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Chagas' disease: an update on immune mechanisms and therapeutic strategies

Silvia Beatriz Boscardin [a], Ana Claudia Troccoli Torrecilhas [a, b], Romina Manarin [c], Silvia Revelli [c], Elena Gonzalez Rey [d], Renata Rosito Tonelli [e], Ariel Mariano Silber [a, *]

[a] *Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil*
[b] *Center of Biological Sciences and Health, Mackenzie Presbeterian University, São Paulo, Brazil*
[c] *Institute of Immunology, School of Medical Sciences, National University of Rosario, Rosario, Argentina*
[d] *Institute of Parasitology and Biomedicine Lopez-Neyra, CSIC, Granada, Spain*
[e] *Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, São Paulo, Brazil*

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Abstract

The final decade of the 20th century was marked by an alarming resurgence in infectious diseases caused by tropical parasites belonging to the kinetoplastid protozoan order. Among the pathogenic trypanosomatids, some species are of particular interest due to their medical importance. These species include the agent responsible for Chagas' disease, *Trypanosoma cruzi*. Approximately 8 to 10 million people are infected in the Americas, and approximately 40 million are at risk. In the present review, we discuss in detail the immune mechanisms elicited during infection by *T. cruzi* and the effects of chemotherapy in controlling parasite proliferation and on the host immune system.

Keywords: Chagas' disease • *Trypanosoma cruzi* • immunity • chemotherapy • immunoparasitology

Introduction

Diseases caused by trypanosomatids constitute a substantial health and socioeconomic problem in several countries, mainly in the Americas, sub-Saharan Africa and tropical and subtropical belt regions. In particular, Chagas' disease, (caused by *Trypanosoma cruzi*) affects 8 to 10 million people in the Americas, with an additional 40 million people at risk (www.who.int/tdr). *T. cruzi* has a complex life cycle involving a reduviid insect vector and a mammalian host (Fig. 1). Insect vectors become infected when they bite an infected mammal that has trypomastigote forms of the parasite circulating in its bloodstream. Trypomastigotes, infective non-dividing forms, are ingested with the blood; in the insect's digestive tube, they differentiate into dividing and non-infective epimastigote forms. In the terminal portion of the digestive tube, epimastigotes differentiate into metacyclic trypomastigotes, which are eliminated in faeces and deposited on mammals' skin while the triatomine bug bites and feeds. Trypomastigotes enter the body and invade host cells; they differentiate into dividing amastigote forms and after proliferating, differentiate into trypomastigotes, passing through a transient epimastigote-like stage. Finally, the trypomastigotes lyse host cells and are released into the extracellular medium, where they can invade other cells or the bloodstream, becoming capable of invading other tissues or a non-infected reduviid insect, thus completing the cycle [1].

Chagas' disease

Chagas' disease presents mainly as two clinical phases in human beings: acute and chronic. The acute phase happens shortly after infection, beginning when the parasite enters the mammalian host. It is either largely asymptomatic or accompanied by non-specific symptoms such as fever and headache. It is characterized by an absence of antibodies and, in most patients, a conspicuous para-

*Correspondence to: Ariel M. SILBER,
Prédio Biomédicas II, Av. Lineu Prestes 1374, Sala 24,
Cidade Universitária (05508-900), São Paulo (SP), Brazil.
Tel.: +55-11-3091-7335
Fax: +55-11-3091-7417
E-mail: asilber@usp.br

