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Targeting T Cells to Treat *Trypanosoma cruzi*-Induced Myocarditis

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http://dx.doi.org/10.5772/55301

1. Introduction

1.1. Myocarditis

In 1995, the last World Health Organization (WHO)/International Society and Federation of Cardiology (ISFC) Task Force on the definition and classification of cardiomyopathies defined myocarditis (also named "inflammatory cardiomyopathy") as an "inflammatory disease of the myocardium associated with cardiac dysfunction" [1]. In myocarditis, the inflammatory infiltrate of the myocardium is associated with necrosis and/or degeneration of adjacent myocytes, which is not typical of – nor consistent with – myocardial ischemic damage seen with coronary artery disease [1, 2]. The clinical presentation of myocarditis is dependent upon the magnitude of myocardial inflammation, thus it may be quite variable. Clinical signs and symptoms may range from subclinical disease (which may initially be unrecognized) to new-onset acute heart failure or sudden death due to ventricular arrhythmias [3]. Moreover, the clinical course of myocarditis may be as variable as its clinical presentations: some individuals may develop acute myocarditis that resolves spontaneously within a few weeks, while others may develop symptoms of chronic heart failure due to dilated cardiomyopathy (DCM) [3]. Although many patients with hemodynamically stable heart failure may respond well to optimal medical therapy, a significant percentage of patients with DCM become medically refractory and progress to irreversible end-stage heart failure for which heart transplantation becomes the only hope of survival. Indeed, it is estimated that acute myocarditis resolves completely in approximately 50% of cases, with an additional 25% of patients having incomplete recovery (i.e.; partial normalization of cardiac function), while the remainder 25% will inexorably progress to end-stage heart failure and death [1, 4-6].

Despite its seemingly over simplistic definition, myocarditis is a disease with multiple heterogeneous etiologies, which in turn lead to a highly variable and very complex pathology. The etiologies of myocarditis can be divided into three groups: infective, immune-mediated and toxic. Infective myocarditis may be bacterial (including gram-positive cocci, gram-negative rods, gram-negative cocci, mycobacterium, mycoplasma); spirochetal (Borrelia, Leptospira); fungal (including Aspergillus, Candida, Histoplasma and Cryptococcus, among others); protozoal (including *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania sp*); parasitic (*Tenia solium*, *Echinococcus granulosus*, *Trichinella spiralis*); rickettsial (e.g., *Coxiella burnetii*); and viral (including adenovirus, influenza A and B, Coxsakievirus, poliovirus, HIV-1, herpes simplex, and varicella-zoster, among many others). Immune-mediated myocarditis may be due to allergens (tetanus toxoid, serum sickness, drugs such as penicillin, cephalosporins, furosemide, isoniazide, tetracycline, among many others); alloantigens (as seen in heart transplant rejection); and autoantigens such as "idiopathic" (or "virus-negative") lymphocytic and giant cell myocarditis, as well as "secondary", i.e., associated with auto-immune disorders such as systemic lupus erythematosus, vasculitides, rheumatoid arthritis, myasthenia gravis, inflammatory bowel disease. Toxic myocarditis may be due to drugs (cocaine, ethanol, lithium, cyclophosphamide, etc); heavy metals (copper, iron, lead); hormones (pheochromocytoma); as well as miscellaneous etiologies such as radiation, certain spider or snake venoms, scorpion sting, arsenic, and carbon monoxide [3].

This extraordinary multitude of ethiopathogenetic agents underscores the fact that proper and accurate diagnosis of myocarditis at the tissue and molecular level is of utmost importance because it may impact therapeutic choices as well as short- and long-term prognosis. Although management of myocarditis should ideally consist of very specific and targeted therapeutic strategies that go beyond symptomatic control of heart failure and temporary reversal of cardiac dysfunction, such therapies are not clinically available for patients with most types of myocarditis.

Myocarditis should be suspected on the basis of clinical presentation and imaging data, and objective diagnosis should be made by endomyocardial biopsy (EMBx) using established histological, immunological and immunohistochemical criteria combined with molecular biological techniques, particularly polymerase chain reaction (PCR) and nested-PCR [1, 2, 7]. Histopathological analysis is essential to reach a classification of myocarditis based on histological criteria (i.e., lymphocytic, giant cell, granulomatous, etc), while semi-quantitative assessments of the specimens with regards to myocyte necrotic damage/inflammatory activity ("grading") and to measure the extension of fibrosis and architectural changes ("staging") have also been proposed [2]. Large panels of antibodies should be performed to characterize the inflammatory cell population and the activated immunological processes. Immunohistochem-istry increases the sensitivity of EMBx, while amplification methods such as PCR are capable of detecting few copy viral genomes even from an extremely small amount of tissue such as an EMBx specimen [2]. A combination of these techniques will most likely reveal the patho-logical nature of myocarditis and help predict which patients may respond to immunomodu-latory therapies or not [8].

