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Cytokines and cell adhesion receptors in the regulation of immunity to *Trypanosoma cruzi*

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Abstract

Pathophysiology of Chagas' disease is not completely defined, although innate and adaptative immune responses are crucial. In acute infection some parasite antigens can activate macrophages, and this may result in pro-inflammatory cytokine production, nitric oxide synthesis, and consequent control of parasitemia and mortality. Cell-mediated immunity in *Trypanosoma cruzi* infection is also modulated by cytokines, but in addition to parasite-specific responses, autoimmunity can be also triggered. Importantly, cytokines may also play a role in the cell-mediated immunity of infected subjects. Finally, leukocyte influx towards target tissues is regulated by cytokines, chemokines, and extracellular matrix components which may represent potential therapeutic targets in infected patients. Here we will discuss recent findings on the role of cytokines, chemokines and extracellular matrix components in the regulation of innate and adaptive immunity during *T. cruzi* infection.
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1. Introduction

Chagas' disease is caused by the protozoan parasite *Trypanosoma cruzi*, being endemic in Latin America. There are approximately 16–18 million infected humans, and nearly 100 million people at risk of infection, representing the largest parasitic disease burden in the continent, with around 50,000 deaths per year [1]. Unfortunately, it remains as a neglected disease in the world, no vaccines are so far available, and the only anti-parasite drug is the nitroimidazole compound, *benznidazole*, which is only efficient in the acute phase of the disease.

Chagas' disease comprises an acute phase characterized by apparent circulating parasites and clearcut tissue parasitism, followed by a chronic phase with very low amounts of parasites in target tissues such as heart, esophagus, colon and peripheral nervous system. The acute episode often self-resolves in about 2 months, although when symptomatic it may lead to variable degrees of heart and/or neurological involvement (myocarditis and meningo-encephalitis, respectively). This episode is followed by a chronic phase that may

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remain silent for 10–20 years, as an indeterminate form, but can evolve to clinically defined cardiac or digestive disturbances.

Chronic cardiomyopathy is generally symptomless at the beginning, though the electrocardiogram may reflect subtle conduction/rhythm abnormalities. In some cases, this scenario changes from mild to moderate heart enlargement, and ultimately progresses to a marked cardiomegaly, cardiac failure, severe arrhythmia and death [2].

At the digestive tract, parts of the lesions are related to the damage in afferent, efferent and/or central neural sympathetic and parasympathetic systems [3]. Moreover, digestive forms are produced by denervation of the enteric nervous system [4]. Patients with advanced megaesophagus often present clinical manifestations of malnutrition, which in turn may predispose to an immunosuppression state with a consequent increase in parasitemia [5].

Although very scarce in the host's tissues, the presence of parasites in the chronic phase of Chagas' disease may be relevant for maintaining specific immune responses, although reactivity against self tissues is comprised in the immune response. Additionally, in chronically infected immunosuppressed individuals, parasites may be responsible for the reactivation of Chagas' disease. Studies in AIDS patients showed reactivation of the chronic asymptomatic phase of the disease [6,7]. Although the pathophysiology of Chagas' disease has not been completely explored, it is widely accepted that the involvement of the immune response is critical in determining the outcome of the disease. In the present review we will focus in the interactions mediated by cytokines, chemokines and extracellular matrix/adhesion molecules, in innate and adaptive immune responses during experimental and human *T. cruzi* infection.

2. Modulation of innate immunity in phagocytic and non-phagocytic cells following *T. cruzi* infection

Innate immunity provides the first line of defense by detecting the type of infectious agent through pattern recognition receptors, including toll-like receptors (TLRs). These receptors recognize multiple microbial patterns and are critical for triggering events necessary for activation of innate immunity and initiation of acquired immunity. It has been reported that stimulation of most TLRs leads to Th1 rather than Th2 differentiation [8]. Microbial-derived antigens, together with effector T cells, NK cells and their secretory products, influence the heterogeneity and state of activation of macrophages [9]. In this respect, the inflammatory cytokine IFN-γ, alone or in combination with TNF-α or microbial products such as LPS, activates macrophages via the classical pathway. This activation pattern is characterized by a polarized type-I response with high production of microbicidal intermediates such as nitric oxide (NO) and reactive oxygen species [10]. On the other hand, cytokines produced in type-II responses such as IL-4 and IL-13 induce the alternative activation of macrophages and antagonize the classic activation pathway. In addition, since IL-10 and glucocorticoids stimulate phenotypes which partially overlap the one induced by IL-4 and IL-13, some authors have proposed to define alternative activation when macrophages are activated only by IL-4 and IL-13 [10,11]. Others have broadened this term to include the phenotype of myeloid cells activated by IL-10 and glucocorticoids [9,12,13]. Here, we refer as alternatively activated macrophages as a general term. This activation pathway is characterized by low production of pro-inflammatory cytokines and a general involvement in type II responses, immunoregulation and tissue remodeling [9].

Infection of mice deficient for the TLR and IL-1R adaptor molecule MyD88 resulted in impaired production of inflammatory cytokines, together with enhanced parasitemia and mortality [14]. This result indicates that *T. cruzi* may be recognized by TLRs and that the activation of cytokine production by these receptors plays an important role in host's defense.

Several parasite antigens alone were able to induce classic macrophage activation and consequent increase in NO levels: glycophosphatidylinositol-anchored mucin-like glycoproteins (GPI) are potent inducers of NO by IFN-γ primed macrophages [15]. The same research group identified TLR2 as the functional GPI receptor, implicated in the induction of IL-12, TNF-α and NO [16]. It has also been demonstrated that the immunization of C57BL/6J mice with recombinant TolA-like surface protein, present in the flagellum of trypomastigotes, generated T $CD4^+$ cells that produced IFN-γ and increased NO levels with reduction of parasite numbers [17]. Another *T. cruzi* antigen involved in classic macrophage activation is the parasite-released molecule –Tc52–, which synergizes with IFN-γ to stimulate NO production [18]. Tc52 signals via TLR2 and confers resistance against lethal infection in BALB/c mice [19].

Cruzipain, the main cysteine-proteinase of *T. cruzi*, is abundantly expressed throughout the parasite life cycle, and is secreted to the extracellular milieu [20,21]. Immunization of BALB/c mice with cruzipain, devoid of enzymatic activity, resulted in an enhanced type II cytokine secretion, with increased levels of IL-4, IL-5 and IL-10. This later was associated with the induction of a $CD11b^+GR1^+$ spleen immature myeloid population that exhibited arginase, but not iNOS activity [22]. Furthermore, cruzipain-stimulated macrophages secreted IL-10 and TGF-β and displayed enhanced arginase activity and subsequent *T. cruzi* growth [23,24]. The later could be due to the induction of ornithine decarboxilase activity, an enzyme involved in the synthesis of polyamines, essential for intracellular parasite replication.

In contrast to the response seen in BALB/c animals, when C57BL/6 mice were immunized with cruzipain, the induced cytokine profile was predominantly type I, with high levels of IFN-γ and low levels of IL-4 [25]. Moreover, when spleen adherent cells from cruzipain-immunized mice were restimulated *in vitro*, an increase in iNOS mRNA and

protein, as well as TLR2 surface expression were observed [Guiñazú et al., personal communication]. Hence, cruzipain immunization in BALB/c mice induced alternatively activated macrophages, whereas in C57BL/6 mice this protein induced classically activated macrophages (Fig. 1).