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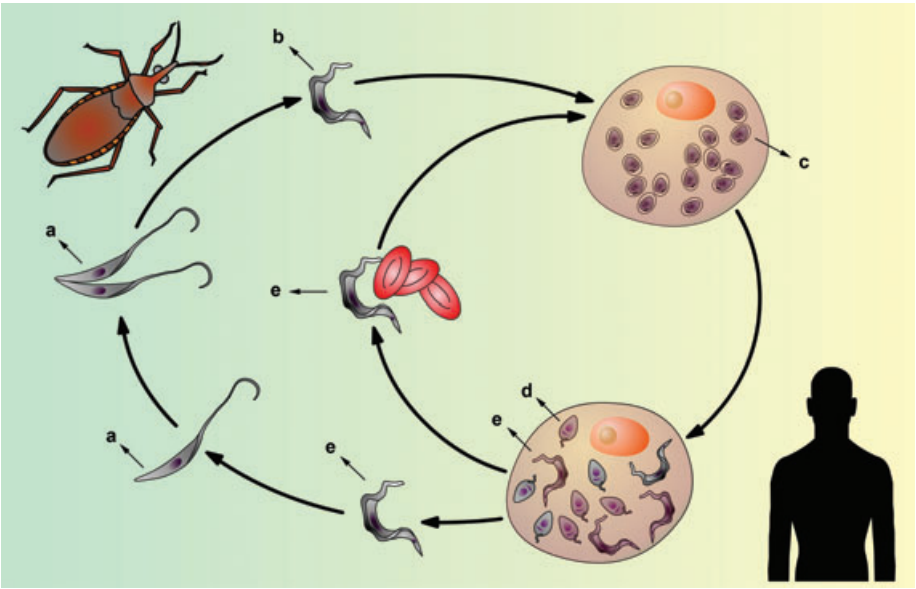
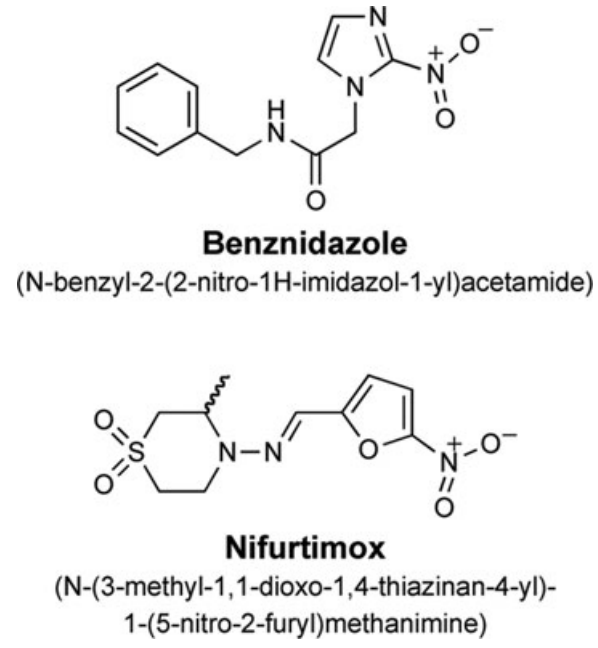


Fig. 1 Schematic representation of the *Trypanosoma cruzi* life cycle. Replicative, non-infective epimastigote forms (**A**), predominantly present in the insect vector, give rise to non-replicative, infective metacyclic trypomastigotes (**B**). Metacyclic forms must invade the host cells and differentiate into replicative amastigote forms (**C**) to establish the infection. These forms give rise to a transient stage called intracellular epimastigotes (**D**), which subsequently differentiate into trypomastigotes (**E**). Trypomastigotes can disseminate in the mammalian host through the bloodstream. The insect vector eventually can take these forms during its bloodmeal. The cycle ends when the ingested trypomastigotes differentiate again into epimastigotes (**A**), which colonize the digestive tube of a new insect.



Benznidazole
(N-benzyl-2-(2-nitro-1H-imidazol-1-yl)acetamide)

Nifurtimox
(N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitro-2-furyl)methanimine)

Fig. 2 Molecular formula of BZL and NF.

sitemia starting 1 or 2 weeks after parasite entry. In some cases, specific symptoms such as lymphadenopathy and splenomegaly, myalgia, malaise, muscle pains, sweating, hepatosplenomegaly or heart failure from myocarditis or pericardial effusion may be present. Less often, meningoencephalitis can occur, which can lead to death [2]. The chronic phase, in principle, can last for the patient's entire lifetime [3], beginning with the decline of parasitemia. It is defined by an initial absence of symptoms. The main chronic forms are indeterminate, cardiac (chronic chagasic cardiomyopathy, or CCC) and digestive. At lower frequencies, the chronic phase can consist of alterations in the peripheral nervous system. The indeterminate form is characterized by the absence of evident tissue damage and organ dysfunction and can last from several months to the patient's entire life, which is the case for approximately 70% of chronically infected people. The remaining 30% develop one of the symptomatic forms, most frequently CCC. This form presents different degrees of severity, ranging from mild symptoms to heart failure (caused by inflammation and fibrosis), frequently followed by sudden death. The main clinical manifestation of CCC is cardiomegaly caused by inflammatory infiltrations, arrhythmias and thromboembolism. The lesions can affect the right ventricle, causing oedema and congestive hepatomegaly [3]. The digestive form consists of two syndromes: megaesophagus, leading to dysphagia and regurgitation, and megacolon, leading to severe constipation and faecal retention [4]. In immunocompromised patients, severe compromise of the central nervous system has been also reported [5]. In conclusion, although the majority of *T. cruzi* infected individuals remain asymptomatic for their entire lives, a percentage of the infected population will develop serious symptoms.

