

Cellular and genetic mechanisms involved in the generation of protective and pathogenic immune responses in human Chagas disease

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Perhaps one of the most intriguing aspects of human Chagas disease is the complex network of events that underlie the generation of protective versus pathogenic immune responses during the chronic phase of the disease. While most individuals do not develop patent disease, a large percentage may develop severe forms that eventually lead to death. Although many efforts have been devoted to deciphering these mechanisms, there is still much to be learned before we can fully understand the pathogenesis of Chagas disease. It is clear that the host's immune response is decisive in this process. While characteristics of the parasite influence the immune response, it is becoming evident that the host genetic background plays a fundamental role in the establishment of pathogenic versus protective responses. The involvement of three complex organisms, host, parasite and vector, is certainly one of the key aspects that calls for multidisciplinary approaches towards the understanding of Chagas disease. We believe that now, one hundred years after the discovery of Chagas disease, it is imperative to continue with highly interactive research in order to elucidate the immune response associated with disease evolution, which will be essential in designing prophylactic or therapeutic interventions.

Key words: pathology - protection - T-cells - immunoregulation - Chagas disease

Chagas disease: contemporary concerns of a century disease

The first records of human infection with *Trypanosoma cruzi* date back nine thousand years, to a time when the first humans peopled the Andean coast. Molecular analyses of tissues extracted from human mummies of the Northern Chile and Southern Peru regions showed the presence of the parasite's kinetoplast DNA in 41% of the samples taken (Aufderheide et al. 2004). Contact of these human groups with other mammals infected with the parasite seemed to have been the main route of disease transmission. It is believed that upon the first human infections, the domiciliation process begun, which provided the vectors with protection to climate changes and predators, consolidating the human disease (Guhl et al. 2000).

Chagas disease was first detected in the Andes and even today it remains mostly restricted to Latin America. Several factors contribute to this restricted distribution, but the most important is likely related to socio-

economic factors. The close contact between the vector, the reservoirs and humans is critical for disease transmission. In fact, recent studies have shown that infected domestic animals are important reservoirs for sustaining natural transmission in endemic areas (Levy et al. 2006, Gurthler et al. 2007). Poor housing conditions offer an adequate nesting environment for the vectors and the proximity to mammals, including man, offers abundant food for these insects. Recent studies have demonstrated both the presence of potential vectors of *T. cruzi* in the United States and a large number of infected animal reservoirs (Beard et al. 2003, Hancock et al. 2005). Despite this, Chagas disease is not endemic in the United States, emphasising the importance of both natural components and socio-economic aspects in maintaining endemicity.

While natural transmission is still the most important form of disease transmission in Latin America, other forms now have epidemiological importance. Infection via blood transfusion or organ transplantation, directly associated with the lack of blood/organ screening for the parasite, has brought the disease to non-endemic countries (Leiby et al. 2002). Moreover, ingestion of contaminated non-pasteurised fruit juices was recently responsible for new cases of Chagas disease in areas where acute cases had not been detected for over 15 years (Steindel et al. 2008), suggesting a repopulation by the vectors in such areas.

Thus, although Chagas disease was discovered 100 years ago, it remains a contemporary public health con-

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cern, even more so because of the threat of its emergence in non-endemic areas and re-emergence in some endemic areas where it was thought to have been controlled. Taken together, these data point to a critical need for Chagas disease control in the following areas: (i) vector control programs that are not restricted to certain areas, but rather extended to all countries with active transmission; (ii) reliable blood bank surveillance, even in areas where the disease is considered non-endemic; (iii) improvement of socio-economic conditions; (iv) efficient parasite-targeted veterinary care and (v) reliable prophylaxis or therapeutic interventions. Multidisciplinary approaches and consistent support are essential for achieving these goals, which will ultimately lead to disease control at all levels.

Clinical progression of human Chagas disease: from asymptomatic to severe forms

Following infection with *T. cruzi*, individuals undergo an acute phase that lasts between two and four months. With the exception of the mucosal edema that may appear at the site of infection (when in the eye, this is referred to as Romaña sign), the signs and symptoms associated to the acute phase of Chagas disease are mostly non-specific, making it difficult to detect infection at early stages. While the acute phase is characterised by high numbers of parasites in the bloodstream, blood smear examination requires expertise and is only performed upon suspicion of *T. cruzi* infection. If the infection is diagnosed, treatment is offered to the patients and cure may be observed in as many as 75% of cases (Sosa-Estani & Segura 2006, Rassi et al. 2007). This highlights the importance of developing reliable and easily available tests for detecting acute infection, as well as providing proper training to health professionals and care to the affected populations. Although a relatively high success rate is observed upon treatment, the currently available drugs are toxic and specific formulations for children, a group in which infection is highly prevalent, does not exist. Therefore, finding new parasitocidal compounds and new formulations will significantly improve disease treatment.

If the infection is not treated and cured, individuals will enter the chronic phase of the disease. The transition from acute to chronic phase is accompanied by a marked decrease in parasitaemia, due to the mounting of a relatively effective immune response, which keeps parasite frequency at below detectable levels in the host throughout the entire chronic phase of the disease. Despite the low parasite levels, it is during the chronic stage that patients may develop the most severe forms of Chagas disease. Figure 1 summarises the clinical progression of human Chagas disease.

