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Putting Infection Dynamics at the Heart of Chagas Disease

Michael D. Lewis^{*,1,2} and John M. Kelly¹

¹Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, UK

²Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, 20892 MD, USA

Abstract

In chronic *Trypanosoma cruzi* infections, parasite burden is controlled by effective, but non-sterilising immune responses. Infected cells are difficult to detect because they are scarce and focally distributed in multiple sites. However, advances in detection technologies have established a link between parasite persistence and the pathogenesis of Chagas heart disease. Long-term persistence likely involves episodic reinvasion as well as continuous infection, to an extent that varies between tissues. The primary reservoir sites in humans are not definitively known, but analysis of murine models has identified the gastrointestinal tract. Here, we highlight that quantitative, spatial and temporal aspects of *T. cruzi* infection are central to a fuller understanding of the association between persistence, pathogenesis and immunity, and for optimising treatment.

Beyond Parasite Persistence

The majority of people infected with *Trypanosoma cruzi* survive the acute phase and progress to a chronic asymptomatic infection. Chagas cardiomyopathy is then estimated to develop at a rate of ~2% per year [1]. Megasyndromes of the gastrointestinal (GI) tract develop in a smaller proportion of cases, sometimes in combination with cardiac disease [2]. *T. cruzi* occurs predominantly in the form of intracellular amastigotes, which replicate in the cytosol of infected cells. These cells are scarce and focally distributed in a range of potential target tissues, making them difficult to detect. The apparent absence of parasites from heart tissue in many people affected by chronic chagasic cardiomyopathy [3, 4] contributed to Chagas disease being regarded as principally an autoimmune pathology. Over the past two decades the consensus has resoundingly changed. Few now support a purely autoimmune aetiology; strong evidence suggests that ongoing infection with *T. cruzi* is necessary to sustain the tissue damage characteristic of the disease. This idea was encapsulated within the parasite persistence hypothesis [5], which stated “the persistence of *Trypanosoma cruzi* at specific sites in the infected host results in chronic inflammatory reactivity”. The evidence base supporting the hypothesis is built on the detection of parasite-derived biomolecules (DNA,

*Correspondence: michael.lewis@lshtm.ac.uk (M.D. Lewis).

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antigen) in chagasic heart tissue [6, 7], the lack of autoimmune reactivity in the absence of concomitant infection [8], and the efficacy of early anti-parasitic chemotherapy [9]. The benefit of treatment is not apparent for those with late stage heart disease [10], most probably because cardiac damage is largely irreversible. The immunopathogenesis of chagasic cardiomyopathy has been comprehensively reviewed [11-14]; here we will focus on the causative agent.

Beyond the requirement that ongoing *T. cruzi* infection is necessary for the development of cardiomyopathy, little attention has been paid to the possible modes of parasite persistence within chronically infected individuals (Figure 1, Key Figure). Emerging evidence suggests that the intensity of infection can vary substantially between different tissues and over time, and that these dynamics may vary further between infected individuals. Multiple factors are likely to underpin this heterogeneity, including genetic diversity of the host and the infecting parasite strain(s) and environmental factors. The *T. cruzi* species comprises six major genetic subtypes (TcI–VI) that have suspected, though largely unproven, associations with many aspects of Chagas disease, including modes of transmission, tissue tropism, severity of outcomes and treatment efficacy (reviewed in [15]). It is likely that the cumulative effect of host-parasite interactions played out over many years, in multiple tissues, ultimately determines clinical outcomes. These include chronic cardiomyopathy of varying severity, digestive megasyndromes, acute meningoencephalitis, and most commonly, the long-term absence of recognized symptoms [2]. In this review, we discuss the spatial, temporal and quantitative dynamics of *T. cruzi* infections. We also explore why a deeper understanding of parasite persistence is necessary to explain the contribution of *T. cruzi* to Chagas disease pathogenesis and to inform the development of more effective treatment strategies.

Measuring Parasite Loads

The number of parasites and their anatomical location over the course of infection are key parameters. Most importantly, an accurate parasite load measurement, or proxy measure, helps to determine curative outcomes after therapeutic interventions (Box 1). There are a variety of techniques to detect and potentially quantify *T. cruzi* in tissue samples, each with benefits and drawbacks (Table 1). Detection sensitivity is a marginal concern in analyses of acute or reactivated infections when parasite loads are high, but becomes critical in the chronic phase, when parasite abundance is decreased by several orders of magnitude [16]. For example, extracellular, non-replicating trypomastigotes are routinely detectable in the blood for a period during acute infection and can be observed by light microscopy. However, these forms become rare after the host establishes control of the infection. Acute blood parasitaemia is not necessarily correlated with tissue parasite burdens or predictive of outcomes in the chronic phase. Isolation of live organisms can be achieved from fresh tissues using *ex vivo* parasite culturing techniques, and this generates enough material for genetic typing [17-19].

Clusters of *T. cruzi* amastigotes can be readily observed in tissue by histological staining. This allows visualisation of infected cells *in situ* and in the context of local pathology. In the chronic phase, such infected cells are typically scarce, which limits the scope of histology for parasite load estimation. Sensitivity can be improved in experimental studies through the

use of parasites expressing reporter genes such as *lacZ* β -galactosidase [20]. Immunohistochemistry has frequently been used to detect *T. cruzi* antigens in tissue sections. In some cases, this eases the difficulty of visualising intact trypomastigotes and lone amastigotes [21], but more often it reveals antigen in the absence of intact parasites [22]. The presence of these antigen deposits is presumed to indicate the remnants of recently destroyed organisms.

