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# L-arginine metabolism in experimental Chagas disease: role of arginase I and iNOS in heart tissue.

# Metabolismo de la L-arginina en la enfermedad de Chagas experimental: papel de la arginasa I y la iNOS en tejido cardiaco.

Memoria presentada para optar al título de Doctora en Biología Molecular por:


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# ABBREVIATION LIST

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| | |
|---|---|
| (q) RT-PCR | **q**uantitative **R**eal **T**ime- **P**olymerase **C**hain **R**eaction |
| ADC | **A**rginine **Dec**arboxylase |
| APC | **A**ntigen **P**resenting **C**ell |
| AZ | **A**nti**z**yme |
| BMM | **B**one **M**arrow Derived **M**acrophages |
| cAMP | **c**yclic **A**denosine **m**ono**p**hosphate |
| CAT | **C**ationic **A**mino-acid **T**ransporter |
| CD | **C**luster of **D**ifferentiation |
| d.p.i | **d**ays **p**ost **i**nfection |
| DFMO | α-**di**fluoro**m**ethyl**o**rnithine |
| Fizz1 | Resistin like alpha |
| Folr2 | **Fol**ate **R**eceptor-2 |
| GM-CSF | **G**ranulocyte and **M**onocyte **C**olony **S**timulating **F**actor |
| IFNγ | **I**nter**f**ero**n** **g**amma |
| Ig | **I**mmuno**g**lobulin |
| IL | **I**nter**l**eukin |
| iNOS | **i**nducible **N**itric **O**xide **S**ynthase |
| L-NMMA | $N^G$-**m**ono**m**ethyl-**L**-**a**rginine |
| LPS | **L**ipo**p**oly**s**accharide |
| Mgl2 | **M**acrophage **g**alactose N-acetyl-galactosamine specific **l**ectin 2 |
| MHC | **M**ajor **H**istocompatibility **C**omplex |
| Mmr | **M**acrophage **m**annose **r**eceptor |
| mPGES-1 | **m**icrosomal **P**rosta**g**landin $E_2$ **s**ynthase-1 |
| MSC | **M**yeloid **S**uppressor **C**ell |
| NK | **N**atural **K**iller |
| NO | **N**itric **O**xide |
| NOHA | $N^{\omega}$-**h**ydroxy-L-**A**rginine |
| Nor-NOHA | $N^{\omega}$-**H**ydroxy-**nor**-L-**a**rginine |
| ODC | **O**rnithine **Dec**arboxylase |

| | |
|---|---|
| Pafah | **P**latelet-**a**ctivating **f**actor **a**cetyl**h**ydrolase |
| PBMC | **P**eripheral **B**lood **M**ononuclear **C**ell |
| PEC | **P**eritoneal **E**xudate **C**ell |
| $PGE_2$ | **P**rosta**g**landin $E_2$ |
| RNOS | **R**eactive **N**itrogen-**O**xide **S**pecies |
| ROS | **R**eactive **O**xygen **S**pecies |
| RQ | **R**elative **Q**uantity |
| Sep | **Se**lenoprotein **P** |
| TCR | **T C**ell **R**eceptor |
| TGF | **T**umor **G**rowth **F**actor |
| TLR | **T**oll-**L**ike **R**eceptor |
| TNF | **T**umor **N**ecrosis **F**actor |
| Treg | **Reg**ulatory **T c**ell |
| Trem2 | **T**riggering **r**eceptor **e**xpressed on **m**yeloid cells 2 |
| WHO | **W**orld **H**ealth **O**rganization |
| Ym | Chitinase 3-like 3 |

# SUMMARY

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In Chagas disease, caused by *Trypanosoma cruzi*, macrophages and cardiomyocytes are the main targets of infection. On the other hand, macrophages can be activated in different ways by Th1 or Th2 cytokines leading to classic or alternative activation, which results in different functional outcomes. Those different ways are often differentiated by the expression of enzymes involved in L-arginine metabolism. Thus, we studied the expression of such enzymes in heart tissue during *in vivo* infection of BALB/c and C57BL/6 mice. We found that expression of inducible nitric oxide synthase (iNOS), arginase I and II as well as ornithine decarboxylase, were much higher in BALB/c compared to C57BL/6 mice and related to parasite burden in heart tissue. Th1 and Th2 cytokines were expressed in heart tissue in both infected mouse strains, but the Th1/Th2 balance was predominantly Th1 in C57BL/6 mice and Th2 in BALB/c mice at the peak of parasite infection. By using mice strains genetically deficient in various cytokines or their receptors, we found that IL-13, probably in cooperation with IL-4, IL-10 and prostaglandin $E_2$, induces arginase I expression. Inducible nitric oxide synthase and arginase II were expressed by cardiomyocytes. Interestingly, heart infiltrated $CD68^+$ macrophages were the major cell type expressing arginase I. When purified, $CD11b^+$ heart infiltrating cells expressing both arginase I and iNOS did not present a clear M1/M2 polarization but showed a myeloid suppressor cell phenotype. In addition, these $CD11b^+$ cells modulated T cell proliferation *in vitro*. Thus, arginase I expression may influence parasite cell survival, and might be also regulating the inflammatory T cell response in heart during infection.

# INTRODUCTION

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## I. Chagas Disease.

### I.1. General aspects and Life cycle.

American trypanosomiasis, or Chagas disease, is a multisystemic disorder that, according to the latest report from the WHO, affects approximately 18 million people, with 120 million at risk. This is an endemic disease in Latin America where it is spread through 18 countries from the southern cone of the continent (Fig. 1), up to Mexico (WHO, 2002).

Transmission of the disease to humans mainly occurs by three different ways: By the insect vector, by blood transfer of infected blood and by vertical transmission.

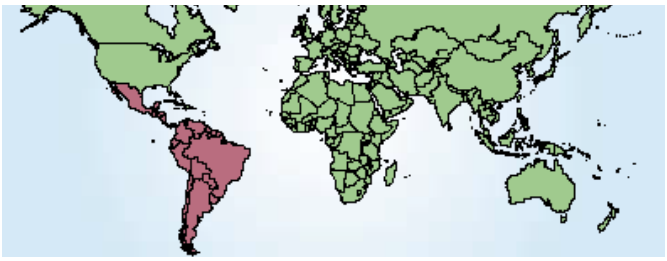

**Figure 1**. *Distribution of Chagas disease*. In purple, is shown Chagas disease distribution from the southern cone of the American continent up to Mexico. From (Morel and Lazdins, 2003)

***Trypanosoma cruzi,*** a flagellated protozoa of the *Kinetoplastidae* family, was first identified by Carlos Chagas in 1909 as the causal agent of the disease which was named after him. This parasite has a complex life cycle (Fig. 2) involving several stages in both vertebrates and insect vectors. *T. cruzi* has three main different morphologies: **epimastigote**, which replicates in the blood-sucking triatomine insect vector; **trypomastigote**, which infects the vertebrate host´s cells, and **amastigote,** which replicates intracellularly inside the host´s cells (Burleigh and Andrews, 1998; Tanowitz et al., 1992). Transmission of *T. cruzi* to humans occurs when feces released by the triatomine bug, while it sucks blood from the vertebrate, containing infective metacyclic trypomastigotes, break through the skin into the bloodstream. There, the flagellated forms of the parasite infect a wide variety of host cells (mainly macrophages and

cardiomyocytes). Once inside the cells, trypomastigote forms transform into amastigotes, which multiply intracellularly. After this, amastigotes break out the cell and give place again to trypomastigotes, which are released into the bloodstream where they spread and infect new cells again. Then, parasites can be sucked from the bloodstream by a triatomine bug while taking a blood meal. Once in the gut of the bug, trypomastigotes transform to epimastigotes, and multiply through the digestive track until they reach the intestines where are transformed into metacyclic trypomastigotes and released with the feces when the bug takes blood from a vertebrate host.

