disease. Our intent here is not to write an exhaustive review of Chagas disease, but rather to focus on individual topics which we believe are of importance in understanding the pathogenesis of this important, but yet neglected, tropical disease.

Life Cycle and Epidemiology of *T. cruzi* Infection

Epidemiology

Chagas disease has been regarded as an exotic and rare "Latin American" disease. It is endemic in Mexico, Central, and South America, where vector-borne transmission of *T. cruzi* usually occurs in individuals in rural areas. The insect vectors (triatomines) invade the primitive houses that are common in rural areas, and feed on people often as they sleep; hence the term "assassin bug." Both domestic and wild mammals can be infected and serve as reservoirs for the parasite. In endemic areas, vector-borne disease has also been observed on the outskirts of large metropolitan areas. Until recently, there were only a handful of indigenous cases in the United States, but recently, 16 cases of autochthonous *T. cruzi* infection were reported [3], and these were most likely acquired from vectors within the country.

Patterns of emigration from Chagas-endemic areas to other areas of the world have now altered our understanding of the epidemiology of this disease in the United States and other non-endemic areas such as Canada, Europe, Australia and Japan. In a landmark article published in 2009, Bern and Montgomery estimated that 300,000 persons living in the United States were chronically infected with *T. cruzi* [4]. In addition, there are Latin American immigrants living in Spain, France and Portugal, as well as Brazilian immigrants of Japanese origin in Japan. The vast majority of serologically positive individuals in these non-endemic areas usually have the indeterminate form of cardiac disease. Although these individuals are not aware of their chronic infection, they remain potential sources of transmission via blood transfusion, organ transplantation and mother-to-child vertical transmission (congenital transmission). In fact, congenital Chagas disease in children of mothers who have emigrated from endemic areas has been reported in Europe [5]. Thus, Chagas disease has indeed gone global [6].

Life cycle and infection

T. cruzi has a complex life cycle consisting of four life stages. First, blood from trypomastigotes circulating in the blood of an infected mammalian host is ingested by the feeding vector. The trypomastigotes then transform first into epimastigotes that divide by binary fission and then into non-dividing, infectious metacyclic trypomastigotes in the hindgut of the vector; they are next deposited with the vector feces during subsequent blood meals. Natural transmission to a new mammalian host occurs when the parasite-laden feces contaminate oral or nasal mucous membranes, the conjunctivae, or wounds in the skin, including vector bites. Once in the mammalian host, the trypomastigotes enter host cells and

transform into the multiplying intracellular forms or amastigotes, which then transform into blood form trypomastigotes. These forms are released into the bloodstream as the host cell ruptures and are then ready to invade healthy cells.

The molecular mechanism(s) of invasion by this parasite and the associated regulatory pathways have been the subject of intense investigation for many years. *T. cruzi* interacts with several mammalian host cell receptors, such as toll-like receptors (TLRs), kinins (B1/B2 sub types), receptor tyrosine kinases, TGF- and EGF-receptors, and the activity of these receptors is required for optimal parasite binding and/or invasion (reviewed in [7]). More recently, it was shown that *T. cruzi* also exploits host cell LDL receptor (LDLr) for their internalization and subsequent fusion of the parasitophorous vacuole with the host cell lysosomal compartment [8]. Parasites directly bind to LDLr, and inhibition or disruption of LDLr resulted in a reduced rate of invasion. Importantly, acutely infected mice displayed a significant decrease in plasma LDL levels and LDL was increased at areas where parasites were present in the heart, which may mean there was an infection-induced increase in phospholipids, triglycerides, and fatty acids that could contribute to the pathogenesis of Chagas disease.

Blood-form trypomastigotes infect adjacent uninfected cells or disseminate via the lymphatics and bloodstream to infect cells at distant sites. Although any nucleated mammalian cell can be parasitized, those commonly infected include cardiac myocytes, peripheral skeletal and smooth muscle cells, endothelial cells, and cells of the nervous and reticulo-endothelial systems and adipose tissue. Besides vectorial transmission, transmission of *T. cruzi* by transfusion of blood or transplantation of organs from infected donors, congenital transmission, and acquisition of the infection via the oral route have been reported [9].

Clinical Chagas Disease and Pathology

Cardiac involvement

Vector-borne acute Chagas disease is usually mild. After an incubation period of 1 to 3-weeks, a newly infected individual may develop fever, chills, nausea, vomiting, diarrhea, rash, and meningeal irritation. A raised inflammatory lesion at the site of parasite entry (a chagoma), unilateral periorbital edema (Romaña's sign), conjunctivitis, lymphadenopathy and hepatosplenomegaly are observed in acute infection. Trypomastigotes are observed in wet preparations of blood and cerebrospinal fluid during acute infection [10]. Intracellular amastigotes are also found in the liver Kupffer cells rather than in the hepatocytes. In most patients, an immune response develops, and acute parasitemia and associated symptoms usually resolve within 2 to 4 months. The mortality rate of acutely, naturally infected patients, often children, is less than 2%, and the common mode of death is usually acute myocarditis and/or meningoencephalitis. The presence of arrhythmias is usually considered a poor prognostic finding, and parasite pseudocysts are readily found in cardiac biopsies of such patients, indicating a high level of infection.

At the conclusion of the acute phase, the majority of infected persons enter the indeterminate clinical form of infection, characterized by a positive serology and absence of clinical manifestations. This phase may last months to an entire lifetime; many chronically infected people never develop clinical manifestations. Approximately 30–40% of infected individuals eventually develop symptomatic Chagas disease. Imaging of the heart employing echocardiography, cardiac magnetic resonance imaging and microPET is extremely useful in the evaluation of disease severity. Dilated congestive cardiomyopathy is an important manifestation of chronic Chagas disease and may present insidiously as heart failure or more abruptly with arrhythmia and/or a stroke [11]. Liver pathology may reflect right-sided heart

failur*e.* Apical aneurysm of the left ventricle is one of the hallmarks of chronic chagasic cardiomyopathy, observed by cardiac imaging and at autopsy (Fig. 1A). Generally, the therapy of chagasic patients with chronic heart disease is similar to that of other patients with congestive heart failure (CHF). Some patients also exhibit destruction of conduction tissue, most commonly right bundle branch block, that results in atrio-ventricular and intra-ventricular conduction abnormalities, and patients may require pacemaker placement. Patients with end-stage CHF may benefit from heart transplantation [12]. Gastrointestinal (GI) manifestations, such as the mega-syndromes, involving tubular structures of the GI tract are not as common, but may be more frequent in certain geographic areas. In addition, patients may exhibit both cardiac and gastrointestinal manifestations.

There are three layers of cardiac myocytes in the heart that are obliquely oriented to each other and then meet at the apex of the heart. Typically, trypomastigotes must pass through the basal laminae and extracellular matrix (ECM) layers of the myocardium and the interstitial matrix layer between the basal laminae layers to reach cardiac myocytes. Studies both in experimental models and chagasic patients demonstrate that myocardial damage results from infection and multiple insults, including ischemia, inflammation, oxidative stress, and necrosis, which contribute to ECM degradation. Indeed, cardiac metalloproteinases are up regulated in the setting of infection, and their inhibition ameliorates myocardial inflammation [13]. ECM degradation gradually results in slippage of the ventricular layers, believed to be the primary event for the formation of the apical aneurysm, which has become the *Sine qua non* of chronic Chagas heart disease (Fig. 1A). Thus, the remodeling of the heart in chronic Chagas heart disease is a result of reorganization in the heart wall resulting from tissue damage from ischemia, necrosis, inflammation and chronic increase in intra-cavitary pressure associated with hypertrophy and dilation of the ventricles. Histological examination of the heart reveals myocytolysis, myonecrosis and contraction band necrosis resulting from hypoperfusion, followed by periods of reperfusion which may be the result of vasospasm of the branches of the coronary circulation. Eventually bands of fibrous tissue and extracellular collagen replace and engulf hypertrophied cardiac myocytes (Fig. 1C&D).

