Infection by the Sylvio X10/4 clone of Trypanosoma cruzi: relevance of a low-virulence model of Chagas’ disease

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Abstract

The physiopathology of Chagas’ disease has been largely defined in murine infections with virulent strains which partially represent parasite diversity. This report reviews our studies with Sylvio X10/4 parasites, a Trypanosoma cruzi clone that induces no acute phase but in C3H/He mice leads to chronic myocarditis resembling the human disease.

Keywords: Chagas’ disease; Trypanosoma cruzi; Sylvio X10/4 parasites; Parasitaemia; Heart pathology; Immune response; Parasite evasion

1. Chagas’ disease

Chagas’ disease caused by Trypanosoma cruzi represents a serious health problem in Latin America, with around nine million people currently infected and an estimated 120 million individuals at risk ([1] and TDR Report of the Scientific Working Group on Chagas Disease, Buenos Aires, Argentina, April 17–20, 2005). The invasion of the human host frequently occurs by entry through damaged skin or intact mucosa of metacyclic trypomastigotes released with faeces/urine of infected hematophagous triatomides after their blood meal. Moreover, infection can also occur by congenital, transfusional and digestive routes. The parasite can be found in vertebrate hosts as intracellular replicating amastigotes, mainly within macrophages, striated muscle and myocardial cells, and as extracellular trypomastigotes that freely circulate in the blood and tissues.

The infection has a self-limited acute phase, which develops unnoticed in many individuals. While a small percentage succumbs to the acute phase [2], in most cases the parasite is controlled. Nevertheless, this constitutes non-sterile control in which the innate and adaptive effector mechanisms fail to totally eradicate the parasite, with the patient remaining infected for life. Years after the infection, a significant proportion (~30%) of the infected human population goes on to develop the severe cardiac or digestive manifestations of the chronic disease: cardiomyopathy that may lead to congestive heart failure, arrhythmias and patient death, or oesophageal or colonic digestive megasyndromes. These are irreversible pathological changes that occur in spite of parasite scarcity.

2. Sylvio X10/4 parasites

Due to the difficulties in working with human beings, much of our understanding on T. cruzi infection has been drawn from experimental animal data. Decades of investigation using animal models have provided us with a comprehensive view of
the immune elements involved in *T. cruzi* control [3,4] and in the development of the pathology [5,6]. One of the main conclusions drawn from these studies is that both host and parasite genetic backgrounds influence disease development [7–9].

In this review, we summarize our data on the overall evolution of the infection, parasitism and development of heart pathology in mice infected with Sylvio X10/4 parasites. This constitutes a low-virulence clone belonging to the type I phylogenetic group of *T. cruzi*, that was originally isolated from a triatomide bug used for xenodiagnosis in a human chagasic patient from Paraíba state, Brazil [10,11]. Mouse infection with this parasite was chosen as an experimental model because it does not cause patent acute disease but induces chronic cardiac lesions incorporating several pathological features of human disease [9,11,12]. The present review outlines the conclusions drawn from data on Sylvio X10/4 infection and speculates on how they fit into the general map of Chagas’ physiopathology.

3. Parasitaemia in Sylvio X10/4-infected mice

In contrast to virulent *T. cruzi* strains, circulating trypanomastigotes are rarely visualized by microscopy examination of blood from Sylvio X10/4-infected immunocompetent mice, although they are detectable by amplification methods [9]. This subpatent parasitaemia partially results from the susceptibility of Sylvio X10/4 parasites to IFN-γ-induced effector mechanisms, inasmuch as in IFN-γ-KO (or IFN-γR-KO) mice, parasites can be easily found in blood smears 2–4 weeks after infection (Fig. 1A and [13]). Interestingly, in IFN-γ-KO mice, parasitaemia shows an oscillatory pattern that could be the result of the discontinuous activation of innate immunity. Mice lacking IL-12p40, CD8, CD4, iNOS (inducible nitric oxide synthase) and CD28 molecules are more resistant than IFN-γ-KO mice to infection by Sylvio X10/4 parasites as they rarely display patent parasitaemias.