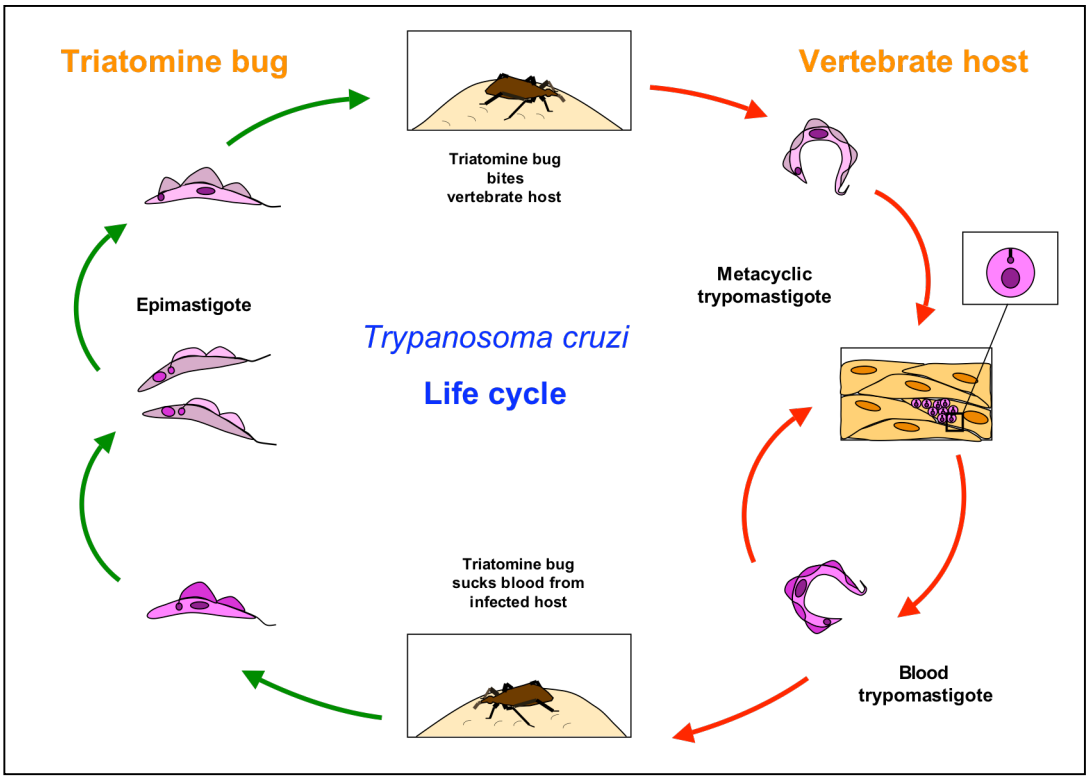


**Figure 2.** *Life cycle of* Trypanosoma cruzi**.** Triatomine bug bites the vertebrate host and releases with the feces metacyclic parasites that enter the host, infect cells, multiply as amastigotes and break out the cell to the bloodstream. When triatome bug bites an infected host, tripomastigotes in the blood are sucked up by the bug and turn into epimastigotes in the insect's gut where they multiply. In the intestine epimastigotes differentiate back to metacyclic trypomastigotes that are released with the feces when the bug feeds on the host.

Individuals residing in rural areas of Latin America are at highest risk of infection, because the bugs live in these dwellings and feed constantly on the inhabitants during the night. The WHO has conducted several programs for the elimination of the insect vector, and for comprehensive health education with marked success in the southern cone where the incidence has fallen by over 70% since 1985. Also blood screening to prevent transmission through transfusion needs to be performed

to prevent and control the disease. Two drugs (benznidazol and nifurtimox) are available for the treatment of the acute phase of the disease but, besides having a high percentage of side effects, are not useful for the chronic phase. Moreover, to date, an effective immunotherapy or vaccine is still lacking.

### I.2. Clinical findings.

Two phases, acute and chronic, can be differentiated in Chagas disease (Kirchhoff, 1993; Prata, 2001; Tanowitz et al., 1992). In the acute phase, few days after infection, a local inflammatory lesion appears at the site of infection where the metacyclic trypomastigotes infect and undergo their first rounds of multiplication. Then, parasite dissemination through the body occurs, and circulating blood trypomastigotes can be easily observed (parasitemia); a small number of patients develop symptoms of cardiac affection, reflecting an underlying severe myocarditis. This might lead to heart failure, which is responsible for the few deaths in the acute Chagas disease. Meningoencephalitis also may occur, especially in some immunosuppressed patients (Hoff et al., 1978). However, the acute phase mainly remains undiagnosed without severe clinical symptoms. In contrast, the common manifestations and most severe pathology of the disease appear many years (10 to 30) after infection with *T. cruzi* in approximately 30% of the infected people, in what has been named chronic phase of the disease. During this phase, circulating parasites cannot be detected in blood, but progressive damage occurs involving esophagus, colon and heart. In the chronic phase, the heart is the most commonly affected organ; cardiomyopathy frequently develops, being congestive heart failure the most common cause of death in these patients.

### I.3. Experimental model for Chagas disease: the mouse.

The mouse, because of its small size and easy maintenance, has been the preferred animal for a great number of experiments. Mice are susceptible to *T. cruzi* infection and develop acute and chronic phase somewhat similar to the pathology described in humans.

However, both mouse and parasite strains, as well as inoculation procedure, determine susceptibility and pathogenesis of Chagas disease in the mouse model, but, despite the differences found among them, mice show an acute phase when blood-form

parasites can be detected in blood by microscopic techniques spanning from 7 to 30 days. After this period, chronic phase begins to develop; no parasites can be detected in blood, and chagasic cardiomyopathy can be observed in histopathological studies of hearts from infected mice.

## II. Immune response in Chagas disease.

*T. cruzi* infection triggers a complex immune response. As an intracellular pathogen, involves an intricate, and not yet well understood, crosstalk of many cellular and humoral mechanisms from the innate and adaptive immunity.

### II.1. Innate immunity

As part of the first barrier that the parasite encounters after entering the host, molecules and effector cells from the innate immunity play a very important role in immune response against *T. cruzi*.

After breaking the skin barrier, the parasite enters the blood and tissues, and triggers the early response of complement and acute phase proteins (C-reactive protein, serum amyloid P component). These molecules, bind to *T. cruzi* and mediate its **receptor-mediated endocytosis**. Also, macrophages and dendritic cells express in their surface mannose receptor, which recognizes carbohydrates with molecular patterns not found in host cells.

*T. cruzi* is also able to actively enter in a variety of cell types, especially macrophages, triggering a diversity of molecular interactions that mobilize the innate immune response of the host (Fig. 3). Macrophages secrete **IL-12** that activates natural killer (**NK)** cells to produce interferonγ (**IFNγ)** (Aliberti et al., 1996), this cytokine acts reciprocally in macrophages which release tumor necrosis factor (**TNF)** to act synergistically with IL-12 and IFNγ to induce the production of nitric oxide (**NO)** by these cells (Abrahamsohn and Coffman, 1996; Munoz-Fernandez et al., 1992). However, there are several evidences supporting the fact that inflammatory response is self-regulated and macrophage activation and NO production is reduced by the so-called anti-inflammatory cytokines. It has been found that **IL-10** is required to prevent an excessive pro-inflammatory response during *T. cruzi* infection (Gazzinelli et al., 1992; Hunter et al., 1997; Reed et al., 1994), and something similar occurs with tumor growth

factor (**TGF)β** (Silva et al., 1991) although this cytokine might have other functions besides immune regulation (Hall and Pereira, 2000; Ming et al., 1995). It has been proposed that **IL-4** is also downregulating IFNγ and inflammation when cooperating with IL-10 (Abrahamsohn et al., 2000), but some other reports showed that this cytokine has similar effects to those of IFNγ as trypanocidal activity (Golden and Tarleton, 1991; Wirth et al., 1989). Although the role of **IL-13** is not clear yet, it has been documented that this cytokine might also be involved in the regulation of IFNγ release (Antunez and Cardoni, 2001).

