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# Pathogenesis of Chagas disease: time to move on

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## 1. ABSTRACT

*Trypanosoma cruzi* is the etiologic agent of Chagas disease. The contributions of parasite and immune system for disease pathogenesis remain unresolved and controversial. The possibility that Chagas disease was an autoimmune progression triggered by *T. cruzi* infection led some to question the benefit of treating chronically *T. cruzi*-infected persons with drugs. Furthermore, it provided the rationale for not investing in research aimed at a vaccine which might carry a risk of inducing autoimmunity or exacerbating inflammation. This viewpoint was adopted by cash-strapped health systems in the developing economies where the disease is endemic and has been repeatedly challenged by researchers and clinicians in recent years and there is now a considerable body of evidence and broad consensus that parasite persistence is requisite for pathogenesis and that antiparasitic immunity can be protective against *T. cruzi* pathogenesis without eliciting autoimmune pathology. Thus, treatment of chronically infected patients is likely to yield positive outcomes and efforts to understand immunity and vaccine development should be recognized as a priority area of research for Chagas disease.

## 2. INTRODUCTION

### 2.1. *Trypanosoma cruzi*-induced myocardial inflammation

Myocardial destruction, characteristic of the most severe cardiac clinical forms of Chagas disease, is caused by a multi-focal progressive inflammatory reaction (1) that is associated with injury to the vascular endothelium (2-4), vasospasm and a reduction in blood flow (5). The search for what characteristics of the host immune response could explain the establishment of protective or pathological responses has driven research over the years. Since the triggering event causing Chagas disease is the infection with *T. cruzi*, the parasite would be expected to have a major role in the development of pathology. Indeed, peripheral blood cells from individuals with Chagas disease respond *in vitro* to parasite-derived antigens (6-9). In experimental animals, the parasite can induce many of the pathological and clinical alterations observed in humans. Moreover, a correlation between the presence of parasite antigens and presence of inflammatory infiltrate was found in the heart of individuals with the cardiac form of Chagas disease (10). However, as some people never develop heart disease despite infection (11),

the precise mechanism whereby parasitism causes tissue damage in the chronic phase is still not completely understood (12).

## 3. AUTOIMMUNITY

### 3.1. What causes the autoimmunity observed?

Although the pathogenesis of Chagas disease is highly variable, it is dependent on both the genotypes of the host and the infecting parasite strain (13). In general, the onset of chronic chagasic heart disease frequently follows a protracted asymptomatic period, the indeterminate phase. As noted, post *mortem* examination of hearts from patients in the indeterminate phase and the asymptomatic chronic phase often appear to be free of parasites by routine histological examination. The primary histopathological feature of chagasic heart disease is chronic inflammation of the myocardium accompanied by myocytolysis, vasculitis, and fibrosis. A variety of auto-antibodies have been observed in these individuals including antibody to cardiac specific antigens such as cardiac myosin. However, even in asymptomatic infections, high anti-parasite antibody titers are maintained (14). Several mechanisms, which are not mutually exclusive, have been put forward to explain the autoimmunity observed. Most studies have tended to be focussed on bystander activation and molecular mimicry but polyclonal activation, cryptic epitopes and epitope spreading have also been suggested as potential mechanisms (15).

The attraction of bystander activation as a mechanism for generating cardiac specific autoimmune responses is based on the observation there is lysis of the parasite in the myocardium during acute infection releasing antigens. It is easy then to envisage that such release in a cytokine rich environment, activated by the presence of the parasites themselves, would overcome tolerance resulting in a degree of autoimmunity. Nevertheless, the observation of potentially shared epitopes between some of the parasite and cardiac proteins has led to the popular idea of cross-reactive proteins to explain the phenomenon. Notably, the B13 epitope of *T. cruzi* has been reported to share peptide sequence with cardiac myosin (12, 16). Since bystander activation seems likely to require live parasites, reports highlighting the ability of killed trypanosome antigens to elicit both cardiac damage (as evidenced by elevated serum cardiac troponin I) and cardiac specific autoimmunity provide support for the mimicry hypothesis (17); particularly as those same lysates have a low toxicity to cultured cardiac myocytes. Interestingly, polyantigenic autoreactivity emerged as a result of epitope spreading in the experimental model employed (17, 18). However the type of immunity elicited by challenge with parasite lysates was distinct from that observed during infection and so it is perhaps most likely that a combination of mechanisms operating during the course of an infection is responsible for the autoimmune reactivity observed.

### 3.2. Is the autoimmune response pathogenic?

Autoimmune reactivity (such as that observed in Chagas disease) is requisite in the description of an autoimmune disease but it is not sufficient for a disease to be described as such. Autoimmune reactivity is often detected in otherwise healthy individuals and hence the critical questions which remain are 1) whether the autoantibody and any autoreactive T-cell responses are actually pathogenic and 2) whether any such pathogenic responses can be maintained, or indeed exacerbated, in the absence of infection (as would be the case in an autoimmune disease)?