Chemotherapy

Despite the fact that Chagas' disease was first described a century ago, only two therapeutic compounds presently in use have been shown to be useful against human infections by *T. cruzi*: benznidazole (BZL) and nifurtimox (NF) (Fig. 2). BZL, a nitroimidazole, was launched in the 1970s; in most Latin American countries, it is the only drug used to treat Chagas' disease. This treatment is effective

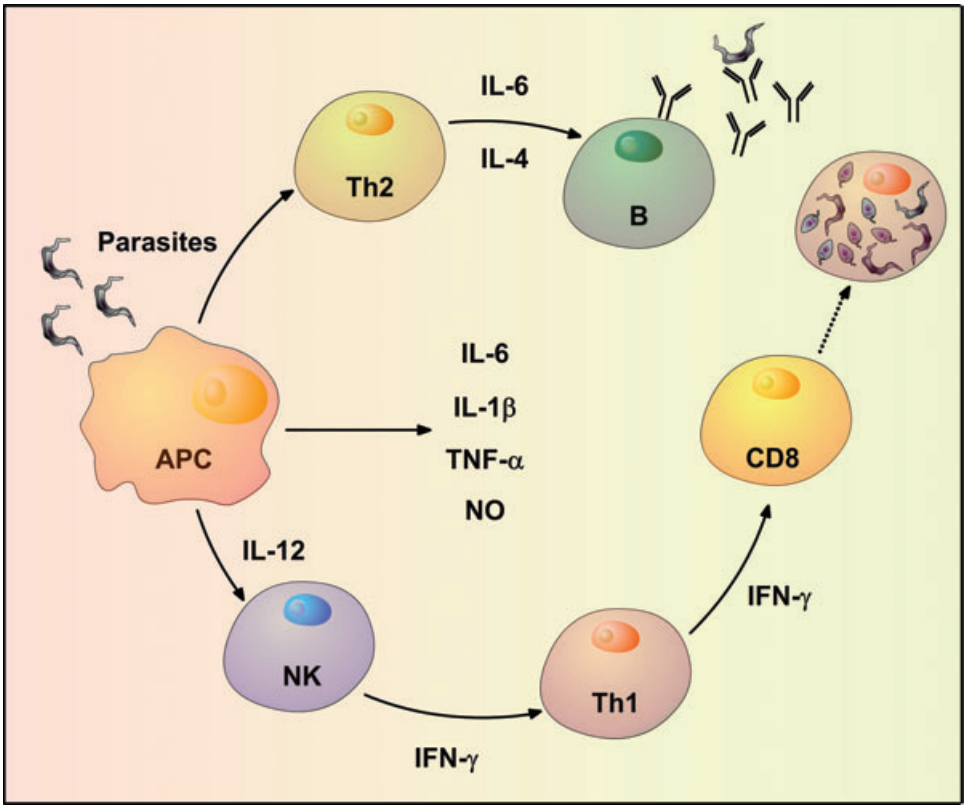


Fig. 3 Schematic representation of the protective immune response during *T. cruzi* acute infection. Antigen-presenting cells are among the first cells that become infected by the trypomastigotes when they enter the mammalian host. Normally, the cells react by up-regulating IL-6, IL-1β, TNF-α, IL-12 and nitric oxide in an attempt to control the infection. NK cells are among the first line of responders and usually produce high levels of IFN-γ when stimulated by IL-12. The stimulus of the Th1 profile and the $CD8^+$ T cells contributes to eliminate the intracellular amastigotes in infected tissues. On the other hand, the parasite antigens stimulate a Th2 profile which contributes to the production of specific antibodies.

for acute phase infections, congenital infections, reactivated infections and early chronic disease. However, its efficacy during the chronic phase is controversial [6]. The key mode of action of BZL seems to be based on interference with the synthesis of macromolecules *via* covalent binding between nitroreduction intermediates and various cellular components such as DNA, lipids and proteins of the parasite. BZL has also been shown to improve phagocytosis, increase trypanosomal death through interferon (IFN)- production and inhibit *T. cruzi* NADH-fumarate reductase [7]. The mechanism of action of NF involves the generation of nitroanion radicals by nitroreductases that, in the presence of oxygen, produce reactive intermediates to which *T. cruzi* is susceptible. Considerable efforts are being made to identify promising targets for new drugs. A detailed discussion of new drugs with chemotherapeutic perspectives is outside the scope of this review. However, some *T. cruzi* specific pathways contain proteins/enzymes that are being validated as targets; several drugs that interfere with these targets are particularly promising [6]. The cysteine proteinases of *T. cruzi*, which participate in cellular processes such as energy metabolism, differentiation, host cell invasion and evasion of the immune system, have been validated as drug targets [8]. In particular, the use of synthetic inhibitors such as vinyl sulfone-derivatized dipeptides has shown promising results *in vivo* [9, 10]. In addition, sterol and polyisoprenoid biosynthesis pathways provide promising targets, because ergosterol (rather than cholesterol) is the main sterol in *T. cruzi* membranes [11]. Specific inhibitors such as azole derivatives [12] and bisphosphonates [13, 14] have been tested *in vitro* or *in vivo*. Inhibitors of *T. cruzi* specific enzymes such as trypanothione reductase [15], arginine kinase [16] and proline racemase [17] have shown promising trypanocidal activities. It was recently shown that proline transporters could also be relevant targets [18]. Allopurinol, an inhibitor of purine (hypoxanthine/guanine)-phosphoribosyl-transferase, has been proposed for treating *T. cruzi* reactivation infection in patients after heart transplantation [19]. Inhibitors of topoisomerases I and II [20, 21], which are involved in nuclear and kinetoplastid DNA replication, were efficient in blocking *T. cruzi* growth. Parasite DNA has also been proposed as a target for intercalators and binders with trypanocidal activity [22–24].