PCR-based methodology is a mainstay of parasite detection in both diagnostic and experimental settings. Two loci in particular, the nuclear 195 bp satellite and kinetoplast DNA minicircles, allow very high sensitivity because there are thousands of copies per genome. Quantitative PCR protocols allow estimates of parasite burdens and comparison across tissues; however, they may overestimate the number of viable parasites. The presence of organisms in blood can confound interpretations of tissue residence and repeated PCR assays can be necessary to ensure accurate results [6]. *T. cruzi* subtypes can be ascertained using PCR-based methods, but this generally requires analysis of lower copy-number genes [23]. The resulting reduction in sensitivity means that direct identification of parasite genotypes in chronic infections remains challenging. New detection tools continue to be developed. For example, nucleic acid aptamers to identify *T. cruzi* secreted antigens in serum may surpass the sensitivity of PCR [24], and lineage-specific serology has the potential to define historical exposure to different parasite subtypes [25].

Experimental studies with predictive animal models can generate insights into *T. cruzi* infection dynamics that are not possible in humans. The techniques mentioned above can be applied to animal samples at specific times post-infection, in combination with defined experimental parameters, e.g. genotypes, inocula or treatments. Broad tissue-type sampling can be easily conducted for groups of identically-treated animals, although in practice most animal studies concentrate on a limited set of tissues. Real-time imaging methods have also been developed that allow serial analysis of *T. cruzi* infections in individual mice [26-29]. These methods employ transgenic parasites expressing luciferases or fluorescent proteins, such that light signals emitted by parasites can be quantified and pinpointed to anatomical locations. The intensity of light emitted from tissue samples *ex vivo* can also be used as a proxy for parasite loads in specific organs. A key development was the introduction of a firefly luciferase gene engineered to emit longer wavelength light, which has enhanced tissue penetrating capacity [30, 31]. The use of *T. cruzi* expressing this red-shifted luciferase enabled highly sensitive imaging of chronically infected mice [16, 32-34]. Drawbacks include the high associated costs and, potentially, a reduced ability to detect parasites that are metabolically quiescent. Interpretation of imaging data also requires careful consideration of the light absorbing and scattering properties of different tissues. For example, peripheral infection foci in sites such as the skin will have a lower associated limit of detection than those in visceral organs.

In summary, there are now multiple sensitive approaches to detect and estimate numbers of *T. cruzi* in diverse tissue types. Emerging technologies could soon increase sensitivity further and generate new insights into the parasite's biology in vivo. For example, aptamers [35] or spliced leader trapping [36] can potentially enrich samples for rare parasites or parasite-derived mRNAs, while ribosomal profiling [37], fluorescence dilution [38] and

isotope labelling [39] approaches could help differentiate between dormant and active parasites. In the following sections we review current understanding of *T. cruzi* infection dynamics and explore why the utility of the parasite detection techniques described above depends on appropriate tissue sampling strategies.

Acute Infection and Tropisms

Upon primary infection from the insect vector, metacyclic trypomastigotes invade various cell types local to the site of inoculation and transform into amastigotes, which then undergo multiple rounds of mitotic replication. This proceeds for approximately one week and is followed by differentiation into bloodstream trypomastigotes and host cell rupture. The release of motile trypomastigotes into the haemolymphatics permits systemic dissemination and the acute phase of infection continues until the immune response brings parasite loads under control several weeks later. *T. cruzi* has the ability to invade and replicate inside almost any type of nucleated mammalian cell *in vitro*. The broad range of infected organs was noted in the earliest autopsy studies of fatal acute *T. cruzi* infection [40]. Analyses of acutely infected mice have shown that diverse parasite strains indeed infect a huge array of cell types in virtually any tissue [16, 20-22, 26, 33, 41-47]. However, the relative abundance of parasites in different cell or tissue types varies greatly. Sites reported to harbour the highest acute infection intensities include skeletal, smooth and cardiac muscle, mononuclear phagocytes and adipose tissues. Conversely, *T. cruzi* is comparatively rare where the blood/oxygen supply is poor, for example in osteocytes and chondrocytes [42], cartilage [41] and in immune-privileged sites including ovaries and testes [43]. It is worth making a distinction between cell and tissue types, which is possible with microscopic detection of parasites, but not with methods that analyse homogenized tissue samples or macro-scale imaging. For example, bioluminescence imaging readily identifies the GI tract and lung as sites of infection [16, 26], but not the infected cell types. Using histological analysis, Guarner *et al.* [21] also described lung infections and found that amastigotes were only localised to the muscular stratum of pulmonary blood vessels. Similarly, within the GI tract, amastigotes can be more easily found in the smooth myocytes or within the myenteric plexus, than in the submucosa [45].

Heterogeneity in site-specific infection intensities with different parasite strains resulted in the concept of tropism being applied to *T. cruzi* [45, 48, 49]. However, the presence or absence of *T. cruzi* in particular cells and tissues may also be influenced by factors other than innate parasite preferences. For example, the route of inoculation, the dose, and the occurrence of other infections could all act to determine which of the viable niches the parasite occupies *in vivo*. Notwithstanding these caveats, all *T. cruzi* strains appear to share the ability to replicate within muscle cells (myocytes), and “myotropic” is something of a default term. It has been suggested that the preference for muscle might be an adaptation to access myoglobin as a source of heme [50]. Myocytes may also be invaded preferentially due to their highly active plasma membrane repair pathway, which *T. cruzi* can hijack to facilitate invasion [51]. Variability has also been described between different muscles, for example, the Brazil strain (TcI) is associated with higher parasite burdens in skeletal than in cardiac muscle, for unknown reasons [52].