Here, the answers become far more equivocal. Indeed, although the presence of mononuclear cells in the heart clearly causes damage and correlates with release of auto-antigens and production of auto-antibodies, it is not entirely clear what draws them to the heart and whether they can be retained in the absence of infection. The role of the innate immune system in directing the initial response to parasitemia is beginning to receive attention, with a role for dendritic cells in parasite sensing and setting up of the adaptive response (19). Toll receptor (TLR 2 and TLR9)-dependent activation of proinflammatory cytokines such as IL-12 and TNF have also been implicated in the successful control of *T. cruz*i infections (20). Toll receptors signalling has also linked to the production of chemoattractants which may initiate immune cell infiltration of the myocardium (20). What has received rather less attention to date is whether the innate immune system is also critical for the advent of the autoimmunity observed.

Recent work has drawn attention to the majority of the immune response being directed against immunodominant epitopes of the trans-sialidase (TS) molecule (21), with the magnitude of the anti-parasite response dwarfing the auto-antibody responses observed and implying that the major part of the immune response arising from infection is appropriate to controlling the infection. This has raised the prospect that variation of these immunodominant epitopes in the TS repertoire of trypanosomes can prevent cross protection between life-cycle stages and has even raised the spectre of antigenic variation (22), a phenomenon much more strongly association with African trypanosomes. Differential expression of TS was associated with difference in thymic lesions in a murine model infected with different strains (23). *T cruzi* strains that produce and secrete higher amounts of TS and induce 100% mortality in mice, cause thymic involution and thymocyte depletion in infected mice (11).

If the autoimmune response is pathogenic, then reconstitution of naïve mice with immune cells, but not parasites, from infected mice would be evidence of the pathogenic potential of the autoimmune response. Early experiments using adoptive transfer suggested that this could indeed be the case, but these have been criticized (24). Nevertheless, reports of successful adoptive transfer experiments in animal models continue to suggest that the autoimmunity observed may be transferred to naïve recipients in the absence of parasites, leaving the question unresolved (25). Inhibition of G-protein signaling reduces both the ability of the parasite to invade cells and the immune and autoimmune response raised against it. Where cannabinoids were used to achieve this, cardiac

inflammation was dramatically reduced, however parasitemia increased and there was no reduction in mortality gained from this class of drug (26).

### 3.3. Can parasite-directed immune responses elicit protective immunity without pathogenicity?

Some have taken the view that parasites are present in the hearts of all patients with chagasic heart disease and that although not necessarily patent from histological investigation they can be detected by more sensitive techniques such as PCR (24). However, others have countered with evidence that intracellular parasites might perish but that their DNA could persist in cardiac myocytes and perhaps even integrate into the host nuclear DNA allowing chagasic antigens to be expressed by heart cells after the death of all parasites. These suggestions have proven controversial(27).

The immune response generated to *T. cruzi* is largely effective at controlling, but not eliminating the infection which is believed to be life-long unless treated with drugs. Following the acute phase of the disease in which parasite levels are relatively high, an asymptomatic, indeterminate phase is normal in which antibody levels to the parasite remain high but blood and tissue levels of the parasite can diminish to sub-patent levels. In model systems the key components involved in modulating this response are parasite specific antibody, CD4+ and CD8+ T cells (28). Not unexpectedly for an intracellular parasite, biasing of the immune system towards a Th1 type response was protective, whereas Th2 biased responses exacerbated disease.

Patient data indicates that for drug-treated patients anti-parasite antibody titers eventually decline which is indicative of cure (29). Two important recent studies have examined in detail the anti-parasite and autoimmune responses following drug cure in animal models. The study of Hyland *et al.* (30) demonstrated that the autoimmune response correlated directly with the presence or absence of parasites. Following cure, autoimmunity declines, but following re-infection with a more virulent strain, it is re-established. As noted, Bustamante *et al* were interested in the persistence of a parasite-directed response mediated by CD8+ T-cells after drug-induced cure (31). In this case the immune memory generated was shown to be protective against subsequent challenge and was more effective in drug cured individuals than in chronically infected. Taken together these two studies strongly suggest that it will be possible to evoke specific long-lasting protective immunity to Chagas disease without necessarily evoking a damaging autoimmune response.

## 4. MOUSE MODELS OF IMMUNITY

Most information concerning the immunity related to *T. cruzi* infection has been derived from studies using experimental models, in particular the mouse. Several important observations have arisen from studies in mice with respect to the role of immune mediators and cells in the control of acute infection. However, although the experimental studies model the acute infection well, they have not provided a clear association with what is observed in chronic human chagasic cardiomyopathy. Over the past decade experimental models have been described to study chronic *T. cruzi*–associated cardiomyopathy (see references (32, 33). The study of the chronic phase has additional complexities, and must take into account the interactions of the immune response, the presence of the parasite and host cardiac physiology (11, 34).