Immune response in experimental *T. cruzi* infection

T. cruzi's success in maintaining its life cycle is dependent on its capacity to cause chronic infection in the host, thus favouring encounters with insect vectors. To maintain latent infection, a balance between parasite and host immune response is necessary.

Table 1 Effect of the absence of different molecules of the immune system in the experimental acute infection by *Trypanosoma cruzi*

KO	Phenotype/absence of:	Effect on parasitemia	Effect on mortality	Reference
Immunoglobulin heavy chain	B lymphocytes	Increase (end of acute phase)	Increase	[35]
μ Chain	Mature B lymphocytes	Increase	Unaltered	[75]
CD4	$CD4^+$ T lymphocytes	Increase	Increase	[26]
CD8	$CD8^+$ T lymphocytes	Increase	Increase	[26]
MHC class I and II	$CD4^+$ and $CD8^+$, T lymphocytes	Increase	Increase	[27]
β2 Microglobulin	$CD8^+$ T lymphocytes and NK cells	Increase	Increase	[27]
δ chain of the T lymphocyte receptor	γδ T lymphocytes	Unaltered	Decrease	[48]
IFN-γ	IFN-γ production	Increase	Increase	[57]
IFN-γ receptor	Activation by IFNγ	Increase	Increase	[55]
NOS2	Nitric oxide production	Increase	Increase	[55,58]
IL-10	IL-10 production	Decrease	Increase	[67]
IL-12	IL-12 production	Increase	Increase	[57, 64]
TNF-α receptor	Activation by TNF-α, reduction of Ig production	Increase	Increase	[65]
Perforin	Perforin	Unaltered	Unaltered	[35]
Granzime B	Granzime B	Unaltered	Unaltered	[35]
Stat4	$CD4^+$ Th1 response	Increase	Increase	[37]
Stat6	$CD4^+$ Th2 response	Unaltered	Unaltered	[37]
MyD88	Absence of signalling through some TLRs	Increase	Increase	[30]
TLR-2	Absence of signalling through TLR 2	Unaltered	Unaltered	[30]
TLR-4	Absence of signalling through TLR 4	Increase	Increase	[28]
TLR-9	Absence of signalling through TLR 9	Increase	Increase	[33]
CD1d	NK T cells	Decrease	Not assessed	[52]

Host resistance to Chagas' disease depends on both innate and adaptive immunity (Fig. 3) [25–27]. Different cell types and molecules are involved in the response against experimental *T. cruzi* infection, as summarized in Table 1. Generally, the absence of some component(s) of the immune response leads to greater susceptibility to *T. cruzi* infection, resulting in higher parasitemia and mortality rates.

With respect to the innate immune mechanisms triggered by the parasite, it has been found that some pathogen-associated molecular patterns derived from *T. cruzi* are recognized by specific receptors known as pattern recognition receptors (PRRs) [28]. The toll-like receptor (TLR) family is the best characterized class of mammalian PRRs [29]. Mice that are unable to signal through most TLRs (MyD88 knockout [KO] or MyD88/TRIF double KO) are highly susceptible to *T. cruzi* infection, suggesting that resistance to acute *T. cruzi* infection is dependent on TLR signalling [30, 31]. *T. cruzi* derived glycosylphosphatidylinositol (GPI) anchors and glycoinositolphospholipids (GIPLs) were shown to have immunoregulatory properties and were present in significant quantities on the parasitic surface. GPIs are recognized by the transmembrane receptor TLR-2, which is associated with CD14. Interestingly, *T. cruzi* infected TLR-2 KO mice are able to produce pro-inflammatory cytokines, yet parasitemia and mortality rates are not different from wild-type animals [30]. It has also been shown that GIPLs are recognized *via* TLR-4, and TLR-4 KO mice are more susceptible to *T. cruzi* infection than wild-type mice [28]. However, the role of these molecules in natural infection has not been assessed. *T. cruzi* DNA binds TLR-9 and has been reported to stimulate macrophages and DCs to express interleukin (IL)-12, tumour necrosis factor (TNF)-α and nitric oxide [32]. In fact, TLR-9 KO mice are highly susceptible to *T. cruzi* infection, and some results suggest that TLR-2 and TLR-9 cooperate to control *T. cruzi*

replication during acute infection [33]. In summary, these data suggest an important role for TLR signalling pathways in the innate immune response to *T. cruzi* infection.