As Sylvio X10/4 parasites are routinely maintained in cell culture, it could be argued that subpatent parasitaemias of infected immunocompetent mice result from the low virulence of *in vitro* propagated parasites. This is not the case, however, since identical results are obtained when those mice are infected with blood trypanomastigotes isolated from IFN-γ-KO mice (Fig. 1B). Moreover, when these blood trypanomastigotes are injected intravenously in C57BL/6 mice, they disappear from the blood within minutes and are no longer observed (Fig. 1C). The basis for the paucity of circulating trypanomastigotes in this infection model remains unknown. There is evidence that parasites inducing subpatent parasitaemias share a closely related genetic background. This is suggested by the observation that amastigote surface protein 2 (ASP2) of Sylvio X10/4 parasites shows limited identity with that of several virulent Tc1, Tc2 and hybrid *T. cruzi* strains, yet shows a high degree of identity to ASP2 of G strain parasites, another Tc1 stock that also induces subpatent parasitaemias in immunocompetent mice [14,15].

4. Immune response to *T. cruzi*: peculiarities of the Sylvio X10/4 infection model

Control of virulent *T. cruzi* parasites has been shown to result from the combined effect of diverse arms of the innate
and adaptive immune responses. TLRs are extremely important for parasite recognition, where very high susceptibility is observed in animals deficient in both the TLR-adaptor MyD88 and TRIF molecules [16]. CD4+ and CD8+ T cells greatly contribute to parasite control [17,18]. The anti-\textit{T. cruzi} activities of CD4+ T cells include B cell help and activation of macrophage trypanocidal activity, two effects fully optimized after Th1 differentiation. CD8+ T cells are thought to operate through cytotoxicity of infected tissue cells and production of cytokines and chemokines. B cells are also important [19] where parasite-specific antibodies act through opsonization and neutralization, and complement-dependent lysis of trypanastigotes is of lesser importance [20]. Among cytokines, IFN-\gamma, produced by NK and T cells [21,22], is a key molecule for \textit{T. cruzi} control [23]. The anti-parasitic activity of IFN-\gamma results from a combination of effects, which includes production of nitric oxide (NO) [24] and other macrophage effector molecules, MHC class II induction, Th1 cell polarization, switch to IgG2a and chemokine production. Moreover, IFN\gamma induces the expression of LRG-47, a GTPase involved in accelerating phagosome maturation and lysosome—phagosome fusion, whose deficiency results in increased susceptibility to \textit{T. cruzi} [25]. The trypanocidal activity of IFN-\gamma-activated macrophages seems largely due to iNOS induction for NO production [24]. Nonetheless, experiments in iNOS-KO mice have revealed contradictory results [26—28] as these mice displayed higher or similar susceptibility to \textit{T. cruzi} to wild-type (WT) mice. Besides TCR-mediated signaling, differentiation of \textit{T. cruzi}-specific T cell clones towards a Th1 pattern seems to depend on provision of IL-12 and co-stimulatory signals, such as those mediated through the CD28 molecule [29].

The low virulence of Sylvio X10/4 parasites allows the relative importance of several of these immune elements to be analyzed over a long period of time [13]. Similarly to the observed in virulent \textit{T. cruzi} strains, IFN-\gamma is crucial for Sylvio X10/4 parasite control, as IFN-\gamma-KO mice die up to a month after infection. Sylvio X10/4-infected IL-12p40-KO mice also die, albeit at a later time point (40—60 days post-infection). On the other hand, 20—30% of CD4-KO and CD8-KO mice and most CD28-KO mice manage to control the infection by Sylvio X10/4 parasites.

As in infections by virulent \textit{T. cruzi} strains [30], parasite-specific IgG response of Sylvio X10/4-infected immunocompetent mice is dominated by IgG2a, an expected isotype pattern considering the Th1 profile of the anti-\textit{T. cruzi} response. Accordingly, the IgG response of Sylvio X10/4-infected IFN-\gamma-KO mice is restricted to IgG1. Since IgG2a is the antibody class with higher opsonizing activity, its absence could be speculated to contribute to the susceptibility of IFN-\gamma-KO mice. Nevertheless, despite producing no specific IgG antibodies [13], most Sylvio X10/4-infected CD28-KO mice survive the infection. This surprising result indicates that IgG is not crucial for Sylvio X10/4 parasite control, provided that other elements such as IFN-\gamma and CD8+ cells compensate the antibody deficit. The fact that IgG is not essential for Sylvio X10/4 control, contrary to the observed in most \textit{T. cruzi} strains [19], is probably due to the low level parasitaemia observed in this case. Interestingly, B cell-deficient mice have been shown to display long-term survival after infection by a low number of \textit{T. cruzi} parasites from the virulent Brazil strain [31].