## 5. TOLL-LIKE RECEPTORS

*T. cruzi* infection is characterized by an acute phase with detection of circulating parasites and an intense inflammatory reaction at the site of parasite penetration. Romaña's sign or "chagoma" is the earliest sign of *T. cruzi* infection in a proportion of naturally infected individuals and is characterized by an intense mononuclear infiltrate at the site of infection (35). The molecular basis of this intense response is well understood but studies suggest that *T. cruzi* can stimulate the host's cutaneous cells to produce mediators that will trigger a local inflammatory response. The initial recognition of invading pathogens is mediated mainly by the Toll-like receptors (TLRs), family of type I transmembrane receptors that recognize a large variety of exogenous ligands. These germline-encoded receptors are defined by the presence of a Toll/IL-1R domain in their cytoplasmic region, and by leucine-rich repeats in the extracellular domain (36). TLRs are conserved and are found in plants, insects, and mammals. To date, 13 TLRs have been reported in mammals, at least 10 of which are clearly expressed in human cells. TLR signaling via the cytoplasmic Toll/IL-1R domain following the recruitment of cytosolic adaptor molecules, including MyD88, which in turn facilitate the assembly of signaling complexes (37, 38) and induces the NFκB activation, production of proinflammatory cytokines, and up-regulates the expression of co-stimulatory molecules, linking innate to adaptive immune responses (39).

TLRs are expressed differently on diverse cell types and appear to respond to distinct ligands. TLR ligands include a growing list of a pathogen-associated molecular pattern (PAMP) (40), as well as endogenous molecules such as heat shock proteins or products of oxidative stress (41-43). The surface of *T. cruzi* contains large amounts of glycoinositolphospholipids (GIPLs), which occur either as GPI anchors for glycoproteins and polysaccharides, or as free GIPLs that contain the identical core structure of GPI (44, 45). The oligosaccharide sequences and lipid structures of the major GIPLs purified from different *T. cruzi* strains, shown heterogeneity in the glycan and lipid composition of GIPLs, both between different strains of the parasite, as well as within a single strain (46-48). GPI purified from *T. cruzi*, trigger proinflammatory cytokine expression in a TLR2-dependent manner (49), and GIPLs engage TLR4-dependent signaling pathways (50). Despite its relative importance for GPI-induced responses, *T. cruzi*-infected TLR2 null mice had no major changes in parasitemia or mortality (51). However, MyD88 null mice were more susceptible to infection with *T. cruzi* (51). This suggests that other TLR(s) and/or receptor(s) that depend on this

adapter molecule act in concert with TLR2 to activate innate immune responses to *T. cruzi* infection. In this regard, Oliveira *et al* (50) reported that TLR4 expression was necessary for the *in vivo* proinflammatory responses to *T. cruzi*, as well as for the *in vitro* activation of NF-κB. TLR4-null mice were defective in their ability to control parasite replication in the early stages of infection, and hence displayed an earlier mortality when compared with C3H/HeN mice (50). More recently, Bafica *et al* reported that TLR2 and TLR9 cooperated in the control of parasite replication and that TLR9 had a primary role in the MYD88-dependent induction of IL-12/IFN-γ synthesis during *T. cruzi* infection (52). Taken together, these studies suggest an important role of a cooperative function of multiple TLRs in the mechanisms leading to control of *T. cruzi* infection in the murine host.

While the role of TLR2 in the modulation of cytokines production and parasite replication during *T. cruzi* infection has been demonstrated, other studies demonstrated that TLR2 functions as the main upstream regulator of hypertrophy triggered in *T. cruzi*-infected cardiac myocytes. Cardiac myocytes are terminally differentiated cells that participate in monitoring conditions of stress in the heart via intrinsic TLRs (41, 53, 54). In fact, cardiac myocytes express TLR2, -3, -4, and -6 (41) and respond to stress by initiating adaptive strategies, including hypertrophy and inhibition of apoptosis (55-57). Cardiac myocyte hypertrophy involves the reactivation of an embryonic pattern of gene expression and increased expression of contractile proteins, followed by an increase in cell size (55). The biochemical and physiologic changes that accompany a hypertrophic response result in increased myocardial contractility which contributes to the maintenance of cardiac function in the short term. If the hypertrophic state is prolonged, it may lead to apoptosis, fibrosis, and dilated cardiomyopathy and ultimately congestive heart failure (55).

The hypertrophic response in *T. cruzi*-infected cardiac myocytes is characterized by re-expression of the gene encoding a fetal contractile protein, myosin heavy chain-β; increased expression of β-actin; and increased cell size (58), consistent with a standard definition of hypertrophy (55). Further characterization of this response identified the proinflammatory cytokine, IL-1β, as an important mediator of *T. cruzi*-induced cardiac myocyte hypertrophy. This was demonstrated by the addition of a specific cytokine trap to infected cardiac myocyte cultures, which blocked the activity of IL-1β and significantly diminished the overall hypertrophic response (58). These studies demonstrated that cardiac myocytes represent an important target of *T. cruzi* infection where invasion, intracellular replication, and persistence are key factors contributing to the pathogenesis of Chagas disease.