To unravel the role of cellular immune responses, initial experiments have been performed in which T cells were adoptively transferred from chronically infected to naïve mice. Significant protection was observed after experimental challenge with *T. cruzi* [34]. Both $CD4^+$ and $CD8^+$ ($\alpha\beta$) T-cell subsets appear to be important for the generation of protective immunity against acute experimental *T. cruzi* infection [26, 27, 35, 36].

Tarleton and collaborators showed that Th1 $CD4^+$ T cells are important for controlling *T. cruzi* infection, while Th2 cells contribute to parasite persistence and increased disease severity [37]. Attempts to obtain protective vaccines against defined *T. cruzi* antigens have also provided valuable information about the role of Th1-type responses during infection. For example, cruzipain, when used as an antigen in immunization protocols, significantly improved immune responses in animals challenged with a lethal dose of the parasite [38] and precluded tissue damage [39].

$CD8^+$ T cells also seem to play a role in *T. cruzi* infection by killing infected cells through the production of perforin and granzyme B or through the Fas/Fas ligand pathway. Acute infection with the Brazil strain of *T. cruzi* did not enhance the susceptibility of either perforin or granzyme B KO mice [35]. By contrast, previously reported data have shown that mice lacking perforin and granzymes A and B are more susceptible to infection with the Tulahuen strain [40]. Increased susceptibility to infection with the Tulahuen strain was also observed in mice deficient in the Fas/Fas ligand pathway [40]. *T. cruzi* antigens that are $CD8^+$ T-cell targets have also been studied using chronically infected mice. These studies showed that epitopes from the *trans*-sialidase family of proteins are immunodominant, and the $CD8^+$ T-cell response is focused mainly on very specific epitopes [41, 42]. When surface markers of some of these cell populations were analysed, it was found that they were central memory T cells that are maintained even during persistent *T. cruzi* infection [43]. Immunization protocols using plasmid DNA coding for the proteins *trans*-sialidase and amastigote surface protein-2 have also shown that both cellular and humuoral immune responses were induced, and the presence of $CD4^+$ Th1 and $CD8^+$ specific T cells was shown. Furthermore, parasitemia and/or mortality reduction was seen in mice infected with the Y [44–46] or Brazil strains of *T. cruzi* [47].

The effective participation of $\gamma\delta$ T cells in the immune response against *T. cruzi* is still controversial. These cells were found to be deleterious for the host in a study in which $\gamma\delta$-KO mice had lower mortality rates and fewer areas with skeletal and cardiac inflammatory lesions compared to wild-type mice [48]. On the other hand, an increase in susceptibility was observed in $\gamma\delta$ T-cell depleted animals after experimental infection associated with a reduction in IFN-γ production [49].

The immune response against *T. cruzi* is also influenced by NK, NK T and regulatory T cells (T reg). The relevance of NK cells in acute *T. cruzi* infection was demonstrated when normal, NK-depleted mice showed a significant increase in parasitemia and mortality rates [50, 51]. NK T cells are activated by glycolipids presented *via* CD1d molecules and act by limiting parasitemia. These cells also seem to influence antibody responses during chronic Chagas' disease [52]. The role of regulatory T cells ($CD4^+$ $CD25^+$) during *T. cruzi* infection was also shown through depletion. Controversial results were obtained with mice infected with different *T. cruzi* strains: no effect was seen when Brazil and Tulahuen strains were used [53], while limited effects were seen with the Colombian strain [54]. No role was observed for these cells during chronic infection of mice with the Colombian strain [54].

The role of IFN-γ during *T. cruzi* infection was demonstrated when IFN-γ and IFN-γ receptor KO mice showed higher rates of parasitemia and mortality [55]. Infected IFN-γ KO mice showed increases in cellular infiltrates in the heart and skeletal muscles and reduced survival [36, 56, 57]. The role of the inducible nitric oxide synthase (iNOS) was also studied. iNOS KO mice showed greater parasitemia in the acute phase and rapid mortality compared to control animals [55, 57, 58]. Additionally, nitric oxide seems to have an effect on the generation of the inflammatory heart infiltrate seen during *T. cruzi* infection by modulating chemokine expression. Cardiomyocytes from iNOS KO mice that were stimulated with IFN-γ and TNF produced significantly higher levels of the chemokines CCL2, CCL4, CCL5 and CXCL2 [59]. Other chemokines and chemokine receptors have also been analysed during acute or chronic *T. cruzi* infections. CXCL9, CXCL10 and CCL5 are expressed in the heart during both phases of *T. cruzi* infection [60, 61]. Their presence correlates with the expression of IFN-γ and TNF-α and the presence of inflammatory cells. However, their ablation did not modulate the severity of heart inflammation [61]. During the acute phase, CCR5 seems to be critical to controlling the migration of $CD4^+$ and $CD8^+$ T cells to the heart [62], but it does not seem to play an important role in maintaining an inflammatory response in the heart during chronic infection [63].