The virtual absence of parasites in the heart of chronically infected individuals has engendered a discussion in the literature regarding the etiology of chronic chagasic heart disease. It has been accepted that interaction between the parasite and its host, well-adapted for more than 9,000 years, results in a balance that favors chronic disease in the great majority of cases. Different strains of the parasite have been associated with distinct clinical outcomes of infection in experimental models [14,15] and human disease [16]. Host factors such as genetic background of the host, mitochondrial dysfunction, oxidative stress and pathological immunity, have also been associated with distinct clinical outcomes of Chagas disease and are briefly discussed in this review.

Vascular compromise in Chagas disease

The vasculature comprises approximately one-third of the myocardium. Yet, *T. cruzi*-induced vasculitis, though described in the early years following the recognition of Chagas disease, was not appreciated until the 1980s. In those years, microvascular compromise was demonstrated as an important factor in the pathogenesis of cardiomyopathies of various etiologies [17], and treatment with the calcium-channel blocker (verapamil) was found to improve the coronary blood flow and outcome and to reduce inflammation and fibrosis in the heart [18,19]. Subsequently, alterations in the sub-endocardial microvasculature, aneurysm formation and vasospasm were observed in an experimental model of *T. cruzi* infection [20], and verapamil was shown to ameliorate reduced blood flow [21] and cardiovascular remodeling [22,23] in infected mice.

In recent studies, Tanowitz and colleagues examined the role of TXA_2 (a bioactive lipid eicosanoid) and the 21-amino acid peptide endothelin-1 (ET-1) in Chagas disease. Both of these agents are known to be pro-inflammatory and cause platelet aggregation and vascular spasm. The synthesis of ET-1 is mediated by endothelin-converting enzyme, which converts precursor ET-1 (31 amino acids) to ET-1, noted in endothelial cells. The biological properties of ET-1 are mediated by the G-protein-coupled endothelin receptors, ET_A and ET_B. It is now appreciated that multiple cell types, such as cardiac myocytes, fibroblasts, astrocytes, and macrophages, can synthesize ET-1. Moreover, *T. cruzi* infection of cultured endothelial cells resulted in an increase in biologically active ET-1 in studies with *T. cruzi*-infected mice in which there was an increased expression of ET-1 protein and mRNA in the myocardium and an increase in plasma ET-1 levels [24]. Treatment of infected mice with phosphoramidon, an inhibitor of endothelin converting enzyme, reduced *T. cruzi* infection-induced right ventricular enlargement [25]. Importantly, mice deficient in ET-1 in either cardiac myocytes or endothelial cells exhibited amelioration in cardiac remodeling that is associated with *T. cruzi* infection [26]. Hassan et al [27] found increased expression of ET-1 in the carotid arteries of *T. cruzi*-infected mice, and indicated the importance of ET-1 in the vasculature changes during infection. Elevated plasma levels of ET-1 have also been noted in patients with chronic chagasic cardiomyopathy [28]. However, it is unclear whether this is a result of CHF in general or chagasic cardiomyopathy in particular.

TXA_2 serum levels are increased in *T. cruzi*-infected mice; the majority being parasite-derived [29,30,31]. TXA_2 regulated vasospasm, thrombosis, vascular permeability, and endothelial cell dysfunction were observed in acute infection. The TXA_2 receptor knockout mice displayed increased tissue parasitism and myocardial inflammation [30]. It is believed that TXA_2 signaling acts as a potential quorum-sensing mechanism regulating intracellular amastigote proliferation. This would prevent the overwhelming of the host during acute infection thus enhancing the transition into a chronic persistent state of infection and inflammation.

Involvement of the central nervous system (CNS)

Soon after the description of *T. cruzi*, CNS involvement in Chagas disease was described. Infants born with congenital Chagas disease may display signs, symptoms and pathological findings of myocarditis and meningoencephalitis [32]. These children may display pathological findings in the brain similar to those observed in other perinatal infections. Trypomastigotes may be present in the cerebral spinal fluid (CSF) during acute infection. In adults, a necrotizing meningoencephalitis is most often observed in the setting of immunosuppression such as that which accompanies HIV/AIDS [33] or the administration of immunosuppressive drugs. As discussed above, stroke is a common finding in patients with chagasic heart disease, especially in those individuals with dilated cardiomyopathy. Neuro-cognitive abnormalities have been reported in experimental Chagas disease [34].

T. cruzi may invade glial cells including astrocytes and microglial cells, while neurons are only infrequently infected [35]. The GI tract is also a target of infection which results in injury to the enteric nervous system. Affected individuals may develop dilation of portions of the GI tract. Although megacolon and megaesophagus are most common, megastomach, megaduodenum, megajejunum, megagallbladder, megacholedochus have all been described. Achalasia, aspiration pneumonia, disturbances of gastric emptying, alterations in intestinal transit, and motility disorders of the colon and gallbladder have also been reported. Also, in one study of colon samples from chagasic patients without megacolon, there was an increase in Foxp3^{+} T cells compared with those with megacolon. This finding may mean that Foxp3^{+} T cells reduce inflammatory cells and prevent neuronal destruction [36]. In this regard, parasite-derived neurotrophic factor (PDNF) may promote neuronal cell survival [37,38].

Others have suggested gene mutations associated with carcinoma are present in patients with chagasic megaesophagus [39], though further research in this area is needed.

Adipose tissue and the adipocyte: Reservoir tissue for parasite

Until recently, adipose tissue and the adipocyte (fat cell) were underappreciated aspects of Chagas disease pathology and pathogenesis. *T. cruzi* was first described as parasitizing adipose tissue and adipocytes in the 1970s [40,41] and 1990s [42]. Combs et al in 2005 [43] published the functional consequences of parasitism of adipose tissue and adipocytes in a mouse model. In the same year, Philipp Scherer and colleagues [44] reported that injection of LPS into mice that were rendered fatless (by using the regulated fat apoptosis murine model) did not result in the immediate death of mice as seen in control mice with a normal component of adipose tissue. This seminal observation set the stage for studies by the Tanowitz group exploring the significance of adipose tissue during acute infection. Nagajyothi et al [45] demonstrated that as early as 15 days after infection, when mice exhibited no detectable blood parasitemia, there was an increase in the number of macrophages (Fig. 2) and a greater parasite load in brown and white adipose tissue, obtained from the interscapular and subcutaneous regions, respectively, when compared with those in other organs such as the spleen and heart. The authors also noted a reduction in lipid content, adipocyte size, and adipose tissue mass, accompanied by increased expression of lipolytic enzymes, pro-inflammatory cytokines, chemokines, toll-like receptors and ERK in adipose tissue obtained from infected mice [45]. By day 30 post infection, the tissue and serum levels of adiponectin were significantly reduced and inflammatory mediators were up regulated in the adipose tissue [43]. The reduction in adiponectin expression and local lipolysis has been linked to up-regulation of inflammatory mediators, insulin resistance [46] and increase in the influx of macrophages into adipose tissue [47], as was demonstrated in experimental of Chagas disease. Thus, the authors propose that the persistence of the parasite and macrophages in adipose tissue, well into the chronic phase of infection, creates a "low-level" chronic inflammatory state similar to that observed in morbid obesity. The experimental data as well as recent studies in humans [48] strongly indicate that adipose tissue is an early target of infection, as well as a reservoir from which infection can recrudesce during periods of immune-suppression.