In addition to the innate and adaptive immune responses mounted by the host, physiologic responses intrinsic to cardiac myocytes are likely to influence the progression of disease. IL-1β and TLR2 function as upstream regulators of hypertrophy triggered in isolated cardiac myocytes by *T. cruzi*. These observations suggest that intrinsic cardiac myocyte TLRs play a role in the rapid response to pathogens and their products and that cardiac myocyte hypertrophy may be an important early consequence of the engagement of this innate system of pathogen recognition in the heart. Thus, in addition to being relevant to the ability of the murine host to deal with *T. cruzi* infection, it appears that TLRs and MyD88 signaling may be relevant for the cardiac remodeling observed in chronic stages of Chagas disease (34)

## 6. CYTOKINES

A protective immune response against *T. cruzi* infection is characterized by a TH1-type response where the cytokines IFN-γ, TNF-α, and IL-12 contribute to the control of parasite replication and infection (59-62). Early in the course of infection, several *T. cruzi*-derived molecules, including GPI mucins from trypomastigotes and DNA (63, 64), stimulate the synthesis of IL-12 and TNF-α by macrophages (65). Once secreted *in vivo*, IL-12 triggers IFN-γ production by NK-cells, which in turn induces more IL-12 production that cycles back to enhance IFN-γ production by NK cells. IFN-γ production is also amplified by IFN-γ-induced production of macrophage TNF-α and IL-1β in a positive feedback loop. More importantly, these cytokines drive the generation of type 1 $CD4^+$ and $CD8^+$ T cells, which produce further IFN-β, a key requirement for inducing and maintaining control of acute infection (59, 66). In mice, the potent microbicidal/microbiostatic activity of IFN-γ appears to be mainly via the upregulation of the expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) by *T. cruzi*-infected macrophages (61, 67-71). In addition, TNF-α provides a second signal stimulating NO production and anti-*T. cruzi* activity in IFN-γ-activated macrophages, and conversely, TGF-β and IL-10 negatively regulate NO production (72). Since *T. cruzi*-infected macrophages and cardiac myocytes produce TNF-α, this cytokine appears to mediate its trypanocidal function via an autocrine pathway (73). The down-regulatory cytokines IL-10 and TGF-β are associated with susceptibility to infection (66, 74) by inhibiting IFN-γ-mediated macrophage activation. Thus, neutralization of endogenous IL-10 leads to an increased *T. cruzi*-induced IFN-γ production and parasite killing (66, 75). These results suggest that IL-10 may be a potent inhibitor of IFN-γ production during infection in mice and that the early resistance to infection is a result of the balance between IFN-γ and IL-10 produced (66). Interestingly, IFN-γ has been demonstrated to be a negative regulator of lymphocyte expansion, preventing uncontrolled lymphoproliferation during the response to infection (76).

A complex program of >1200 genes is induced by IFN in host cells (77), including a new group of IFN-induced genes that belong to a family encoding a series of GTPases (78), described to play a important role in host control of intracellular pathogens (79, 80) and to be expressed by a variety of cell types in response to stimulation by both type I and type II IFNs. So far, six proteins have been described in the mouse, LRG-47, IRG-47, TGTP/Mg21, IIGP, GTPI (79-81) and inducibly

expressed GTPase (IGTP), (77). Recent studies demonstrated that IGTP KO mice were resistant to *T. cruzi* (82). In contrast, LRG-47 KO mice were extremely susceptible to *T. cruzi* infection (83).

LRG-47 is a membrane-associated protein that in resting cells is localized to the Golgi by a C-terminal amphipathic helix (84). During phagocytosis, LRG-47 is recruited to the plasma membrane where it becomes associated with phagocytic cups (84) and in a recent study was demonstrated to play an important role in accelerating phagosome maturation and lysosome-phagosome fusion (85). *T. cruzi*-infected LRG-47 KO mice exhibited unimpaired proinflammatory responses, and displayed a rebound in parasite growth and all succumbed to the infection. During *T. cruzi* infection, LRG-47 deficiency induced severe splenic and thymic atrophy, anemia, and thrombocytopenia not observed in their wild type littermates. The studies *in vitro* demonstrate that IFN-γ-stimulated LRG-47 null macrophages display defective intracellular killing of amastigotes despite normal expression of TNFα and iNOS and that both iNOS and LRG-47 are required for optimum IFN-γ-dependent restriction of parasite growth (86). The results of these studies taken together indicate that IFN-γ signaling has pleiotropic effects on immune defense and homeostasis as well as hemopoietic function (87-89). In addition, they suggest that LRG-47 is a critical regulator of this shared pathway, and that the study of LRG-47 function may therefore shed important light on how IFN jointly regulates these diverse immunological and hematological parameters. Furthermore, in humans, the levels of IFN-γ were correlated with the severity of cardiac tissue damage (72). In addition, both parasite products and parasite-driven endogenous IFN-γ production are the main factors responsible for control of *T. cruzi* infection. It was recently demonstrated that IL-17, a cytokine produced by TH17 cells, plays critical roles in host defense against *T. cruzi*. Indeed, during this infection in mice, was proposed that IL-17 reduce IL-12 production and decrease the production of TNF-α and IFN-γ regulating parasite-induced myocarditis (90). IL-17A null mice show greater susceptibility to *T. cruzi* infection (91) and, *T. cruzi*-infected wild-type mice treated with anti-mouse IL-17 monoclonal antibody, developed a premature mortality, reduction in cardiac parasitism, enhanced production of TNF-α, IFN-γ, chemokine and chemokine receptors and increase inflammatory infiltrates compared with untreated-infected-wild type (90).