As in most parasitic diseases, the vast majority of the individuals who are infected develop a relatively mild form of the disease, in which no clinical symptoms or signs are observed. Past or present infection with the parasite in many cases is only identified by the presence of specific antibodies directed against the pathogen. These individuals, who are serologically positive for anti-*T. cruzi* antibodies, are classified as indetermi-

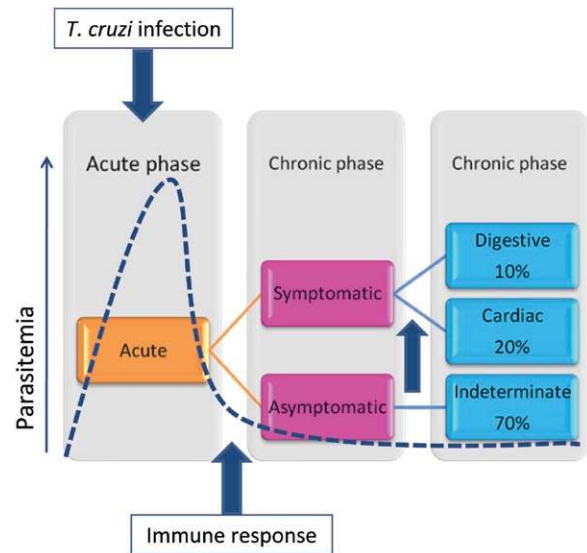


Fig. 1: clinical evolution of human Chagas disease. Upon infection with *Trypanosoma cruzi*, individuals undergo an acute phase that lasts between 2-4 months and is characterized by high parasitemia. Detection of acute disease is one of the challenges of human Chagas disease, since individuals who live in endemic areas often do not display symptoms and, if symptoms appear, they are usually unspecific. As infection is detected, treatment is offered to the patients and about 75% of the treated acute patients are cured. In the case that disease is not cured (or detected early on), patients will enter the chronic phase of disease. The transition from acute to chronic phase is accompanied by a marked decrease in parasitemia, as a result of the host's immune response. Most patients within the chronic phase are asymptomatic, classified as indeterminate. However, a significant percentage of the patients become symptomatic and develop pathology associated to cardiac or digestive tissues, which may lead to death. The reasons why some individuals remain asymptomatic while others develop severe pathology are not completely understood to date.

nate and often harbour the parasite throughout their entire lives without ever developing clinical disease. Thus, indeterminate patients represent a dramatic example of co-adaptation between host and parasite in Chagas disease and, considering the long co-evolution of the host-parasite relationship in this case, it makes sense that the majority of infected individuals remain asymptomatic. Still, a considerable percentage of infected patients will develop severe forms of Chagas disease. Destruction of neuronal and muscle fibres in the digestive system leads to the digestive form of Chagas disease which, in very severe cases, leads to dilation of digestive structures and loss of motility of the oesophagus and intestine. Among symptomatic individuals, the most frequent and morbid form is the cardiac clinical form, which presents as a spectra of different diseases. Chagas patients classified as cardiac may have relatively mild heart commitment, only detectable by refined clinical exams such as Doppler Echocardiography and 24-h Holter monitoring. Other patients, also classified as cardiac, present with severe heart disease as a result of damage to conductive or muscle structures, or both. In cardiac patients, it is common to

observe heart enlargement and loss of contractile function, characteristic of chronic chagasic cardiomyopathy, which often leads to death (Rocha et al. 2007).

The differences in the clinical courses observed in chronic Chagas disease, as well as the variation observed within the same clinical form, suggest that different pathogenic mechanisms are associated with the clinical status of the patients. Thus, refined clinical characterisation, obtained through well-defined patient groups, is a key element to be considered when analysing immunological as well as epidemiological data. The combination of clinical and basic research is essential in determining strategies for better understanding disease progression and pathology.

The million-dollar question in human Chagas disease research is "how can we halt the establishment of pathology?" In order to answer this question, researchers must first establish what factors mediate the different clinical progressions among patients. Given the complex nature of the host-pathogen relationship, this is not an easy task. However, it poses an exciting challenge and the pursuit of this knowledge will lead to concrete benefits to the large contingent of infected patients, as well as to those at risk of infection. In the following paragraphs, we will discuss the theories of pathology development and the immune response associated with pathology or protection in human Chagas disease.

Mechanisms of pathogenesis in human Chagas disease: controversies and consensus

There are two main hypotheses seeking to explain the mechanisms of pathogenesis in human Chagas disease. The first of these defends the pivotal role of parasite's persistence in the host as a major cause of pathology, while the other postulates that an immune response against self antigens is responsible for the tissue damage observed in affected organs of chagasic individuals (Kierszenbaum 2005, Hyland & Engman 2006, Dutra & Gollob 2008).