Importantly, Nagajyothi et al [49] found that *T. cruzi*-infected cultured adipocytes displayed a reduction in levels of adiponectin and PPAR-γ and a concomitant increase in many inflammatory mediators. These observations may mean that the inflammatory phenotype observed in adipose tissue obtained from *T. cruzi*-infected mice can be attributed to infection of adipocytes *per se*. Thus, the chronic inflammatory state observed in adipose tissue may lead to insulin resistance and enhance heart disease in patients with Chagas disease. This remains an active area of investigation.

Innate Immunity to *T. cruzi* Infection: Experimental Studies

Control of *T. cruzi* depends both on innate and acquired immune responses which are triggered during early infection, critical for host survival, and involve macrophages, natural killer cells, T and B lymphocytes, and the production of pro-inflammatory Th-1 cytokines such as IFN-γ, TNF-α, and IL-12. Excellent recent reviews have discussed in detail the B and T cell immunity to *T. cruzi* in experimental models (e.g., [50,51,52]). Herein, we focus on innate immune responses to *T. cruzi* infection that have primarily been studied by using experimental models of infection and have provided important information regarding mechanisms of cell activation and parasite control [53,54].

Macrophage activation and cytokine/chemokine response

Studies in experimental models have shown that macrophages, dendritic cells and natural killer cells play an important role in parasite control via elicitation of an intense inflammatory reaction, accompanied by an up regulation of cytokines and chemokines [55,56,57]. The interaction of *T. cruzi* with macrophages and other cell types involved in the innate immune response are mediated by pathogen recognition receptors such as Toll-like receptors (TLRs) and a family of type I trans-membrane receptors. Upon recognition of pathogen-associated molecular patterns (PAMPs), TLRs transmit the signal via cytoplasmic Toll/IL-1R domains for the recruitment of cytosolic adaptor molecules including myeloid differentiation primary-response protein 88 (MyD88), and subsequently induce nuclear factor-κB (NFκB) activation, leading to the production of inflammatory cytokines and linking the innate to the adaptive immune response [56]. *T. cruzi*-derived molecules, such as glycosylphosphatidylinositol (GPIs) and mucins stimulate the synthesis of IL-12 and TNF-α by macrophages [58,59]. The mucin-linked GPI anchors induced TLR2-dependent leukocyte recruitment via CCL2 [58,60]. The parasite also expresses cruzipain, a kinin-releasing cysteine protease, which induces dendritic cell maturation via the activation of bradykinin (BK) B_2 receptors (B_2R) [59,61]. TLR2 activation by *T. cruzi* links early-phase inflammation to dendritic cell-driven mechanisms that stimulate Th1 responses via the cruzipain/kinin/B_2R pathway [59,62]. Neutrophils expressing TLR2 induce the extravasation of plasma proteins into interstitial spaces, thus allowing for the cruzipain-mediated release of the dendritic cells and the stimulatory peptide BK at the downstream end of the inflammatory process. It has been suggested that *T. cruzi* evokes interstitial edema through the sequential activation of TLR2/CXCR2/B_2R characterized as an innate axis pathway fueled by the cooperative effects of exogenous and endogenous danger-type signals [59]. TLR4 and TLR9 likely recognize parasite-derived GIPLs and DNA, respectively, and cooperate in the activation of host innate immune response leading to infection regulation [58,63]. Other TLRs have been suggested to be activated by as yet unrecognized *T. cruzi*-derived components [63,64].

T. cruzi-induced up regulation of IL-12 mediates IFN-γ production through activation of NK cells and the induction of the Th1 cells [63]. IFN-γ activates the macrophage expression of iNOS and nitric oxide (NO) production [63,65,66]. TNF-α provides a second signal stimulating NO production and anti-*T. cruzi* activity in IFN-γ activated macrophages as well as infected cardiac myocytes [56,66,67,68], and thus mediates the trypanocidal function via an autocrine pathway.

Others have shown that the immuno-regulatory cytokines IL-10 and TGF-β with a susceptibility to acute infection [69,70]. By using genetic knockout mice or antibodies for depletion of specific immune molecules, it was shown that blockage of type 1 cytokines (IFN-γ, TNF-α) correlates with an increased susceptibility to *T. cruzi* infection [71,72] (reviewed in [73]). Neutralization of endogenous IL-10 leads to an increased *T. cruzi*-induced IFN-γ production and parasite killing, which may point to IL-10 as a potent inhibitor of IFN-γ production during infection in mice, and the resistance to infection is a result of the balance between IFN-γ and IL-10 produced [69]. Indeed, a complete absence of Th2 or anti-inflammatory cytokines had severe negative effects on the infected host; IL-10-deficient mice infected with *T. cruzi* developed a syndrome similar to that of endotoxic shock due to the enhanced production of TNF-α and IFN-γ [74].

Non-immune cells also respond to *T. cruzi* infection by cytokine production. For example, infection of endothelial cells with *T. cruzi* causes the direct induction of IL-1β and IL-6 [75]. Trans-sialidase, a released surface protein of *T. cruzi*, induced IL-6 production in isolated endothelial cells [76]. Our finding of increased TNF-α and IL-1β mRNAs in infected cardiac myocytes suggested to us that cardiac myocytes also respond to *T. cruzi* by

inflammatory cytokine production [77]. Whether the cytokine response by the non-immune cells is a component of innate immunity or a bystander effect to *T. cruzi* infection is not known, and it remains to be investigated in future studies. Collectively, these results underscore the importance of both inflammatory and anti-inflammatory responses during *T. cruzi* infection, and indicate that IL-4 + IL-10/TNF-α + IFN-γ ratio may be an important determinant of desirable outcome.

The cytokines synthesized during *T. cruzi* infection (IFN-γ, TNF-α, IL-1α TGF-β and IL-10) are capable of inducing or regulating the production of chemokines in infected macrophages and cardiac myocytes [68]. The enhanced expression of CCL3 (MIP-1α), CXCL10 (IP-10), and CCL5 (RANTES) at both the mRNA and protein levels has been noted in macrophages and myocardium of *T. cruzi*-infected experimental models [78,79,80]. Chemokines stimulated the infected macrophages in an autocrine manner, enhancing NO release and NO-depend killing of the parasites [68]. Chemokines and their receptors also affect T-cells proliferation, Th1/Th2 differentiation [81] and resistance to infection [68,82]. CCR5 and CXC3 are immunological preferential markers of the Th1 response, and CCR3 and CCR4 are preferentially associated with Th2 response [83,84]. Others have suggested that chemokines contribute to the pathogenesis of *T. cruzi* infection due to their effects on leukocyte migration and activation [82].