In parallel, during infection spleen macrophages from BALB/c mice secreted lower concentration of nitrites than C57BL/6 mouse cells, indicating that impaired function of myeloid cells in susceptible mice could contribute to the persistence of infection [26]. These and other studies indicate that host genetic background is a further determinant, influencing the nature of the immune response and the infection outcome [27]. Thus, it seems clear that classical activation of macrophages by pro-inflammatory cytokines plays a central role in the control of parasitemia and mortality in the acute phase of *T. cruzi* infection [28].

Chemokines are molecules able to influence macrophage activation pathways. The CC chemokines CCL3/MIP-1α, CCL4/MIP-1β (macrophage inflammatory protein 1-α and 1-β), and CCL5/RANTES are able to induce NO production by human macrophages similarly to IFN-γ. Interestingly, these chemokines activated human macrophages to kill *T. cruzi* via NO [29]. In addition, *in vivo* neutralization of CCL3 resulted in a dramatic reduction of macrophage influx into the spleen of acutely infected mice, as ascertained by counting of the total macrophages numbers within the organ. Nevertheless, an increase in the numbers of macrophages was recorded in the myocardium [30]. These findings suggest that, in the context of *T. cruzi* infection, the dynamics of this major innate immunity cell type is partially under chemokine control. Such control seems to be specific to one or few chemokines: *in vivo* simultaneous neutralization of the chemokines CXCL9/MIG and CXCL10/IP-10 (both being ligands for the receptor CXCR3) did not generate any change in macrophage infiltration of the heart tissue [31].

Another molecule that participates in host response to *T. cruzi* infection is galectin-3, a β-galactoside binding protein which showed immunoregulatory activity in different infectious processes [32]. Recent data showed that this lectin, as well as its ligands, is up-regulated in murine splenic dendritic cells following *T. cruzi* infection, with modulation of cell adhesion and migration [33]. Targeted disruption of the galectin-3 gene reduced phagocytosis by macrophages *in vitro* [34] and attenuated peritoneal inflammatory responses [35]. Moreover, we showed that these mice are more susceptible to *T. cruzi* infection when compared to wild-type controls [36]. Additionally, extracellular matrix protein may be involved in infection by *T. cruzi*, as exemplified by the effect of galectin-3 in the infection of human macrophages and muscle cells [37,38]. Taken together, these data show that macrophage response to *T. cruzi* infection is under the control of a complex network of molecules including cytokines, chemokines and extracelllular matrix proteins, exemplified by galectin-3.

In addition to classical cell types of the innate immunity, cumulative findings indicate that cardiomyocytes are actively integrated in the inflammatory response during *T. cruzi* infection, releasing NO, cytokines and chemokines,

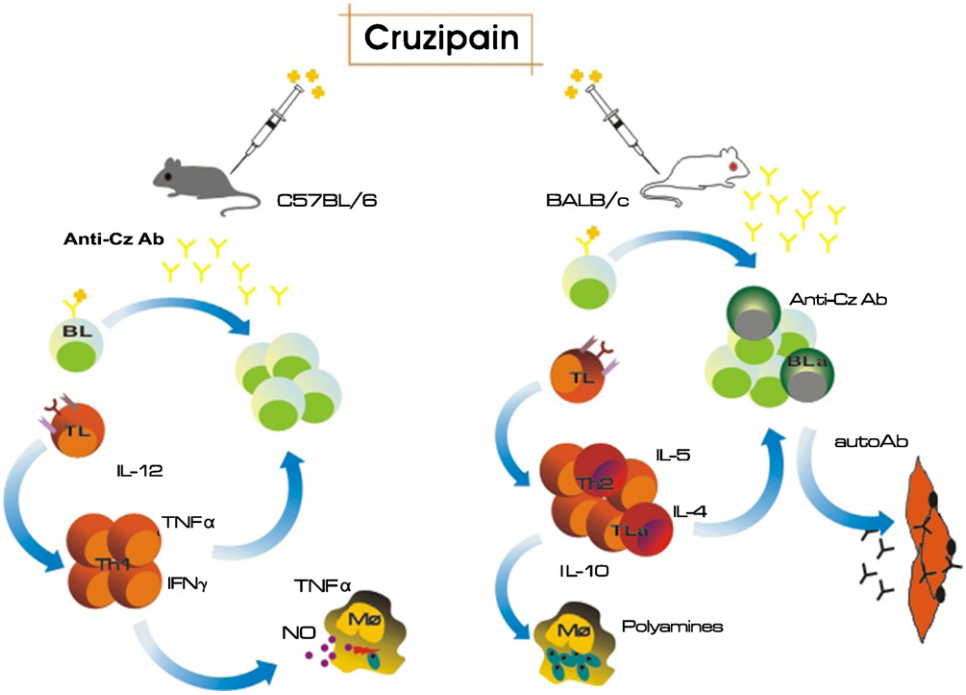


Fig. 1. Immune response to cruzipain. BALB/c and C57BL/6 mice immunization activates T and B lymphocytes that react with cruzipain (Cz). In BALB/c mice, these cells also recognize cardiac myosin. Activated T cells from BALB/c mice secrete type-2 cytokines which contribute to macrophage (Mϕ) alternative activation, while C57BL/6 mice show a type-1 cytokine profile which contributes to classical activation.

which in turn, might attract leukocytes to the inflammatory site and control intracellular parasite replication. It was shown that heart tissue collected from *T. cruzi*-infected rats expressed IL-6, IL-1β, TNF-α, and iNOS [39]. Hearts from mice infected with *T. cruzi* also expressed iNOS mRNA and pro-inflammatory cytokines [40]. Moreover, parasite-infected cardiomyocytes express mRNA for TNF-α, IL-1α and iNOS, as well as CXCL3/MIP-2β and CCL5/RANTES among other chemokines [41].

The mechanisms by which iNOS expression is induced by *T. cruzi* in cardiac myocytes remain to be determined. It is possible that parasite-secreted products induce the enzyme directly. Alternatively, iNOS expression may result from autocrine/paracrine stimulation by cytokines and chemokines released from cardiomyocytes and/or inflammatory cells following *T. cruzi* infection. Accordingly, it has been reported that cardiomyocytes infected and stimulated with IL-1β plus TNF-α induced considerable iNOS-derived NO [41]. Furthermore, NO synthesis strikingly correlated with trypanocidal activity exerted by cardiac cells, showing that cardiomyocyte-derived NO is effective enough to kill the parasite [41,42].

Although the IFN-γ-induced cardiomyocyte trypanocidal effect has been indisputably demonstrated, the molecular mechanisms involved in such effect remain uncertain. In the rat cardiac myocyte model, IFN-γ exerted a trypanocidal action, although neither iNOS expression nor significant NO production were recorded [42]. Conversely, *T. cruzi* infected embryonic mouse cardiomyocytes, produced parasiticidal levels of iNOS-derived NO in response to IFN-γ [41]. In this regard, it was also reported that treatment of neonatal cardiomyocytes with IFN-γ plus LPS strongly increased NO production [43]. In any case, the ability of cardiac myocytes to limit the growth of *T. cruzi in vitro*, in response to cytokines, clearly reveals an active role for these cells in resistance against the infection.

Exposure of cardiac cells to pro-inflammatory cytokines in the heart may precondition the myocardium to temporarily protect cardiomyocytes from growth factor deprivation induced apoptosis, and such an effect was due to an increase in Bcl-2 [44]. Interestingly, protection was also enhanced by the pretreatment with cruzipain devoid of enzymatic activity [45].

There are at least two signal transduction pathways responsible for the antiapoptotic properties of cruzipain, the phosphatidylinositol 3-kinase (PI3K)-Akt and MEK-1/ERK pathways. Interestingly, as illustrated in Fig. 2, these mediators are also triggered by *T. cruzi* infection of cardiomyocytes [45]. In this regard, ERK phosphorylation was also relevant to up-regulate parasite entry in human macrophages by another parasite antigen, the trypomastigote-cloned surface ligand, gp83 trans-sialidase [46]. These results indicate that *T. cruzi* uses released antigens to signalling target cells to its own benefit.