A subset of strains, e.g. Y (TcII), have been described as reticulotropic because, in addition to infecting muscle, they have a greater capacity to parasitize both resident and inflammatory mononuclear phagocytes compared to other strains, at least in acutely infected mice [45, 48, 53]. Targeting these cell types allows these strains to infect a wider variety of tissues and may be related to increased virulence.

In some circumstances, *T. cruzi* is able to cross the blood-brain barrier, potentially leading to fatal meningoencephalitis. A subset of TcI strains, ~20%, have been associated with this phenotype in mice [44, 54, 55]. Nevertheless, central nervous system (CNS) infections in humans are rare; they tend to follow immunosuppression [56] and can be caused by other lineages [57, 58]. The data available are not sufficient to conclude whether CNS involvement is a consequence of parasite-intrinsic virulence factors, a result of increased host susceptibility, or a combination.

Elusively Reclusive: *T. cruzi* in the Chronic Phase

The heart

Evidence for parasite tropisms is based almost exclusively on acute infections. The long-term dynamics of *T. cruzi* infection have remained vague because of the difficulty in detecting rare parasite foci during the chronic phase. This has limited progress in understanding factors that influence chronic parasite loads in the heart and their connection to pathogenesis. Histological studies typically identified *T. cruzi* amastigotes in fewer than 30% of chagasic human hearts [3, 4, 59-64]. Higher sensitivity molecular detection methods indicated the presence of *T. cruzi* DNA or antigen at frequencies of 50 – 95% [6, 61, 65-70]. The presence of *T. cruzi* or derived material frequently co-occurs with myocarditis [65, 67, 69, 70], but quantitative correlation has not been demonstrated. Importantly, inflammation is only one of several pathological processes that contributes to the development of chagasic heart disease. Limited data suggest that an association between infection dynamics and fibrosis or tissue re-modelling is absent [65, 71], and evidence is lacking for links to denervation and conduction or microvascular abnormalities. Comparative analyses of human cases tend to rest on the presence or absence of parasites in samples from small cohorts of patients who had died or required a heart transplant. Causation is therefore uncertain because parasite loads at these times may not accurately reflect the preceding asymptomatic and early symptomatic period.

Experimental studies have also generated valuable data. Non-human primates, dogs, rabbits, rats and guinea pigs are all useful to study chronic cardiac infection [72], but for practical and ethical reasons mice are by far the most commonly used models. Drawing firm conclusions from the existing literature is complicated by variations in experimental design. This includes widespread use of different strains of parasites and mice, inoculum sizes, routes of inoculation, end-points, methods of parasite detection and parameters for pathology. Only a few mouse models of chronic *T. cruzi* infection have been described that involve heart parasite burdens high enough for consistent detection using histology [73, 74]. Similar to some human data, Zhang and Tarleton [22] found that the presence of *T. cruzi* kDNA was qualitatively associated with co-localized inflammatory infiltrates, indicative of ongoing immune responses against parasites persisting within the hearts of chronically

infected mice. Estimates of cardiac parasite loads made using qPCR range from below the limit of detection [16, 33], 2 – 80 per 50 ng of host DNA [52, 75], 20 per mg of tissue [76] to 1 per 200 host cells [77]. However, direct comparisons of qPCR loads with pathology at the level of individual animals have not been reported.

The recent application of real-time bioluminescence imaging to quantify tissue-specific parasite burdens in multiple mouse models has generated unexpected insights into the dynamics of chronic infections. For the CL Brener strain (TcVI), cardiac-localised parasite bioluminescence was not detected in C57BL/6, and only ~10% of BALB/c or C3H mice, but frequencies of 40%, 50% and 88% respectively, were detected for a TcI strain (JRcl4) [16, 34]. Cardiac fibrosis was consistently observed, but there was no correlation with parasite loads in individual animals. The host-parasite genotype combinations used in these studies had differing frequencies of infection in multiple organs other than the heart (see below). Furthermore, models exhibiting the most broadly disseminated infections had significantly higher levels of cardiac fibrosis. This correlation, together with additional lines of evidence, implied that infection of the heart is likely to be sporadic and repetitive, rather than continuous, and to occur at a frequency determined by the overall systemic distribution of parasites [34] (Figure 2). Further work is required to validate this hypothesis and to determine whether it can help to explain the relationship between cardiac parasitism and pathogenesis.

Other Sites

In humans with established infections, *T. cruzi* is clearly not restricted to cardiac tissue. For example, approximately half of Chagas disease patients that receive heart transplants develop symptomatic reactivation of *T. cruzi* infection [78]. Transmission via infected blood transfusions and the practice of xenodiagnosis demonstrate that parasites can be present in the blood after the acute phase [2]. Parasitemia clearly fluctuates because PCR analysis is not consistently positive or negative in individuals over time [10]. *T. cruzi* can also cross the placenta leading to congenital transmission in ~5% of cases, with some geographic variability linked to *T. cruzi* genotype [79]. As outlined below, parasites have been detected in a wide range of other organs, but whether any of these sites serve as genuine long-term reservoirs, or simply become transiently infected is far from clear.