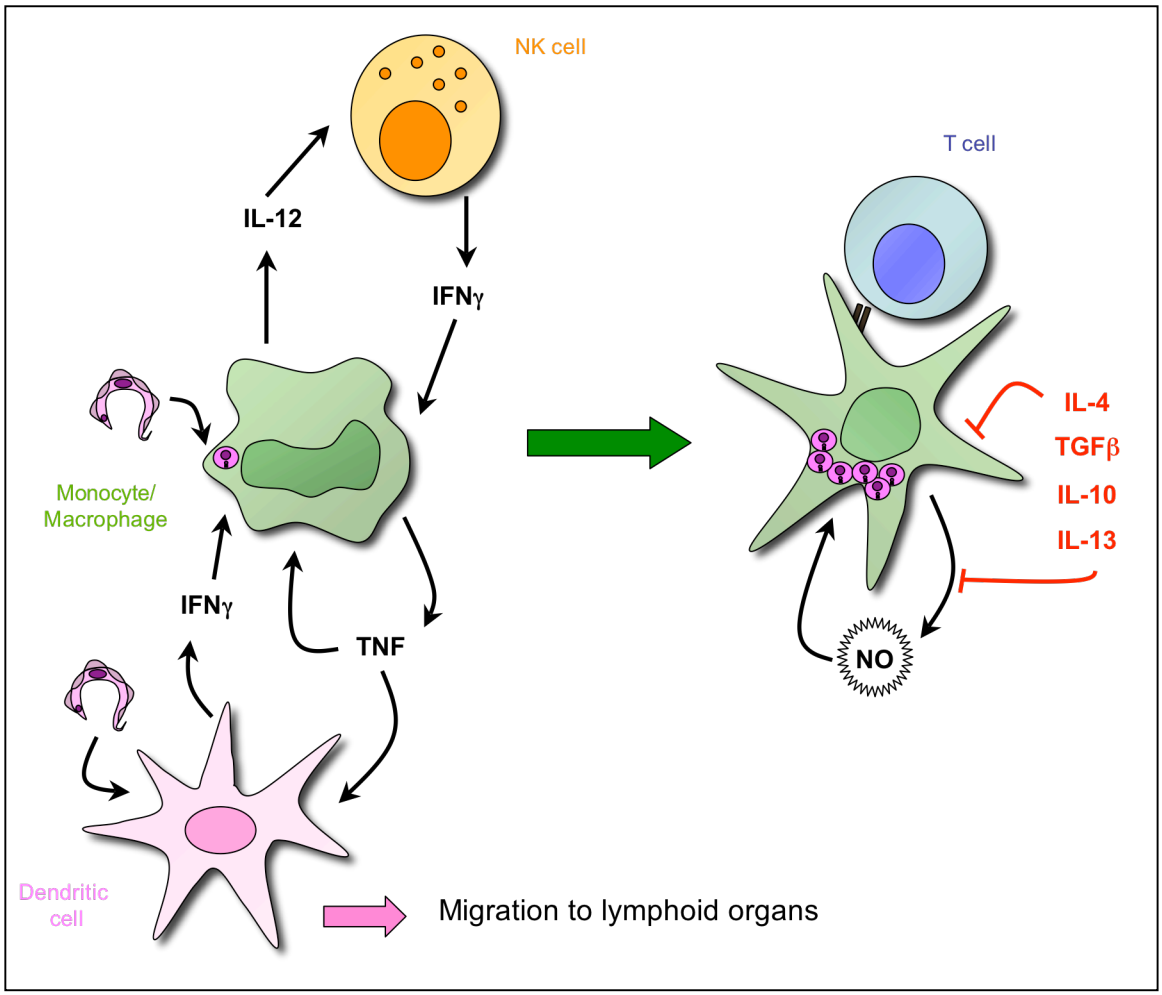


**Figure 3**. *Scheme of innate response to* Trypanosoma cruzi.

Macrophages exposed to IFNγ become very effective as antigen presenting cells (APCs) and interact and present *T. cruzi* peptides to T lymphocytes. Also, TNF is capable of inducing mature dendritic cells and their migration from the skin and mucosa to the lymphoid organs to start and activate the expansion of lymphocytes specific for

microbial antigens (Fearon and Locksley, 1996). But not only professional APCs are able to present parasite peptides, the ability of *T. cruzi* to invade a high number of mammalian cell types involves them into the inflammatory and immune responses. Cardiomyocytes have been shown to respond to the infection releasing NO, cytokines and chemokines (Fichera et al., 2004; Machado et al., 2000).

## II.2. Adaptive immunity

Immune response against *T. cruzi* also involves activation of the humoral and cellular components of the adaptive response (Fig. 4). Acute experimental *T. cruzi* infection in the murine model triggers an intense and polyclonal activation of **B lymphocytes**, which implies an overproduction of **immunoglobulins (Ig)**. The response against parasite antigens in this phase represents only a very small fraction of the total Ig produced (Minoprio et al., 1989). This polyclonal response is widely dependent on $CD4^+$ helper T cells (Minoprio et al., 1989).

The importance of the two main subpopulations of **T lymphocytes,** $CD4^+$ and $CD8^+$ cells, in controlling infection has been widely established. *T. cruzi* is able to infect almost any type of cell in the host tissues (Lenzi et al., 1996); after escaping the parasitophore vacuole in the cytoplasm (Nogueira and Cohn, 1976), parasite antigens will be processed and presented in the context of MHC class I molecules which will be recognized by effector T **$CD8^+$** cells. On the other side, dead parasites, soluble parasite antigens, or parasites that did not escape the parasitophore vacuole (McCabe et al., 1984), will be presented only by professional APCs through MHC class II to the **$CD4^+$** T cells, which will cooperate with B cells to induce antibodies. Moreover, despite the fact that B cell deficient mice succumb to infection (Kumar and Tarleton, 1998), the protective immune response seems to mostly depend on $CD8^+$ cells that produce IFNγ. Cytotoxic $CD8^+$ T cells can control infection through perforin/granzyme mediated killing of infected cells and/or FAS-mediated apoptosis (Kumar and Tarleton, 1998). However, there are reports indicating that $CD8^+$ T cells cannot completely control infection because they become unresponsive (Martin and Tarleton, 2004).

**Cytokines** play a key role in regulating both the induction and the type of immune response, but no clear polarization into Th1 *versus* Th2 was seen when studying mice susceptible or resistant to *T. cruzi* challenge (Powell et al., 1998; Zhang

and Tarleton, 1996a, 1996b). Thus, the role of Th1/Th2 cytokines during infection is not yet clearly established.