Another deficiency that could contribute to the susceptibility of IFN-\gamma-KO mice is a failure to activate iNOS for NO production. Infection of iNOS-KO mice by Sylvio X10/4 parasites reveals a gender effect, with females presenting subpatent parasitaemias, mild heart pathology and 100% survival rates, while the males remain susceptible, 60% dying during the first month of infection with low, but detectable, parasitaemias [13]. The phenotypes exhibited by Sylvio X10/4-infected iNOS-KO mice suggest that NO is indispensable in conditions of low resistance, such as those exhibited by male mice during the early infection. Yet, in females, or in males after full development of the specific antibody and T cell responses [27], NO becomes superfluous.

In summary, our studies in immunodeficient mice allow effector elements participating in the control of Sylvio X10/4 parasites to be ranked. We confirm the central role of IFN-\gamma for \textit{T. cruzi} control [23], where the partial susceptibility of CD4-KO and CD8-KO mice could be largely related to the fact that T cells are important sources of this cytokine. Our observation that a fraction of CD8-KO mice survive Sylvio X10/4 infection agrees with data showing that only a proportion of MHC class I-deficient mice die after infection by low virulence M/80 and M/78 clones of \textit{T. cruzi} [32]. IL-12 and/or IL-23 are also necessary for survival of Sylvio X10/4-infected mice, an unexpected finding considering that IL-12p40-KO mice produce IFN-\gamma and anti-\textit{T. cruzi} IgG2a antibodies [13]. The reason behind the susceptibility of IL-12p40-KO mice appears to be related to the fact that IL-12 and/or IL-23 are crucial for the elimination of Sylvio X10/4 parasites in the central nervous system, as we observed that these mice present lesions at the spinal cord and manifest a neurologic syndrome of progressive paralysis that culminates in the animals’ death [13]. Finally, concluding the ranking of the immune elements analyzed, neither iNOS, IgG, or CD28-mediated co-stimulation seem to be essential but rather, elements that can be compensated in \textit{T. cruzi} control.

5. Similarities between chronic heart pathology in chagasic patients and Sylvio X10/4-infected C3H/He mice

Considerable efforts have been dedicated towards finding experimental models of chronic myocardopathy in Chagas’ disease. Infection of C3H/He (LPS-responsive C3H/HePas or C3H/HeN) mice by Sylvio X10/4 parasites may represent one such model, inasmuch as a large proportion of these mice develop intense myocarditis during the chronic phase, a process not observed in C57BL/6, A/J, BALB/c and DBA mice [9]. Chronic heart pathology in Sylvio X10/4-infected C3H/He mice incorporates many features of the human disease, with mononuclear cell infiltrates in the myocardium (Fig. 2A), endocardium and pericardium, occasional amastigote nests (Fig. 2B) and fibrosis (Fig. 2D), with infiltrates showing CD8+ T cell predominance [33]. IFN-\gamma seems to be
an essential mediator of Sylvio X10/4 control at the heart. Thus, not only did we find a strong upregulation of mRNAs for IFN-\(\gamma\) and IFN-\(\gamma\)-induced chemokines ITAC, MIG and IP-10 (Table 1) in the hearts of chronic C3H/He mice, but cardiac parasitism was notably increased in Sylvio X10/4-infected IFN-\(\gamma\)-KO mice (Fig. 2C). Meanwhile, leucocytes in the heart of Sylvio X10/4-infected chronic C3H/He mice have been shown to produce both pro-inflammatory and anti-inflammatory cytokines[34], suggesting that the effector activity of heart-infiltrating cells is tightly regulated.