The data discussed herein clearly show that *T. cruzi* belongs to one of the most striking examples of how a parasite benefits itself in several ways from manipulating the apoptotic pathways of host cells, favoring its own survival in the parasitized host (for more details on the role of cytokines and apoptosis in parasite growth see DosReis et al. in this issue).

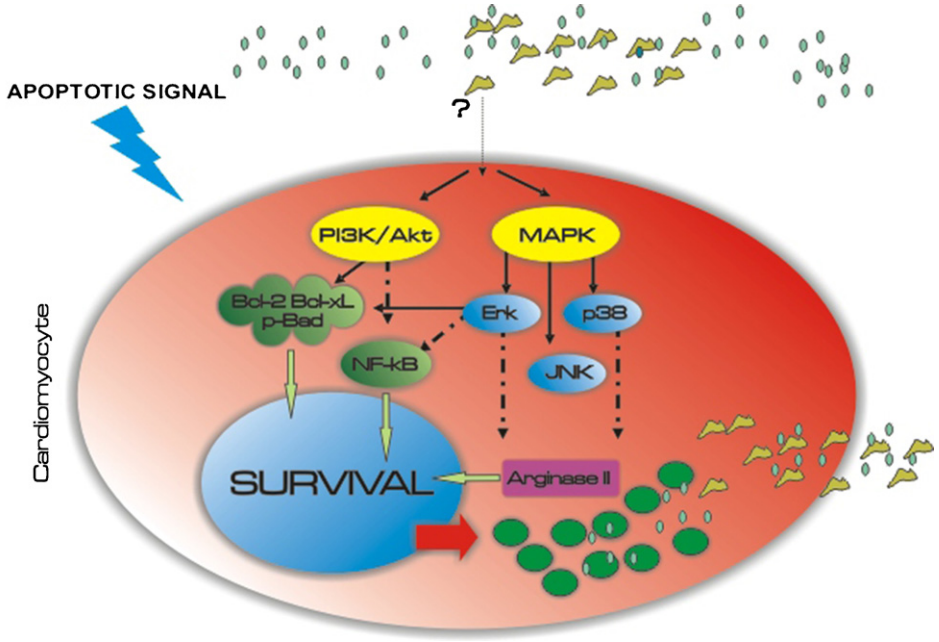


Fig. 2. Molecular components of signalling pathways triggered in cardiomyoytes by *Trypanosoma cruzi* and cruzipain. In this schematic diagram the parasite or cruzipain activates PI-3K/Akt and MEK1/ERKs pathways. They can differentially modulate Bcl-2 family members, resulting in cell protection from apoptosis exerted by serum deprivation. Additionally, cruzipain activates arginase. Altogether these events maintain the target cell alive, favoring parasite survival and replication.

3. Adaptative immunity in *T. cruzi* infection: role of cytokines in protective and pathogenic immune responses

Divergent disease outcome during *T. cruzi* infection is thought to result from complex interactions between the parasite and components of the anti-parasite immune response. Thus, the BALB/c and C57BL/6 murine models were found to be useful in view of their respective susceptibility and resistance to a series of intracellular pathogens, among them *T. cruzi*. Compared to the C57BL/6 background, BALB/c mice seemed to be more susceptible when challenged with the Peru strain of *T. cruzi* [47]. Nevertheless, other authors have shown that both mouse strains were equally susceptible to the Peruvian strain of the parasite, whereas C57BL/6 mice were more resistant to the *T. cruzi* Colombian strain [48]. By contrast, increased mortality of C57BL/6 mice was reported for Tulahuén strain infection [49]. This finding was confirmed with C57BL/6 mice which exhibited cachexia and a progressive fatal disease, whereas 60% of the BALB/c mice recovered [50]. Moreover, the disease severity seen in C57BL/6 mice was not linked to an increased parasitemia, myocardial parasite nests or amastigote number in peritoneal macrophages, which did not differ from the data recorded in BALB/c animals. Interestingly, C57BL/6 mice showed increased TNF-α levels in serum and cultures of *T. cruzi*-infected macrophages. In agreement with this finding, low levels of IL-10 during the early and intermediate phases of the acute disease were found [51]. This observation is in line with data showing that TNF-α exerts a deleterious effect in American trypanosomiasis [52]. On the other hand, IL-10 may play an important immunoregulatory role in this experimental infection, as demonstrated by the role of this cytokine in balancing the TNF-α-driven proinflammatory response in *T. cruzi*-infected mice [53]. In any case, the essential role of IL-10 in the immunomodulation of *T. cruzi* infection was illustrated by experiments showing that infection of IL-10-deficient mice resulted in decreased parasitemia, but increased mortality [54,55].

No clear polarization into Th1-cytokine versus Th2-cytokine responses was seen when studying mice susceptible or resistant to *T. cruzi* challenge. Among the possible mechanisms responsible for the pathogenesis of chronic Chagas' disease, autoimmunity is the one that has received much experimental support but also elicited most controversy during the last 20 years [56–59]. Nevertheless, there are studies suggesting that parasite persistence in the host tissues is relevant in the pathogenesis of the disease, since, anti-parasite treatment results in a decrease of disease severity [58]. In this context, Girones and Fresno [60] postulated that *T. cruzi* persistence, together with the specific immune response induced by multiple myocardial antigens, may participate in heart damage. These authors found that autoreactivity found in the chronic phase of *T. cruzi* infection is linked to the parasitemia; the antibody titers and reactive T cells against the *Cha* autoantigen isolated by screening of a library with chagasic sera, were significantly lower in C57BL/6 than in BALB/c mice [57].

Diverse mechanisms may explain how an infectious organism could break immunological self tolerance. Among them, the molecular mimicry, bystander activation and polyclonal lymphocyte activation have been widely documented in both murine and human Chagas' disease [56,57,59]. There is evidence demonstrating that at least two variables could influence the outcome of infection and the induction of autoimmunity, the genetic backgrounds of both parasite and host [56]. Immunization with different *T. cruzi* antigens (cruzipain or ribosomal P proteins) induced altered electrocardiograms despite the fact that inflammatory lesions were not detected in immunized mice [56,61,62].

In humans, *T. cruzi* infection induces both humoral and cellular autoimmunity to several host antigens [59,63,64]. In this respect, elimination of parasite in acute phase could potentially abrogate the induction of autoimmunity [56]. In fact, anti-*Cha* as well as anti-*T. cruzi* antibody titers decreased in parallel with anti-parasite treatment and positively correlated with clinical symptoms [65].

Cunha-Neto et al. [63] proposed that myosin cross-reactive T lymphocytes infiltrating heart tissue are involved in cardiac damage. Moreover, they demonstrated that peripheral blood mononuclear cells from chagasic patients with overt heart disease produced high levels of IFN-γ, but decreased IL-4 production after *in vitro* stimulation with B13 myosin peptide, suggesting that heart damage could result from inflammatory cytokines. The persistence of inflammation may be related to cytokines produced by intralesional heart antigen specific $CD4^+$ T cells upon recognition of *T. cruzi* cross-reactive heart antigen epitopes (exemplified by cardiac myosin) constitutively presented by macrophages [59].

More recently, it was reported a very high frequency of parasite specific IFN-γ producing $CD8^+$ T cells among chronic patients with mild clinical disease compared to those with the most severe form of the disease, supporting a link between the strength and nature of the anti-parasite response and the severity of the disease [66]. In conclusion, it is likely that the parasite acts as a triggering agent, whereas anti-self or anti-parasite T and B cells are the actual effector cells in the pathogenesis.