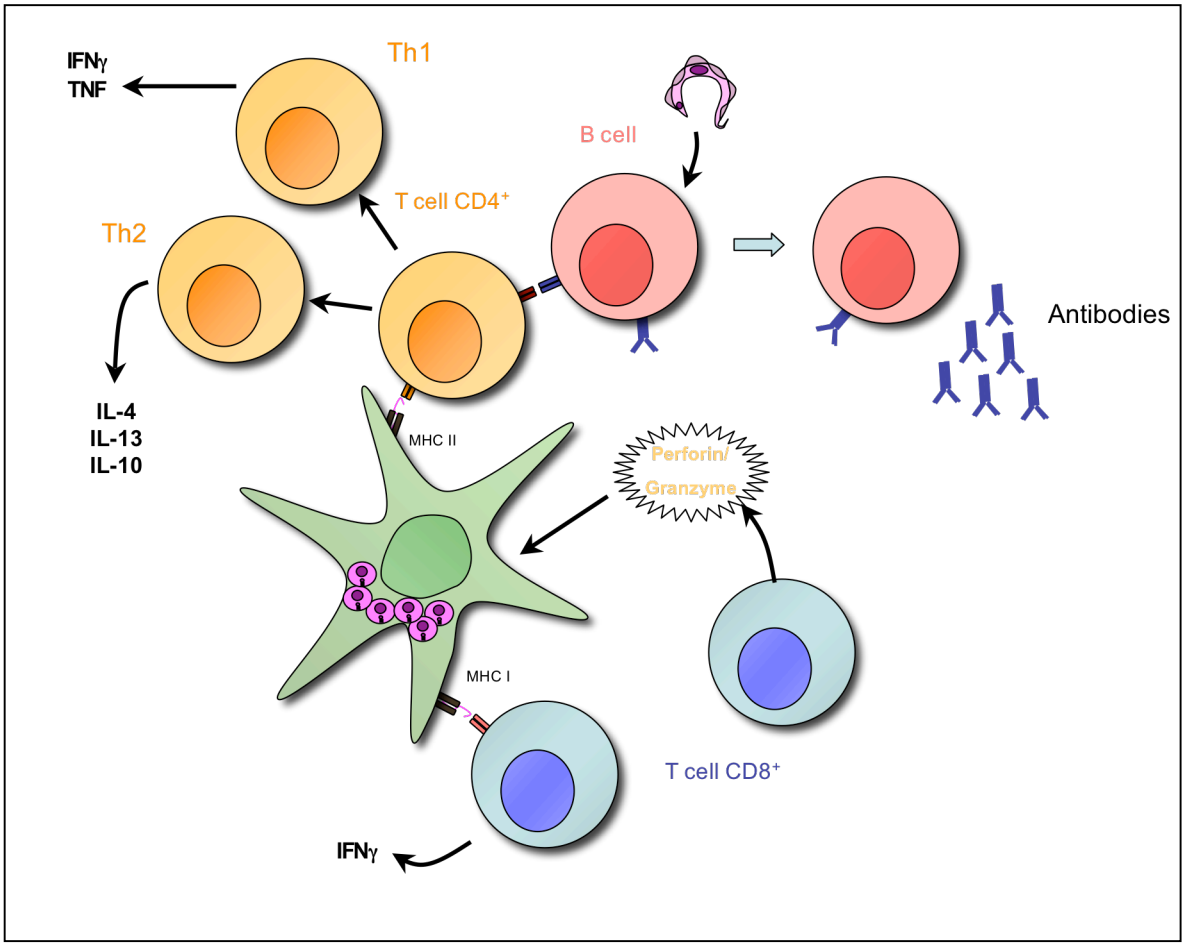


**Figure 4.** *Scheme of adaptive response to* Trypanosoma cruzi

Also, a variety of **chemokines**, have been suggested to play a key role in the influx of the immune cells to target tissues during infection, reviewed in (Silva et al., 1991; Teixeira et al., 2002). CCR5 and its ligands play a central role on T cell migration to the heart (Machado et al., 2005) and parasite proliferation (Hardison et al., 2006a), CXCL9 and CXCL10 do not affect leukocyte migration but when blocked with neutralizing antibodies resulted in a decrease in parasite load in the heart (Hardison et al., 2006b). Also chemokines as CXCL12 and CCL4 have been implicated in thymocyte migration and development (Mendes-da-Cruz et al., 2006). Overall, there is a complex network in which specific chemokines play different roles in the pathophysiology of the disease.

## II.3. Autoimmunity and immunomodulation.

Among the possible mechanisms responsible for the pathogenesis of chronic Chagas disease, **autoimmunity** is the one that has received more experimental support but also elicited more controversy (Girones et al., 2005; Girones and Fresno, 2003; Kierszenbaum, 1986, 1999; Levin, 1996; Soares et al., 2001; Tarleton, 2001, 2003). On the other hand, there are studies suggesting that **parasite persistence** in host tissue is the key in the pathogenesis of the chronic form of the disease since anti-parasite treatment decrease disease severity (Tarleton, 2001).

Several mechanisms might explain how an infectious pathogen can break immunological self-tolerance. Among them, molecular mimicry, bystander activation, and polyclonal lymphocyte activation have been widely documented in Chagas disease (Girones et al., 2005; Kierszenbaum, 2003) . However, none of these three mechanisms is exclusive and any combination of them or the three could be taking place.

An important issue, usually not taken into account, is the role of regulatory T cells (Tregs), which have been described to suppress autoimmune responses. In addition to the classical Tregs that leave the thymus, there are others, Tr1 and Th3, which acquire regulatory capacity on the periphery (Battaglia et al., 2002). Thus, a definition of the role of Tregs during *T. cruzi* infection, may contribute to shed some light to the mechanisms that control the pathogenesis observed in chronic Chagas disease.

# III. Regulation of immune responses by L-arginine metabolism

L-arginine can be metabolized inside the cells by two main enzymes (Fig. 5), arginase and nitric oxide synthase (NOS) to give urea and L-ornithine, and L-citrulline and NO, respectively.

L-arginine in cells can also be metabolized through a less known pathway by **Arginine Decarboxylase** (ADC) that produces Agmatine, which can regulate both NO production and polyamine synthesis (Satriano, 2004).

The **cationic amino-acid transporters** CAT-1, CAT-2 and CAT-3 are a family of transmembrane proteins that transfer L-arginine (among other cationic amino-acids) from the extracellular, to the intracellular compartment to compensate the degradation produced by the arginase and inducible NOS (iNOS) enzymes. Expression of CAT-3 is restricted to the brain (Verrey et al., 2004) but CAT-1 and CAT-2 are present in

macrophages and in some cases have been shown to play an important role in regulating macrophage activation in response to parasites (Wanasen et al., 2007; Yeramian et al., 2006).

### III.1. Arginase

To date, two arginase isoforms have been described in mammals. These isoforms are encoded by different genes and have different subcellular location (Jenkinson et al., 1996).

**Arginase I,** also known as hepatic arginase, is located in the cytoplasm, and is highly expressed in liver, but also, in a restricted way, in other cell types, as macrophages. This isoform is mainly induced by Th2 cytokines (IL-4, IL-13) (Corraliza et al., 1995; Munder et al., 1998), and also IL-10, TGFβ, granulocyte and macrophage colony stimulation factor (GM-CSF) and prostaglandin $E_2$ ($PGE_2$) (Boutard et al., 1995; Corraliza et al., 1995; Jost et al., 2003).

**Arginase II** is located in the mitochondria; it is expressed in a wide variety of tissues and cell types, mainly in kidney (Levillain et al., 2005), but also prostate, small intestine (Gotoh et al., 1997), and cardiomyocytes (Aoki et al., 2004). This isozyme is mainly induced by LPS and dibutyryl cAMP (Gotoh et al., 1996).

Both arginase isoforms catalyze the same reaction, and no clear distinct function has been described for each of them although this is still debated (Cederbaum et al., 2004).

Arginase is involved in the Urea cycle catalyzing the conversion of L-arginine into L-ornithine and urea. Further on, L-ornithine can be metabolized by Ornithine aminotransferase to increase L-proline, which is required for collagen synthesis. On the other hand **Ornithine Decarboxylase** (ODC), is the key enzyme of the polyamine pathway; it enhances the production of polyamines, needed for proliferation of all eucaryotic cells. ODC is a highly regulated eukaryotic enzyme, with one of the lowest half lives known in mammals, mainly due to the binding of the antizyme (AZ) that directs ODC degradation by the 26 S proteasome (Pegg, 2006).