2. Pathophysiology and ciinical presentation of *Trypanosoma cruzi*-induced myocarditis

In the particular case of myocarditis induced by *Trypanosoma cruzi* infection, there is a distinct disturbance in myocardial microcirculation with both vasoconstriction at the arteriolar level and coronary vasodilation, as well as microaneurysm formation and ventricular fibrosis which ultimately lead to congestive heart failure and ventricular arrhythmias [9]. Left ventricular apical aneurysm is considered to be pathognomonic of Chagas disease, consisting of thinning of the left ventricular apex, with a clear reduction of the myocardium due to fibrosis. Mural thrombus is a frequent finding. Depending on the severity of cardiac dysfunction in infected patients, the heart may maintain its normal volume or be mildly enlarged. However, patients who die of chronic advanced or acute heart failure oftentimes have severe DCM with or without hypertrophy and intramural thrombosis in the right atrium and left ventricular apex. These patients usually have rounded hearts, venous congestion, and dilated chambers mainly on the right side [10].

In this review we will focus on the importance of the acquired immune response to the control of *T. cruzi*-induced myocarditis and discuss the possibility of targeting T cells to treat the disease.

3. *Trypanosoma cruzi* infection

In 1909, Brazilian physician Carlos Chagas, M.D., identified a hemoflagellate parasite in a child's blood, leading to the discovery of the American Trypanosomiasis, or Chagas disease (named in his honor). Dr. Chagas accomplished a unique feat in the history of medicine: not only did he identify a new disease, but he also discovered the invertebrate vector and its biological characteristics; isolated the causative agent – *Trypanosoma cruzi* (named in honor of his mentor, Dr. Oswaldo Cruz) – and described its life cycle; identified the epidemiological characteristics of the disease and its symptoms; and defined the disease's diagnostic criteria. Many years later, the disease was also found to be prevalent in many other Latin American countries. Because the chronic manifestations of Chagas disease (particularly chronic heart disease) affect patients in their most productive years of life, the disease carries a heavy social and economic burden.

The disease can be transmitted by transplacental infection or during childbirth, organ trans-plantation, laboratory accidents with contaminated sharp objects, blood transfusion, or ingestion of food or drink contaminated with infected vectors or their feces. During the process of natural infection in endemic areas, *T. cruzi* parasites are transmitted by the infected feces of blood-sucking reduviidae bugs, mainly *Triatoma infestans* and *Rhodnius prolixus*. These insects typically live in poorly-constructed homes with cracks and crevices on the walls and roof, and are very active at night, when they feed on human blood [11]. The bugs defecate while biting exposed areas of the skin and, despite the injection of anesthetics and inhibitors of blood clothing, the person instinctively smears the bug feces into the bite. The parasite then gains

access to adjacent tissue through skin breaks or mucosal surfaces such as eyes and mouth. Infective metacyclic trypomastigote forms invade macrophages and other cell types and differentiate into proliferative amastigote forms [12]. These cytoplasmic forms differentiate into trypomastigote forms that disrupt the host cell membrane and are free to be transported by blood and infect other cells, such as cardiomyocytes.

The infection is followed by a typically benign acute-phase that lasts up to two months. In this period, high numbers of circulating parasites are observed in blood. Symptoms, when present, may include fever, headache, enlarged lymph nodes, pallor, muscle pain, difficulty in breathing, swelling and abdominal or chest pain. All patients will then enter a chronic phase, which starts with a so-called "indeterminate" asymptomatic period. Most chronic patients will remain asymptomatic throughout their lives. However, about 10% will develop digestive tract (enlargement of the esophagus and/or colon, known as "megaesophagus" and "megacolon"), neurological or mixed symptoms; and about 30% will develop Chagasic myocarditis, the most common cause of death in infected patients [13].

Twenty years ago, the number of infected people was estimated at 16-18 million, with about 100 million people at risk of contracting the disease [14]. This dire epidemiological situation has improved thanks mostly to a combined effort by many Latin American countries to control the burden of transmission through insecticide spraying and serologic screening in blood banks. Contemporary estimates indicate that approximately 10 million people are infected with *T. cruzi* worldwide, and about 25 million people are considered at risk of contracting the disease [15]. Despite the reduction in the number of infected people, the dynamic movement of human populations from and to endemic areas in Latin America, the recrudescence of vector-borne transmission, the risk for domestication of silvatic species of invertebrate hosts, and the increased importance of secondary vector species still make the infection an imposing challenge [14].

An important aspect of the infection in the current globalized world is the broader geographic distribution of infected patients. In the last decades, many cases of Chagas disease were reported in the USA, Canada, Europe and some Western Pacific countries. Most of those cases were considered "imported" because they originated from infected Latin American immigrants [16]. This changing geographical distribution highlights the increasing necessity to heighten efforts to combat the spread of the disease and to develop new strategies to treat *T. cruzi*-infected patients.