A further potentially important issue to take into account is the role of regulatory T cells (Treg), which suppress autoimmune responses. In addition to naturally arising Treg cells that leave the thymus as $CD4^+CD25^+$, there are others, Tr1 and Th3, which acquire regulatory capacity in the periphery, and are defined by the cytokine profile: Tr1 cells produce high levels of IL-10 and significant levels of IFN-γ, IL-5 and TGF-β whereas Th3 cells produce high levels of TGF-β [67]. Thus, a definition

of the role of Treg cells during *T. cruzi* infection may contribute with new insights for preventing or controlling the lesions observed in patients with chronic Chagas' disease.

3.1. Role of the cytokine-hormone network during *T. cruzi* infection

As discussed above, several factors are likely to underlie the establishment of Chagas' disease, being dependent on the parasite and the host's immune responses. Endocrine factors are known to affect homeostasis of cell-mediated immune reactions and as such, might be involved in the outcome of trypanosomal infection. Pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, are known to stimulate the production of corticotropin-releasing hormone (CRH) in the hypothalamus which in turn leads to pituitary production of adrenocorticotropic hormone (ACTH), followed by glucocorticoid hormone secretion by the adrenal cortex. These hormones inhibit cell-mediated immunity since they suppress IL-2 and IFN-γ production by inhibiting the transcription of IL-2 and IFN-γ and promote Th2-cytokine production [68].

Recent studies performed in C57BL/6 and BALB/c mouse strains revealed differential hormonal susceptibility upon *T. cruzi* infection. An intense stimulation of the hypothalamus-pituitary-adrenal (HPA) axis was observed 3 weeks after infection in both strains, but corticosterone levels at the first week post-infection were 2–3-fold higher in BALB/c strain. Moreover, blockage of glucocorticoid receptors with the antagonist RU486 accelerated death in C57BL/6J mice and increased lethality to 100% in BALB/c mice, suggesting that the endocrine response accompanying the development of the immune process strongly affect the progression of the disease [69].

Studies performed in BALB/c mice also revealed that at the peak of parasitemia, CRH contents in the hypothalamus were 5-fold lower than controls. Nevertheless, despite the decrease seen in CRH contents, the circulating levels of ACTH in *T. cruzi* acutely infected mice did not change significantly [70]. Overall, these findings point to an unbalance of the HPA axis in acute Chagas' disease. Some pro-inflammatory cytokines such as IL-6 are able to directly stimulate both glucocorticoid and ACTH release [71]. In fact, an increase in the serum levels of IL-6 in infected mice was found, and the *in vitro* infection of ACTH-producing cells resulted in an enhancement of *IL-6* gene expression [70].

Moreover, IL-2 production by these cells was also enhanced after *in vitro T. cruzi* infection [72]. Such effect may promote the local activation of T cells infiltrating the organ. Whether it also modulates the pituitary gland itself is a subject that remains to be determined. Therefore, in addition to the fact that nervous and endocrine systems are targets in Chagas' disease; it is likely that disturbances in neuroendocrine circuits themselves contribute to the pathophysiology of the disease, an issue that certainly deserves further investigation.

4. Dynamics of lymphocyte populations in experimental Chagas' disease

Along with the evolution of Chagas' disease, lymphocyte populations undergo a series of changes in their dynamics, including expansion, differentiation, death, and migration. In particular, lymphocyte death and migration will be detailed below, focusing in their relationship with cytokines, chemokines and adhesion receptors.

4.1. Lymphocyte death in acute infection

It is clear that, as in any infectious condition, there is an expansion of stimulated peripheral lymphocytes following *T. cruzi* infection; some cells differentiate into effector and memory cells, although the majority of them die. Thus, cell death is also relevant in the general pathophysiological process.

4.1.1. Massive thymocyte death in acute Chagas' disease

A common feature seen in a variety of acute infections is a severe atrophy of the thymus, largely reflecting intense lymphocyte depletion secondary to apoptosis. In some cases, thymocyte loss is so severe that the cortical region of thymic lobules virtually disappears [73]. Several research groups demonstrated that mice acutely infected with *T. cruzi* develop a progressive atrophy of thymus with massive death, particularly of cortical thymocytes, bearing the immature $CD4^+CD8^+$ phenotype [48,74–80]. Thymocyte depletion becomes noticeable during early infection to increase progressively in parallel with parasitemia and pro-inflammatory cytokine levels until death, while showing a partial recovering in resistant mice which succeeded in controlling the acute phase. As mentioned above, one mechanism that may be incriminated for such a severe loss of cells in the thymus is apoptosis. Yet, other mechanisms can be involved, such as increase in the export of cells from the organ; decrease in proliferation and low income of bone marrow-derived precursors.

The contribution of apoptosis for thymocyte loss is consistent since the first observation of apoptotic bodies in cortical region of thymuses from acutely infected mice was reported by Savino et al. [74]. Yet, the mechanisms involved in triggering apoptosis are not completely understood. One candidate is the increase in the circulating glucocorticoid hormone levels [69,70,75]. In C3H/HeJ mice infected with the CL strain of the parasite, adrenalectomy did not prevent *T. cruzi*-induced cortical thymocyte depletion [75]. Nevertheless, recent findings revealed that in C57BL/6 mice infected with the Tulahuen strain of *T. cruzi*, a thymocyte loss was stress-related, since inhibition of corticosterone action partially prevented massive $CD4^+CD8^+$ depletion [69].

Recently, the putative role of pro-apoptotic pathways in the generation of thymocyte apoptosis in murine Chagas'

disease was evaluated. Thymic atrophy was seen following acute *T. cruzi* infection in Fas-deficient *gld/gld* as well as in perforin knockout animals, thus ruling out the involvement of the Fas/Fas-L or perforin pathways in this process [81]. In addition, the TNF-α pathway was also investigated: thymuses from mice that have been double-knocked out for type-I and type-II TNF-α receptors exhibited a profound thymic atrophy following *T. cruzi* infection [Perez et al, personal communication]. However, as detailed below, TNF-α is involved in $CD8^+$ T cell apoptosis, seen in mesenteric lymph nodes of *T. cruzi*-infected mice [82].

Analysis of other putative thymocyte depletion mechanism revealed that thymic atrophy was not observed in galectin-3-deficient mice [36], suggesting an important role for this carbohydrate-binding protein in infection-induced thymocyte loss. In keeping with this hypothesis, exogenous galectin-3 did induce apoptosis in thymocytes. Similar finding was seen in peripheral T cells, and in this model, the effect was mediated by the binding to CD29 (β1-integrin chain) and CD7 [83]. Taken together these data strongly indicate that the role of extracellular galectin-3 on thymocyte death is related to integrin signaling, triggering the activation of intracellular apoptotic pathways [84]. In a second vein, recent data showed that trypomastigote forms of *T. cruzi* make use of their surface glycoproteins to bind galectin-3 and to adhere to laminin, with this interaction being crucial for infection [37]. Similarly, human galectin-3 was shown to promote *T. cruzi* adhesion to human coronary artery smooth muscle cells [38]. These data can be placed in the context of the presence of parasites within the thymus, and fact that this galectin-3 binds to laminin (whose intrathymic contents are also increased following infection). Accordingly, it is conceivable that the absence of galectin-3 in the thymic microenvironment of knockout mice would impair the local binding of trypomastigotes to extracellular matrix, thus favoring thymocyte survival to infection.