## 7. CHEMOKINES

Chemokines are inflammatory mediators with potent chemoattractant activity both *in vitro* and *in vivo*. Based on the position of the first two conserved cysteine residues, chemokines have been grouped into the CXC (α), CC (β), C (γ), and $CX_3C$ (δ) subfamilies, that act on G protein–coupled serpentine receptors on target cells (92). Besides playing an important role in leukocyte recruitment and migration, chemokines and their receptors affect T-cell proliferation (93), Th1/Th2 differentiation (94), and resistance to infection (95-97). CCR5 and CXC3 are important immunological markers of Th1 response, while CCR3 and CCR4 are associated with Th2 response (11). Because of these effects on leukocyte migration and activation, chemokines likely play a role in the pathogenesis of *T. cruzi* (95). *T. cruzi* infection or *T. cruzi*-derived molecules, including tGPI mucins and DNA (63, 64), stimulate the synthesis of cytokines and chemokines by different cell types. When macrophages important in the host-parasite relationship (98), are infected in culture or stimulated with tGPI mucins they produce and secrete several CC and CXC chemokines (99-101). Cardiac myocytes, an important target of infection, also synthesize large amounts of chemokines when infected with this parasite (97). Interestingly, several of the cytokines that are synthesized during *T. cruzi* infection, including IFN-γ, TNF-α, IL-1β, IL10 and TGF-β, are capable of inducing or regulating the production of chemokines by infected macrophages and cardiac myocytes (73, 97). For example, IFN-γ enhanced the expression of mRNA for CXCL10 and CCL5, which were elicited in response to infection of macrophages or by activation with tGPI mucins (73, 100). It is noteworthy that chemokines, especially CCL2, enhanced the ability of macrophages and cardiac myocytes to kill *T. cruzi* (97, 99). Indeed, chemokines synthesized during *T. cruzi* infection *in vitro* stimulate cardiac myocytes or macrophages in an autocrine manner so that they release NO and kill the parasite in an NO-dependent manner. Overall, these studies show that macrophages and cardiac myocytes can produce and respond to chemokines after infection with this parasite.

Chemokines facilitate the production of TNF-α by infected macrophages and act synergistically with IFN-γ to mediate parasite killing (99). One possibility resulting from these *in vitro* observations is that the chemokines produced after infection of target cells might play a role during the early innate protective response against the parasite and drive the early influx of leukocytes. Since chemokines are effective modulators of dendritic cells and lymphocyte trafficking *in vivo*, and can modulate the production of cytokines (96); these initial events might influence the ensuing adaptive and more-effective immune response against the invading parasite. When the adaptive immune response occurs, chemokine production may be modulated by IFN-γ. Chemokines may then influence T-helper-cell recruitment and local IFN-γ production. Together, these events will lead to the production of chemokines in finely regulated way, which is likely to result in defining of the inflammatory infiltrate and the adequacy of the immune response in the hearts of infected individuals (95).

### 7.1. Interaction between cytokines and chemokines

In recent years, the results of many studies are consistent with the notion that cytokines might modulate the production of chemokines *in vivo* during *T. cruzi* infection. In this regard, it was reported that mice infected with the Y strain of *T. cruzi* had a sustained production of IFN-γ-inducible chemokines during the acute and chronic phase of infection. In addition, there was a clear relationship between the migration of leukocyte to the heart and the expression of particular chemokines, including

those induced by IFN-γ, for example CXCL10 and CXCL9 (73, 100, 102). IFN-γ null mice infected with *T. cruzi* displayed a reduction in inflammatory infiltrates which correlated with the altered expression of chemokines in this tissue (73), confirming the involvement of IFN-γ in eliciting favorable environment for leukocyte migration and the myocarditis found in experimental Chagas disease.

In the setting of acute *T. cruzi* infection there was marked expression of chemokines, including CXCL1, CXCL9, CXCL10, CCL2, CCL3, CCL4 and CCL5 in the heart tissue, and that CXCL9, CXCL10, CCL3, CCL4 and CCL5 were co-detected at sites of $CD4^+$ and $CD8^+$ T-cell infiltration (73, 103). The chemokines CXCL9, CXCL10 and CCL5 were produced throughout the course of infection including the chronic phase even when tissue parasitism was scanty (100), and high levels were detected concomitant with an increase in the infiltration of activated $CD8^+$T cells. $CD8^+$T cells comprise large proportion of inflammatory infiltrate found in the heart of *T. cruzi*-infected mice (103) as well in the heart of chagasic patients. $CD8^+$T cells present in the heart tissue obtained from infected mice expressed the chemokine receptor CCR5 (103-105), which we believe contributes to myocardial damage in *T. cruzi*-infected individuals (10, 98, 106).