Several cases of transmission caused by transplantation of livers or kidneys from seropositive donors have been recorded, although the risk is lower than for hearts [60]. Parasites can be frequently detected in skeletal muscle in some chronic mouse infection models [20, 52, 75, 80, 81]. This site is poorly documented in humans – some physiological abnormalities have been described, but not the presence of parasites or inflammation [82]. The smooth muscles of blood vessels have also been identified as a site of chronic infection. For example, parasites were detected in the central vein of the adrenal gland in several small autopsy studies at frequencies of 5% [6], 30% [4] and 50% [83]. Progressive multi-organ vasculitis has been reported in mice infected with the high virulence Colombiana (TcI) strain [84].

Adipose tissue has also been suggested as a site of persistence. *T. cruzi* readily parasitizes adipocytes *in vitro* and lipid rich tissues can have high parasite loads in acutely infected

mice [16, 41, 77, 85]. Indeed, adipose tissue was a major site of *T. cruzi* persistence associated with posaconazole treatment failure in acutely infected mice [33], and panniculitis (inflammation of the fatty subcutaneous tissue) is a common symptom of reactivated Chagas disease [78]. The majority of C3H mice with long-term TcI infections have parasites in adipose tissue [34, 77], but this is less frequent for other genotype combinations [34]. A small study that analysed subcutaneous fat samples from ten seropositive patients found that three of them contained *T. cruzi* kDNA [86]. It is not known which cell type(s) within adipose tissue harbour *T. cruzi* in chronically infected subjects, nor whether infection is continuous or sporadic.

T. cruzi might parasitize the peripheral, enteric and central nervous systems in settings other than acute or reactivated infection, but direct evidence from humans is scarce. Amastigotes have been found in the sciatic nerve and lumbar spinal cord of C3H mice 8-10 months post-infection [20, 87]. Neuronal damage and loss within the affected organs are important features of both cardiac and digestive Chagas disease, but this is more likely to be collateral to active inflammation in adjacent tissue compartments [88].

Owing to the focal pleiotropism of *T. cruzi*, studies on persistence have frequently been associated with tissue sampling biases. The development of sensitive real-time imaging has largely overcome this issue, at least for mouse models. Recent work in our laboratory revealed that the GI tract, specifically the large intestine and stomach, is the predominant site of parasite persistence for multiple mouse-parasite genotype combinations [16, 34]. One reason for this may be a local trade-off between incentives for the host to limit parasite numbers and to avoid bacterial translocation, which can occur in an inflamed gut [89]. Particular features of the intestinal microenvironment that might contribute to *T. cruzi* persistence include macrophage populations that are refractory to activation, as well as high levels of IL-10 and abundant regulatory T cells, which dampen inflammatory responses [90, 91]. These data raise the possibility that ongoing GI infection continuously contributes to the development of digestive forms of Chagas disease. A lack of data on chronic GI infections in humans means this will remain an open question (Box 2). Nevertheless, in the context of drug development strategies, it appears prudent to consider the ability of compounds to reach effective concentrations in GI tissues as an important parameter.

These imaging studies also identified *T. cruzi* in a variety of other sites, including the heart, lung, skeletal muscle, skin and visceral fat. Unlike the GI tract, these infection foci were only ever detected in a subset of animals. Different combinations of parasite and mouse strains were associated with varying levels of disseminated infection foci outside the GI tract, but without any evidence of differential tissue tropism. For example, C57BL/6 mice infected with a TcVI strain rarely had dissemination outside the gut, whereas C3H mice infected with a TcI strain typically had multiple systemic parasite foci. Importantly, models with a higher degree of dissemination had significantly more severe cardiac fibrosis - a key marker of Chagas cardiomyopathy [34] (Figure 2). These findings highlight the importance of understanding both tissue-specific interactions between host and parasite, as well as the potential interconnectedness of infection between sites over time.

Re-Invasion as a Route to Parasite-Driven Cardiopathogenesis

Adaptive immune responses, particularly those mediated by CD8⁺ T cells, are critical to maintain a stable long-term host-parasite equilibrium [92]. Humans with reduced immune function (e.g. HIV co-infection or immunosuppressive treatment), typically have lowered ability to control *T. cruzi* and often experience pathology in atypical sites (CNS, skin, GI tract) [56-58, 93]. Antibody-mediated depletion of T cells leads to exacerbated heart parasite loads and myocarditis in mice [94], and chronically infected mice treated with the immunosuppressant cyclophosphamide experience rapid systemic expansion of parasite loads, which resembles the acute phase pattern of infection [34]. Therefore, the niches available for *T. cruzi* within the chronically infected host are primarily defined by host responses rather than parasite tropism.