4. Pathogenesis of *Trypanosoma cruzi*-induced myocarditis

In the acute phase, many cardiomyocytes are parasitized [17]. This process typically occurs in close proximity to extensive and diffuse inflammatory foci, which consists mostly of mononuclear cells. However, opposite to what is observed in the acute-phase of the disease, parasites are much less frequently found in the heart of symptomatic chronic patients, despite the persistence of extensive mononuclear inflammatory foci. Contrary to what was previously hypothesized, chronic heart involvement in Chagas disease most likely does not rely on

autoimmune mechanisms, but on parasites persistence [18]. However, the reason why most patients will not develop chronic myocarditis and heart failure is unknown to this date. It is postulated that the final outcome of the infection results from a complex and random combination of pathological characteristics, including microcirculatory derangements; micro ischemia; significant impairment of the autonomic nervous system due to ganglia cells death; deregulation of the immune system balance; progressive cardiomyocytolysis induced by parasite nests; individual genetic background; malnutrition; and comorbidities.

Experiments using murine infection and *in vitro* systems showed that the innate immune response takes over the control of the infection shortly after the contact with the parasite, with NK cells producing high levels of gamma interferon (IFN-γ), which then controls the early replication of parasites in host cells [19]. Macrophages are very important to control the infection, producing nitric oxide (NO) that limits the burden of intracellular parasites. Mast cells are also very important in this scenario and we have recently published that infected CBA mice treated with cromolyn, a mast cell stabilizer, have much greater parasitemia and IFN-γ levels, and higher mortality rates, myocarditis, and cardiac damage [20].

With regards to acquired immunity, a number of published reports support the role and importance of both CD4 and CD8 T cells in the control of the infection. Experimental approaches can be used to deplete sub populations of lymphocytes, including the use of thymectomized mice; injection of neutralizing antibodies; or the infection of *nude/nude* mice [21-23]. On the other hand, human data is based on the identification of T cell subsets in postmortem specimens, which generally shows the predominance of CD8+ lymphocytes with few macrophage-like, NK or plasma cells [24]. The predominance of CD8+ T cells starts in the early acute-phase of the infection and extends to the chronic phase both in experimental models and human patients. Although chronic *T. cruzi*-induced myocarditis seems to have a very complex pathology, the immune system, especially CD8+ T lymphocytes, is considered a key player in this condition. Despite many efforts, it is still not clear which cytotoxic cells and molecular pathways employed by T lymphocytes may be contributing to the death of cardiomyocytes. We tested whether perforin, a major cytotoxic molecule employed by CD8+ T lymphocytes, was important to the death of cardiomyocytes during the infection [25]. However, we observed that the molecule was important for myocarditis control, because in the absence of this cytotoxic pathway, cardiac cellular infiltration was much more intense, but without increased signs of damage to myocytes. In this review, in this review we will summarize some data from the literature discerning biochemical pathways that target T lymphocytes migration and their effector function in the myocardium, and the possibility of targeting these cells to treat *T. cruzi*-induced myocarditis.

5. Treatment of *T. cruzi* infected patients

During the 1960s, two new drugs proved to be effective *in vitro* and *in vivo* in the treatment of Chagas disease: nifurtimox, a nitrofuran [3-methyl-4-(5-itrofurfurilidenoamino) tetrahydro-4H-1, 4-tiazin-1,1-dioxide, Bayer 2502]; and benznidazole [N-benzyl-2-nitroimi-

dazole acetamide, RO 7-1051]. Although these drugs have been widely used since then, therapeutic efficacy varies according to the phase of the disease (acute or chronic), duration of treatment, patient's age and geographical area of original infection [13]. The best results are obtained with recently infected patients, when cure rates of 60 to 80% can be achieved, as opposed to cure rates no greater than 10% in chronic patients, depending on the severity of cardiac dysfunction [26].

The side-effects of nifurtimox include anorexia, weight loss, insomnia, nausea, vomiting, and others. Benznidazole-associated side-effects are classified in three types: (i) hypersensitivity manifestations, such as dermatitis with cutaneous eruptions, periorbital or generalized edema, fever, lymphadenopathy, and muscular and articular pain; (ii) depression of the bone marrow, among which neutropenia, granulomatosis, and thrombocytopenic purpura: (iii) peripheral polyneuropathy, in the form of paresthesia and polyneuritis.

More recently, new progenitor cell-based therapies have been developed with good and promising results. In this therapy, total bone marrow cells are collected from individual patients and a mononuclear cell-enriched preparation is slowly injected into the left and right coronary systems. No adverse effects have been described with this procedure [27] and a few months after treatment some patients had improved cardiac function. However, it is still necessary to characterize the phenotype of the transferred cells and the mechanisms underlying such improvement in cardiac function.