Two other mechanisms have been involved in the thymic atrophy during experimental Chagas' disease. A non-virulent strain of the parasite did not induce thymocyte depletion, suggesting that parasite-derived factors could be involved. In accord, specific treatment of the acute disease with benznidazole prevents intrathymic $CD4^+CD8^+$ cell depletion [85]. Moreover, Mucci et al. [79] observed that apoptosis seen in thymic nurse cell (TNC) complexes from infected mice could be reproduced with the parasite-derived enzyme trans-sialidase. These findings fit the *in vivo* data showing the intrathymic presence of the parasite in both epithelial and nonepithelial cells of the thymic microenvironment [74,86]. Lastly, thymocytes from infected mice were particularly sensitive to the pro-apoptotic action of extracellular ATP [87]. All together, these data indicate that depletion of thymic lymphocytes during *T. cruzi* infection result from multiple interactions, involving both parasite-derived moieties and endogenous products such as cytokines and adhesion receptors. Downstream signaling pathways involved in triggering these pro-apoptotic events within thymocytes deserve further investigation.

Although the occurrence of thymic atrophy in chagasic patients has not yet been investigated, abnormalities in intrathymic T-cell selection have been observed, suggesting that thymic abnormalities observed in experimental models, might be present also in humans, as discussed below in Section 4.3.

4.1.2. Lymphocyte death in peripheral lymphoid organs

Experimental acute infection by *T. cruzi* is believed to promote T and B lymphocyte polyclonal activation, with general cell expansion in peripheral lymphoid organs, as originally reported by Minoprio et al. [88]. Nonetheless, recent studies strongly indicate regional differences in such a pattern. Although subcutaneous lymph nodes and spleen expand in total cell numbers, an opposite behavior is seen in mesenteric lymph nodes, due to extensive lymphocyte apoptosis, as ascertained by the numbers of $TUNEL^+$ cells, as well as *in vitro* sensitivity to cell death of freshly isolated lymphocytes. Interestingly, such atrophy was independent of the infection route, parasite load or the mouse strain used. Moreover, studies in Fas-L deficient ($gld/gld^{+/+}$) and in type-I TNF receptor ($p55^{-/-}$) knockout mice indicated that both molecules were involved in mesenteric lymph node atrophy, promoting $CD4^+$ and $CD8^+$ lymphocyte apoptosis, respectively. In contrast, perforin does not play any role, as lymph nodes from perforin-deficient animals do not behave differently from the corresponding wild type littermates [82]. These data support the concept that, even in a systemic infection, differential responses can be mounted in distinct lymph nodes, implying the existence of intrinsic regulatory mechanism(s) in these organs.

4.2. Lymphocyte migration disturbances in *T. cruzi* infection

Cumulative data indicate that in addition to the progressive cell death seen in the thymus of acutely infected individuals, the migration patterns of thymocytes are also altered. Such hypothesis was first raised with the observation that the contents of extracellular matrix (ECM) molecules were dramatically augmented in the thymic microenvironment of infected animals [74]. In fact, BALB/c mice infected with Colombian (clonotype I) or CL (clonotype II) strains of *T. cruzi* responded with an increase in the expression of ECM ligands, such as fibronectin and laminin. Moreover, cell surface expression of fibronectin and laminin receptors was increased in thymocytes from infected animals [80]. Considering that ECM ligands and receptors play a role in physiological cell migration within the thymus [89], it was conceivable that intrathymic alterations in their contents might result in changes of thymocyte migration. The first direct evidence appeared with the studies on TNC. These are lymphoepithelial complexes in which one epithelial cell can harbour variable amounts of thymocytes, particularly those

bearing the immature $CD4^+CD8^+$ phenotype [90]. The epithelial component of TNC produces ECM components and ECM-mediated interactions are relevant for the exit of thymocytes.

A decrease in the number and size of TNCs in infected mice was found and the thymocyte release from the remaining lymphoepithelial complexes was faster than the corresponding controls, an event likely due to the enhancement of ECM production that occurs under the infection condition. Similar results were observed *in vitro* [91]. Considering that most intra-TNC thymocytes are immature cells, it is possible to speculate that the migratory capacity of these cells should be enhanced in murine Chagas' disease. A more direct evidence of an abnormal thymocyte migration in *T. cruzi*-infected animals was provided by experiments showing that fibronectin-driven migration was significantly increased in $CD4^+CD8^+$ thymocytes from infected animals [80].

In addition to typical ECM glycoproteins, there is evidence that galectin-3 is also involved in the migration of these cells. Interestingly, galectin-3 is augmented in the thymus of infected animals, and in cultures of thymic epithelial cells, infected *in vitro* [36].

It is well established that chemokines play a role in regulating thymocyte migration [92,93]. Moreover, it is noteworthy the existence of chemokine-ECM association: for example, CXCL12 binds to and is presented by fibronectin, promoting receptor polarization and enhanced lymphocyte migration [94]. Additionally, fibronectin and laminin enhanced the chemotactic activity of CXCL12 to immature $CD4^+CD8^+$ thymocytes *in vitro*, suggesting the involvement of chemokine-ECM interactions in normal thymocyte migration and development [95,96]. In the context of *T. cruzi* infection, an increase in the intrathymic deposition of CXCL12 was detected, with enhanced co-expression of this chemokine and fibronectin (Fig. 3). Similar data were found regarding the expression CCL4 in the thymus of infected animals.

Concomitantly to the enhanced chemokine ligands, thymocytes from infected animals exhibited higher membrane levels of CXCR4, and similar findings were seen in respect to CCR5—one of the CCL4 receptors. When the CD4/CD8-defined thymocyte subpopulations were analyzed, thymocytes from infected mice showed higher densities of both CXCR4 and CCR5, particularly in the immature $CD4^+CD8^+$ subset. Functionally, thymocytes from infected animals migrated significantly more than control thymocytes, particularly when CXCL12 was added as a chemoattractant. Furthermore, CXCL12 plus fibronectin showed significant synergy upon $CD4^-CD8^-$ and $CD4^+CD8^+$ immature thymocyte migration, and this response was correlated with the enhanced expression of CXCR4 receptor and fibronectin receptors (particularly VLA-4) during *T. cruzi* infection [97].

Overall, these data suggest that multiple cellular and molecular interactions involved in cell migration are altered within the thymus as a consequence of *T. cruzi* infection. Such changes are likely involved in the abnormal release of immature thymocytes. In this regard, the export of cells escaping from the process of intrathymic negative selection may have consequences in the immunopathology of the disease.

4.3. Escape of immature T cells from the thymus in *T. cruzi* infection

As a consequence of the abnormal thymocyte migration, the thymus releases immature $CD4^+CD8^+$ T lymphocytes in the acute and chronic phases of murine Chagas' disease. It was initially suggested that the increase in peripheral $CD4^+CD8^+$ T lymphocytes could be prevented in animals that were thymectomized prior to infection [80]. More direct evidence came with the analysis of the so-called ''recent thymic emigrants'' (RTEs), which can be tracked in the periphery, following an intrathymic labeling of lymphocytes with a fluoresceinated conjugate [95]. Mice infected with the clonotype II *Tulahuen* strain revealed enhanced numbers of RTEs bearing the $CD4^+CD8^+$ phenotype (Fig. 4). Thymic depletion is a complex phenomenon involving death and escape of thymocytes that, when altered, may have important functional consequences in impairing normal central tolerance, thus favoring the development of autoimmune responses. The thymus is the major source of naturally occurring-regulatory T cells involved in organ-specific autoimmunity [98]. In this respect, their loss may worsen the outcome of Chagas' infection. Accordingly, chronically *T. cruzi*-infected rats subjected to adult thymectomy showed an exacerbation in myocardial damage [99]. This result indicates that circumventing thymic output in the adult host may interfere with the generation of T cells capable of down-regulating immunopathological responses. Moreover, the enhanced output may not be restricted to αβ thymocytes, but also to the γδ subpopulation. In this regard, it has been showed that continuous thymic export in the adult is a requisite to sustain suppressor γδ T cell activity during acute *T. cruzi* infection [100].