The dynamic nature of chronic phase parasite distribution means it is important to distinguish between repeated short-term infection and long-term persistence within defined tissues, because alternative modes of infection can be expected to provoke different types of immune response. Data from animal imaging models has led us to propose a model of continual parasitism of the GI tract that is tolerated by the host, combined with repeated, sporadic reinvasions of the heart and other sites, which provoke effective host responses. There is good evidence that chronically infected mice can efficiently control systemic parasites. C57BL/6 mice generate plentiful *T. cruzi*-specific CD8⁺ T cells with a cytotoxic, non-exhausted phenotype [80]. The number of interferon- γ (IFN- γ) producing T cells increases after high-dose intravenous secondary infection, concomitant with rapid clearance of parasites from the re-infected spleen, lung and liver [19]. Similarly, neither homologous nor heterologous superinfections of chronically infected C3H mice had a lasting impact on muscle tissue burdens [52]. Immunohistochemical detection of *T. cruzi* in chronic phase hearts typically reveals debris-like antigen deposits, and not intact organisms [21]. In terms of pathogenesis, the reinvasion model (Figure 1, Key Figure) could explain the lack of consistent correlation between tissue fibrosis (a cumulative, largely irreversible pathology) and ‘snapshot in time’ measurements, such as inflammation and local parasite load [34]. It may also lead to a better understanding of why the majority of Chagas disease deaths are attributed to sudden arrhythmic events or embolism, rather than chronic heart failure [95].

The logical next questions concern the source and mechanism of active (re-)infection foci and the consequent inflammatory responses in particular sites. Addressing this question is likely to be difficult given the requirement to track the movement of individual parasites between distant sites *in vivo*. It is not known how trypomastigotes transit between tissues and the circulation. The trypomastigote surface glycoprotein gp85 does have high avidity for vascular endothelial cells [96] and vasculitis can be a feature of chronic infections [84]. Nevertheless, superinfection experiments indicate that the majority of parasites entering the blood of chronically infected hosts are expected to be rapidly opsonized and cleared, mainly in the liver by Kupffer cells [19]. This would be particularly true for parasites exiting the GI tract into the portal venous system. Trafficking via the lymphatics could allow *T. cruzi* to circumvent the liver, promoting both dissemination and transmission. Pertinently, parasites or infected cells exiting the GI tract by a lymphatic route would drain into the subclavian vein and then directly encounter the right atrium. Imaging shows that mesenteric tissue is a

relatively frequent site of infection in mice [34], but it remains unclear whether live parasites can evade the firewall function of the mesenteric lymph nodes. Besides reinvasion by free trypomastigotes, several studies provide circumstantial evidence that trafficking of parasitized myeloid cells into organs could be important. *T. cruzi* antigens often localize to interstitial dendritic cells in the heart [97]. Peripheral infection foci typically appear and disappear over the course of hours, consistent with trafficking of infected host cells [16]. Furthermore, in a fatal acute infection model, parasite burdens in the heart depended on the ability of *T. cruzi* to replicate specifically in myeloid cells expressing *Slamf1* [98], a surface receptor with a pro-migratory function [99, 100]. Lastly, it is important to acknowledge the possibility that reinvasion may occur alongside reactivation of (quasi)dormant parasite foci. Indeed, evidence for *in vivo* metabolic heterogeneity in other intracellular pathogens [38, 39] suggests this could be a vital, but largely unexplored aspect of *T. cruzi* biology.

Concluding Remarks

T. cruzi is a fascinatingly versatile microorganism. It parasitizes diverse cell types in multiple tissues, in hundreds of different mammal hosts, and is transmitted by dozens of triatomine vector species. This promiscuity makes *T. cruzi* infections challenging to study in both clinical and experimental settings (see Outstanding Questions). Nevertheless, increasingly sophisticated parasite detection technologies are leading to a better appreciation of the dynamic nature of chronic infections and how this intersects with Chagas disease pathogenesis. Host immune responses clearly enforce a dramatic restriction of the niches within which *T. cruzi* can persist. However, the mechanism(s) of long-term immune evasion within individual hosts remains largely unknown. Different modes of persistence may occur within and between organs, including continual low-level infection, dormancy-reactivation and episodic re-invasion. Distinguishing the contribution of these processes to long-term tissue-specific infection dynamics will require careful experimental investigation. An even greater challenge will be to analyse them in humans and define their contribution to the development of both cardiac and digestive Chagas disease.

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Box 1: Cured or not cured?

Identifying whether a patient or experimental animal has been cured of *T. cruzi* infection is a critical question, whether assessing the effectiveness of the front-line drugs (benznidazole or nifurtimox), or testing new chemotherapeutic agents and immunotherapies [72]. Detection of *T. cruzi* in humans is usually restricted to blood. Consequently, prediction of parasitological cure is normally based on the presence or absence of parasite-specific immunoglobulins or parasite DNA. Post-cure conversion to a seronegative status may take years to occur. Quantitative PCR can be sufficiently sensitive to detect a single parasite in 5 ml of blood [101]. However, the main utility of PCR is to confirm failure to cure, because even consistently negative results cannot prove that tissue parasites have been completely eliminated [2]. Ongoing investigation of a number of other biomarkers may expand the options available to monitor clinical interventions e.g. [24].

The increased sampling possibilities available for experimental animal models can potentially generate a greater degree of confidence in predicted cure rates [72]. Quantitative PCR is a powerful tool, but suffers from limitations arising from the focal and dynamic nature of chronic infections. Bioluminescence imaging models can overcome this problem, yet are subject to their own detection limits and will have a restricted ability to detect metabolically quiescent parasites. Dormant forms of *T. cruzi* are currently hypothetical and their role in disease progression would probably be minor. They could still be important for sustaining persistence though, and would be particularly concerning in the context of drug treatment. The use of immunosuppression in tests involving predictive animal models is therefore seen as an essential procedure because it leads to rapid expansion and dissemination of *T. cruzi* [33, 34, 75, 80, 94]. The most common protocol used in drug efficacy studies involves treatment of mice with cyclophosphamide, which is cytotoxic to many leukocyte populations. It is important to note that cyclophosphamide dosing regimens have never been systematically validated in the context of chronic *T. cruzi* infection. Nevertheless, consistently negative parasite detection tests conducted on multiple tissues, even after cyclophosphamide-induced immunosuppression, is currently the most convincing criterion of cure in murine models.