The lack of effective treatments for most chronic symptomatic patients reinforces the need for new drugs and strategies for treating *T. cruzi* infected patients. This could include the development of anti-parasite drugs based on the elucidation of biochemical pathways of the parasite and/or on particular aspects of the immune response triggered by the infected host.

6. Molecular therapies

Advances in basic research that focus on interconnected molecular pathways in the immune system led to the design of more specific therapeutic strategies. Many autoimmune and inflammatory diseases can be treated using humanized or fully human-derived antibodies; fusion proteins targeting co-stimulatory molecules; or injection of competitive ligands. Neutralization of molecules involved in endothelial transmigration (CD11a/CD18, for example), T lymphocyte activation (CD80/CD86 and CD28; CD25) or function (CD2, lymphocyte function-associated antigen 3 (LFA-3) and cytotoxic T-lymphocyte antigen 4 CTLA-4) are now being used with very good results [8]. However, in the case of *T. cruzi* infection, the inflammatory response is very important to control parasite burden and to maintain the immunological equilibrium during the infection. This means that an effective treatment would have to be specific enough to silence the pathogenic components of the immune system but still allow a protective response, especially to the heart. The importance of inflammation in the control of the infection is illustrated by a number of experimental approaches that block normal T cells ontogeny/development (infected nude *nu/nu*, RAG$^{-/-}$, and thymectomized mice), endothelial transmigration (blockage of adhesion molecules such as ICAM-1 and CD11a), or

function (IFN-$\gamma^{-/-}$ and perforin$^{-/-}$ mice) [28, 29, 25]. In all these models, after T lymphocyte inactivation, the infection was much more aggressive with higher mortality rates and increased blood and intracellular parasitemia. Previous results indicate that this delicate balance between an efficient or harmful inflammatory response relies on multiple aspects of the normal physiology of T lymphocytes, and these may be targeted for future therapeutic strategies.

7. T lymphocyte-based possible targets for treating *T. cruzi*-induced myocarditis

7.1. T lymphocytes senescence

Immunological senescence of memory T lymphocytes is a very interesting aspect of the immune response against pathogen-based and sterile inflammation in general, not only in *T. cruzi* induced myocarditis. Normal temporary exposure of naïve T cells to antigens in an appropriate context of activation signals leads to cellular proliferation and differentiation into effector and memory T cells. Memory T lymphocytes are generated in much smaller quantities and are retained for longer periods of time to fight against a potential subsequent exposure to the same antigen, eliciting a more rapid and effective response. However, prolonged exposure of T lymphocytes to pathogen-derived antigens or endogenous danger signals leads to the accumulation of a heterogeneous memory T cell population with unique characteristics regarding the phenotypic profile and functional activities. These memory T cells are generally regarded as CD8$^+$/CD28$^-$ (or CD8$^+$/CD57$^+$) T cells, as the loss of CD28 is counterbalanced by the expression of CD57 in this population [30]. The loss of CD28 and gain of CD57 expression on T cells during persistent immune stimulation is characteristic of humans and non-human primates but probably not of mice. Although CD8$^+$/CD28$^-$ T cells are seen in mice, they are not the result of chronic antigenic stimulation, do not express CD57 and represent a distinct subset of naturally occurring CD8$^+$ T cells. Amongst this population (CD8$^+$/CD28$^-$), there is a sub population of memory T cells that was described to be increased in severe *T. cruzi*-induced myocarditis (CD8$^+$/CD27$^-$/CD28$^-$) and this particular phenotype is expressed by cells that are at the latest stage of memory activation. This means that they are closest to memory terminal differentiation and senescence, differentiated to a point where co-stimulatory signals are no longer sufficient to induce normal memory T cell response. It seems that the phenotypic sequence of memory stages is CD27$^+$/CD28$^+$; CD27$^-$/CD28$^+$ or CD27$^+$/CD28$^-$; and CD27$^-$/CD28$^-$ for cells that are 'early', 'intermediate' and 'late' stages of memory CD8$^+$ T cells, respectively [31].

It was first shown that chronic patients with cardiac enlargement and clinical or radiological evidence of heart failure have a higher frequency (%) of late activated memory CD8$^+$ T cells (CD27$^-$/CD28$^-$) in blood, when compared with patients that present mild cardiac alterations [32]. Accordingly, the frequency of early activated CD27$^+$/CD28$^+$/CD8$^+$ T cells in the total memory CD8$^+$ T cell population decreases, as disease becomes more severe. The authors hypothesize that there is a gradual clonal exhaustion of this sub-population of early activated

memory CD8$^+$ T cells, perhaps as a result of continuous antigenic stimulation by persistent parasites