The parasite persistence hypothesis is supported by evidence showing that *T. cruzi* is present close to, or within, damaged areas (Fuenmayor et al. 2005). Although immunohistochemical/histological techniques often fail to reveal parasites at lesion sites, studies using sensitive techniques such as polymerase chain reaction (PCR), and in situ hybridisation have shown *T. cruzi* persistence in affected organs (Jones et al. 1993, Vago et al. 1996). Benvenuti et al. (2005) detected *T. cruzi* DNA in endomyocardial biopsies after heart transplantation using PCR. In situ hybridisation using human cardiac tissue provided little evidence for the presence of intact *T. cruzi* at sites of marked inflammation. Nevertheless, remnants of both *T. cruzi* kinetoplast and nuclear DNA were detected (Elias et al. 2003). Furthermore, the transmission of *T. cruzi* via blood transfusion from chronic chagasic donors and the observation that there is reactivation of parasitaemia in immuno-suppressed patients both support the assertion that parasites persist in many hosts (Bocchi et al. 1996, Ferreira et al. 1997). Consistent with the parasite persistence hypothesis is the observation that patients treated with drugs that decrease

parasite load display concomitant decrease in disease severity. Viotti et al. (2006) observed that patients presenting with chronic disease and no heart failure treated with benznidazole exhibited reduced progression of Chagas disease and increased negative seroconversion. Reduction of electrocardiogram abnormalities were observed in chagasic patients treated with itraconazole or allopurinol (Apt et al. 2003). Thus, it seems beyond doubt that parasite presence is strongly tied to pathology, since *T. cruzi* infection is the initial event responsible for triggering Chagas disease, persists in the host, and because an anti-parasite response is observed in chronic patients.

On the other hand, the contrast between the severity of the lesions observed during the chronic phase of Chagas disease and the low parasite load in the blood and tissues of chagasic patients suggests that the response to the parasite alone is insufficient to account for the observed pathology and that autoreactivity may contribute to disease aggravation. This idea led to the hypothesis that autoimmune responses take place during the development of pathology. Favouring this hypothesis is the fact that epitopes of parasite antigens elicit antibodies that cross react with epitopes of host tissues (Girones et al. 2005, Cunha-Neto et al. 2006). Furthermore, it was demonstrated that anti-parasite host-derived antibodies can mediate cellular reactivity (Gazzinelli et al. 1990, Reis et al. 1993a, Dutra et al. 2000). In addition to the presence of auto reactive antibodies, many studies demonstrated the existence of auto reactive T-cells in Chagas patients (Benoist & Mathis 2001, Cunha-Neto et al. 2006). To this end, molecular mimicry has been suggested to exist between components of the host and *T. cruzi* and, thus, strongly supports the participation of autoimmune reactivity in the pathogenesis of Chagas disease (Cunha-Neto et al. 1996).

Although the theories seeking to explain the mechanisms underlying the pathogenesis of Chagas disease are controversial, autoreactivity and parasite persistence theories are not mutually exclusive. It is clear that, as the studies are not decisive in excluding one another, both should be considered when attempting to understand the establishment and maintenance of Chagas disease pathology. Regardless of the origin/source of the antigens that trigger the immune response during chronic infection, there is a consensus that the host's immune system, particularly T-cell subpopulations, plays a central role in pathology development. The increasing technical ability of researchers to phenotypically and functionally define T-cell subpopulations has provided more information about the role of different T-cells during disease evolution, allowing for a more refined clinical classification of patients. This, in turn, is critical for defining what leads to protective *versus* pathogenic responses.

The role of different cell populations and cytokines in establishing pathogenic versus protective responses: association with clinical aspects

The pathological manifestations of Chagas disease, both in the cardiac and in the digestive form, are associated with the occurrence of an inflammatory reaction.

Interestingly, the parasite is present in symptomatic as well as in indeterminate patients and, to date, there is no published evidence that parasite load is higher in the symptomatic groups as compared to the indeterminate groups. Moreover, T-cells from all groups of patients, regardless of the clinical form, display characteristics of activation (Dutra et al. 1994, 1996, Lemos et al. 1998) and are capable of proliferating *in vitro* in response to parasite antigens (Dutra et al. 2000). The apparent lack of differences amongst patients with distinct clinical forms of Chagas disease led to the idea that particular parasite populations could lead to the establishment of different clinical forms. Molecular genetic analyses of different *T. cruzi* isolates have demonstrated that distinct parasite populations are associated with different clinical forms of Chagas disease (Vago et al. 2000), suggesting that genetically distinct populations display characteristic tissue tropism and, thus, influence disease outcome. However, the observation that genetically similar parasite isolates have been found in different organs suggests that the host immune response is a critical factor in determining the outcome of infection (Lages-Silva et al. 2006). Also, as more refined clinical criteria are used, important immunological differences can be associated with patients with distinct clinical outcomes. Although studies of the human disease are still scarce in comparison to those using animal models, a collection of data has been made available in the literature describing aspects of the immune response observed in patients with different clinical forms. We will present some of these data below, with emphasis on the cellular immune response.

Indeterminate form: equilibrium between host and parasite - The indeterminate clinical form represents the ideal situation for both the host and the parasite. Individuals with this clinical form harbour the parasite, as demonstrated by a variety of methods, but have no symptoms of disease whatsoever. Thus, the type of immune response induced in these individuals seems to be critical in maintaining a "healthy" balance between the parasite and host.