Chemokines are important in cell recruitment, including $CD8^+$ T cells. Therefore, blockade of chemokine receptors might represent a target for drug treatment of the inflammation. In this regard, Machado *et al* (103) and others (104, 105) demonstrated an important role for CCR5-acting chemokines in the control of leukocyte migration and parasite replication during the acute phase of experimental infection. *T. cruzi*–infected mice lacking CCR5, which recognizes CCL3, CCL4 and CCL5, results in an increase in both parasitemia and parasitic burden in the heart which correlates with a paucity of infiltration of T-cells (103, 104). Treatment with Met-RANTES, a selective CCR1 and CCR5 antagonist, was reported to improve survival, decrease heart inflammation and reduce cardiac deposition of an extracellular matrix component involved in fibrosis, without enhancement of parasitemia and myocardial parasitism in *T. cruzi*-infected mice (105). Late pharmacological blockade of CCR1 and CCR5 ameliorated the pathology and lethality and absence of CCR5 was associated with loss of the ability to mount an inflammatory response and an inability to control the infection. It is unclear whether the differences in these studies rely on the relevance of CCR1 for infection outcome or the degree of receptor coverage achieved by treatment with the antagonist in comparison to the absence in CCR5-deficient mice. Other studies employing neutralizing antibodies in mice demonstrated the critical importance of CXCL9 and CXCL10 chemokines during *T. cruzi* infection. Mice treated with neutralizing antibodies specific for CXCL9 and CXCL10 resulted in increased parasitemia, indicating that these chemokines also play an important role in generating a protective immune response (107). However, blocking these chemokines does not ultimately alter the severity of chronic cardiomyopathy as characterized by both the parasitic burden and chronic inflammation in the heart. The functional roles of particular chemokines and other chemokine receptors in host resistance to infection and in the pathogenesis of Chagas disease remain to be investigated in mouse model.

The *T. cruzi*-infected rats have also been used to model the human disease. The rat appears to clear the infection more effectively and develop a more silent disease, mimicking the indeterminate form of *T. cruzi* infection observed in humans (108, 109). Recently, employing the rat model, it was demonstrated that neutralization of CCL4, a CCR5 ligand, using a DNA vaccine encoding CCL4 was deleterious to the *T. cruzi*-infected host, evidenced by the increase in myocardial inflammation and higher collagen deposition (110). In contrast to the results found in *T. cruzi*-infected CCR5 deficient mouse, CCL4 in the rat model appeared not to be important in the control of acute infection. This suggests that CCL4 plays a role in preventing excessive inflammation and pathology rather than in controlling parasite replication. It also underscores the fact that there may be varying results with different animal species and parasite strains. It is likely that in the absence of CCL4, other chemokines, such as CCL3 and CCL5, would still be available for activating the CCR5 receptor, which is essential for leukocyte influx during *T. cruzi* infection (103). Indeed, Petray *et al* (111) demonstrated an increase in inflammatory lesions in mice treated with anti-CCL3, and there was also an increasing trend in the number of amastigote nests in the myocardium of treated mice compared with controls. Further studies evaluating the apparent discrepant role of CCL4 in rat versus CCR5 in mice are necessary to resolve these issues. A recent study has shown that combined blocked of CCL3 and CCL5 was associated with greater parasitemia in the rat (Roffe *et al.*, 2010). The loss of control of *T. cruzi* infection in CCR5 deficient mice or in mice treated with neutralization CCL3 antibody are consistent with the studies that recognize that chemokines exert antimicrobial effects against various types of pathogens, including parasites (112). It is noteworthy that chemokines, including CCL2, CCL3, CCL4 and CCL5 *in vitro* stimulate cardiac myocytes and macrophages to release NO, which result in the killing of *T. cruzi*. These observations taken together with the fact of these chemokines are produced *in vivo* during *T. cruzi* infection (100, 107) suggest that, by controlling *T. cruzi* replication, chemokines may play a role in resistance to this parasitic infection. Moreover, the CCL2 receptor CCR2 also is expressed in the heart after infection with *T. cruzi*. This suggests that in addition to CCR5, CCR2 plays a role in the chemokine-chemokine receptor signaling axis, thereby promoting host defense. A possibility that arises from these studies is that the chemokines produced after infection of target cells might play a role during the early innate protective response against this parasite and also drive the early influx of leukocytes.

These experimental studies demonstrate that several *T. cruzi* antigens and/or DNA interact with and participate in the modulation of chemokine production during infection. The functional roles of particular chemokines and chemokine receptors in host resistance to

infection and in the pathogenesis of Chagas disease remain largely unclear. The chemokine-cytokine interaction is currently being examined in several laboratories.

## 8. MODELING CHRONIC HUMAN DISEASE

Chagas disease is a lifelong infection in humans and is manifested many years, often decades, after the initial infection. Moreover, inflammation disproportional to parasite load characterizes chronic myocardial lesions in chagasic individuals. The chronicity of lesions, the low levels of tissue parasitism and cardiac dilatation and conduction defects are important aspects of the human disease that are difficult to consistently model in experimental animals. The human cardiomyopathy is characterized by inflammatory infiltrates that are composed of $CD4^+$ and $CD8^+$ T cells and macrophages, with $CD8^+$ T-cells being the predominant cell type (10, 98, 103, 106). Whereas experimental studies display infiltration of T cells and macrophages into the heart during acute infection is essential for controlling parasite replication (113, 114), the persistent inflammation in the heart in pathological changes characteristic of Chagas disease often resulting in cardiac remodeling. It has been reported that various inflammatory cytokines including IFN-γ, IL-4 and TNF-α are associated with inflammation and disease progression (61, 62, 73, 115, 116). These studies have been carried out in murine models of acute *T. cruzi* infection. To address the lack of association between acute experimental models of *T. cruzi* infection and the chronic human disease several studies have attempted to model the chronic infection in experimental animals. Recently, it was demonstrated that TNF blockade with Etanercept (human TNF- receptor 2) during the chronic phase of *T. cruzi* infection did not increase cardiac parasitism or levels of expression of cardiac hypertrophy-related genes (ANP and A20) in a Syrian hamster model of chronic chagasic cardiomyopathy. However, after 2 months of Etanercept treatment during chronic infection there was a worsening of the cardiomyopathy. These results suggest that absence of TNF signaling may be necessary to compensate for the function of the failing chagasic heart. Further studies are necessary to point the precise TNF-triggered pathway in the heart of these patients (117).