The positive and negative thymic selection shapes the TCR-repertoire. The diversity of the T cell repertoire in the periphery is established by competition between different T-cell subpopulations including RTEs [101]. Moreover, it is known that T cells expressing different variable domain of TCR-β chain generally exhibit functionally distinct characteristics [78]. Hence, changes in TCR selection events may be relevant for the resolution of *T. cruzi* infection [102]. In addition, different studies showed that thymus of *T. cruzi*-infected mice and peripheral T cells of chagasic patients have qualitative changes in the expression of Vβ families suggesting that the selection of the T-cell repertoire was altered during the infection [73,103,104]. In this regard, increased numbers of cells bearing the potentially autoreactive phenotypes $CD4^+CD8^+TCRV\beta5^+$ and $CD4^+CD8^+TCRV\beta12^+$ were found in subcutaneous lymph nodes of acutely or chronically infected BALB/c mice (that normally delete these Vβ families within the thymus) challenged with the clonotype I Colombian strain [105].

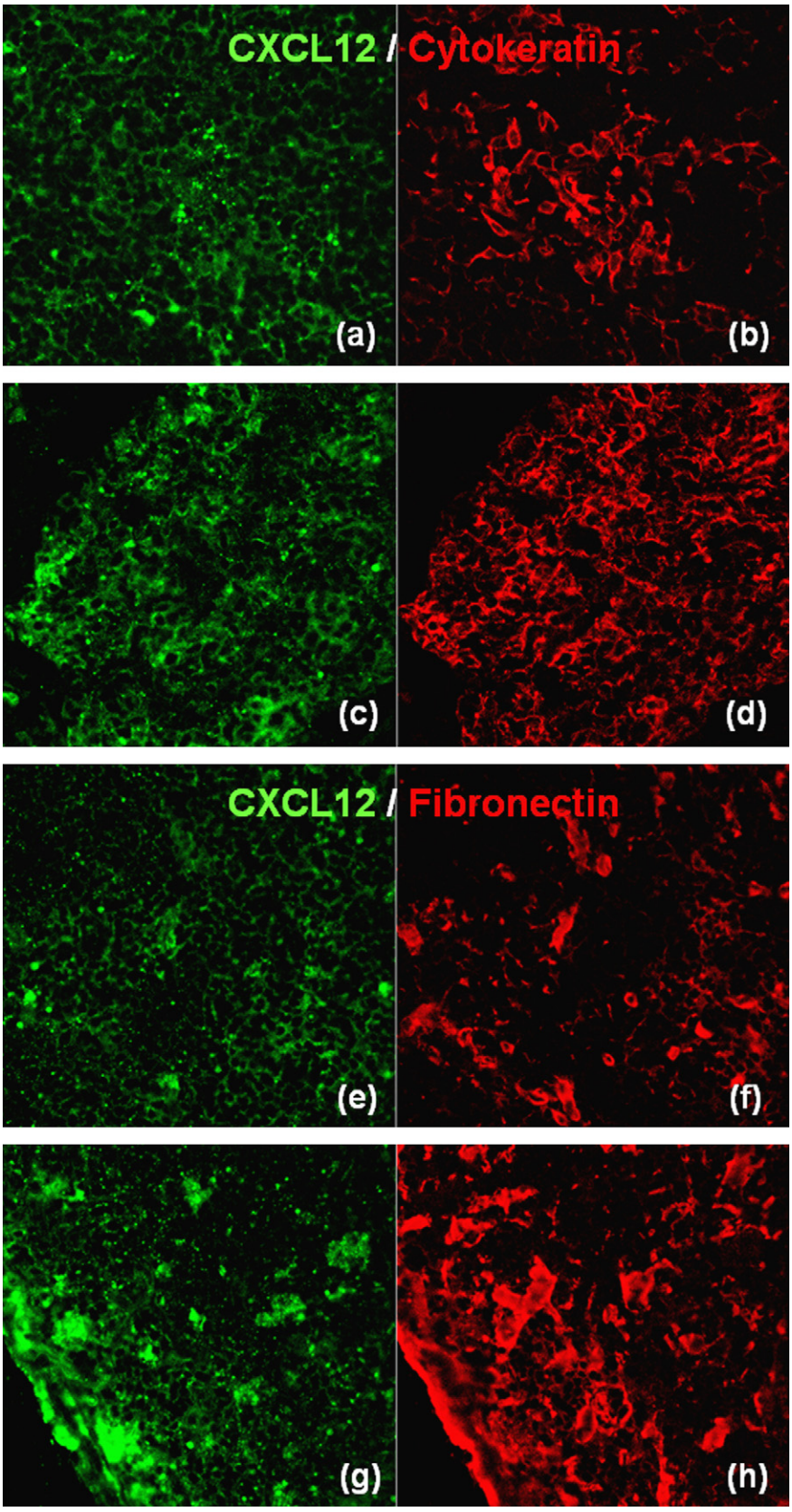


Fig. 3. Enhancement of CXCL12 and fibronectin contents in the thymus *T. cruzi* acutely infected mice. Confocal microscopy immunofluorescent images show co-localization of CXCL12 with thymic epithelial cells (labeled with the anti-cytokeratin antibody in control (a, b) and acutely infected mice (c, d). In both cases CXCL12 is essentially produced by the thymic epithelium but chemokine labeling is more intense after infection. The set of bottom images reveals co-localization of CXCL12 with fibronectin in thymus sections from control (e, f) and infected (g, h) mice. Co-localization of both molecules is apparent. Magnification ×400.

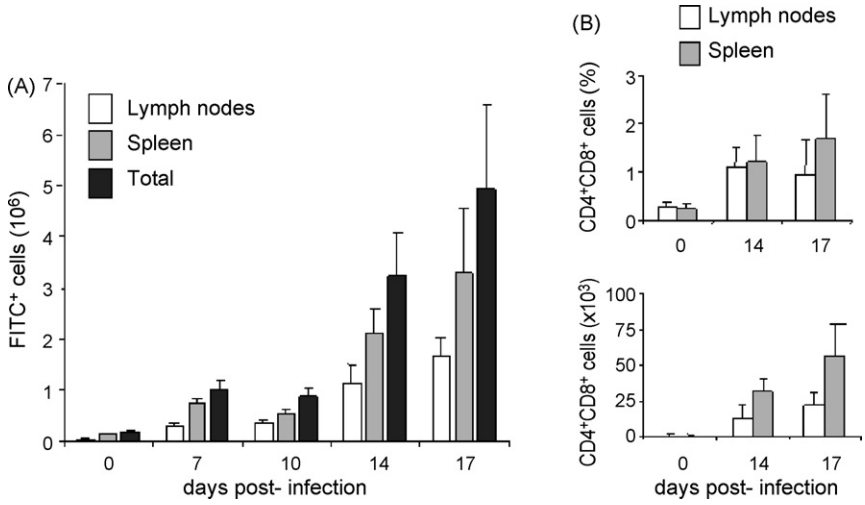


Fig. 4. Increased export of $CD4^+CD8^+$ lymphocytes from the thymus of *T. cruzi* infected mice. Control and *T. cruzi* (*Tulahuen* strain) acutely infected mice received intrathymic injection of FITC, and were analyzed 16 h later by flow cytometry, so as to detect recent thymic emigrants ($FITC^+$ cells) in peripheral lymphoid organs. Panel A clearly shows that the absolute numbers of recent thymic emigrants progressively increased with the infection. Such increase comprises the abnormal release of immature $CD4^+CD8^+$ lymphocytes, whose relative and absolute numbers are progressively higher, in both lymph nodes and spleen as seen in panel B.