It is still not known if there is indeed a causative relation between the increase of CD8$^+$/CD27$^-$/CD28$^-$ memory cells in chronic *T. cruzi* infected patients and the more severe clinical status of myocarditis and cardiac dysfunction. However, it is interesting to speculate that these cells could have a suppressive activity over protective CD8$^+$ T lymphocytes (Fig. 1). If this is true, the death or functional suppression of protective CD8$^+$ T lymphocytes observed in severely affected patients could be a result of late stage senescent memory T cells. A similar interaction has been described for tumor cells [33]. In this case, CD8$^+$/CD27$^-$/CD28$^-$ have a suppressive activity over the proliferation of (protective) effector T lymphocytes, and this function requires cell-to-cell contact. In fact, *T. cruzi* specific late stage memory CD4$^+$/CD27$^-$/CD28$^-$ T lympho-cytes are also increased in more severely affected cardiac patients, when compared with patients with mild myocarditis, as observed in the CD8$^+$ compartment [34]. It is important to highlight that these senescent memory T cells, which can be CD8 or CD4 T cells, are distinct from CD4$^+$ T regulatory (TReg) cells that express the transcriptional factor FoxP3 [35].

Although this immunological characteristic of memory T lymphocytes senescence would probably be hard to be used as a target for treatment, these peripheral blood mononuclear cells (PBMC) markers could be used as a predictive tool for the severity of potentially developing myocarditis in chronic patients in the undetermined stage.

7.2. Chemokines and T lymphocyte migration to infected myocardium

One very important aspect of the myocarditis induced by *T. cruzi* infection is to know which chemotactic mediators are produced by the cardiac tissue and which effector cells migrate to the tissue. Ultimately, the cardiac microenvironment will determine the balance between the control of parasite growth and avoidance of inflammatory secondary damage and cardiac dysfunction. In this regard, it was shown that cardiomyocytes do not act as passive players facing the infection. Indeed, these cells become activated and secrete NO, through the activity of the induced NO synthase (iNOs) enzyme; chemokines; and pro-inflammatory cytokines [36]. These mediators destroy intracellular parasites or act on inflammatory cells in the vicinity [37]. Among these mediators, we find tumor necrosis factor (TNF), interleukin (IL)-1beta (IL-1β), and chemokines growth-related oncogene (GRO or CXCL1), monokine induced by interferon-gamma (MIG or CXCL9), macrophage inflammatory protein-2 (MIP-2), interferon-gamma-inducible protein (IP-10 or CXCL10), monocyte chemotactic protein (MCP-1 or CCL2), and regulated and normal T cell expressed and secreted (RANTES or CCL5). Moreover, inflammatory cells composing cardiac inflammatory foci also produce cytokines and chemo-kines, composing an environment that is rich in pleiotropic inflammatory mediators.