IL-12 is also extremely important for controlling the infection, as IL-12 KO mice show higher rates of parasitemia and mortality compared to controls [57, 64]. The role of TNF-α is controversial: TNF receptor p55 KO mice [25, 65] showed increased parasitemia, while parasitemia and mortality rates in TNFR1 KO mice did not differ from wild-type [66]. IL-10 deficiency leads to parasitemia reduction; however, mortality is accelerated due to a dramatic increase in acute pathology [67]. IL-4 seems to have different effects according to the parasite strain. IL-4 KO mice infected with the Y strain did not differ from wild-type in terms of parasitemia and mortality [68]. However, when the Colombian strain was used, IL-4 KO mice showed reduced parasitemia and mortality rates [57].

The importance of antibodies for controlling chronic infection was demonstrated when sera from chronically infected chagasic patients or mice were transferred to naïve mice, significantly reducing parasitemia and prolonging survival after challenge with *T. cruzi* [69, 70]. Protective antibodies are also able to agglutinate trypomastigotes *in vitro* [70], lyse them in a complement-mediated fashion [71], facilitate phagocytosis/opsonization [72] and mediate antibody-dependent cellular cytotoxicity [73, 74]. During acute *T. cruzi* infection, B cells also play fundamental roles in both the recruitment of $CD4^+$ T cells and $CD8^+$ T cells to the spleen

and in the generation and maintenance of central memory and effector T cells. KO mice lacking mature B cells (μMT KO) produced decreased amounts of inflammatory cytokines and fewer central and memory $CD4^+$ and $CD8^+$ T cells compared to wild-type *T. cruzi* infected mice. An increase in parasitemia was observed in *muMT* KO mice, but no difference was seen in terms of survival [75]. B cells are also able to participate in the cross-priming of specific $CD8^+$ T cells, inducing systemic and mucosal protective immunity against experimental infection [76]. Taken together, these results suggest the important participation of both innate and adaptive immune responses during experimental *T. cruzi* infection.

Immune response in human beings infected with *T. cruzi*

During the chronic phase of Chagas' disease, the majority of individuals show potent cellular and humuoral immune responses [77, 78]. The relevance of a strong immune response for parasite control has been shown by the fact that chemically immunosuppressed individuals [79] and AIDS patients can develop symptomatic forms of the disease [80]. The mechanisms underlying the transition from asymptomatic to clinically symptomatic are still unclear. Several factors may be involved, such as differences in parasite strain, parasite load, infection time, host genetic background and immune response. In animal models, different parasite strains, mouse backgrounds and re-infections can play a role in the development of heart pathology and/or protection [81–83].

Regarding human infections, a study with patients acutely infected with *T. cruzi* has shown that $CD4^+$ and $CD8^+$ T cells are present in endomyocardial biopsies where myocarditis was also detected in 100% of the cases, reinforcing the role of the immune response in acute pathology [84]. With respect to the chronic phase, a predominance of activated $CD8^+$ T cells was found in myocardial biopsy fragments from patients with CCC [85, 86]. It is also of interest that peripheral blood mononuclear cells (PBMCs) from chronically infected chagasic patients were able to produce IFN-γ upon stimulation with recombinant *T. cruzi* derived proteins [87, 88]. Furthermore, PBMCs from CCC patients produced more IFN-γ and less IL-10 [87, 89]. IL-10 expression in monocytes from patients with the indeterminate form was higher than in cardiac patients [89, 90]. By contrast, analysis of IFN-γ-producing $CD8^+$ T cells present in infected patients (with undetectable, mild or more severe forms of clinical disease) showed that there was a negative correlation between the capacity of their cells to respond to *T. cruzi* amastigote antigens and disease severity [91]. A careful analysis of these cells showed that in responding individuals (individuals with milder heart disease), there were early differentiated ($CD27^+CD28^+$) and effector memory ($CD45RA^-CCR7^-$) $CD8^+$ T cells. On the other hand, individuals with more severe forms of the disease presented fully differentiated ($CD27^-CD28^-$) $CD8^+$ T cells [77]. The authors suggest that this profile is compatible with the hypothesis that as the disease progresses, there is a gradual clonal exhaustion of the $CD8^+$ T-cell population, probably due to continuous antigenic stimulation. Another study showed that patients with cardiac forms of Chagas disease display a high frequency of circulating $CD4^+$ and $CD8^+$ T cells lacking the CD28 surface molecule [78]. Two cytokines (IL-7 and IL-15) were suggested as being important for the survival of $CD8^+$ T cells in the cardiac infiltrate [92].