Lipid mediators of immune responses

In addition to cytokines and chemokines, eicosanoids, i.e., lipid mediators, also play a role during *T. cruzi* infection. The arachidonic acid derivatives, including leukotrienes (LTB_4) and cysteinyl lymphotoxins (LTC_4, LTD_4 and LTE_4) are produced during experimental *T. cruzi* infection [85,86,87]. The protein responsible for the synthesis of leukotrienes is the enzyme 5-lipoxigenase (5-LO), which is primarily expressed in macrophages, granulocytes, mast cells, and dendritic cells. Resident and recruited leukocytes are capable of synthesizing LTB_4 during infection and activate macrophages to kill intracellular forms of *T. cruzi* [88]. Deficiency of 5-LO resulted in a dramatic increase in the peak of parasitemia; however, 5-LO knockout mice were eventually able to control parasites and exhibited increased survival and reduced cardiac damage and scarce tissue parasitism [89].

5-LO induces Lipoxin (LX) A_4 production that triggers the AhR receptor altering the expression of suppressor of cytokine signaling (SOCS) 2 and serves as an anti-inflammatory eicosanoid. Esper et al [90] recently demonstrated the role of SOCS2 in an experimental model of Chagas disease; *T. cruzi* infection induced SOCS2 expression in the heart and spleen of mice which was, in part, dependent on the activation of the 5-LO pathway. Moreover, SOCS2 deficiency resulted in a reduction in peripheral parasitemia, but not in heart parasitism, and in the down-modulation of pro-inflammatory cytokines, including TNF-α, IL-12 and IFN-γ, associated with a reduction in myocardial inflammation. Although IFN-γ was reduced, SOCS2-deficient macrophages were hyper-responsive to this cytokine and produced increased levels of NO and dealt with infection more efficiently. In addition, *T. cruzi*-infected SOCS2 knockout mice displayed an enhanced number of T regulatory cells and levels of LXA_4 associated with decreased inflammatory responses and reduced production of pro-inflammatory mediators [90]. In the absence of SOCS2, there was myocardial hypertrophy and cardiac myocyte dysfunction. These results may indicate that production of a 5-LO-derived molecule, presumably LXA_4, contributes to SOCS2 expression during experimental *T. cruzi* infection. It should be noted that LXA_4 may function in a SOCS2-dependent and independent manner during infection. As the lipid was enhanced in the absence of SOCS2, it is clear that SOCS2-independent pathways could be operational in the latter animals to mediate any anti-inflammatory actions of LXA_4 during infection. Interestingly, the phenotype of 5-LO-deficient mice infected with *T. cruzi* is, in part, similar to those observed in SOCS2-deficient mice. TXA_2, another eicosanoid, has also

been shown to participate in regulation of intracellular amastigote proliferation, providing opportunities to survive from infection, but result in transition into a chronic persistent state of disease (discussed above).

Reactive oxygen species

The two major reactive oxygen species (ROS) producers relevant in Chagas disease are NADPH oxidase (gp91phox), renamed NOX2, and mitochondria (Fig. 3). NOX2 catalyzes rapid ROS production by the one-electron reduction of O_2, referred to as a respiratory burst that serves as the first line of host defense against microbes. Early studies demonstrated cytochemical detection of NOX2 at the plasma membrane of peritoneal mouse macrophages during interaction with *T. cruzi* [91]. Others have used *in vitro* assay systems or animal models and demonstrated that *T. cruzi*-mediated macrophage activation results in increased levels of $O_2^{\bullet-}$ formation, likely by NOX-dependent oxidative burst [92,93,94]. We have extended these observations and shown that splenocytes of infected mice and *in vitro* cultured macrophages respond to *T. cruzi* infection by activation of NOX2 and a substantial increase in ROS production (Dhiman & Garg, unpublished data). A robust response of splenocytes of infected mice to *T. cruzi* antigenic lysate led us to speculate that parasite factors (and not active invasion) are sufficient to activate NOX and ROS generation. Indeed, *T. cruzi*-derived components are recognized by TLRs and NOD-like receptors, implicated in NOX activation [63]. Yet, further studies are required to identify the *T. cruzi*-generated stimuli that activate TLR- and NOD-signaling mechanisms and initiate translocation of cytosolic components (p47phox, p67phox, and G protein) and NOX assembly during Chagas disease. In the heart, in response to *T. cruzi* infection, infiltrating activated neutrophils and macrophages are a major source of NOX- and myeloperoxidase-dependent ROS during the acute stage, though mitochondrial release of electrons and superoxide production in infected cardiac myocytes is also noted [95]. Superoxide and NO promote peroxynitrite-mediated killing of *T. cruzi* in macrophages [96,97]. The reactive oxygen species (ROS) are also important regulators of parasite control, modulating the cytokine responses and splenic inflammatory cell proliferation during *T. cruzi* infection, as well as playing an essential role in the formation of acquired immunity for parasite killing [98], discussed below.

ROS signaling of cytokine responses

ROS are critical signaling intermediates linking the innate and adaptive immune systems by triggering the production of proinflammatory cytokines (TNF-α, IL-1β) by macrophages and dendritic cells (DCs) of the innate immune system. Inhibition studies with cultured and primary macrophages showed that NOX/ROS was a critical regulator of cytokine production in response to *T. cruzi* infection. *In vivo* studies using splenocytes of *T. cruzi*-infected mice, with or without *in vitro* stimulation with parasite antigens, validated the above observations and demonstrated that the inhibition of NOX by apocynin or use of ROS scavengers substantially blocked the activation and proliferation of phagocytes and inflammatory mediators (IL-1, IL-6, IFN-γ, and TNF-α) [99]. Subsequently, inhibition of NOX/ROS resulted in an increased susceptibility to *T. cruzi*, a finding that suggested that redox status plays an important role in immune activation and control of *T. cruzi*, also validated by studies in p47phox$^{-/-}$ mice, in which *T. cruzi* infection, even at a dose 10-fold lower than that used for wild-type mice, was lethal (Dhiman & Garg, unpublished data). Further studies will be required to delineate if NOX/ROS signal the nuclear transport and activation of transcription factors (e.g., NF-κB and AP-1) and promote cytokine gene expression; or if NOX/ROS elicit immune cell proliferation and thereby indirectly alter the cytokine profile in infected mice.