Box 2: Chagas enteropathy, a most neglected disease?

Digestive forms of Chagas disease are characterised by progressive dilation of sections of the digestive tract, usually but not exclusively the oesophagus or colon. Cases are most common in Bolivia, Chile, northern Argentina and southern Brazil; and despite fragmentary data, the overall incidence is estimated at 10-15% of infected people [2]. Understanding of the molecular and cellular basis of pathogenesis lags far behind the advances made for chagasic cardiomyopathy. Dilation is associated with loss of neurons, dysperistalsis and hypertrophy, inflammation and fibrosis of the smooth muscle layers [102, 103]. The most common symptoms include difficult or painful swallowing, abdominal pain, constipation and faecaloma. Treatment options are limited to palliative, dietary and surgical interventions [2].

Anti-parasitic chemotherapy has not been considered justifiable for seropositive individuals with digestive symptoms, but normal heart function [104]. This is primarily because clinical trials have not addressed the efficacy of treatment in the context of digestive outcomes. It is also influenced by a prevailing view that megasyndromes result from irreversible enteric denervation during the acute phase [102, 105], in which anti-parasitic inflammatory responses are thought to cause iNOS-dependent collateral damage to neurons [88]. Further age-related denervation is posited to gradually unmask the parasite-driven losses, leading to progressive organ dysfunction [105]. The finding that the colon and stomach are the primary reservoirs of *T. cruzi* infection in mice [16, 34] raises the possibility that local infection may in fact continue to influence the development of digestive forms of Chagas disease into the chronic phase. Indeed, histological analyses have identified persistence of parasites in GI samples in 20-50% of megaesophagus cases [106, 107] and, using PCR-based strategies, other authors have found *T. cruzi* DNA in 100% of such samples [108]. Long-term infection in the dog is considered a useful animal model of chagasic megasyndromes [109] and some features of nascent enteropathy can also be observed in experimentally infected mice [110]. These studies, amongst others, now form a framework for further experimental investigation, not only of the role of *T. cruzi* in digestive pathogenesis, but also of the ability of specific chemotherapy targeting the parasite to treat this type of Chagas disease.

Outstanding Questions

- Why is *T. cruzi* preferentially able to parasitize myocytes in chronic phase infections?
- What are the mechanisms of long-term immune evasion?
- Is there a metabolically quiescent or dormant form of *T. cruzi* that may have a role in long-term persistence and perhaps treatment failure?
- Which modes of persistence occur in humans and how closely do they match the mouse model e.g. does the GI tract serve as a 'safe haven' for parasites and is heart infection episodic or continuous?
- What is the source of parasites when reactivation of Chagas disease occurs under immunosuppression? For example, are cutaneous and CNS pathologies due to local expansion of underlying infection or to re-invasion from other sites?
- What is the mechanism that drives the development of digestive megasyndromes and is it dependent on chronic persistence of *T. cruzi* within the GI tract?
- What is the identity and phenotype of the cells in the GI tract that act as reservoirs of infection?
- Will elimination of parasites from the gut reservoir lead to a sterile cure of chronic infections?
- Is immunotherapy an option for inducing immune-mediated killing of parasites in the sites of persistence?

Trends Box

- Advances in the sensitivity and accuracy of molecular and imaging technologies are leading to a better understanding of quantitative, spatial and temporal variation in *Trypanosoma cruzi* infections.
- *T. cruzi* is pan-tropic in acute phase infections, with some strain-specific heterogeneity in parasite loads between cell and tissue types.
- The gastrointestinal tract serves as the main parasite reservoir in mice during chronic infections; there is currently insufficient evidence to define long-term reservoirs in humans.
- Targeting myeloid cells for infection may allow *T. cruzi* to evade adaptive immune responses, re-invade tissues and achieve transmission.
- A model of repeated reinvasion of the heart has the potential to better explain chagasic cardiac pathology than one of continual local persistence.