Such cells abnormally released from the thymus of infected animals, have bypassed intrathymic negative selection events, and apparently differentiate in the periphery into single positive cells. In this respect, $CD4^+$ T cell-mediated autoreactivity against myocardial cells has been demonstrated in murine as well as in the human Chagas' disease.

The mechanisms involved in the premature release of immature thymocytes into the periphery of *T. cruzi* infected subjects have not yet been analyzed. In this sense, multiple molecular interactions may contribute to this abnormal export. For example, $CD4^+CD8^+$ peripheral T cells seen in experimental Chagas' disease exhibited higher amounts of ECM receptors including the integrins VLA-4, VLA-5 and VLA-6, indicating that abnormal ECM-mediated interactions could favour the release of immature thymocytes [80].

The accumulation of galectin-3 in the thymus of infected animals may also contribute to the escape of immature cells from the thymus. Recent studies revealed that galectin-3 induces activation of α5β1 (VLA-5) fibronectin receptor and stimulates α5β1-integrin-mediated activation of focal adhesion kinase and PI3-K leading to increased fibronectin polymerization and cell motility [106]. These results are supported by the data showing that thymus-derived $CD4^+CD8^+$ cells are not seen in peripheral lymphoid organs of *T. cruzi*-infected galectin-3 null mice [36].

If there are consistent data on the participation of ECM-mediated interactions in the abnormal export of immature thymocytes in *T. cruzi*-infected animals, much less is known on the putative role of cytokines and chemokines. Evidence indicates that, at least TNF-α is involved. In fact, TNF-α expression is enhanced in thymuses from fetuses and offsprings from infected mothers [107], and in thymus treated with trans-sialidase [108]. Additionally, mice subjected to a LPS desensitization protocol before challenge with the Tulahuen strain showed diminished TNF-α levels after infection and a significantly reduced thymic atrophy [109]. Moreover, some studies reported that TNF-α enhanced cell adhesion to ECM [110,111] as well as chemokine and chemokine receptor expression [112–114]. In this regard, preliminary data indicate that TNF-α enhances fibronectin-driven migration of thymocytes from infected animals [Perez et al., personal communication]. Whether chemokines are also involved in immature thymocyte release remains to be determined.

4.4. Influx of leukocytes towards T. cruzi target tissues

A variety of soluble (chemokines) and unsoluble (ECM) secretory factors, have been suggested to play a key role in the influx of immune cells to target tissues in murine and human Chagas' disease. CCR5 and CXCR3 expression may be regarded as markers of type-1 and type-2 immune responses [115]. Studies to evaluate the co-expression of these chemokine receptors on $CD4^+$ and $CD8^+$ T cells producing type-1 or type-2 cytokines from chagasic patients revealed that the percentage of single positive cells coexpressing CCR5 and IFN-γ, CXCR3 and IFN-γ, and CXCR3 and TNF-α were higher in patients with cardiopathy than in indeterminate individuals. By contrast, the proportion of T $CD4^+$ or $CD8^+$ cells coexpressing CCR3 plus IL-10 or coexpressing CCR3 plus IL-4 were lower in the former group, implying that chemokines may be involved in the early development of chronic cardiomyopathy [116]. In keeping with this notion, a significant reduction in cardiac cellular infiltration was seen in CCR5 knockout mice, as

well as normal mice, infected with *T. cruzi* and treated with the CCR5 inhibitor, met-Rantes [117,118].

Recent studies revealed the abundance of CXCL9, CXCL10 and CCL5 within the cardiac tissue of mice acutely and chronically infected with the clonotype I Colombian strain. Moreover, simultaneous blockage of CXCL9 and CXCL10 with neutralizing antibodies resulted in a consistent decrease of parasitemia and parasite load in tissue, although the degree of inflammation within the heart was not altered [30]. Differently, neutralization of CCL3/MIP-1α using specific antibodies reduced macrophage influx toward the spleen, but increased heart inflammation, and did not yield significant changes in tissue parasitism [29]. Overall, these findings clearly indicate a complex network, in which specific chemokines play distinct roles in the pathophysiology of the disease.

Extracellular matrix-mediated interactions also seem to play a relevant role in the influx of leukocytes towards the cardiac tissue in Chagas' disease. The putative role of laminin was approached in a mouse model of heart transplantation. When newborn-derived syngeneic hearts were grafted into the subcutaneous tissue of a chronically infected adult recipient, the grafted was rejected through a $CD4^+$ T cell-dependent mechanism. [119]. Migration of activated anti-myocardial $CD4^+$ cells into the grafted tissue could be prevented by treating the cells with anti-CD49f (α-chain of the laminin receptor VLA-6) or the surrounding tissue of the host with anti-laminin antibodies. [120]. This is in keeping with the increase in membrane levels of VLA-6 in peripheral lymphocytes from chagasic mice, and associated with the rejection process, there was an increase in the local amounts of laminin. Importantly, such an increase could be prevented when the connective tissue surrounding the graft was pre-treated with anti-laminin antibodies [121]. Together, these data show that in experimental Chagas' disease, laminin-mediated interactions are involved in the influx and/or activation of anti-myocardial $CD4^+$ cells within the target tissue. Moreover, the fact that galectin-3 is used by *T. cruzi* trypomastigote forms to adhere to laminin and to human coronary artery cells [37,38] suggests the existence of a coordinated participation of ECM and ECM-related proteins in the pathology of cardiac injury following infection.

Considering that laminin is able to bind both IFN-γ and TNF-α [122,123], it is possible that this matrix glycoprotein may concentrate these cytokines in cardiac lesions, allowing them to be better presented to cells of the immune system. The relationship between the laminin contents and the inflammatory process were performed by evaluating the ear lobes of transplanted mice, treated with anti-laminin prior to injection of $CD4^+$ T cells from chronically infected mice. In this group, the amounts of lymphocytes within the grafts were always smaller when compared with the age-matched grafts of control antibody injected animals.

In order to verify whether the treatment with the anti-laminin antibody resulted in modulation of pro-inflammatory laminin-bound cytokine expression, heart grafts were analyzed for cytokine detection, particularly IFN-γ and TNF-α. Pre-treatment with the anti-laminin reagent significantly reduced the number of IFN-γ^+ cells [121]. Presumably, the binding of cytokines to ECM molecules provides the signal(s) necessary to the anchorage of responsive cells during inflammatory responses. These data indicate an entrapment of pro-inflammatory cytokines by enhanced laminin deposition in the inflamed site, thus favoring the influx of effector cells (Fig. 5). In this context, laminin-mediated interactions should be regarded as a further potential target for immune intervention in Chagas' disease.

A clearcut relationship between ECM contents and inflammatory reaction has also been defined in neuroendocrine tissues of *T. cruzi* infected animals. The presence of inflammatory cells in brain areas of infected mice has been reported, being particularly rich in $CD8^+$ T lymphocytes [124]. Moreover, pituitary gland and adrenals of infected animals also exhibit clusters of $CD8^+$ T cells, and to a lesser extent, $CD4^+$ cells and macrophages [70]. This effect was associated with an increase of fibronectin and laminin deposition in both endocrine glands of infected mice. Moreover, i*n vitro* infection of ACTH-producing cells resulted in an enhancement of ECM production, suggesting a direct effect of the parasite and/or parasite-derived antigens on the endocrine cells [70].