On the other hand, there is also evidence that activated T cells are involved in Chagas disease pathology. Cells from patients with chronic infections (either symptomatic or asymptomatic) express both inflammatory and anti-inflammatory cytokines [93, 94], suggesting that there is probably immune regulation during the chronic phase. However, preferential expression of TNF-α and IFN-γ (both inflammatory cytokines) was observed in cardiac lesions [33, 86] and has been associated with progressively severe cardiac disease [89, 95]. Few reports have focused on the identification the $CD8^+$ T-cell targets in chronic chagasic patients [41, 91, 96–98], probably due to the fact that these responses are not very strong. $CD8^+$ T-cell responses against *T. cruzi* peptides derived from the proteins cruzipain, FL-160, KMP-11 and the *trans*-sialidase family were detected in infected individuals [41, 97, 98]. Peptides derived from the *trans*-sialidase protein family were able to bind to six of the most common class I HLA supertypes, and the stimulated cells showed a lack of polyfunctional cytokine responses, producing only IFN-γ [96].

Evidence has also led to the proposal of an autoimmune hypothesis for the disease, suggesting that the symptoms presented by infected individuals are a consequence of the triggered immune response rather than parasite persistence [99]. However, autoimmunity is not sufficient to explain the multifocal nature of myocarditis and the preferential location of fibrosis in certain regions, such as the apical or posterior left ventricular wall in the cardiac form of Chagas' disease [100]. Moreover, as mentioned above, frequent positive xenodiagnosis during the chronic phase of the disease and episodes of reactivation in immunocompromised patients has provided evidence that the parasite is present even under active control of the host immunological system [79]. In fact, recent studies have described a positive correlation between myocardial parasite persistence and high-grade myocarditis [101, 102]. These studies reinforce the notion that a combination of immune response and parasite persistence determines the development of Chagas' disease pathology. However, the specific mechanisms that trigger the symptoms seen during the chronic phase are still elusive.

Immune response in the treatment of chagasic infection

As previously mentioned, the resistance developed during experimental Chagas' disease depends on innate immune responses as well as on a prevalent Th1 response at the beginning of infection.

In addition, the antibodies produced by a Th2 response can contribute to the control of *in vivo* parasite replication (schematized in Fig. 3). Several works have shown that chemotherapy acts by unbalancing the equilibrium between the host immune response and the parasite in favour of the host [66, 103]. However, it is important to stress that the results obtained with both drugs used for treatment, BZL and NF, differ strongly according to the disease phase, the time extension and the drug dose, as well as the patient's age and geographical origin [104]. *In vitro* phagocytosis and intracellular destruction of trypomastigotes by mouse peritoneal macrophages collected from animals treated with NF and BZL are remarkably increased compared to untreated controls [105]. The effects of *in vivo* treatment with BZL on parasite-macrophage interaction have been studied using the Y strain of *T. cruzi.* Drug-resistant and drug-susceptible parasites from this strain were used, and it was observed that BZL enhanced phagocytosis, parasite destruction and cytokine release by macrophages when a drug-sensitive strain was used. Splenocytes from these animals produced very high levels of TNF-α and reactive nitrogen intermediates [106]. BZL treatment was also evaluated in IFN-γ, IL-12, TNF receptor and iNOS KO mice. Although BZL treatment was able to cure 100% of wild-type mice, it was not as effective when various KO mice were used [107]. The induction of a stable parasite-specific $CD8^+$ T-cell population with the characteristics of central memory was observed in chronically infected mice treated with BZL. These cells also expand more rapidly and provide greater protection after challenge compared to cells from non-treated chronically infected mice [108]. It was also observed that splenomegaly persisted in spite of amelioration of clinical and parasitological signs in infected and BZL-treated mice. This was due to a preferential expansion of effector and memory $CD8^+$ T cells but not of recently activated $CD4^+$ and $CD8^+$ T cells, suggesting that BZL directly affects immunoregulation in *T. cruzi* infected mice [109]. The effect of BZL treatment was also studied in two CCC mouse models, showing contradictory results. One model showed that treatment was able to reduce the severity of cardiac autoimmune disease [82], while in the other, early treatment with BZL did not alter the intensity of CCC [110].