Others have demonstrated that trypomastigotes (or *T. cruzi*-derived proteins, e.g. trans-sialidase) activate NF-κB in a number of cell types, including epithelial cells, endothelial cells, myocytes, and fibroblasts [100,101,102]. NF-κB activation increased the resistance to infection in many of these cell types. Except for myocytes, these studies, however, did not attempt to determine if ROS signaled the NF-κB-dependent cytokine gene expression in non-phagocytic cells invaded by *T. cruzi*. Subsequently, the Garg group reported that ROS of mitochondrial origin elicited cytokine gene expression in cardiac myocytes infected by *T. cruzi* via multiple mechanisms. One, mtROS enhanced the nuclear translocation of RelA (p65), thereby activating NF-κB-dependent gene expression of inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-1β) [77,103]. Two, ROS caused 8-hydroxyguanine (8-oxoG) lesions and DNA fragmentation that signaled polyadenosine ribose polymerase 1 (PARP-1) activation, evidenced by poly-ADP-ribose (PAR) modification of PARP-1 and other proteins in infected cardiac myocytes. PARP-1 signals DNA repair via PARylation of histones; however, its hyper-activation may have pathophysiological effects ranging from the catalytic activation of inflammatory and hypertrophic gene expression, depletion of NAD^+ pool, and cell death [104,105]. Inhibition of PARP-1, by using RNAi or a chemical inhibitor (PJ34), or by removing ROS with an antioxidant, was beneficial in blocking mtROS formation and DNA damage [77]. Importantly, we found that PARP-1 inhibition also regulated cytokine gene expression, albeit via a different mechanism. PARP-1 did not directly interact with p65, and it did not signal RelA (p65) translocation to nuclei in infected cardiac myocytes. Instead, PARP-1 contributed to PAR modification of RelA (p65)-interacting nuclear proteins and assembly of an NF-κB transcription complex. These studies pointed to the possibility that the ROS-PARP-1-RelA signaling pathway contributes to inflammatory cytokine production in cardiac myocytes infected by *T. cruzi* (Fig. 3). It remains to be seen whether mitochondria serve as an activator of an innate defense response by cardiac myocytes upon *T. cruzi* exposure or if these events are bystander effects of *T. cruzi* infection of the host cells.

Oxidative stress in Chagas disease

In addition to pro-inflammatory cytokines, pro-oxidants also affect cardiac function in chagasic conditions. Studies in experimental models and infected humans demonstrate that an infected host sustains oxidative stress due to *T. cruzi*-elicited, splenic NOX/ROS and the enhanced mitochondrial release of ROS in the myocardium [95]. Our studies demonstrate that the host responds to acute *T. cruzi* infection by up regulating its glutathione antioxidant defense constituted by GPx, GSR, and GSH. However, in the chronic phase, the pro-oxidant milieu of the heart was evidenced by a) increased ROS levels, b) decreased activity of MnSOD, c) insensitivity of glutathione defense to oxidative stress, and d) increased GSSG, and lipid (MDA) and protein (carbonyl) oxidation products [106]. A similar pro-oxidant status in seropositive humans has been reported and demonstrated by a) increased GSSG and MDA contents; b) decreased MnSOD, GPX activity and GSH contents [107,108]; and c) inhibition of CIII activity [109]. Moreover, the treatment of *T. cruzi*-infected animals with an antioxidant tipped the balance in favor of preserving mitochondrial and cardiac function. *T. cruzi*-infected mice and rats, treated with an antioxidant, exhibited a significant decline in the myocardial accumulation of oxidative adducts concurrent with improved mitochondrial function as evidenced by increased ATP synthesis and decreased ROS production [110]. Importantly, preventing the oxidative injuries during the chronic stage preserved the cardiac hemodynamic state that otherwise was compromised in chagasic rats [111]. Others have shown a decline in oxidative stress in human chagasic patients given Vitamin A [112]. All of these observations support the idea that antioxidant depletion and inefficient scavenging of ROS, resulting in sustained oxidative stress, are of pathological importance in human chagasic cardiomyopathy progression (Fig. 3).

The re-expression of fetal genes (ANP, BNP, αsk-Actin and β-MHC) is a hallmark of hypertrophic remodeling, and a considerable body of evidence shows the redox regulation of various signaling cascades and remodeling responses in cardiac diseases of various etiologies [113]. Current research findings support the involvement of the following pathways: (i) ERK-1/2 [114] and the small GTPase Ras [115] in response to α-adrenergic agonist stimulation and angiotensin II [116]; (ii) MAPKs in pressure-overload hypertrophy [117]; and (iii) NF-κB and apoptosis-signal-regulated kinase 1 (ASK-1) in response to angiotensin II infusion [118]. ASK-1 is upstream of p38MAPK and JNK in the MAPK-signaling cascade, and both of these have been shown to be activated by NOX/ROS [119]. The inhibition or scavenging of free radicals has been shown to modulate ERK signaling and hypertrophic responses in neonatal and adult cardiac myocytes [120,121].

Mice and cultured myoblasts infected with *T. cruzi* display increased expression of ERK and cyclin D1 [122,123], as well as of ERK activator protein 1 (AP-1) and NFκB, and a reduced expression of Cav-1 and Cav-3 [124,125]. The protein caveolin (Cav) is a negative regulator of both ERK and cyclin D1 [126]. Cav-3 is expressed only in cardiac myocytes, while Cav-1 is expressed in cells other than cardiac myocytes. Cav-1 and Cav-3 null mice and Cav-1/Cav-3 double null mice display a cardiomyopathic phenotype associated with cardiac myocyte hypertrophy and interstitial fibrosis [127,128,129]. These observations, along with the knowledge of a decline in the expression of hypertrophic markers and collagen deposition in response to antioxidant treatment, suggest that ROS signals pathological hypertrophic remodeling in chagasic myocardium, likely through ERK/AP-1 signaling. The role of ROS from a mitochondrial, but not inflammatory, origin in signaling hypertrophy in chagasic hearts was evidenced by the observation that NOX and MPO, the classical mediators of inflammatory ROS, were equally depressed upon treatment of infected rodents with an anti-parasite drug (benznidazole) and ROS scavenger (PBN), while a hypertrophic phenotype was depressed in PBN-treated rodents only [111]. Inflammatory cytokines (e.g., TNF-α, IL-1β, and MCP-1) have been shown to also promote myocardial hypertrophy and contribute to the development and progression of heart failure [130]. Further studies are required to identify whether inflammatory cytokines, noted to be enhanced in chagasic experimental animals and human patients (reviewed in [73,131]), synergistically enhance the ROS-mediated signaling cascades involved in the activation of hypertrophic responses in chagasic hearts.

Human Immunity To *T. cruzi* Infection

The contact between *T. cruzi* and cells from the human host triggers an immune reaction that leads to the control of parasite levels. Yet, chronic persistence of parasites, albeit at low levels, and inflammation are hallmarks of Chagas disease in humans. In this section, we focus on human responses to *T. cruzi* (Fig. 4) and briefly present studies in experimental animals.

Two main strategies have been employed to understand the immunological mechanisms triggered by the infection of human cells with *T. cruzi*: (1) *in vitro* studies of infection of different human cell types with trypomastigote forms of the parasite, and (2) analysis of the immune response in acutely infected patients. That acute infection is not always clinically apparent presents a challenge when performing studies during this phase of the disease. Despite this difficulty, a few studies were recently performed and the findings seem to indicate that innate immune cells, such as natural killer cells (NK) and macrophages, mediate the control of parasite replication in the early stages of human infection. In addition to the role of these cells, antibody production seems to also play an important part in the early control of parasitemia.

Humoral responses

Two types of antibodies are produced during infection: diagnostic antibodies, which form the basis for the serological diagnosis of Chagas disease, and lytic antibodies, which have been defined as antibodies that mediate lysis of the infective forms of the parasite [132,133]. Regardless of the type of antibody produced, it is clear that *T. cruzi* triggers a vigorous B cell response in acutely infected individuals [134]. Lytic antibodies were first identified in mice and demonstrated to correlate with protection to infection. Later studies showed that humans with apparent or in-apparent, acute Chagas disease had similar levels of complement-fixing antibodies [135]. IgM and IgG anti-galactose antibodies against glycoylated epitopes of parasite surface proteins has been a useful tool to discriminate between early, indeterminate and late stages of acute infection in humans due to changing ratios and specificities of the two types as disease progresses [136]. More recently, an association between the presence of lytic antibodies and a protective response in chronic patients was described and strongly indicated that patients in the indeterminate phase displayed higher levels of lytic antibodies compared with those in patients with chagasic heart disease. These data pointed to a possibly protective role for these antibodies [137]. Lytic antibodies have also been brought to attention as a possible marker of parasitological cure, since treated patients that displayed negative hemo-cultures for over ten years did not have circulating lytic antibodies, despite occasionally testing positive by conventional serology [138]. While lytic antibody production depends on the presence of active infection, the loss of such markers does not occur immediately after effective treatment. Thus, they cannot be used as markers for parasitological cure immediately after treatment, but may be useful years after treatment when clearance of preformed antibodies has been achieved.