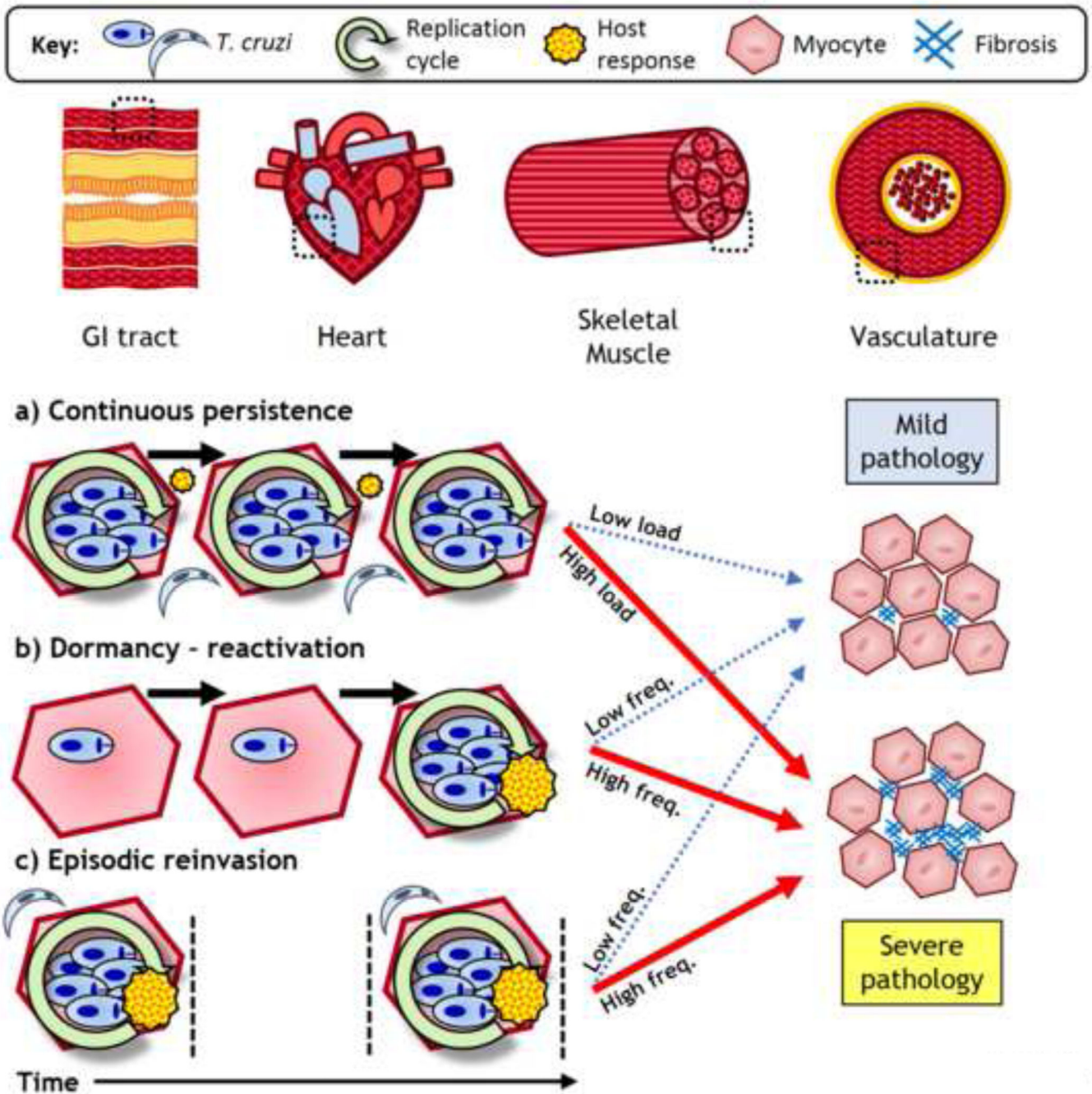


Figure 1. Key Figure. Modes of parasite persistence in long-term *Trypanosoma cruzi* infections
 In chronic infections *T. cruzi* predominantly parasitizes myocytes. These infected cells are typically scarce and focally distributed; they can be within cardiac, skeletal or smooth muscle tissues, such as those from the vasculature or the gastrointestinal (GI) tract. The heart is the most common site of pathology. Parasite persistence within an individual host may occur through different modes: a) Continuous persistence describes an ever-present, low abundance parasite load that is sustained as a locally contained equilibrium between intracellular parasite replication and host immune responses; b) Although they have not been

proven to exist, dormant forms of *T. cruzi* may reside within tissues and evade host immunity. As seen for other pathogens, reactivation into typical replication cycles could occur on an intermittent basis; c) Due to the ability of *T. cruzi* to invade multiple tissues and migrate between them, an organ may be subject to discrete episodes of infection by reinvasion. These three modes are not mutually exclusive and may overlap to different degrees at different times. Over time, the cumulative parasite load is likely to dictate the frequency and intensity of local inflammatory responses, which, depending on their quality, result in differing degrees of pathology. The figure is intentionally simplified and does not convey the molecular and cellular complexity of Chagas disease immunopathogenesis.