Several studies have examined the role of certain chemokines such as CCL3, CCL4 and CCL5, in models of chronic *T. cruzi* infection. Using magnetic resonance imaging and centerline analysis to track changes in heart contractility, we demonstrated that infected C57BL/6×129sv mice had segmental wall motion abnormalities in regions of the heart similar to those observed in affected humans. Dyskinesis was not observed in infected mice lacking the chemokine CCL3 (32). These studies highlight the usefulness of the method for monitoring regional left ventricular wall motion in mice and the important role of CCL3 in mediating cardiac remodeling after *T. cruzi* infection.

The role of chemokines in mediating cardiac remodeling in the setting of *T. cruzi* infection was underscored by studies evaluating Met-RANTES, a selective CCR1 and CCR5 antagonist in *T. cruzi* infection (105). Met-RANTES treatment did not interfere with parasitism but significantly decreased the numbers of CD4+ and CD8+ T cells, CCR5+, and interleukin-4+ cells invading the heart, paralleling the diminished deposition of fibronectin. Moreover, Met-RANTES treatment resulted in increased survival of infected animals, compared with saline treatment. These studies suggest that CC chemokine receptors may be a therapeutic target in this infection. A cautionary note, however, was raised by studies evaluating the role of CCL4 in *T. cruzi*-infected rats. In the latter animals, blockade of CCL4 function with a DNA vaccine was associated with an exacerbation of myocardial inflammation and fibrosis, although alterations in parasitemia and myocardial parasitism were not observed. These data suggest that CCL4/MIP-1β plays a role in preventing excessive inflammation and pathology rather than in controlling parasite replication (110). Further studies in chronic models of *T. cruzi* infection are clearly needed to understand in detail the role of chemokines in mediating cardiac remodeling and dysfunction.

A model of chronic infection was also used recently to evaluate the effects of the infection on myocardial β-adrenergic and muscarinic receptors and the relation of these receptors with cardiopulmonary dysfunction. The study demonstrated that chronic *T. cruzi* infection caused alterations in cardiac receptor density and fibrosis which could be associated with cardiac conduction abnormalities, diastolic dysfunction and lower exercise capacity (118). It was also demonstrated that treatment with benznidazole in the chronic phase of infection prevented the development of severe chronic cardiomyopathy, despite the lack of complete eradication of the parasite (119). These studies are consistent with the role of autonomic dysfunction in the pathogenesis of Chagas disease. Moreover, they suggest that even treatment in the chronic phase may be associated with amelioration of disease progression, even in the absence of complete eradication of the parasite. It is without doubt that these models of chronic infection will open important doors for the further understanding of the pathogenesis of Chagas disease.

### 8.1. Humoral immune response in chagasic patients

Anti-*T. cruzi* antibodies are found in the sera of infected individuals and form the basis of the serological tests that are used for the diagnosis of Chagas disease (120). Because the presence of parasite-specific antibodies has always been associated with infection, several studies were performed to determine the role of these molecules in the dynamics of the disease. Antibodies that react with galactose epitopes were found in the sera of chronic chagasic patients (121) and shown to mediate the lysis of trypomastigotes, hence the name 'lytic antibodies' (LA) first given to murine counterparts (122), suggesting a protective role for these antibodies. In addition to this putative protective role, levels of LA are higher in the sera of indeterminate patients (123) and shown to be a good predictive factor for parasitological cure of Chagas disease as treated patients that displayed negative hemocultures for over a period of ten years did not have any circulating LA, despite occasionally positive conventional serology (124).

Further studies are required to confirm the relevance of LA for the pathogenesis of Chagas disease and their usefulness as correlates of disease severity and response to treatment. Several studies have also shown that sera from chagasic patients are able to react with certain epitopes in the host (125, 126), including epitopes present on beta adrenergic receptors (127), suggesting a role for these molecules in mediating heart dysfunction (reviewed by (12, 128). Other studies have found that the levels of antibodies against adrenergic and cholinergic receptors correlate with parameters of autonomic function but not with the severity of Chagas heart disease (129). Recently, it has been demonstrated that these antibodies are also associated with other dilated cardiomyopathies (130, 131). Thus, despite their putative role in facilitating progression of heart disease, anti-adrenergic and cholinergic antibodies do not appear important in pathogenesis of chronic disease.