### III.2 iNOS

Together with the other NOS isoforms (nNOS in neuronal tissue, and eNOS in endothelial cells), iNOS catalyzes the oxidation of L-arginine to L-citrulline and NO, with the intermediate formation of $N^{\omega}$-hydroxy-L-Arginine (NOHA).

iNOS isoform is found in a diversity of cell types in the immune system (Wu and Morris, 1998) and also in cardiomyocytes (Tsujino et al., 1994). The most common inducer for iNOS is IFNγ combined with LPS, but also other Th1 cytokines as IL-12, IL-1, and TNF.

iNOS generates both NO and superoxide ion ($O_2^-$) which leads to peroxynitrite ($ONOO^-$) formation (Xia and Zweier, 1997). However, very low L-arginine concentrations result in low NO formation and high generation of superoxide, that is very unstable and is converted to hydrogen peroxide ($H_2O_2$) and oxygen (Kusmartsev et al., 2004).

**NO**, reactive nitrogen-oxide species (**RNOS**) as $ONOO^-$ and reactive oxygen species (**ROS**) as $O_2^-$ and $H_2O_2$, produced by NOS are known as very efficient mechanisms to fight against pathogens, but they also have high autotoxicity and, when excessive, can result in pathology.

### III.3 Arginase and iNOS balance.

There is an important cross-regulation between iNOS and arginase products. NOHA, an intermediate form in the NO synthesis, has been shown to inhibit arginase (Daghigh et al., 1994) and it has been reported that polyamines can affect iNOS expression (Mossner et al., 2001; Sonoki et al., 1997); also arginase has been implicated in downregulating iNOS by depleting L-arginine, since decreased availability of this amino acid blocks iNOS protein synthesis and stability (El-Gayar et al., 2003; Lee et al., 2003). It is noteworthy that iNOS and arginase enzymes share L-arginine as a substrate and, although the affinity constant of iNOS for L-arginine is 1000-fold higher that of arginase, the Vmax of arginase is more than 1000-fold faster than that of iNOS which results in a similar utilization of L-arginine by both enzymes (Wu and Morris, 1998).

Due to the substrate sharing, these enzymes have been involved in the regulation of the Th1/Th2 balance during immune processes, and have been used as markers for macrophage activation (Mills et al., 2000).

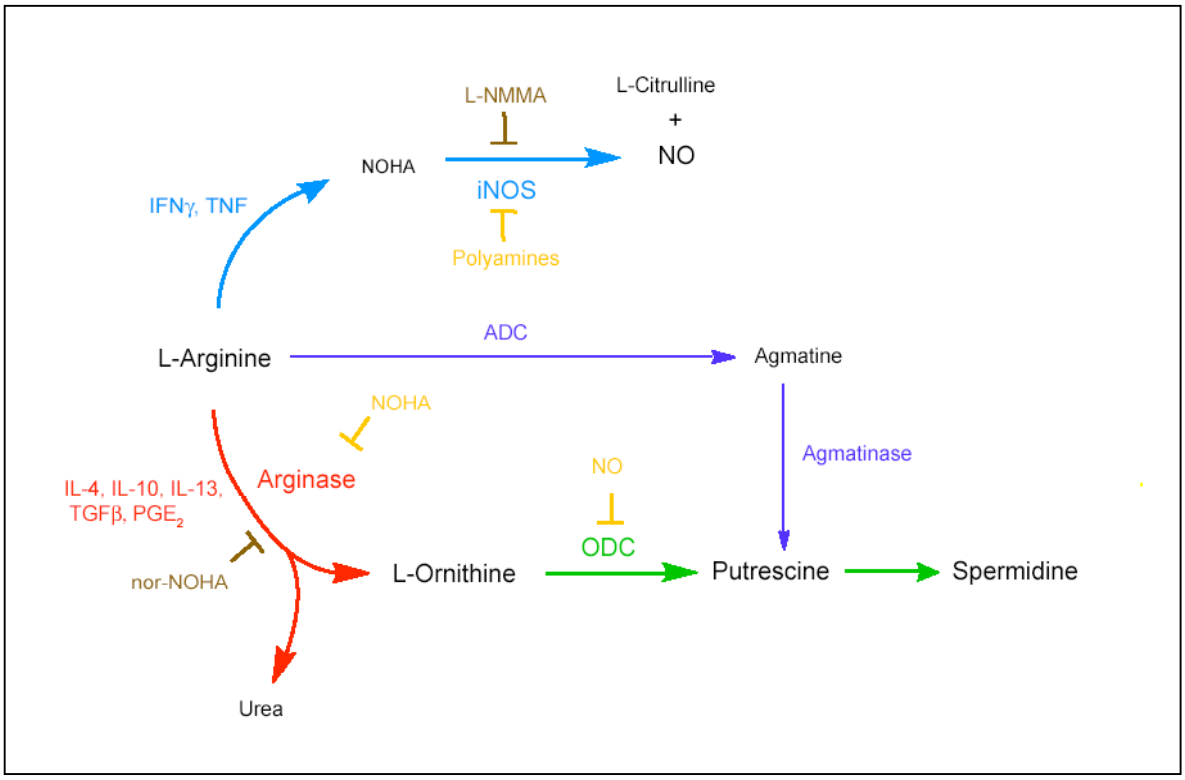


**Figure 5**. *Scheme of different pathways for L-arginine metabolism*

## III.4 L-arginine metabolism in parasitic diseases.

Several infections by intracellular bacteria can have their outcome modified by the modulation of the routes mentioned above; this is the case of *Helicobacter pylori* (Gobert et al., 2002; Gobert et al., 2001), *Chlamydia sp.* (Huang et al., 2002).

The arginase-iNOS balance also modifies several protozoan infections; Macrophage arginase during *Trypanosoma brucei* infection has been shown to deplete concentrations of L-arginine, and by doing so, avoid NO dependent killing of the parasite (Gobert et al., 2000); also in *Trypanosoma congolense* infection, arginase and alternative activation of macrophages have been described to play a crucial role in the development of a chronic infection (Noel et al., 2002). During *Leishmania sp. in vitro* (Iniesta et al., 2001; Iniesta et al., 2002) and *L. major in vivo* infection (Iniesta et al.,

2005; Kropf et al., 2005) it is clear that arginase inhibitors reduce pathology by lowering parasite replication inside macrophages.

*T. cruzi* triggers cytokines and chemokines in infected cardiomyocytes (Machado et al., 2000) and macrophages (Bergeron and Olivier, 2006) that induce potent nitric-oxide dependent trypanocidal activity (Gazzinelli et al., 1992; Metz et al., 1993; Munoz-Fernandez et al., 1992; Pakianathan and Kuhn, 1994; Plasman et al., 1994), but results in infected mice are still unclear (Cummings and Tarleton, 2004; Girones et al., 2006; Holscher et al., 1998). Moreover, when excessive, NO can also have a cytotoxic effect in the host and lead to immune suppression of T cells (Goni et al., 2002).

On the other side, peripheral blood monocytes showed decreased nitric oxide production during acute Chagas disease in rats, due to the increase of arginase activity (Fabrino et al., 2004). In addition, macrophages infected *in vitro* (and macrophages from infected mice), by interaction with apoptotic cells through the vitronectin receptor, increase TGF-β and $PGE_2$ release which enhance ODC activity in macrophages, promoting parasite proliferation (Freire-de-Lima et al., 2000; Lopes et al., 2000). Also, cruzipain, a *T. cruzi* antigen, up-regulates arginase activity in macrophages and promotes intracellular growth of the parasite (Giordanengo et al., 2002; Stempin et al., 2002; Stempin et al., 2004). In addition, it promotes arginase II induction in cardiomyocytes acting as a survival factor because it is able to rescue them from apoptosis (Aoki et al., 2004).