PBMCs from cured patients treated with BZL during the acute phase showed stronger proliferative responses and IFN-γ production compared to non-cured patients. IFN-γ could be acting synergistically with chemotherapy to eliminate parasites [111], as previously suggested [112]. It was also demonstrated that most individuals with an indeterminate clinical form show a dominant regulatory cytokine profile, whereas individuals with CCC display a dominant inflammatory cytokine pattern. Interestingly, an inversion of the cytokine profile (from an inflammatory to a regulatory profile or *vice versa*) was found after *in vivo* treatment of indeterminate and cardiac individuals with BZL [113]. Cytokine expression in *T. cruzi* infected children treated with BZL shifted toward a type 1 modulated immune profile, and IFN-γ was mainly produced by NK cells and $CD8^+$ T lymphocytes [103]. Although a pro-inflammatory immune response is commonly related to Chagas' disease pathogenesis, it is also important for treatment effectiveness. A longitudinal study performed to evaluate immunological status following BZL treatment during early indeterminate Chagas' disease demonstrated that BZL treatment induced substantial T- and B-cell activation [114].

As discussed above, several lines of evidence show that BZL, in addition to having trypanocidal activity, acts as an immunomodulator. More specifically, the fact that BZL is known to affect mammalian host cells raised the hypothesis that it might be affecting macrophage metabolism. Revelli and collaborators showed that BZL down-regulates nitric oxide and pro-inflammatory cytokine synthesis (IL-6, IL-1β) by lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages and leads to an inhibition of iNOS gene expression [115] through the inhibition of NF-κB activation [116]. Moreover, in a rat model of acute *T. cruzi* infection, systemic treatment with BZL led to a marked reduction of nitric oxide derived metabolites, suggesting that the beneficial properties of BZL may depend on both trypanocidal action and immunomodulating effects [117]. These properties of BZL have been further demonstrated by its ability to increase survival and decrease serum levels of IL-6 and TNF-α in C57BL/6 mice challenged systemically with LPS [118]. In an infectious-based situation of systemic inflammatory response (cecal ligation and puncture, CLP), mice treated with BZL had an increased survival rate and a significant reduction of TNF-α levels and bacteremia 24 hrs after CLP [119]. Such beneficial properties may broaden the potential use of BZL in hyperacute cases where inflammatory responses become harmful.

There are limited data concerning the effects of NF on immune responses, mainly because treatment with this drug was discontinued in the 1980s. At the experimental level, no gross changes in cell-mediated immunity were recorded in NF-treated mice [120]. However, an impaired PPD skin reaction was seen in guinea pigs when this compound was administered [121]. From a clinical standpoint, chronically infected human beings who were treated for two months with NF had a detectable peripheral leukocyte migration inhibition test to *T. cruzi* antigens. Migration was lower in untreated patients, and further treatment improved such responses [122]. The effect of NF treatment on re-infection has also been evaluated. Animals that were parasitologically sterilized by NF treatment and re-infected with a small number of parasites showed parasitemia and mortality rates similar to control animals (infected, non-treated). However, when re-infection was performed with a large number of parasites, parasitemia and mortality were increased compared to controls. Immunological studies have shown that NF treatment reduces the levels of antibodies engaged in parasite destruction, reducing either complement-dependent lysis or antibody-dependent cytotoxicity. No difference was observed when treated and non-treated mice were compared in terms of T-cell mediated immunity. Therefore, it seems that NF treatment leads to a loss of resistance to re-infection with a high number of parasites [120].

The need for new therapeutic alternatives for Chagas' disease

During the last 40 years, therapy for Chagas' disease has been based solely on two drugs: NF and BZL. The search for new trypanocidal drugs as well as other strategies to improve immune responses during infection (*i.e.* therapeutic vaccines) are currently under way [123, 124]. Treatment of the symptomatic complications of Chagas' disease, mainly related to heart failure, is also effective in improving quality of life. Heart transplantation [125] and cellular therapy with stem cells [126] are therapeutic options for patients with advanced CCC, but both require optimization of the etiological treatment. As more data become available regarding the relevance of parasite persistence in the development of chronic infection, the search for new treatments becomes extremely critical. Future challenges for developing new drugs for Chagas' disease reside in finding compounds that show both trypanocidal and immunomodulatory activities. Despite the vast literature regarding the anti-*T. cruzi* activity of myriad compounds, only a small fraction of studies explore the characteristics mentioned above. Furthermore, drugs with modest anti-*T. cruzi* activity could be evaluated as co-adjuvant drugs, interacting synergistically with NF or BZL. This strategy would increase the spectrum of available combinations and possibly contribute to reducing the dose and thus avoiding the toxic effects of the drugs currently in use.

Conclusions

In summary, the drugs that are available to treat diseases caused by trypanosomatids are somewhat effective. However, for the reasons discussed above, new drugs that are able to act alone or in concert with current treatments as well as with strategies such as immunotherapy and vaccines are urgently needed. These are relevant goals because they could aid in reducing undesired secondary effects, thereby optimizing the quality of life of patients and diminishing treatment evasion. In this way, funding of all steps related to the development of new therapies, from validation of new targets to evaluation of new drugs (from the bench top to clinical trials), is a necessity.

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