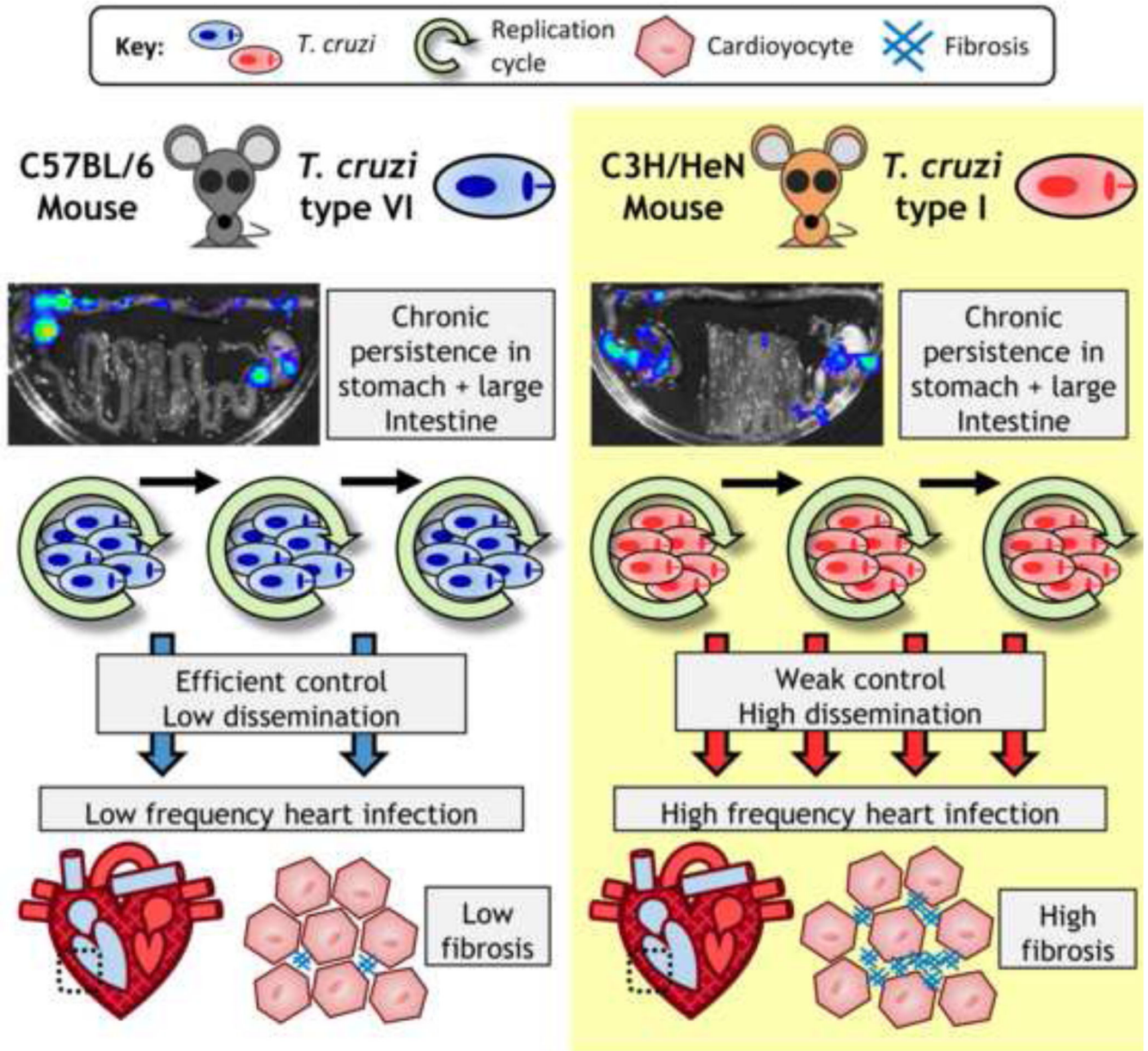


Figure 2. Model for a link between host-parasite genetics, infection dynamics and chagasic cardiac pathology

In experimental murine models of chronic *Trypanosoma cruzi* infection, varying host and parasite genotype combinations can generate different severities of cardiac pathology. Real-time *in vivo* imaging studies of tissue-specific infection dynamics [16, 34] have suggested that an important factor is the extent of parasite dissemination. Regions of the gastrointestinal (GI) tract serve as permanent reservoirs of infection, especially the proximal large intestine and stomach, regardless of the host-parasite genotype combination. The photos show ex vivo imaged GI tracts overlaid with pseudocolour heat-maps of bioluminescence intensity, which is used as a proxy for parasite numbers. Other tissue sites, including the heart, are actively infected more sporadically. Some models, such as C57BL/6

mice infected with a *T. cruzi* type VI strain (CLBR) have low parasite burdens outside the gut, heart infection is relatively infrequent and cardiac fibrosis is relatively mild. Other models, particularly C3H/HeN mice with chronic *T. cruzi* type I infections, have more broadly disseminated infections, frequent infection foci localized to the heart and more severe cardiac fibrosis.

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Table 1
Common methods for detection of *Trypanosoma cruzi* in mammalian hosts

Method	Target	Main Advantages	Main Drawbacks	Example Refs
Serology	Anti-parasite antibodies	Simple, low cost Diagnostic gold standard	Indirect, qualitative Long time to seronegativisation in cured subjects	[9, 10, 25, 58, 79, 97]
Fresh blood microscopy	Live trypomastigotes	Definitive Fast result	Mainly restricted to acute phase Blood poor proxy for tissue	[98]
Haemoculture	Live parasites	Low cost Definitive	Long time until result Low sensitivity	[18]
Xenodiagnosis	Live parasites	Low cost Definitive	Long time until result Requires triatomine colony	[111]
Histology	Intact fixed organisms	Parasites seen directly <i>in situ</i> Morphological and pathological context available	Low sensitivity Sampling bias ^a Snapshot data	[3, 4, 19, 20, 41, 59, 61, 73, 98]
Immunohistochemistry	Parasite antigen	More sensitive than routine histology Semi-quantitative Morphological and pathological context available	Careful optimisation of signal and background needed Sampling bias ^a Snapshot data	[21, 61, 66, 69, 97]
PCR	Parasite DNA	High sensitivity Lowest limit of detection Can be quantitative Some targets allow discrimination of <i>T. cruzi</i> subtypes	Sampling bias ^a Snapshot data Risk of contamination or non-specific amplification generating false positive results	[6, 16, 17, 19, 22, 23, 52, 57, 61, 66-68, 75-77, 84, 101]
Bioluminescence / Fluorescence Imaging	Transgene expression in live parasites	Allows serial evaluation Can be highly sensitive Minimal tissue sampling bias Only live parasites detected	Animal models only High cost Requires optimisation of transgene expression	[26-29, 32-34]

^aOwing to the variability in distribution of parasite foci over time and between tissue sites.