In brain areas of infected animals, an increase of ECM deposition has also been documented, in agreement with the inflammatory infiltrate [125]. In this respect, adhesion of infection-induced activated T cells on tissue slices of brain areas, as well as penetration of these cells after passive transfer *in vivo*, could be abrogated with anti-CD49d receptor antibody, which is able to block VLA-4 interaction with VCAM-1, as well as fibronectin [125]. In this same line, *in vitro* experiments co-culturing spleen-derived T cells from control or infected mice, with neuronal cells revealed an increase in ECM-deposition in infected cultures. Moreover, adhesion of T cells derived from *T. cruzi*-infected mice to neuronal cells was enhanced, being dependent on ECM-mediated interactions [72].

Taken together, these findings indicate that ECM-mediated interactions are also functionally relevant to drive the influx of T cells towards nervous and endocrine tissues of *T. cruzi*-infected mice. A detailed study of the cytokines and chemokines involved in this process is an interesting open field for further investigation. Table 1 summarizes the data showing that, at least in the murine Chagas' disease, chemokine- and adhesion receptor-mediated interactions are involved in the progression of the inflammatory infiltrate seen in target tissues.

It should be noted that ECM-mediated interactions are likely important in the intracellular infection. In this regard, expression of cell surface receptors for fibronectin [126] and laminin by the parasite have also been documented [127,128]. Moreover, as mentioned above *in vitro* infection enhances ECM production [72,129], and RNAi-mediated blockage of the intracellular production of α_1-laminin chain

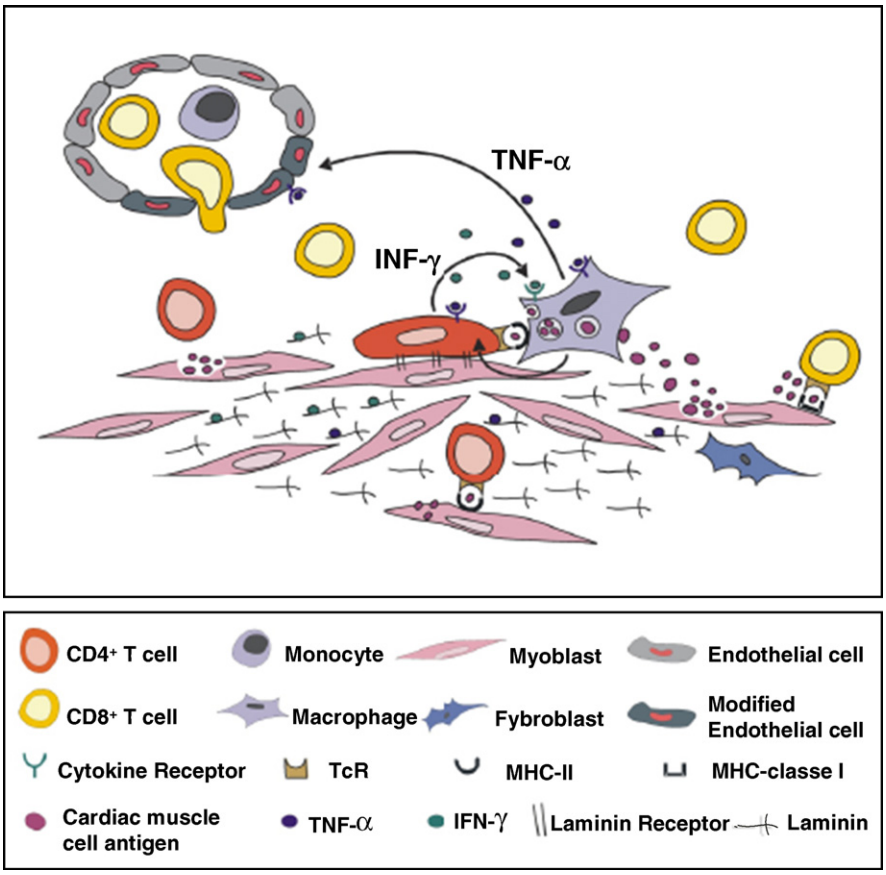


Fig. 5. Relationship between the inflammatory infiltrate, laminin contents and deposition of pro-inflammatory cytokines. In this diagram, we propose that, once activated, $CD4^+$ T cells reach the heart, the levels of IFN-γ produced by these cells can trigger a chemotatic migration of mononuclear cells including macrophages, dendritic cells, and cytotoxic $CD8^+$ T cells. IFN-γ activates macrophages to produce iNOS, TNF-α and IL-1, and modulate the expression of MHC class I molecules on inflammatory and myocardial cells. Once laminin content is locally augmented – through synthesis by stromal cells and/or cardiomyocytes –, the leukocyte recruitment is further maintained through the haptotactic effect of this protein. The influx of effector cells to the grafted area is diminished, as well as the local deposition of inflammatory cytokines, when the local laminin expression is reduced, suggesting a potential role of this molecule in regulating the autoimmune response against cardiac muscle cells.

or thrombospondin [130,131] resulted in significantly decreased infection.

We can therefore speculate that ECM-mediated interactions might play a multi-faceted role in the pathophysiology of the disease, contributing to parasite infection of host cells and concentrating cytokines/chemokines thus enhancing the inflammatory infiltrate.

Finally, data obtained in genetically engineered mice indicate that cell–cell membrane interactions also play a relevant role in the influx of inflammatory cells to the cardiac tissue of infected animals. Acutely infected-*ICAM-1-deficient* mice exhibited a large reduction in the cardiac inflammatory infiltrate, as compared to corresponding wild types [132].

Table 1
Control of leukocyte influx towards target tissues in *Trypanosoma cruzi* infected mice: role of chemokines and adhesion receptors

Target receptor	Target ligand	Experimental approach	Methods of evaluation	References
CCR5	CCL3 and CCL5	Injection of receptor antagonist	Immunohistology of the cardiac inflammatory infiltration	[118]
CCR5	CCL3 and CCL5	Infection in CCR5 knockout mice	Immunohistology of the cardiac inflammatory infiltration	[119]
VLA-6 (CD49f/CD29)	laminin	Treatment with neutralizing antibodies	Heart graft rejection and immunohistology of the cardiac inflammatory infiltration	[120,121]
VLA-4 (CD49f/CD29)	VCAM-1 and fibronectin	Treatment with neutralizing antibodies	Immunohistology of the brain inflammatory infiltration	[125]
ICAM-1	LFA-1	Infection in ICAM-1 knockout mice	Immunohistology of the cardiac inflammatory infiltration	[132]

5. Concluding remarks and perspectives

The extensive data discussed herein clearly indicate that cytokine/chemokine-mediated circuits, as well as interactions involving adhesion receptors, play a crucial role in the pathophysiology of Chagas' disease, thus placing these molecular interactions as potential targets for therapeutical approaches.

Nevertheless, there are a large number of missing points that, once resolved, shall be helpful in designing new therapeutic strategies for this disease. Among those, we can list: (a) comparative studies on the immunology of Chagas' disease due to type-1 versus type-2 *T. cruzi* clonotypes, including both innate and adaptative immune responses; (b) better definition of the imbalance of cytokine network during the evolution of Chagas' disease; (c) the use of immunodeficient animals for better understanding the immunology/immunopathology of human Chagas' disease; (d) better understanding of how the immune system operates in the context of co-infection involving *T. cruzi* and other(s) infectious agent(s); (e) levels of cell migration-related receptors in distinct cells of the immune system, including in the switch from the asymptomatic to cardiac or digestive forms of chronic Chagas' disease; (f) clear-cut definition of potential targets to immunological-based therapy in Chagas' disease; (g) search for immunological markers of the disease (e.g. cytokine and chemokine levels). These still unresolved issues should be included in an agenda for future investigation.

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