It's worth mentioning that in other trypanosomatids, as *Trypanosoma brucei* and *Leishmania donovani,* replication is controlled by the polyamine synthesis inhibitor α-difluoromethylornithine (DFMO) through inactivation of parasite ODC. However, *T. cruzi* lacks arginase (Camargo et al., 1978) and ODC, and DFMO treatment has been shown to be ineffective. The parasite relies on external source of polyamines for growth, probably from the host. Alternatively, *T. cruzi* could synthesize polyamines from L-arginine via agmatine (Persson, 2007). Also, during *T. cruzi* infection in mouse macrophages and rat myoblasts, pharmacological inhibition of ODC reduced invasion and intracellular proliferation and these effects were reverted by the exogenous addition of agmatine or putrescine (Kierszenbaum et al., 1987).

## IV. L-Arginine metabolism in mononuclear phagocytic cells. Regulation of immune responses

### IV.1 Monocyte-Macrophage activation: M1 & M2 macrophages.

Cells from the monocyte-macrophage lineage are known to be a very heterogeneous cell type depending on the differentiation and activation state, site and tissue distribution.

Traditionally, activation of macrophages was considered when they exerted cytotoxic response against intracellular pathogens and also when soluble mediators of the immune response were released. Nowadays, the capacity of macrophages to eliminate intracellular pathogens is known to be mediated by NO (Hibbs, 2002), RNOS and ROS (Kusmartsev et al., 2004; Xia and Zweier, 1997), and to be induced by Th1 cytokines (as IFNγ and TNF) alone or with microbial products. These macrophages also show capacity to produce high levels of IL-12 (in humans IL-23 too), IL-1, TNF, IL-6, and low levels of IL-10 (reviewed in (Mosser, 2003)). Such an activation state leads the scientific community to call these cells **classically activated macrophages** or **M1** macrophages (in analogy to Th1).

In opposition to the type of activation mentioned above, mononuclear phagocytes, which do not show cytotoxic properties and release high levels of IL-10 instead of IL-12, are named **alternatively activated macrophages.** This terminology was conceived for those macrophages that are activated by IL-4/IL-13 cytokines and expressed high arginase I levels, which some authors named **M2** in analogy to the M1 type, but also to those ones that show "deactivation" state in presence of IL-10. Other authors also distinguish amongst the alternative activation what they call type II activated macrophages (Anderson and Mosser, 2002), mononuclear phagocytes exposed to immune complexes and LPS; these cells secrete high IL-10 and low IL-12 levels, and trigger Th2-like responses in T lymphocytes (Anderson et al., 2002).

Due to the fact that some consensus is needed to establish a common nomenclature for these mononuclear phagocytic cells, Mantovani et al. (Mantovani et al., 2004) propose the following (Fig. 6):

M1 corresponds to the classic activation described above. The generic term M2 is used to define macrophage activation other than M1 based in common functional properties (low IL-12 secretion and involvement in Th2 responses, immunoregulation

and tissue remodeling). Also the three well defined forms of M2 will be referred to as M2a, induced by IL-4/IL-13; M2b, induced by immune complexes and agonists of Toll-like receptors (TLRs) or IL-1R; and M2c induced by IL-10 and glucocorticoid hormones.

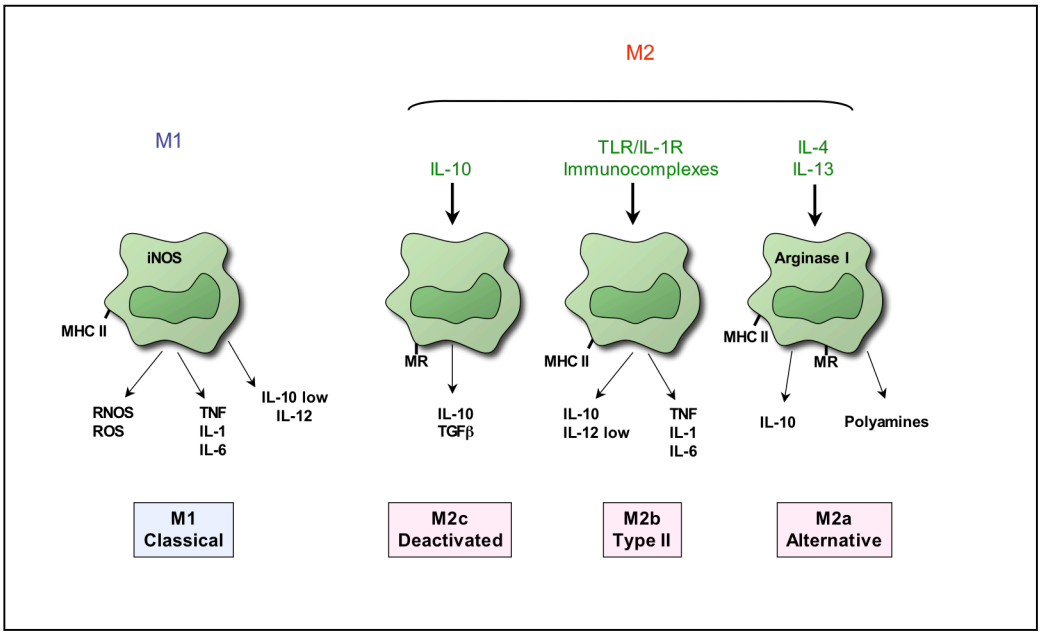


**Figure 6.** *Different subpopulations for polarized monocyte/macrophage cells according to their functional properties.*

Ghassabeh et al. (Ghassabeh et al., 2006) identified a common gene signature for generic M2 cells elicited in different pathological conditions analyzing several genes grouped in specific functional clusters (anti-inflammation, wound healing, angiogenesis, cell-cycle regulation, and others).

## IV.2. Myeloid suppressor cells

Back in the 1970s and early 1980s researchers described what they called natural suppressor cells; non-lymphocyte cells, capable of suppressing lymphocyte response to immunogens and mitogens in an MHC-independent manner (Strober, 1984). Nowadays, it is known that these cells are a subset of myelomonocytic cells called **Myeloid Suppressor Cells (MSCs)**, which accumulate during acute and chronic immune responses to pathogens, immune stress (after exposure with super antigen and

subsequent extensive T-cell activation), tumor growth and development, and others. MSCs are equipped with extremely efficient mechanisms for destroying invading pathogens, and are also highly efficient in suppressing activated T-cells. Reviewed in (Bronte and Zanovello, 2005).

These cells are commonly identified in mice by the expression of **CD11b** and **Gr-1** surface markers; in addition to these markers MSCs might also express a wide variety of myeloid-cell differentiation markers as Ly-6C, CD31 (Bronte et al., 2000); also, there are important differences in the phenotype depending on the anatomical site where they are located or in the pathological conditions. A rigid cell-surface-marker classification for MSCs is not available at present moment; it would need to separate the continuum that is myeloid differentiation into discrete steps. (Bronte and Zanovello, 2005)

## T-cell suppression and L-arginine metabolism

Arginase and iNOS, either separately or in combination, are used by MSCs to inhibit T-cell responses. (Fig.7)

**Arginase**: Loss of CD3ζ chain is the only mechanism, to date, that has been described and proven to have an effect on T-cell function. When MSCs were isolated from tumors, arginase I activity caused depletion of L-arginine amino acid in the extra cellular microenvironment. This inhibited re-expression of the ζ-chain of CD3 after its TCR signaling-induced internalization by antigen stimulated T-cells, thereby impairing the function of these cells (Baniyash, 2004; Rodriguez et al., 2004).

**iNOS:** NO, does not impair the early events triggered by the TCR, but acts instead at the level of IL-2 receptor signaling, blocking the phosphorylation and activation of signaling molecules (Bingisser et al., 1998; Mazzoni et al., 2002).