Chemokines are small (8-14 kDa) constitutive or inducible inflammatory cytokines, compris-ing four protein subfamilies (CXC or α, CC or β, C or γ, and CX3C or δ) that act through trans-membrane spanning G protein-coupled receptors expressed on the surface of several leukocyte and other cells. Chemokines are mostly known by their chemotactic capacity, but they also play a role in angiogenesis; dendritic cell maturation; tumor growth and metastasis; and others.
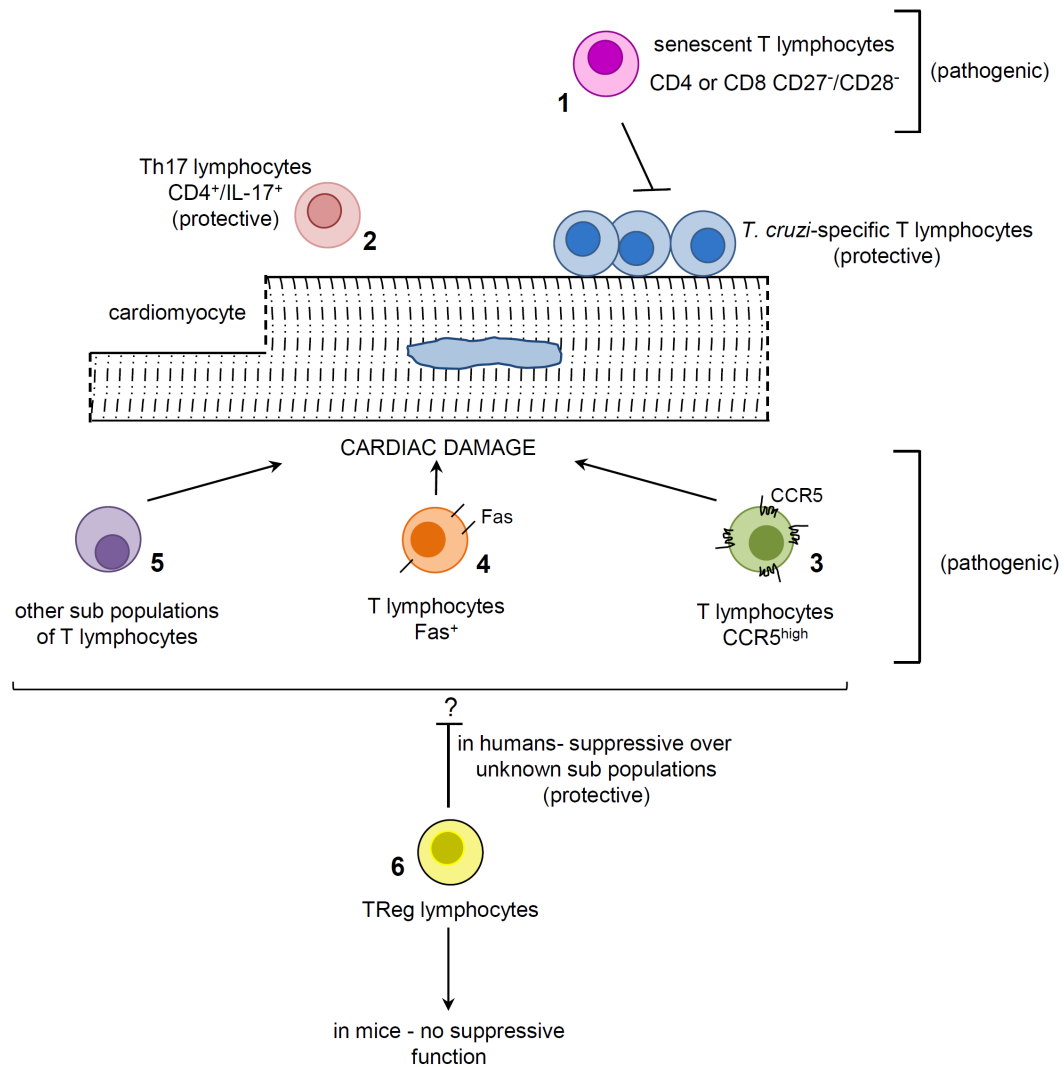
Figure 1. Cardiac pathogenic and protective T lymphocytes in *Trypanosoma cruzi* infection. Myocarditis and cardio-myocyte damage are considered pivotal in the progression of cardiac dysfunction. Therefore, the balance between pathogenic and protective T lymphocyte sub populations may determine the severity of the cardiac pathology induced by the infection. Senescent (1) CD4 and CD8 late stage memory T lymphocytes (CD27-/CD28-) are enriched in blood of patients more severely affected. It has been hypothesized that these cells act as suppressor cells over protective T lymphocytes, as observed in some tumors. CD4+ T lymphocytes secreting IL-17 (Th17) (2) are protective for *T. cruzi*-induced myocarditis, as the inactivation of this cytokine leads to increased susceptibility and cardiac inflammatory infiltration. Some directly pathogenic T lymphocyte sub populations are enriched in the heart of patients with more severe myocarditis. This was observed for T lymphocytes expressing high levels of the chemokine receptor CCR5 (3), T lymphocytes (and maybe other cells, like macrophages) expressing Fas (4) and possible other pathogenic T lymphocytes that remain to be uncovered (5). T regulatory (TReg) lymphocytes apparently do not play a role in the control of myocarditis in murine infection (6), but are enriched in blood of chronic asymptomatic ones (6), when compared with cardiac symptomatic patients. Although still not known what sub populations of T lymphocytes are suppressed by TReg lymphocytes, we suggest some sub populations in the diagram. (?) Means not experimentally tested.

These functions are mostly mediated by the activation of many protein kinases, increased cytoplasmic Ca^{++} and mainly activation of transcription factors [38].

In the case of non-experimental infection with *T. cruzi*, it was found that patients with severe chronic chagasic cardiomyopathy have higher levels of TNF and CCL2 (CCR2 ligand), when compared with patients with mild cardiac dysfunction [39]. Conversely, enhanced expression of CCR5, a chemokine receptor for some CC chemokines (CCL3/MIP1α, CCL4/MIP-1β, CCL5/RANTES), and CXCR3 was found in PBMC from patients with cardiomyopathy, when compared with asymptomatic patients [40]. Taken together, these data suggest that not only cytokines, but also chemokines and their receptors, may be involved in the cardiac pathogenesis associated with *T. cruzi* infection, especially CCR5^{high} T lymphocytes (Fig. 1), what could be explored in future therapeutic designs. This is illustrated by human polymorphisms that show that migration of CCR5$^+$ T lymphocytes to the heart is associated with a more severe human and experimental cardiomyopathy. Namely, studies of CCR5 59029A/G gene polymorphism in Peruvian and Venezuelan patients revealed that the G allele, which reduces CCR5 expression, is found more frequently in asymptomatic than in symptomatic chronic patients [41].