Indeterminate patients present positive serological standard tests (at least 2 positive results using different methods) and display no clinical signs and symptoms related to Chagas disease. According to WHO (2002) criteria, indeterminate patients have normal electrocardiogram and radiological examination of the chest, oesophagus and colon. Patients with this form of the disease may progress to symptomatic forms eventually. However, many never develop clinical disease and die later in life of unrelated causes. Interestingly, despite the complete lack of clinical disease manifestations, indeterminate patients display quite a robust immune response. The idea that these individuals do not develop disease because they do not display cellular reactivity, therefore, is incorrect. Rather, the quality of this cellular reactivity is what seems to set these patients apart from other groups.

Most studies concerning the cellular immune response in Chagas disease, especially in indeterminate

patients, who do not display associated lesions, have been performed using peripheral blood cells (PBC). Early studies have demonstrated that PBC from indeterminate patients proliferate upon stimulation with *T. cruzi*-derived antigens and that this proliferative response does not differ quantitatively from those observed in patients with the cardiac clinical form of the disease (Morato et al. 1986, Dutra et al. 2000). Also, cells from indeterminate chagasic patients proliferate when stimulated with anti-epimastigote antibodies derived from patients with Chagas disease (Gazzinelli et al. 1990, Dutra et al. 2000). However, this particular response to the antibody stimulus seems to be lower in patients with the indeterminate form compared to cardiac patients. Reis et al. (1993a), studying the response to anti-epimastigote antibodies of chagasic individuals, showed that anti-epimastigote antibodies derived from indeterminate patients displayed a lower stimulatory capacity than antibodies isolated from cardiac patients. These data suggest that, although indeterminate patients do display anti-epimastigote antibodies in their bloodstream that can stimulate T-cells, these antibodies are less stimulatory, which may contribute to a lower cellular response *in vivo*.

Analysis of the expression of activation markers by T-cells showed that indeterminate patients have a high frequency of CD4⁺ and CD8⁺ T-cells expressing HLA-DR and CD45RO (Dutra et al. 1994). Moreover, the vast majority of these T-cells do not express the co-stimulatory molecule CD28 (Dutra et al. 1996, Menezes et al. 2004, Albareda et al. 2006). Because CD28-negative T-cells were so frequent in indeterminate patients, further studies were designed to better characterise these cells with regards to their immunoregulatory potential through the analysis of cytokine expression. Interestingly, we found a positive correlation between the frequency of CD4⁺CD28⁻ T-cells and the expression of the anti-inflammatory cytokine IL-10 in indeterminate patients (Menezes et al. 2004). This suggested that this subpopulation of CD4⁺CD28⁻ activated T-cells from indeterminate patients displayed down modulatory capacity. It is known that, upon activation and consequent down-regulation of CD28, T-cells express the co-stimulatory molecule CTLA-4. This molecule recognises the same ligands as CD28 but, instead of leading to cell activation, it leads to modulation of T-cell responses. When we evaluated the expression of CTLA-4 in T-cells from indeterminate patients, we observed an upregulation of CTLA-4, especially within the CD8⁺ T-cell population (Souza et al. 2007). These data suggest that CD8⁺ T-cells from indeterminate patients may be self-regulated, possibly due to intrinsic regulation via CTLA-4. Given that CD8⁺ T-cells seem to be the best candidate for tissue destruction, as we will discuss below, it is possible that this regulatory mechanism, working in tandem with others, helps prevent pathology in indeterminate patients. Thus, activated T-cells from indeterminate patients, although present at similar levels as those in cardiac patients, are associated with modulatory capacities (e.g., IL-10 and CTLA-4 expression).

An effective T-cell response requires appropriate stimulation via antigen-presenting cells. Antigen pre-

senting cells are mechanistically essential for T-cell activation and cytokines produced by these cells may create an environment that will influence T-cell function. Since unique T-cell characteristics have been observed in indeterminate patients, the question arises as to whether they were associated with characteristics of antigen presenting cells. It was observed that in vitro infection of monocytes from indeterminate patients with the trypomastigote form of *T. cruzi* led to a decrease in the expression of HLA-DR and, at the same time, an increase in the expression of CD80 (Souza et al. 2004). While the lower expression of HLA-DR may help keep T-cell activation at lower levels (since this molecule is important for antigen presentation), the increase of CD80, a ligand for CTLA-4 which is increased on the T-cells from these patients, will likely lead to a modulation of the T-cell response. Importantly, the exposure of monocytes from indeterminate patients to the parasite in vitro leads to a high expression of IL-10, consistent with a modulatory response. Other researchers have also shown that monocytes from indeterminate patients are an important source of this immunoregulatory cytokine (Gomes et al. 2003). An interesting study by Vitelli-Avejar et al. (2006), evaluating children at the early stages of the indeterminate form of Chagas disease, showed a high frequency of proinflammatory monocytes and regulatory cells, as compared to non-infected children. Thus, different kinetics of cytokine expression may be important for determining the fate of infection. Considering all these data, we hypothesise that, at early stages of indeterminate disease (which follows recent infection), expression of inflammatory cytokines is important to help control parasite levels. However, later on, it is critical to establish modulation of the inflammatory response to avoid tissue destruction. In this later stage, IL-10 may play an essential role in controlling disease. A recent study performed by our group demonstrated a biased distribution of the high expression the IL-10 allele amongst indeterminate chagasic patients (Costa et al. 2009). Thus, the ability to express IL-10 at sufficiently high levels may be genetically determined and may influence disease outcome.