The physiopathology of chronic heart lesions in chagasic patients remains controversial, the main hypotheses being autoimmunity[5,35,36] and immune reactivity towards locally persistent parasites[37–39]. While the main evidence supporting the autoimmune hypothesis is parasite scarcity at the affected heart, lesions are thought to occur by T cell reactivity against myosin and other heart proteins[36], as well as by humoral reactivity to beta-1-adrenergic and M2 cholinergic receptors that lead to autonomic system imbalance[40]. However, a body of evidence suggests, that, independently of any contribution of an autoimmune component, pathology results from persistence of \(T. cruzi\) parasites in the heart, where they evoke a chronic inflammatory process with fibrosis and myocardial cell loss. In this respect, significant positive correlations have been established between the occurrence of myocarditis and heart parasitism measured by immunohistochemistry[39], PCR[37,38] or hemoculture[9]. Incidentally, the absence of inflammatory infiltrates in neonatal hearts transplanted to an ear pocket in Sylvio X10/4-infected chronic C3H/He mice is considered a solid proof of the parasite persistence hypothesis over the autoimmune theory[41].

Further, in a histopathological study involving Sylvio X10/4-infected chronic mice, we observed amastigote nests in the hearts of 9 out of 26 C3H/He mice, but in none (0 out of 24) of the A/J mice that did not develop chronic cardiomyopathy (Fig. 3A and [9]). Differences in heart parasitism were confirmed by culture of cardiac tissue samples in liver-infusion-tryptose (LIT) medium, an amplification method that revealed heart parasitism in 38.5% and 0% of chronic C3H/He mice.

Table 1
Chemokine transcription in heart of Sylvio X10/4-infected chronic C3H/He mice.

<table>
<thead>
<tr>
<th>mRNAsa</th>
<th>Control</th>
<th>Chronic</th>
<th>Ratiob</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP1(a)/CCL3</td>
<td>0.1</td>
<td>1.4</td>
<td>10.8</td>
</tr>
<tr>
<td>MIP1(b)/CCL4</td>
<td>4.3</td>
<td>48.8</td>
<td>11.3</td>
</tr>
<tr>
<td>RANTES/CCL5</td>
<td>3.0</td>
<td>294.6</td>
<td>98.0</td>
</tr>
<tr>
<td>C10/CCL6</td>
<td>43.4</td>
<td>72.6</td>
<td>1.7</td>
</tr>
<tr>
<td>JE/CCL2</td>
<td>35.0</td>
<td>84.3</td>
<td>2.4</td>
</tr>
<tr>
<td>6cKINE/CCL21</td>
<td>20.3</td>
<td>4.4</td>
<td>0.2</td>
</tr>
<tr>
<td>ITAC/CXCL11</td>
<td>0.1</td>
<td>29.3</td>
<td>407.7</td>
</tr>
<tr>
<td>MIG/CXCL9</td>
<td>1.8</td>
<td>726.4</td>
<td>395.9</td>
</tr>
<tr>
<td>IP-10/CXCL10</td>
<td>2.4</td>
<td>89.2</td>
<td>37.0</td>
</tr>
<tr>
<td>Fract/CXCL1</td>
<td>8.2</td>
<td>16.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a The relative expression of each chemokine was calculated versus the relative abundance of cDNA encoding for ubiquitin.

b Ratios between chronic infected mice and control mice.
and A/J mice, respectively (Fig. 3B and [9]). Meanwhile, 28.6% and 38.5% of blood cultures were positive in C3H/He and A/J chronic mice (Fig. 3B), providing an indication that systemic parasitism is similar in the two mouse strains. Taken together, these results reveal a correlation between the occurrence of infiltrates and the presence of parasites in the cardiac tissue, supporting the hypothesis that, at least for this *T. cruzi* strain, local parasite persistence is the primary cause of pathology [37].

In addition, as blood parasitism is similar in chronic C3H/He and A/J mice, our data on heart parasitism suggest these strains differ in terms of local tissue factors that influence parasite invasion/colonization of heart cells, or in the efficacy of the natural or adaptive immune effector mechanisms involved in the *in situ* destruction of parasites. Examples of mechanisms that could differ in C3H/He and A/J mice are NO production by myocytes, expression of class I MHC molecules, presentation of parasite peptides by myocytes and/or endothelial cells, and local production of chemokines or other molecules involved in leukocyte recruitment [42,43].

Another important feature for heart lesions in Sylvio X10/4-infected C3H/He mice is their late development. Thus, in a kinetic study of the development of heart pathology in this host–parasite combination, we observed discrete inflammatory infiltrates up to day 110 post-infection, which dramatically increased at days 160 and 225 (Fig. 3C). The development of heart pathology in these mice correlates with increased positivity of LIT cultures from the blood and heart tissue. This slow and progressive increase in parasite load contrasts with its evolution in infections by high virulence *T. cruzi* strains, where there is an early increase in parasitaemia and tissue parasitism that progressively declines at the end of the acute phase as a result of the activation of the immune system [30].