**Arginase and iNOS:** When both enzymes are induced, depletion of extra cellular L-arginine occurs. This leads the iNOS enzyme to switch his NO producing effect to mostly superoxide ion production, and subsequently ROS and RNOS. These species can have multiple inhibitory effects on T cells. Combined activity of arginase and iNOS enzymes was shown to be important in MSCs from mice with tumors, but also in chronic infections with helminthes (Bronte et al., 2003; Brys et al., 2005). This indicates that co-expression of iNOS and arginase might be a molecular marker for MSCs (Bronte and Zanovello, 2005). It is important to consider in this issue, that some

authors describe a mechanism of down regulation for iNOS mRNA when levels of L-arginine are low; what will result in a decreased iNOS protein stability (El-Gayar et al., 2003).

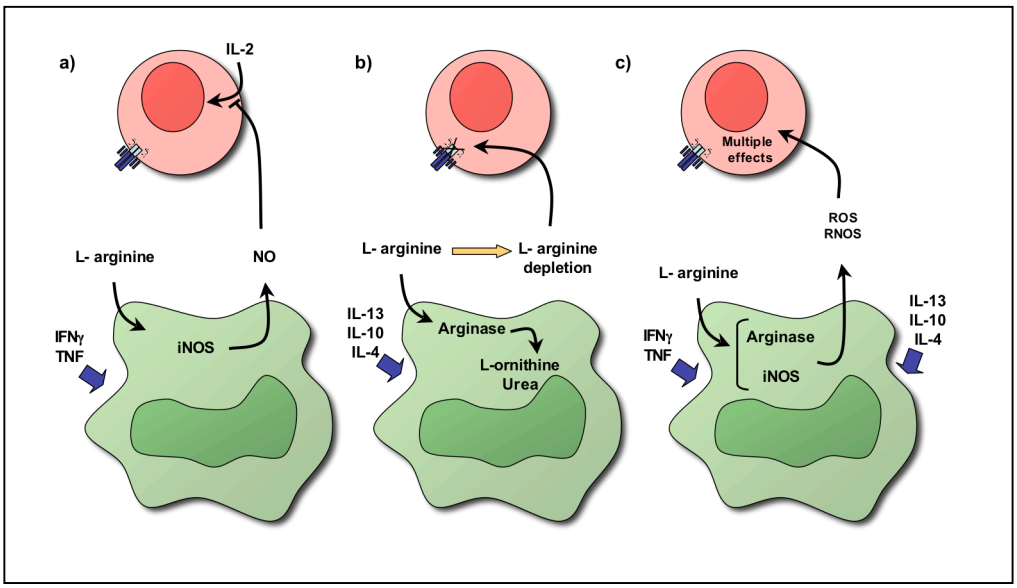


Figure 7. *Arginase and iNOS inhibit T cell response in different ways*. a) MSCs expressing iNOS, b) MSCs expressing arginase, c) MSCs expressing both arginase and iNOS enzymes

## MSCs in parasitic infections

There are several evidences of the role of MSCs in parasitic diseases. Human T cells stimulated in the presence of a crude extract of *H.pylori* had reduced proliferation and this correlated with decreased CD3ζ expression (Zabaleta et al., 2004). Also, results from experimental infections with *Schistosoma* spp. (Terrazas et al., 2001), *Candida albicans* (Mencacci et al., 2002), and *T. cruzi* (Abrahamsohn and Coffman, 1995; Goni et al., 2002) illustrate the existence of an IFNγ induced, NO dependent mechanism of immunosuppression, mediated by MSCs that colonize the spleen during the acute phase of the disease.

# OBJECTIVES

# OBJECTIVES

1. Analyze the expression of arginase and other L-arginine metabolic enzymes in hearts of susceptible and resistant strains of mice infected with *T. cruzi.*

2. Determine the agents responsible for arginase I induction in hearts of *T. cruzi* infected mice.

3. Study the role of arginase I in hearts of *T.cruzi* infected mice.

# MATERIALS AND METHODS

# MATERIALS AND METHODS

## I. Parasites and mice

Work was performed with two different *T. cruzi* strains: Y and Tulahuen (obtained from Dr. John David, Department of medicine, Harvard Medical School, Boston, Massachusetts, U.S.A.). Blood trypomastigotes of the two *T. cruzi* strains were routinely maintained by infecting mice and purifying them from their blood. Young adult (6 to 8-week-old) BALB/c and C57BL/6 mice were purchased from Harlan, Interfauna Iberica. iNOS deficient mice ($NOS2^{tm1\ Lau}$), TNF deficient mice (B6;129S6-$Tnf^{tm1Gkl}$), hybrid B6129SF2/J (wild-type, control mice for TNF deficient mice), IL-10 deficient mice (129-$Il10^{tm1Cgn/J}$), 129Sv, control mice (129S1/SvImJ, wild type for IL-10, and IFNγ-R deficient mice), all purchased from The Jackson Laboratory, Bar Harbor, ME. IL-4 deficient 129Sv mice (Kopf et al., 1993) and IFNγ receptor-deficient 129Sv mice (Huang et al., 1993) were a gift from Manfred Kopf, (Max-Planck-Intitute for Immunobiology, Freiburg), and IL-4R deficient BALB/c mice (Mohrs et al., 1999) were a gift from Dr. Beschin (VUB, Brussels) All mice were maintained under pathogen-free conditions in the animal facility at the Centro de Biología Molecular, Universidad Autónoma de Madrid (Spain).

Groups of 3-5 mice were infected with $2x10^3$ typomastigotes per mice (unless stated different) by intraperitoneal injection. At different days post infection (d.p.i.), mice were euthanized in a $CO_2$ chamber and blood and various tissues were collected. Samples were processed for RNA and protein expression, enzymatic activity, immunohistochemistry, confocal immunofluorescence analyses and magnetic cell sorting.

For *in vitro* infections, Y strain parasites were co-cultured with Vero cells (CCL-81, ATCC) and trypomastigotes were purified from supernatant.

## II. Real time PCR for parasite detection

Hearts were minced into small pieces and DNA was isolated with High Pure PCR Template preparation Kit, Roche. For *T. cruzi* detection, we followed the PCR assay described by (Piron et al., 2007). Different amounts of DNA purified from Y

strain epimastigotes were used to generate the standard curve. Experimental heart tissue PCR reactions contained 100 ng genomic DNA.

### III. mRNA analysis by quantitative RT-PCR

Total RNA was extracted from heart tissue mechanical disruption in TRIzol reagent (Invitrogen) as indicated by the manufacturer. For quantitative real time (q) RT-PCR analysis, reverse transcription of total RNA was performed using the components of the High Capacity cDNA Archive Kit (Applied Biosystems) and amplification of different genes (arginase I, arginase II, iNOS, CAT-1, CAT-2, ODC, IFN-γ, TNF-α, IL-4, IL-10, IL-13, TGF-β, mPGES-1 and Ribosomal 18S) was performed using TaqMan MGB probes and the TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900 HT instrument (Applied Biosystems). All samples were ran in triplicate. Quantification of gene expression by real-time PCR was calculated by the comparative threshold cycle ($C_T$) method following the manufacturer's instructions. All quantifications were normalized to the ribosomal 18S control to account for the variability in the initial concentration of RNA and in the conversion efficiency of the reverse transcription reaction. RQ= $2^{-\Delta\Delta C_T}$

(q)RT-PCR of $CD11b^+$ purified cells was performed as follows: Total RNA was extracted with TRIzol reagent following manufacturer instructions. Reverse transcription was carried out by using 1-2 μg total RNA, 0,5 μg oligo(dT) and 200 units Superscript II reverse transcriptase (all from Invitrogen) in a total volume of 20 μl following the manufacturer recommendations. (q) PCR was performed using a total volume of 25 μl, containing Bio-Rad iQ SYBR Green Supermix, 4μl of (1/25) diluted cDNA reaction as template and 0,2 μM of each primer, in a Bio-rad iCycler (Bio-Rad Hercules, CA). Each PCR cycle consisted of 1 min denaturation at 94ºC, 45 sec annealing at 55ºC and 1 min extension at 72ºC. PCR primer sequences were described in (Ghassabeh et al., 2006). For (q) PCR analysis, the values were normalized against the house-keeping gene S12.