In addition to their possible role in parasite control and maintenance of protective responses in chagasic patients, antibodies may also contribute to the pathologic alterations observed in this disease. Several studies have shown that individuals within the chronic phase of Chagas disease produce antibodies capable of recognizing several host proteins, which may lead to tissue damage. Among these self-reactive antibodies, the anti-beta adrenergic receptor and the anti-myosin antibodies have been associated with auto-reactive responses [139,140]. The mechanisms by which such antibodies are produced have not been completely clarified, though evidence of *T. cruzi*-derived cross-reactive molecules exists [141]. A recent study proposed that cardiac antigens exposed to *T. cruzi*-induced oxidative stress produce neo-antigens eliciting self-directed antibody response [142]. Others have shown that anti-epimastigote antibodies purified from the sera of chronically infected patients are capable of triggering proliferative responses in peripheral blood mononuclear cells from Chagas patients [143,144,145]. CD5^{+} (B1) cells, the main cell type that proliferate upon stimulation with such anti-epimastigote antibodies [146], have been demonstrated to correlate with pathology development in experimental models of *T. cruzi* infection [147]. This finding may mean that anti-parasite antibodies or antibodies against host-derived neo-antigens could serve as a constant source of stimulation, providing a mechanism that would perpetuate cellular activation during the chronic phase when parasitemia is particularly low. The antigens responsible for differential B cell activation, the mechanisms that lead to the production of lytic versus conventional antibodies, and the role of antibodies in the cross talk between different cell types are questions that require further investigation.

Natural killer cells

Recent observations point to NK cells as important in the regulation of parasitemia. For example, Sathler-Avelar et al. analyzed patients classified as being in the early acute, late acute and early chronic phases of Chagas disease, based upon the expression of anti-gal IgM and IgG antibodies [148]. These authors reported that patients in the early stages of the acute disease displayed an expansion of B cells, but no significant changes in the frequency of NK

cell population. However, during the late acute stage, patients displayed a selective increase in a distinct lineage of NK cells ($CD16^+CD56^-$), as well as a persistent expansion of B cells, possibly indicative of a relationship between B cell activation and a subset of NK cells. Recent studies demonstrated that NKT cells provide help for the development and response of lipid-specific B cells in the mouse [149]. While the response to lipid antigens remains poorly understood in Chagas disease, the relationship between lipid metabolism and inflammatory response in *T. cruzi* infection has been demonstrated, as discussed above, and is an area that merits greater investigation.

T cell responses

There are few studies characterizing the T cell responses during acute infection in humans (Fig. 5). Early investigations suggested that *T. cruzi* infection leads to inhibition of T cell responses, since acutely infected patients failed to mount a delayed type response to a series of non-related antigens [135]. The mechanism of this impaired response was later associated with a low expression of IL-2 receptor, induced by *T. cruzi* [150]. A study of the T cell repertoire in acutely infected patients from Bolivia showed a marked decrease in the frequency of T cells expressing the variable region 5 (Vβ5) of the T cell receptor (TCR) [151] perhaps indicating that a vigorous T cell response, followed by clonal exhaustion, could lead to the decrease in the frequency of Vβ5$^+$ T cells. The impaired T cell response observed in acutely infected patients could reflect a temporarily suppressed or exhausted response rather than the lack of a response. Interestingly, Vβ5$^+$ TCR-expressing T cells are highly expanded in chronic chagasic patients, observed in both freshly isolated PBMCs as well as after *in vitro* stimulation with parasite antigens [151,152], possibly indicative of the pathologic significance of vβ5 containing TCR. Finally, the involvement of T cells in the acute phase may be signaled by the identification of T cells in the inflammatory infiltrate in the hearts of acutely infected patients. These patients presented with a myocarditis associated with the presence of CD4$^+$ and CD8$^+$ T cells, as well as parasite antigens [153], which may indicate the pathologic significance of T cells in acute myocarditis. Results in studies in the murine model of *T. cruzi* infection could point to a protective role for CD8$^+$ T cells in parasite control during acute infection [52,154]. To our knowledge, there is little evidence to support this finding in human disease.

In patients progressing to the chronic phase, a robust expansion of T cell response to parasite and host-derived antigens has been clearly demonstrated [146,155,156,157]. A high frequency of activated T cells was found in peripheral blood of indeterminate and cardiac patients [157,158] and in inflammatory infiltrate observed in the hearts of cardiac patients [159]. Detection of high levels of ICAM-1 expression by endothelial cells in the heart [160] and of the chemokine receptors CCR5 and CCR7 by infiltrating inflammatory cells in the myocardium [161,162] may mean that these molecules play a role in the mechanisms of T cell recruitment to the heart of chagasic patients. The CD8$^+$ granzyme$^+$ T cells were the main cell type found in infiltrating infiltrate in the myocardium. Based upon these observations, it is currently believed that CD8$^+$ T cells play an important role in tissue destruction in Chagas disease [163,164]. However, others have suggested that CD8$^+$ T cells undergo immunological exhaustion in chronic patients and, thus, their lack of activity could contribute to the establishment of pathology [165]. In summary, it is difficult to define a single role for CD8$^+$ T cells in human Chagas disease, and the evidence points to the involvement of these cells in several distinct processes during disease progression including; a) playing a role in parasite control during acute infection, b) mediating pathology in the chronic phase, and c) a possible lack of activity aiding in the establishment of pathology. The body of work already generated concerning CD8$^+$ T cells in human Chagas disease leads to speculation that distinct populations of CD8$^+$ T cells may co-exist during the course

of Chagas disease and play different functions. Future studies would be required to test this hypothesis and further our understanding of the role of T cells in Chagas disease.

CD4+ T cells and monocytes/macrophages act as key orchestrators of the cellular response during chronic Chagas disease. These cells are capable of producing inflammatory and anti-inflammatory cytokines after stimulation with parasite-derived antigens or upon *in vitro* infection with trypomastigote forms of *T. cruzi*. Several studies have demonstrated a correlation between the production of inflammatory cytokines by CD4+ T cells and monocytes of patients with the cardiac form of the disease, and the production of IL-10 by the same cells of clinically asymptomatic patients [166,167]. For example, peripheral blood mononuclear cells from chronic chagasic patients produce more IFN-γ and less IL-10 than do those from indeterminate patients [167]. Accordingly, chagasic patients exhibit a Th1 type (IFN-γ) cytokine profile with suppression of Th2 type cytokines (IL-4, IL-10), and elevated plasma levels of TNF-α in the chronic stage [168,169].