6. Failure of the immune system to completely eradicate *T. cruzi* parasites

One of the main unresolved issues of *T. cruzi* infection is the immune system’s inability to totally eradicate the parasite. It is intriguing why, in spite of the high levels of immunity reached during infection, the organism fails to attain sterility. A particular situation that illustrates this failure is depicted in Fig. 2B, which shows an amastigote nest within the myocardial fibers of a C3H/He mouse infected by Sylvio X10/4 parasites for nearly a year, that is, of an animal with a high level of anti-*T. cruzi* immune effector activity. A notable feature in this picture is the size of the nest and most importantly, the absence of cellular infiltrates surrounding it. This suggests deficiency in signaling for leukocyte recruitment, a condition that allows Sylvio X10/4 parasites to temporarily evade the immune system. Therefore, although there is no clear understanding of the mechanisms that lead to elimination of tissue parasites, based on the simple observation of the presence of parasite nests undetected by the host immune system we suggest that the failure to attain sterile immunity may to a large extent result from an intrinsic defect of the infected cell in sensing the intracellular parasite. Our observation is not new; in fact, undetected parasite nests were observed by G. Vianna, as early as 1911 [44], one year after the description of Chagas’ disease. But how are the infiltrating cells recruited, and more specifically, the effector CD8⁺ cells that recognize MHC I-peptide complexes in infected cells? For recruitment to occur, the infected heart cell must have the capacity to signal its infected condition through secretion of chemotactic molecules. *In vitro* work has shown that *T. cruzi*-infected cardiomyocytes transcribe the chemokine genes for MCP-1, RANTES, KC/GRO, MIP-2, MIG and IP-10, together with those for the cytokines TNF-α and IL-1β [43,45]. But is this same process occurring during chronic infection? The picture in Fig. 2B indicates otherwise for Sylvio X10/4-infected C3H/He mice. In sum, although we do not rule out the occurrence of intracellular detection, we know that in certain host–parasite combinations this is inefficient, because some infected cells are ostensibly ignored by infiltrating leukocytes. The extent to which the Sylvio X10/4-C3H/He mice model reflects an exception to the rule, or a frequent situation, is a matter for future research to elucidate.

In other *T. cruzi* models, the intracellular parasite is known to interfere with cardiomyocyte apoptosis, an effect that occurs at different intensities for different stocks [46]. This is thought to occur by parasite-driven activation of NFkB and PI3K/Akt and MEK1/ERK1/2 pathways in the host cell, which leads to...
increased expression of anti-apoptotic Bcl-2 molecules and arginase [47]. In partial conflict to this, Hall et al. [48] showed that Sylvio X10/4 parasites induce NFκB activation in epithelial and endothelial cells, but not in rat skeletal or cardiac muscle cell lines, thus, inversely correlating cell susceptibility to infection and NFκB induction. Incidentally, the authors discussed that, besides granting enhanced susceptibility to infection, failure of the myocyte to respond to trypomastigotes could limit production of immune mediators by the infected cell. Therefore, the possibility that in certain T. cruzi-host combinations the intracellular parasite interferes with the production of signaling molecules with pro-inflammatory and/or chemotactic properties by the infected cell should be considered as an explanation for the immune system’s failure to detect tissue nests.

Nevertheless, a deficit in intracellular parasite detection, although important, is insufficient to guarantee T. cruzi evasion in the chronic host. This is so because sooner or later, an undetected amastigote nest will spontaneously disrupt releasing extracellular parasites that, upon detection by antibodies, cause complement and resident macrophages/dendritic cells to generate mediators for leukocyte recruitment. Thus, for parasite perpetuation, a deficit in extracellular T. cruzi destruction must also be occurring, guaranteeing that at least a few released parasites re-invade neighboring or distal cells. The outcome of extracellular trypomastigotes recently released into tissue of a chronically infected host resembles a hide-and-seek game where the rapidity of leukocyte recruitment and the efficiency of phagocytosis and destruction of opsonized parasites are key elements in preventing parasite colonization of neighboring cells, a location where a fraction of the amastigote nests could remain temporarily hidden.