The idea that some CC chemokines, and particularly CCR5 receptor, could be involved in the pathogenesis of *T. cruzi*-induced myocarditis has been tested in experimental infection [42, 43]. Chronically infected mice were treated with N-terminal-methionylated RANTES (Met-RANTES), a selective CCR1/CCR5 antagonist, and the treatment led to a reduction in the number of cardiac parasite nests, fibrosis, and cardiomyocytes damage, as ascertained by creatine kinase (CK-MB) levels in blood. Moreover, there was an increase in the expression of connexin 43, a major component of gap junctions in the heart, and iNOs. These results are very important as a possible alternative for myocarditis treatment, especially if we consider that these mice were treated in the chronic phase, when the cardiac dysfunction is many times irreversible.

7.3. Th17 immune response

When a naïve CD4$^+$ T lymphocyte encounters an antigen presenting cell (APC), it has the potential to differentiate into a T (helper) h1; Th2; Th3 (secreting mostly TGF-β and IL-10 and usually found in mucosa); inducible regulatory T lymphocyte (iTReg - cited in a following item); or Th17 lymphocyte. This commitment is mostly based on the cytokines secreted by the APC, which will interact with cognate cytokine receptors on the lymphocyte's surface and lead to the activation of the JAK/STAT (Janus kinases/Signal Transducers and Activator of Transcription proteins) pathway. The differentiation of cellular subtypes induced by the cytokines is mostly based on different combinations of JAK proteins and STAT transcription factors. In mammals, there are four members of the JAK family (JAK1, JAK2, JAK 3 and Tyk2) and seven members of the STAT family (STAT1-4; 5A; 5B; 6). These signaling molecules will ultimately induce the expression, or repression, of many genes that will orchestrate the final cellular differentiation, including the panel of cytokines that will be secreted by the final lineage committed CD4$^+$ T lymphocyte [44]. Th1 cells are mainly induced by IL-12 and produce mostly IFN-γ, TNF-α, IL-2 and IL-12; while Th2 cells are mainly induced by IL-4 and produce IL4, IL5, IL-6, IL-10, and IL-13. In humans, the cytokines that instruct Th17 cell lineage development likely include IL-6; IL-21; IL-23; and IL-1β, with TGF-β playing a role in the suppression of Th1

cell lineage commitment. Then, STAT3 is necessary for gene clusters transcription, ultimately leading to the expression of their lineage-defining transcription factors, which are some retinoid orphan receptors (ROR). Th17 cells secrete mainly IL-17A, IL-17F, IL-21, IL-22, IFN-γ, IL-4, IL-10, IL-9, and IL-26 [45] and were initially described as destructive cells that induced autoimmunity and inflammatory diseases. However, more recently it became clear that they also play a role as protective cells, at least in the case of pathogenic infection with *C. albicans* and *S. aureus*.

Targeting IL-17 alone with Secukinumab (AIN457) or Ixekizumab, both fully human neutralizing antibodies against IL-17A, has been shown to lead to clinical improvement in patients with psoriasis, rheumatoid arthritis, and other auto-immune diseases. On the other hand, in the case of experimental *T. cruzi* infection, Th17 response appears to be protective against the infection (Fig. 1). IL-17A-deficient mice infected with *T. cruzi* have a lower survival rate, display prolonged and higher parasitemia, multiple organ failure, and increased markers of tissue injury when compared with infected C57BL/6 (wild type) mice [46]. Moreover, mice treated with neutralizing antibodies against IL-17 showed signs of more severe myocarditis, with more mononuclear cells migrating to the tissue [47]. According to these results, IL-17 secretion plays a role in the control of the infection and, differently from other inflammatory diseases, should not be treated by neutralizing IL-17.

7.4. Cell membrane fas/fas-L interaction

Fas agonistic stimulus was formerly a synonym of apoptosis. However, Fas/Fas-L interaction can no longer be inextricably associated with cell death. Fas-linked downstream pathways can lead to cellular survival; proliferation and/or activation, cytokines and chemokines secretion; genes transcription; inflammatory regulation; etc [8]. The Fas molecule is a type I membrane protein that belongs to the tumor necrosis factor (TNF) family, and is normally distributed as monomers on cell surface. These monomers spontaneously and temporarily group into non signaling oligomers, but agonistic activation through trimers of Fas-L leads to conformational changes and trimerization/coupling of Fas to intracellular signaling pathways. With regards to apoptosis, it has been demonstrated that two adjacent trimeric Fas complexes are sufficient to induce a functional response [48]. Alternative splicing of Fas generates soluble molecules (sFas) that retain the ability of binding to Fas-L and inhibit Fas-L-dependent responses. Fas-L is a type II membrane protein belonging to the TNF receptor family and can also exist as a membrane (mFas-L) or a soluble molecule (sFas-L). SFas-L is generated by matrix metalloproteinase (MMP7) and sFas-L monomers have no proapoptotic activity, as long as they do not induce Fas trimerization. On the other hand, sFas-L shows proinflammatory functions, acting as a strong chemotactic factor for polymorphonuclear cells, although not involved in neutrophils activation [8].