Whether the immunological characteristics associated with the protective response observed in indeterminate patients can be achieved via the use of immunological interventions is still unclear. Adding to the complexity of the host-parasite interaction is the host's genetic background, which may be a key factor in disease development. However, it is clear that some elements of the overall immune response, such as IL-10, are consistently associated with the generation of this partially protective phenotype. Dissecting the mechanisms that control IL-10 expression, the expression of its functional receptor and the subsequent intracellular signalling triggered by IL-10 will certainly lead to important information on how to achieve (or maintain) this desirable response.

Immune-pathology of megaesophagus and megacolon: consequences of neuronal destruction - Megaesophagus and megacolon are the major causes of

morbidity in the digestive clinical form of chronic Chagas disease. Pathologically, both the oesophagus and colon exhibit striking luminal enlargement and muscular hypertrophy. Microscopically, inflammatory infiltrates and fibrosis are found associated with lesions of muscle cells and of the intramural nervous system (Koberle 1968, Adad et al. 2001). The inflammatory infiltrates are composed mainly of CD3⁺CD4⁺ T lymphocytes, CD20⁺ B lymphocytes, CD57⁺ NK cells and CD68⁺ macrophage-like cells (Corbett et al. 2001, d'Avila Reis et al. 2001). The observation that *T. cruzi* kDNA persists in the chronic lesion suggests a role for the parasite in the maintenance of cell activation and of the late inflammatory process (Jones et al. 1993, Vago et al. 1996, Vago et al. 2000).

Denervation, characterised by a striking reduction in the number of neurons, has been considered the hallmark of the chronic digestive disease (Adad et al. 1991, 2001). A reduction of about 85% in the number of neurons is necessary for the development of megaesophagus, while megacolon is associated with a neuronal loss of at least 50% (Koberle 1968). Assessment of denervation in chagasic megacolon and megaesophagus has also been performed by computerised morphometric analyses after immunolabelling with anti-PGP-9.5 monoclonal antibody, specific for neurons and nerve fibres (da Silveira et al. 2005, 2007c, 2008a). In patients with megacolon or megaesophagus, these studies have demonstrated both decreased expression of the integrated PGP-9.5 area and thinning of the nerves, which has been interpreted as a loss of axons in the fibre bundles.

More recently, the denervation process in chagasic megacolon has been further analysed by immunophenotyping the enteric neurons in the colon. Since the enteric nervous system contains between 10-100 million neurons with a great variety of neurotransmitters and/or neuropeptides (Furness 2000), destruction of certain selective neuronal classes in Chagas disease could easily affect the peristalsis and vascular tonus, favouring the development of pathology. In fact, in chagasic megacolon, inhibitory motor neurons (VIP and NOS immunoreactive) are preferentially destroyed. This may explain, at least in part, the partial inability of the involved colon and internal anal sphincter to relax, which seems to induce a mechanical obstruction and dilation of the organ (da Silveira et al. 2007b, 2008b).

The cause of neuronal destruction in Chagas disease has been debated in the literature. In the acute phase, when *T. cruzi* is present in high numbers in the tissue, the parasite may be responsible for the neuronal lesions. In contrast, the parasite load is very low in chagasic lesions during the chronic phase; moreover, the frequent occurrence of ganglionitis and periganglionitis in patients developing megaesophagus and/or megacolon points to participation of immune system cells in these processes (da Silveira et al. 2005). A strong association between the denervation rate and presence of cells with potential cytotoxicity has been demonstrated. Chagasic patients with megacolon present increased numbers of eosinophils and masT-cells compared with both non-infected individuals and chagasic patients without mega-

colon (da Silveira et al. 2007a). It is well known that these cells, when associated with inflammatory processes, can participate in tissue injury through the secretion of cytokines such as IL-1, TNF-alpha and IL-6, which activate the cytotoxic process (Cardoso et al. 2006). In this context, it has already been demonstrated that NK cells and TIA-1+cytotoxic lymphocytes are prevalent in the oesophagus and colon of chronic chagasic patients, with or without digestive disease (d'Avila Reis et al. 2001, da Silveira et al. 2005, 2007a). Moreover, eosinophils and masT-cells themselves can cause tissue injury by secreting, when activated, a variety of enzymes, nitric oxide and free radicals. MasT-cells and eosinophils are probably also implicated in the development of fibrosis, another important factor in the pathogenesis of megacolon and megaesophagus (Adad et al. 1991, 2001).

Another important co-factor in cell-mediated pathology of megaesophagus and megacolon is the enteric glial cell. These cells are activated by inflammatory activity and may contribute actively to inflammatory pathology via antigen presentation and cytokine synthesis. The participation of enteric glial cells in the pathology of megacolon was suggested primarily by the demonstration that these cells have different phenotypes based on whether or not they are associated with an intense inflammatory process, and whether they are in dilated or non-dilated portions of the organ. The non-dilated portion of chagasic megacolon exhibits increased expression of glial fibrillary acidic protein (GFAP) compared with both the dilated portion of the colon in the same patient and also with the colons of non-infected individuals (da Silveira et al. 2007c, 2009). As a constituent of the intermediate filaments, one of the functions of GFAP is to increase the cohesion between enteric glial cells (Steinkamp et al. 2003, von Boyen et al. 2004). It is thus tempting to speculate that the increased expression of GFAP on glial cells in chagasic patients creates a barrier of protection for the neuronal cell bodies and represents an attempt to protect the neurons against destruction by the inflammatory processes. Studies designed to identify signalling molecules, including cytokines, neurotransmitters and neurotrophic factors, which lead to GFAP expression by the enteroglia cells, are crucial for understanding, not only the pathogenesis of chagasic megacolon, but also the feedback of the enteric nervous system under inflammatory conditions.