Increased myocardial expression of the adhesion molecules MCP-1, IP-10 and MIG, their receptors CCR2 and CXCR3, and cytokines IFN-γ, TNF-α, IL-15, IL-6 and IL-4 has been reported in chagasic patients by several researchers [167,170]. High levels of TNF-α have been associated with worse cardiac function. Gene expression profiling of myocardial tissue from chagasic experimental animals and human patients showed that 15% of the genes known to be selectively up regulated are IFN-γ-inducible [171]. These observations point to the pathologic significance of IFN-γ and TNF-α in chagasic cardiomyopathy. Notably, the regulatory FoxP3+IL-10+ T cells are predominantly found in indeterminate patients, providing evidence for the importance of IL-10 producing cells in the regulation of pathogenic responses in asymptomatic patients [172].

It has been demonstrated that T cells that do not express the co-receptors CD4 and CD8 (referred to as double negative T cells) have emerged as abundant producers of immunoregulatory cytokines in chagasic patients [173]. The double-negative T cells appear at a low frequency in the peripheral blood of chagasic patients, but are highly expanded by exposure to *T. cruzi* trypomastigotes and express preferentially inflammatory and anti-inflammatory cytokines in cardiac and indeterminate patients, respectively. This finding relates to the possible role of lipid antigens in the *T. cruzi* – host interaction, given that many double-negative T cell populations are preferentially activated by lipid antigens. Given the particular characteristics of these cells to mount fast responses and to tolerate chronic stimulation, it is possible that double-negative T cells also represent a link between innate and adaptive responses in Chagas disease [174]. Thus, the regulatory networks established during the chronic phase of Chagas disease are critical for the clinical evolution of patients into indeterminate or cardiac disease stage. The indeterminate patients are capable of producing inflammatory and anti-inflammatory cytokines; the balance between these molecules in chronic phase may ultimately determine disease pathogenesis [163].

A critical question is what drives the anti-inflammatory balance observed in indeterminate patients towards an inflammatory environment observed in cardiac patients? (Fig. 5). It is possible that cardiac patients display a genetic predisposition towards the establishment of inflammatory responses. Supporting this hypothesis are several studies that have shown polymorphism of genes encoding cytokines, which affect protein expression levels, is associated with the indeterminate and cardiac forms of the disease [167]. Of note, a functional polymorphism in the promoter region of *IL-10*, which leads to the low expression of this cytokine, is associated with the cardiac form of Chagas disease [175]. Additionally, the occurrence of a promoter region polymorphism in the *TNF-α* gene leading to high expression of this cytokine is associated with worse prognosis and progression to death for cardiac chagasic patients who undergo a heart transplant [175]. However, it is important to

consider that, even patients who display genetic susceptibility profiles to develop cardiac disease still go through the indeterminate form. Thus, it is possible that external factors will combine with genetic risk factors to trigger the shift in cytokine balance from the controlled, regulatory profile observed in indeterminate patients to the exacerbated inflammatory profile observed in cardiac patients. Epigenetic mechanisms and gene regulation studies may help clarify this question.

Summary and Future Perspectives

The literature strongly suggests that infected host responds to *T. cruzi* infection by eliciting inflammatory cytokines (TNF-α, IFN-γ) and ROS production. A sustained ROS generation of inflammatory and mitochondrial origin, coupled with an inadequate antioxidant response, results in the inefficient scavenging of ROS in the heart and leads to long-term oxidative stress. Thus, while ROS are essential for activation of inflammatory responses and pathogen control at an acute stage, the persistent oxidative stress denies the control of the inflammatory state. Further, intracellular *T. cruzi* or Tc-antigens that persist during late or chronic infection might interact with the immune and non-immune cells in the myocardium and subsequently activate signaling cascades (e.g NF-κB pathway) that trigger the production of inflammatory cytokines (TNF-α, IL-1β), ROS induced-DNA damage, and hypertrophy in cardiac myocytes. Importantly inflammatory cytokines and ROS create a complex feedback mechanism that can positively sustain stress responses and, thus, play an important role in cardiac remodeling and the evolution of chronic Chagas disease. We propose that a substantial effort should be made in delineating the complex interrelationships between oxidative stress and inflammatory mediators, wherein the promise of antioxidant and anti-inflammatory therapies in controlling progressive chagasic cardiomyopathy can be realized.

As noted, there remains a dearth of data on the immune response in human Chagas disease, and these data are required to understand the pathogenesis of this important disease. In addition, these data are needed for vaccine development as well as for the development of new therapeutic agents. The current therapies are prolonged and have unacceptable side-effects. We do not know after years of efforts whether these agents actually prevent the evolution to chronic disease in humans.