The existence of this local hide-and-seek game does not conflict with the participation of other evasion mechanisms. For many years, it has been known that trypomastigotes escape lysis from the complement system [49] and that internalized parasites of various T. cruzi strains escape the phagocytic vacuole of unprimed resident macrophages [50]. More recently, a new escape mechanism has been proposed for intracellular T. cruzi infection holding that the parasite is able to survive inside myocardial or striated muscle cells, not because of lack of leukocyte recruitment, but due to the fact that following migration to the tissues, CD8+ cells lose their cytotoxic and IFN-γ-producing capacities [51].

7. Development of the indeterminate and cardiac forms of Chagas’ disease

A closely related subject is the development of heart pathology in only 30% of chronically infected individuals. If we assume that chronic heart pathology mainly results from immune reactivity towards locally persisting parasites, we may consider that individuals with chronic heart lesions (mice or men) are those unable to mediate complete elimination of T. cruzi parasites in the heart. Conversely, individuals in the indeterminate phase who show no chronic heart lesions are either those that never had T. cruzi in their heart or those that have completely eliminated the parasite from this organ, even though the parasite might persist in other locations such as skeletal or smooth muscles. Thus, without ignoring the complexity of this issue, we can draw a parallel between the human casuistic and the chronic infection by Sylvio X10/4 parasites, and extrapolate that C3H/He mice on one hand and AJ mice on the other, are murine equivalents of human patients in the chronic and indeterminate phases of Chagas’ disease, respectively.

The two main conclusions taken from our data with Sylvio X10/4 parasites are, first, that chronic heart pathology seems to result from immune reactivity towards locally persisting parasites and, second, that a proportion of infected heart cells may remain invisible to the immune system. Based on these premises we can analyze the argument commonly posed in support of the autoimmune origin of Chagas’ pathology, namely, the paucity of T. cruzi parasites in the middle of inflammatory infiltrates. According to our view, parasite nests will successively develop at different points of the heart, in a continuous cycle of nest rupture, destruction of most released trypomastigotes by recruited effector leukocytes and successful re-invasion of neighboring cells by surviving trypomastigotes (Fig. 4). Secondary to the presence of extracellular T. cruzi antigen, the inflammatory foci will develop in an itinerant fashion at the sites of nest rupture. Therefore, assuming that leukocyte infiltration mostly represents the reaction to ruptured nests, rather than the coexistence of amastigotes and infiltrates in the same space, we should expect most inflammatory infiltrates to be devoid of viable nests, where the location of the infiltrates reflects the point occupied hours, days or even weeks earlier, by an infected cell. In this respect, the observation of diffuse staining for T. cruzi antigen in inflammatory infiltrates at the heart of Sylvio X10/4-infected chronic C3H/He mice [37] supports our interpretation.

Finally, it is important to mention that for a given T. cruzi stock, two chronic patients (or two mouse strains) displaying incomplete parasite control in the heart and thus with similar local parasite burdens, can differ in terms of pathology. Theoretically, where two chronic hosts are unable to completely eliminate the parasite in the heart, the host inducing the greatest local inflammatory response will pay a higher price by causing more damage to infected tissue [52]. In these individuals, the strength of the local immune response would clearly represent a detrimental factor in the induction of pathology, thus explaining the reported associations between high levels of cardiac dysfunction and genotypes of high reactivity [53].

8. Concluding remarks

Two of the main obstacles that hinder our understanding of the physiopathology of Chagas’ disease are the heterogeneity of T. cruzi parasites and the fact that many of the human cases are diagnosed years after the onset of the infection. These limitations prevent us from knowing the real impact the infection has during the first weeks. Do all infected individuals manifest a flourished acute phase with patent parasitaemias, or is the acute phase restricted to individuals infected by high virulence parasites? Is there any relationship between the
intensity of the early infection and the development of chronic pathology? Due to our need of understanding the immunology of Chagas’ disease, experimental studies have used infection by *T. cruzi* stocks of high virulence, which have proved extremely valuable for determining the immunological mechanisms involved in parasite control. Yet, we must not withdraw our attention from low virulence infection models given they could represent a significant proportion of individuals with Chagas’ disease.

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