The alterations suffered as a result of neuroimmune integration have been the subject of research in several different diseases that affect the gastrointestinal tract. In Chagas disease, it is possible that the study of the enteric nervous system, as well as its association with the inflammatory process, could provide a basis for understanding neuroimmune alterations that may somehow be involved in the development of mega-organs, allowing for the design of interventions to control immune and nervous cells, and prevent disease.

Cardiac disease: a lack of proper immunological modulation? - The pathology that characterises chronic chagasic cardiomyopathy is associated with the presence of an intense inflammatory infiltrate in the myocardium

of the patients, especially at sites where *T. cruzi* antigens are observed (Fuenmayor et al. 2005). This inflammatory infiltrate is mainly composed of mononuclear cells, especially CD8⁺ T-cells (Reis et al. 1993b). These CD8⁺ T-cells display characteristics of activated cells, since they are associated with the expression of inflammatory cytokines and cytotoxic molecules, such as TNF-alpha and granzyme A (Reis et al. 1993b). Recent studies have suggested that cytokines such as IL-7 and IL-15 are critical for maintenance of these cells and of their activation state in the heart tissue of cardiac chagasic patients (Fonseca et al. 2007).

The T-cell activation observed in situ is also observed in the circulating cells of cardiac patients. Several studies have shown that PBC from cardiac patients proliferate in vitro upon exposure to both parasite and host-derived antigens (Dutra et al. 2000). Both CD4⁺ and CD8⁺ circulating T-cells from cardiac patients display high expression of HLA-DR and lower expression of CD28, similar to what was described in indeterminate patients (Dutra et al. 1994, 1996). However, significant differences at the functional level distinguish these activated cell populations between the two clinical forms. While a modulatory profile in activated T-cells from indeterminate patients has been observed, CD28⁻ T-cells from cardiac patients are associated with the expression of inflammatory cytokines such as TNF-alpha (Menezes et al. 2004). A correlation between serum levels of TNF-alpha and the occurrence of severe chagasic cardiomyopathy has also been established (Ferreira et al. 2003). These data were expanded by findings demonstrating an inverse correlation between high levels of TNF-alpha or the chemokine CCL2 and the left ventricular ejection fraction (lower fractions, as assessed by echocardiography, indicate worse heart function) in severe cardiac chagasic patients (Talvani et al. 2004). Interestingly, the activated T-cells from cardiac patients, which lack CD28 at the same levels as cells from indeterminate patients, do not up-regulate CTLA-4 (Souza et al. 2007). While this molecule is expressed intracellularly, it is not seen on the cell membrane, suggesting a defect in CTLA-4 expression by T-cells from cardiac chagasic patients. This event could determine a lack of control in T-cell responses and aid in tissue destruction. This mechanism is under investigation in our laboratory.

Our lab demonstrated that CD4⁺ T-cells from indeterminate and cardiac patients display a biased expression of the T-cell receptor region Vbeta5, suggesting the response to a dominant peptide or to a superantigen in the chronic phase of Chagas disease (Costa et al. 2000). We observed a positive correlation between the frequency of CD4⁺CD28⁻Vbeta5⁺ T-cells and the frequency of TNF-alpha and IL-10 producing cells in indeterminate patients, while amongst cardiac patients the same T-cell population was only correlated with the expression of TNF-alpha producing cells (Fig. 2). We have previously described a similar finding in human leishmaniasis, where the patients with the mild form of the disease (co-etaneous) presented a co-regulation of expression of inflammatory and anti-inflammatory cytokines, suggesting a balanced control of the response, and patients with