Many groups have published that Fas activation in the heart of experimental models or human patients leads to enhanced inflammation, cardiac dysfunction, and hypertrophy. Accordingly, lack of Fas/Fas-L interaction results in less severe myocarditis and cardiac involvement. To date, it has been shown that murine myocarditis induced by coxsackievirus B3 was reduced in mice treated with anti-Fas-L, in Fas-deficient mice (*lpr/lpr*), and in Fas-L-deficient mice (*gld/*

gld). In infected wild type mice, γδ T lymphocytes selectively kill protective Th2 CD4$^+$ T cells through a Fas-based pathway, enriching the inflamed heart in pathogenic Th1 cells [49]. When the Fas/Fas-L pathway is silenced, Th2 cells are enriched in the organ, what counterbalances the activity of Th1 cells and reduces cardiac inflammatory response and damage [50].

With regards to a possible molecular therapy for myocarditis that modulates Fas/Fas-L interaction, a likely alternative would involve the blockage of the pathway, which however is very complicated. The injection of competitive ligands or neutralizing Abs can mislead to general Fas inactivation and important side effects could be induced by indiscriminate lack of apoptosis, such as tumor growth and metastasis, and reduced normal turnover of cells. Moreover, the Fas/Fas-L pathway is coupled to many different cytoplasmic signaling mole‐ cules that lead to a number of different cellular responses in different populations. This makes very difficult to predict what kind of side effects could be observed [8].

In the case of myocarditis induced by *T. cruzi* infection, we observed that infected *gld/gld* mice have a very modest cardiac inflammatory infiltration, when compared with infected wild type mice, suggesting a pathogenic role for Fas-bearing cells (Fig. 1)However, despite this promis‐ ing finding, we observed that both lineages have high mortality rates [51]. Apparently, the death of infected *gld/gld* mice is due to a more severe and earlier renal inflammatory infiltra‐ tion/damage, while the death of infected wild type mice seems to be mostly related to myo‐ carditis and cardiac dysfunction [52]. There are complex organ-specific modulatory roles played by Fas/Fas-L interaction, and more studies are necessary to approach this pathway therapeutically. If possible, one of the most promising options would be the injection of non-agonistic humanized Abs against Fas to avoid cardiomyocytes death through this pathway [8]. This would probably not induce bystander cell death or trigger the proinflammatory activities of this pathway. Another alternative could be the inactivation of downstream signaling molecules of the Fas pathway to reduce cardiac inflammation, hypertrophy, and dysfunction. Inhibition of Fas-1,4,5-inositol triphosphate cascade with genistein, xestospongin C, or herbimycin A prevented apoptotic and non-apoptotic cardiac dysfunction. This pathway is functionally interconnected to the PI3K/AKT/GSK3beta pathway that acts in concert to cause nuclear factor of activated T cells (NFAT) nuclear translocation. The elucidation of these Fas-based biochemical pathways responsible for unwanted outcomes in the cardiac function may help to design more efficient therapies in the future. On the other hand, it is noteworthy that any prolonged treatment blocking the Fas pathway could be dangerous.

7.5. Regulatory T cells

Regulatory T cells (TReg) were first described by Sakagushi et al [53] and consist of a thymus-derived sub-population of T lymphocytes (natural TReg cells) that have suppressive activity over effector peripheral T cells, avoiding autoimmunity. However, TReg cells can be generated in the periphery, and these cells are known as induced TReg cells. TReg cells were phenotyp‐ ically described as CD4$^+$/CD25$^+$ and use a molecular arsenal to silence peripheral effector T cells, such as membrane IL-10 and TGF-β; CTLA-4; and others [54].

In the particular case of *T. cruzi*-induced myocarditis, there is a controversy regarding these regulatory T cells when considering animal models and results obtained from human subjects

(Fig 1). Apparently, in mice these cells have no regulatory function over effector cardiac T cells [55]. On the other hand, it was observed that these cells are enriched in chronic asymptomatic patients, when compared with chronic symptomatic ones [56]. A better discrimination of the phenotype of these cells shows that $CD4^+/Foxp3^+/CD25^{high}$ TReg cells from chronic non-cardiomyopathy patients produce higher levels of IL-17, IL-10 and granzyme B. This correlates with increased apoptosis of effector (pathogenic) cardiac T cells and maintenance of a better cardiac function [57]. Regulatory T cells would probably not be targeted for myocarditis therapy, but instead could be used as a prognostic marker for cardiac dysfunction.

8. Conclusion

All molecular pathways cited here could potentially be used to silence pathogenic T lympho-cyte sub-populations that lead to myocarditis, or as a predictive tool for patients that have the potential to develop myocarditis and cardiac dysfunction. Despite this targeted modulation of sub-compartments of the immune system, the capacity of controlling the infection should in general terms be preserved to ensure infection resistance.

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