Acknowledgments

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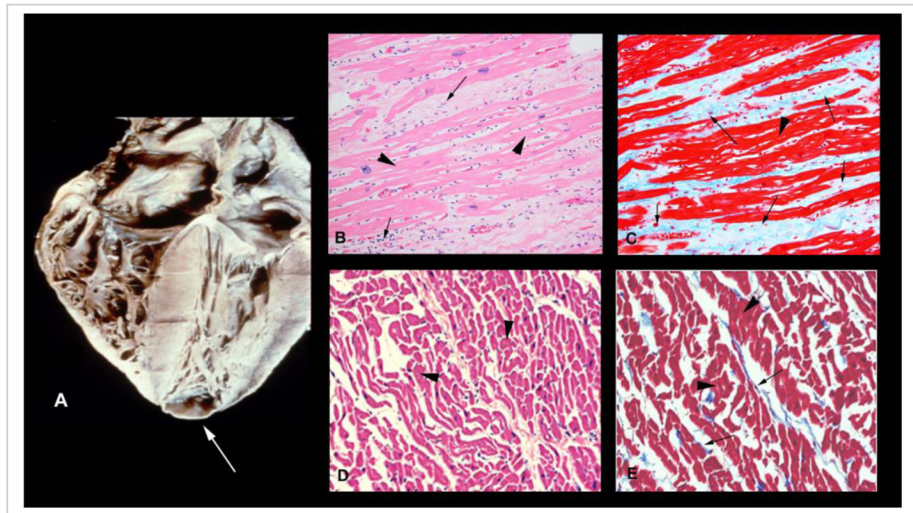
Fig. 1. Heart of a patient with chronic chagasic cardiomyopathy
(**A**) Shown is 4-chamber enlargement of the heart in a chagasic patient. Apical aneurysm is marked with an arrow (printed with permission from the Armed Forces Institute of Pathology). (**B**) H&E-stained myocardium from a patient with chronic chagasic cardiomyopathy showing fibrosis and chronic inflammation (arrow). (**C**) Myocardium of the same patient sample stained with Masson's trichrome showing significant fibrosis (blue color, marked by arrow). H&E (**D**) and Masson's trichrome (**E**) staining of the myocardium of an uninfected healthy donor are shown for comparison. Arrow-heads in all panels mark the myocardium.
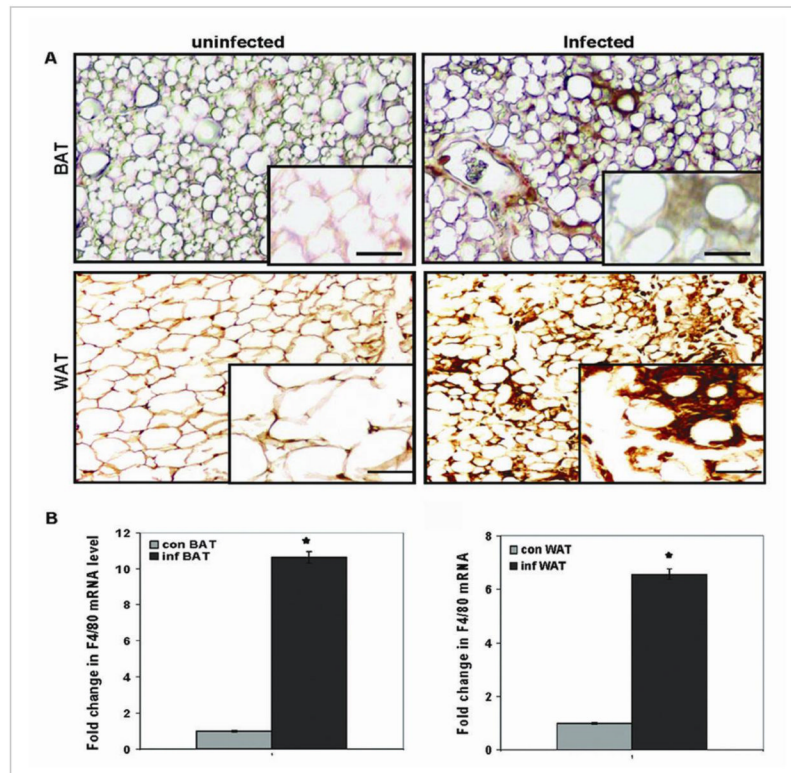
Fig. 2. Macrophages in adipose tissue
Immunohistochemical analysis using antibody against ionized calcium-binding adaptor molecule 1 (Iba1) of adipose tissue obtained from mice. (**A**) Brown adipose tissue (BAT), obtained from the interscapluar region and white adipose tissue obtained from the subcutaneous region (WAT). Note the increase in macrophages in the infected adipose tissue. (**B**) Macrophage-specific F4/80 messenger RNA (mRNA) demonstrating the increase in macrophages in both BAT and WAT, as determined by real-time quantitative polymerase chain reaction. Con=control; Inf=infected (reproduced from Nagajyothi et al. *Journal of Infectious Disease* [45] with permission from the Journal and the Oxford University Press).
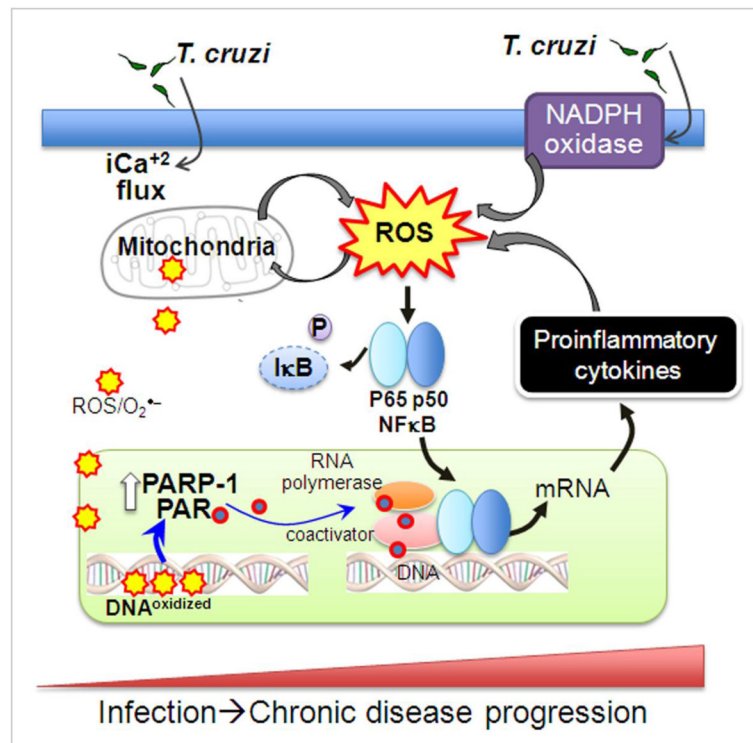
Fig. 3. Oxidative stress in Chagas disease

T. cruzi infection elicits generation of superoxide ($O_2^{\bullet-}$) through activation of NADPH oxidase in phagocytic cells. T. *cruzi* invasion of other cells elicits intracellular Ca^{+2} flux affecting mitochondrial membrane potential and results in increased leakage of electrons from the electron transport chain to molecular oxygen and formation of $O_2^{\bullet-}$ and other reactive oxygen species (ROS). Two pathways of ROS signaling of the NFκB pathway are envisioned. One, ROS directly promotes phosphorylation and transport of p65 (RelA) and p50 to nucleus, assembly of transcription complex, and expression of proinflammatory cytokines (e.g. IL-1β, TNF-α). Two, ROS-mediated oxidation of DNA may signal activation of poly (ADP-ribose) polymerase (PARP-1). PARP-1 cleaves NAD^+ to form ADP-ribose and polymerizes the latter onto nuclear acceptor proteins (e.g. histones), transcription factors, and PARP itself, and contributes to DNA repair and genomic stability. Binding of poly ADP-ribose (PAR) molecules activates NF-κB transcription complex and inflammatory cytokine gene expression. The feed-back loop of ROS generation and cytokine gene expression contribute to persistence of inflammatory responses and oxidative damage, and progressive evolution of cardiomyopathy in Chagas disease.
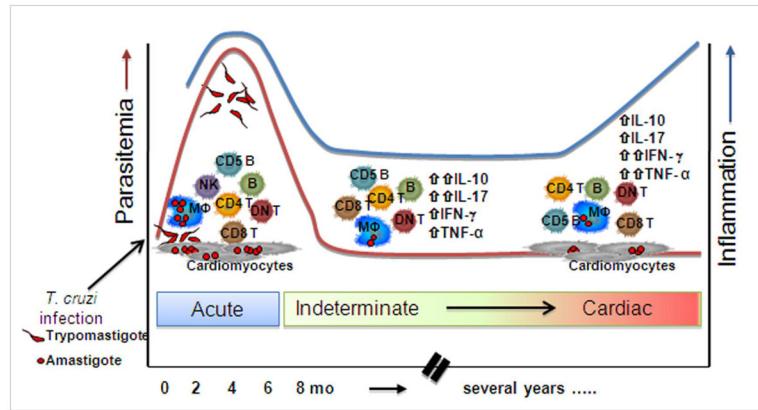
Fig. 4. Progression of human Chagas disease
Following infection with *T. cruzi* the majority of individuals develop parasitemia associated with a robust cellular and humoral immune response involving cells of the innate and adaptive immune response. This stage can present with severe clinical features ranging from high fever to arrhythmia and death. Upon successful control of the initial infection by the immune response, parasitemia wanes and symptoms subside, as the individual enters the chronic phase of Chagas disease. Here the majority of individuals enter the indeterminate (asymptomatic) clinical form, associated with a balanced inflammatory and regulatory immune response (low inflammatory index). Approximately 30% of the individuals will progress to the cardiac clinical form of disease associated with a higher inflammatory state. Cell types referred in the figure: CD4, CD8 and double negative (DN) T cells, macrophages (MΦ), Natural killer (NK) cells, CD5 (IgM-secreting) B cells. The increased inflammation refers to data demonstrating an up regulation of the inflammatory response (cytokines, chemokines).