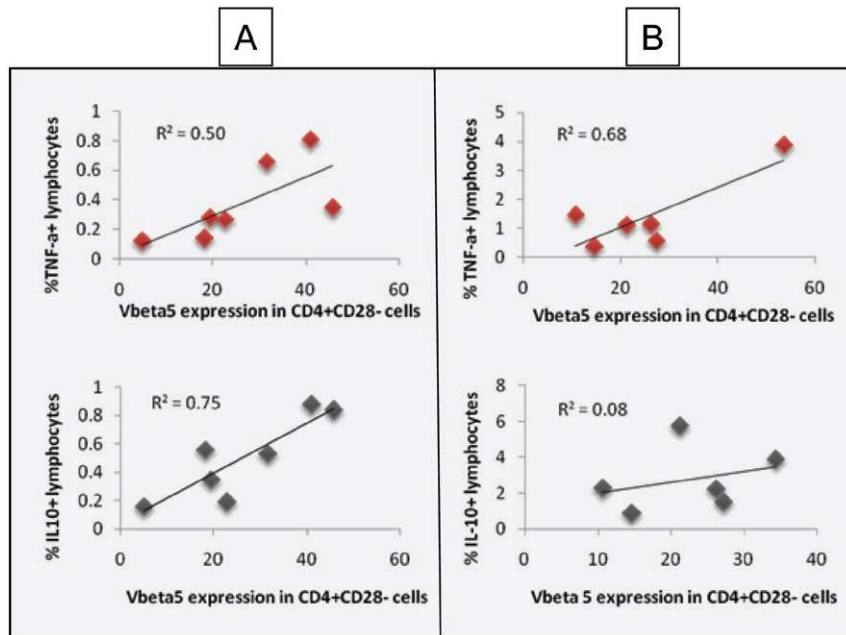


Fig. 2: immunoregulation in human Chagas disease: distinct functions of CD28- T-cells. Correlative analysis of the frequency of CD4⁺CD28-Vbeta5⁺ T-cells and TNF-alpha or IL-10-expressing cells was performed in indeterminate (A) and cardiac (B) chagasic patients. Peripheral blood mononuclear cells were obtained from indeterminate and severe cardiac chagasic patients and analyzed using flow cytometry to determine the values obtained in X and Y axis, as previously done by us (Menezes et al. 2004). The data showed a positive correlation between the frequency of CD4⁺CD28-Vbeta5⁺ cells and IL-10 as well as TNF-alpha in indeterminate, suggesting a balance in the expression of these cytokines by this cell sub-population, leading to a co-regulation of inflammatory/anti-inflammatory responses. On the other hand, a positive correlation was only observed between CD4⁺CD28-Vbeta5⁺ cells and TNF-alpha, but not IL-10, in cardiac patients. This suggests a predominance of inflammatory function of these cells in cardiac patients, which could be associated to the establishment of an unregulated immune response, favoring pathology development.

the severe form (mucosal) did not display this balance (Antonelli et al. 2004, Gaze et al. 2006, Gollob et al. 2008). The same rationale can be used to interpret these data, where patients with cardiac disease would display a lack of immunoregulatory control that may contribute to the establishment and maintenance of pathology.

Activated T-cells are not the only important source of TNF-alpha; monocytes from cardiac patients produce this important inflammatory mediator as well. We have shown that in vitro exposure to *T. cruzi* trypomastigotes induces expression of TNF-alpha by monocytes of cardiac patients, as opposed to IL-10 preferentially expressed by monocytes from indeterminate patients, under the same conditions (Souza et al. 2004).

Another inflammatory cytokine consistently associated with cardiac disease is IFN-gamma. It has been shown that PBC from cardiac patients express higher levels of IFN-gamma as compared to PBC from indeterminate patients (Menezes et al. 2004) and that there is a direct correlation between disease severity (as determined by different degrees of cardiomyopathy) and expression of IFN-gamma (Gomes et al. 2003). In addition, it has been shown that T-cell clones derived from the heart of cardiac patients produce predominantly IFN-gamma (Abel et al. 2001). It has been shown that the main sources of IFN-gamma in chagasic patients

are CD4⁺ T-cells (Gomes et al. 2003). However, recent studies in our laboratory have suggested that other cell populations such as CD4-CD8- T-cells are an important source of this cytokine in cardiac chagasic patients (unpublished data), which again was paralleled by a finding from our group in human leishmaniasis (Antonelli et al. 2006). Taken together, these findings concerning the expression of IFN-gamma and TNF-alpha are consistent with the inflammatory immune response observed in situ. However, others have found an opposite correlation between the expression of IFN-gamma and cardiac disease (Lauccella et al. 2004). Moreover, Bahia-Oliveira et al. (2000) demonstrated that the levels of IFN-gamma were higher in cured former chagasic individuals than in those submitted to therapy, but not cured, suggesting a role for IFN-gamma in the mechanisms of disease resolution. Again, this is suggestive that the balance of inflammatory and anti-inflammatory cytokines determines the fate of infection and the progression of disease.

Although cells from cardiac patients are able to produce IL-10, the ratio of this cytokine to TNF-alpha seems to be lower in cardiac patients (Souza et al. 2004). The lower expression of IL-10 has been associated with the occurrence of a gene polymorphism in the promoter region of the *IL-10* gene (Costa et al. 2009). The association of this polymorphism with cardiac Chagas disease

points to an important genetic susceptibility factor that could influence the outcome of the immune response in these patients.

Genetic susceptibility to development of cardiomyopathy has been described with regards to polymorphism in genes that code for molecules involved in the control of the immune response, especially cytokines. Recently, an

association with polymorphisms in lymphotoxin, *MCP-1*, *Bat-1* and *NFkB* genes, among others, was described for cardiac Chagas disease in the Brazilian population (Ramazawmy et al. 2006a, b, 2007, 2008). Taken together, these data show that genetic predisposition can also influence the outcome of Chagas disease. Thus, typing of gene polymorphisms could be an important approach