The involvement of antibodies as mediators of cellular reactivity in human Chagas disease was suggested by the demonstration that idiotypic anti-epimastigote antibodies (Id) isolated from the sera of chagasic patients were able to elicit cellular proliferation in vitro (8). Id isolated from cardiac patients as opposed to indeterminate chagasic patients displayed stronger stimulatory capacity and did not require processing (106). Interestingly, recent studies demonstrated that CD5+ B cells were particularly stimulated by these Ids (9). These studies suggest that Id could be involved in the immunopathology of Chagas disease, representing a constant self-stimulatory element able to elicit cellular responses.

**8.2. Cellular immune response in chagasic patients**

T-cells comprise the great majority of the cells in the inflammatory infiltrate in the myocardium of cardiac chagasic patients (10, 106) and an extensive number of studies have demonstrated their ability to react to parasite (6, 7) and host antigens (8, 9) related to Chagas disease. As discussed above T cells and T cell-derived products, especially IFN-γ, play a critical role in the ability of the murine host to deal with the infection. It is believed that T-cells are major regulators of the immune reactivity in the disease and is important in perpetuating the inflammatory response in the myocardium. A plethora of studies have attempted to characterize phenotypically and functionally T-cells from chagasic patients and to understand their role in the establishment of pathological or protective responses (reviewed by (132). Overall the available clinical studies show that chagasic individuals: (i) have T-cells that are activated (133), (ii) display a high frequency of circulating CD4+ and CD8+ T cells lacking the CD28 molecule on their surface (134). Activated T-cells, particularly the CD8+ T cell sub-population, were also found in the inflammatory infiltrate of cardiac lesions (10, 106) and (iii) possess a T cell repertoire with preferential expression of V-beta 5 by CD4+ T cells (135). However, *in situ* analysis failed to demonstrate a biased T-cell V-beta repertoire, whereas a V-alpha bias was observed (136). It is of note that none of the latter studies describe quantitative differences in terms of cellular reactivity when comparing chagasic patients with and without cardiomyopathy.

Several studies have evaluated qualitative phenotypic differences between the responding cell populations in indeterminate and cardiac chagasic patients. The major drawback of these studies is that they evaluate patients in whom the disease was already established. Thus, any conclusions obtained from such studies are limited because it is difficult to separate the effects of the immune system on the development of disease versus the effect of ongoing disease. For example, it has been shown that chagasic patients with heart disease have greater levels of TNF-α in the circulation that those with the indeterminate form (137 , 138). However, the levels of TNF-α associated with the degree of heart failure and brain natriuretic peptides (137). The expression of the chemokine receptors CCR5 and CXCR4 on leukocytes of patients with chronic chagasic cardiomyopathy correlates with the degree of cardiac function, such that those with reduced cardiac function display a reduction in the expression of either receptor (139). Therefore, it appears myocardial dysfunction rather than Chagas disease per se may account significantly for the changes in immunological parameters.

T regulatory cells (Treg) have recently emerged as a critical factor in the modulation of various immune responses. Presence of Treg in an inflammatory site is generally regard as contributing to pathogen persistence, because it maintains an attenuated immune response against infectious agents (140). The role of Treg in the infection by *T. cruzi is* controversial (141). There are studies demonstrating a higher frequency of circulating Tregs, with the majority of CD4+CD25+ cells expressing FoxP3, in patients with the indeterminate form in relation to the cardiac form of the disease (142). The increase frequency of Treg cells were also found during the acute phase of the experimental infection in mice (141). Sales el al (143) demonstrated that depletion of Treg cells, using anti-CD25 antibodies, does not confer resistance to infection in mice. In addition, the data demonstrate that during acute *T. cruzi* infection there is migration of Treg cells to the heart of infected mice and, the treatment with anti-CD25 resulted in increased mortality (144). The conflicting results may be due to experimental protocols, distinct strains of mice and/or differences in the parasite strains employed (144). In summary, Treg cells may play an important role during *T. cruzi* infection participating in the immunological balance during this infection. More studies are necessary to elucidate the precise roles of Treg cells during *T. cruzi* infection.

Despite these potential confounding variables, the available data from clinical studies have provided important clues as to the mechanisms associated with *T. cruzi*-infected patients. Overall, it appears that a lower production of IFN-γ by parasite antigen-stimulated lymphocytes is observed in patients with the indeterminate form of the disease (145, 146). Conversely, T-cells and monocytes from indeterminate chagasic patients produce more IL-10 than monocytes than those with cardiac disease (145, 147). Thus, in agreement with the studies in mice, a balance between IFN-γ and IL-10 may determine the fate of human infection. In the experimental acute infection, IFN-γ is important for the control of infection, an effect

modulated by IL-10. In humans, levels of IFN-γ were higher in cured former chagasic individuals than in those submitted to therapy but not cured, suggesting a role for IFN-γ in the control of the infection (148). In the chronic human disease, however, persistent production of IFN-γ and diminished regulation of by IL-10 appears to be associated with progression to fibrosis of the heart and remodeling. Other studies have suggested that CCR5 positive T-cells are the major producers of IFN-γ in *T. cruzi*-infected patients with cardiac disease (149). The latter findings are consistent with the ability of the blockade of CCR1/CCR5 receptors to ameliorate pathology in *T. cruzi*-infected mice (105).

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