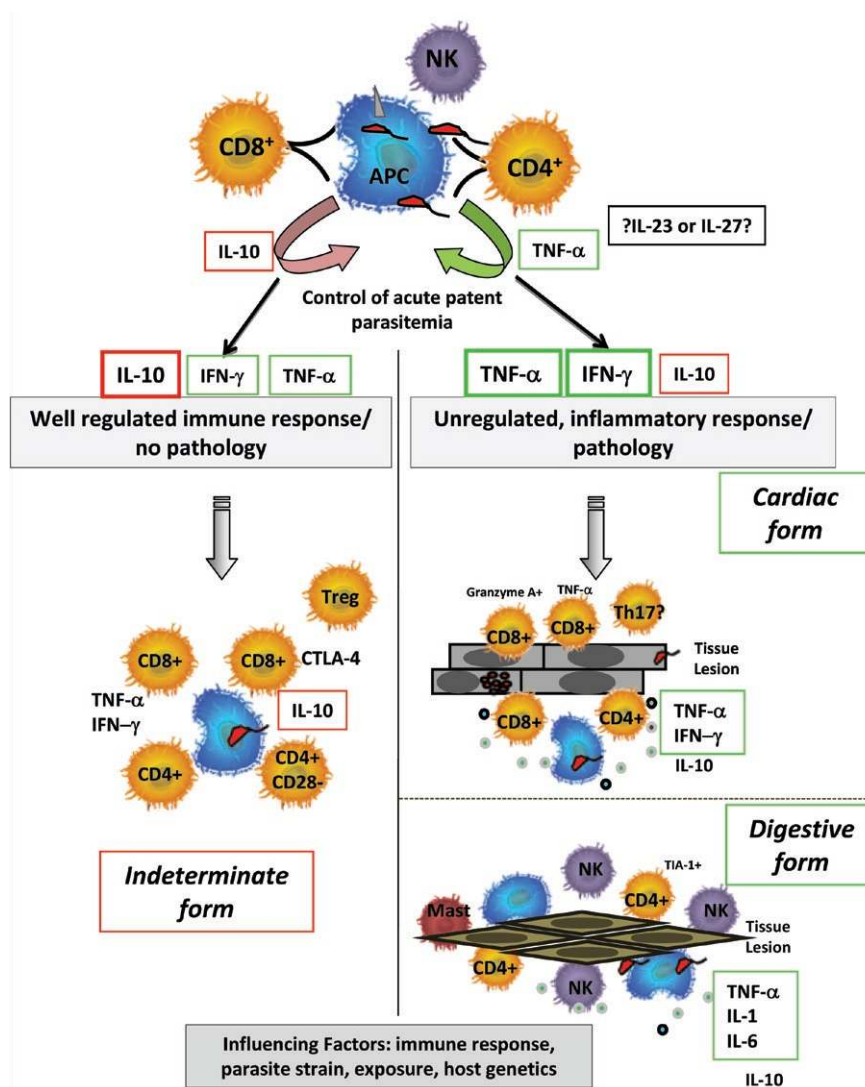


Fig. 3: cytokines and cell populations involved in the generation of protective and pathogenic responses in chronic Chagas disease. Shortly following infection by *Trypanosoma cruzi*, patent parasitemia will be controlled and patients enter the chronic phase of disease. During this phase, differential immune responses may be the defining factor which allow establishment of a well controlled immune response for maintaining the parasite in check (indeterminate clinical form) vs. a response which continues to control patent parasitemia, yet leads to pathology (cardiac and digestive clinical forms). The initial interaction between the parasite and the host is likely key in establishing effective control of patent parasitemia and at the same time is critical in the formation of cytokine microenvironments which could orchestrate subsequent differentiation of regulatory and effector T-cell populations. Depending on the balance between biologically active T-cell subpopulations and their relative life spans, activation thresholds and functional activity, the overall response will be successful in maintaining the indeterminate clinical form or progressing into the more severe cardiac or digestive forms. Several studies have demonstrated that activated CD4 and CD8 T-cells are present in all clinical forms with the production of inflammatory and regulatory cytokines, however, recent studies have also demonstrated differences among clinical forms in terms of relative production of inflammatory cytokines, expression of IL-10 and expression of regulatory molecules such as CTLA-4. To date, the roles of Th17 cells in pathology, or of Treg cells in controlling inflammation are unknown in human Chagas disease. In addition to differential immune responses, several other factors likely influence the differential progression of individuals into distinct clinical forms of Chagas disease including the parasite strain, the strength of inoculation, environmental factors, such as previous immunological experience and nutrition, and host genetics.

for identifying groups of individuals at risk of developing severe disease.

The current picture shows that cardiac chagasic patients display an inflammatory cytokine profile, consistent with the tissue damage observed in these individuals. Moreover, specific cell populations are involved in the establishment of the cytokine environment that favours inflammation. Most importantly, all of the data, taken together, suggest that a lack of control of the inflammatory response is a major cause of pathology establishment in cardiac Chagas disease.

A critical analysis of the data presented in this review suggests that the generation of protective or pathogenic responses in human Chagas disease is highly influenced by the complexity of the immune response generated during *T. cruzi* infection. The anti-parasitic response, crucial for chronification of infection, may work as a double-edged sword if not properly modulated. It is clear that while an activated, inflammatory, response may be beneficial in the early stages of infection, lack of control of this response later on will allow for the establishment of pathology. Fig. 3 summarises the cellular immunological characteristics of the different clinical forms. Parasite, environmental and genetic factors are the key players in the establishment of the different responses. The use of genetic studies to identify groups at risk of developing severe disease opens new perspectives for disease surveillance. However, it is likely that more than one approach needs to be taken towards designing strategies for disease control and prevention. Our ongoing quest for efficient diagnosis, therapy and prevention is fully justified.

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