## IV. Protein expression analyses

Protein extracts from heart tissue were prepared by mechanical disruption of the tissue in phosphate buffered saline (PBS) containing 0,1% Triton X-100 (Merck), 100 μg/ml of pepstatin, 100 μg/ml of aprotinin and 100 μg/ml of antipain (all protease inhibitors from Sigma-Aldrich) utilizing a PT 1300 D homogenizer (Polytron). Protein concentration was determined by the bicinchonic acid method (Pierce). Western blot analyses were performed as follows: 15 μg of tissue extract were fractionated on SDS-10% polyacrylamide gel and transferred to a Nitrocellulose membrane Hybond-ECL (Amersham Biosciences). Primary antibodies were diluted as follows: Goat anti-mouse Arginase I (V-20, Santa Cruz Biotechnology) 1:1000, Purified anti-mouse iNOS (BD Transduction) 1:500, mouse monoclonal anti-mouse ODC (clone ODC-29, Sigma) 1:500, goat anti-mouse Actin (I-19, Santa Cruz Biotechnology) 1:1000. Then, the membranes were incubated with horseradish peroxidase (HPO) conjugated rabbit anti-goat (Sigma) 1:10000, or goat anti-mouse (Pierce) 1:1000 as secondary antibodies. Detection was carried out with Supersignal detection reagent (Pierce) and photographic film exposure. For cell-cultured cells, samples were treated as mentioned above except for the mechanical disruption.

## V. Measurement of arginase activity

Arginase activity was measured in cell and tissue lysates as previously described (Corraliza et al., 1994). Briefly, the mixture of 50 μl of 10 mM MnCl2 and 50 mM Tris-HCl, pH 7.5, plus 50 μl of heart tissue or cell extract were heated at 55°C for 10 min to activate the enzyme. Arginine hydrolysis was carried out by the addition of 50 μl of 0.5 M arginine, pH 9.7, to a 50 μl of the lysate. Incubation was performed at 37°C for 60 min, and the reaction was stopped by the addition of 400 μl of an acid mixture containing $H_2SO_4$ (96%), $H_3PO_4$ (85%), and $H_2O$ (1:3:7, vol/vol/vol). After adding 25 μl of 9% μ-isonitrosopropiophenone (ISPF; Sigma) and heating at 100°C for 45 min, the formed urea was colorimetrically quantified at 540 nm. A calibration curve was prepared with increasing amounts of urea solution.

## VI. Immunohistochemistry

Hearts from mice infected with $10^4$ *T. cruzi* trypomastigotes (T strain) were removed at different times, fixed in 10% neutral buffered paraformaldehyde and embedded in paraffin. Four micrometer-thick sections were mounted on gelatine coated glass slides. Samples were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in PBS for 10 min. Slices were permeabilized with 1% Triton X-100 in PBS for 10 min. The sections were then washed and non-specific binding prevented by incubating with 2% bovine serum albumine (BSA) for 30 min. Samples were incubated with anti-arginase II, or anti-iNOS antibodies, or isotypic antibodies (all purchased from Santa Cruz Biotechnology, Inc.) diluted 1:100. After washing with PBS, the samples were incubated with anti-rabbit HPO (Sigma Aldrich) diluted 1:250. The antigen-antibody complexes were visualized by the diaminobenzidine/hydrogen peroxide technique and counterstained with hematoxylin. The slides were observed under a light microscope (Carl Zeiss, Germany) and photographed (Axiocam HRc).

## VII. Confocal immunofluorescence

Organs were removed from mice at different times post infection, fixed in a 4% paraformaldehyde solution and after 1h placed in a 30% sucrose solution over night. Hearts were then embedded in Tissue-Tek (Sakura) and frozen. 10-15 µm sections were cut using a cryostat. Slides were fixed in acetone for 10 min at room temperature, and incubated 10 min with $NH_4Cl$ to avoid autofluorescence; then were washed and incubated over night at 4ºC with the following primary antibodies diluted to 10 µg/ml in 100mM Tris-HCl pH 8.0, 300mM NaCl, 0,1% Triton X-100, 5% normal mouse serum: goat anti-mouse arginase I (V-20, Santa Cruz Biotechnology), rat anti-mouse CD68 (clone FA-11, Serotec), rat anti-mouse CD11c (clone N418, e-Bioscience), rat anti-mouse F4/80 (clone BM8, e-Bioscience). Rat IgG2a (clone R35-95, BD Pharmingen), and ChromPure Goat IgG (Jackson Immunoresearch) were used as isotypic controls. Secondary antibodies were used at the following dilutions: F(ab´)2 Goat anti-rat IgG fluorescein isothiocyanate (FITC; Serotec)1:100, Cy 5-conjugated Donkey anti-goat IgG (Jackson Immunoresearch) 1:50. Stained slides were subjected to confocal

microscopy using a LSM510 META confocal laser coupled to an Axiovert 200 (Zeiss) microscope.

## VIII. Neonatal mouse primary cardiomyocyte culture

Neonatal cardiomyocytes were obtained following a procedure previously described (Wang et al., 1999). For this, one to three-day-old neonatal mice were euthanized, hearts were removed aseptically and kept in Hanks' balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$ (HBSS) (in g/l: 0.4 KCl, 0.06 $KH_2PO_4$, 8.0 NaCl, and 0.05 $Na_2HPO_4$, pH 7.4) on ice. The tissues were washed three times and minced into small fragments. The remaining pieces were dissociated with trypsin (0.25% wt/vol in HBSS) at 37°C. The cells released after the first digestion were discarded, whereas the cells from subsequent digestions were added to an equal volume of cold HBSS with $Ca^{2+}$ and $Mg^{2+}$ plus 10% fetal bovine serum (FBS; Biowittaker) (in g/l: HBSS plus 0.14 $CaCl_2$, 0.047 $MgCl_2$, 0.049 $MgSO_4$, 0.35 NaHCO3, and 1.0 D-glucose, pH 7.4) until all cardiac cells were isolated. The resulting cell suspension was centrifuged at 200 g for 8 min, and the cells were resuspended in complete Dulbecco's modified Eagle's medium (DMEM; Sigma), containing 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 1000u/ml and 0,1 mM non-essential amino acids and supplemented with 10% FBS (vol/vol). To exclude non-muscle cells, the isolated cells were first plated in tissue culture dishes at 37°C for 2 h under a water-saturated atmosphere of 5% $CO_2$. The suspended cells were then collected and plated at a density of 1.0 x $10^5$ cells/$cm^2$ in 10% FBS-complete DMEM. More than 90% of cells were cardiomyocytes as detected by immunostaining with antibody to mAchR M2 (Aoki et al., 2004). Cells were cultured for 24 h and then the culture medium was changed to 10% FBS-complete DMEM containing different stimuli: IL-4 (20 ng/ml), IFNγ+LPS (25U/ml and 1µg/ml, respectively) and 5:1 (parasite: cell ratio) trypomastigotes. Recombinant mouse IL-4 and IFNγ were purchased from BioSource, International. LPS (*Escherichia coli* serotype O26:B6) was purchased from Sigma-Aldrich. Cells where then incubated for 48h at 37ºC in 7% $CO_2$ atmosphere.

## IX. PBMCs, spleen and lymph node cell suspension

Spleen and inguinal lymph nodes were aseptically removed from BALB/c mice and disaggregated in 40μm nylon cell strainers (Falcon). Blood was obtained by cardiac punction and treated with heparin. Single suspension cells were depleted of erythrocytes by hypotonic lysis, washed with cold PBS and processed for SDS-PAGE as described above.

## X. Peritoneal cells culture and infection

Peritoneal cavity from BALB/c mice was washed with 7 ml of a solution of sucrose (116g/l) in PBS. Cells were then plated in complete RPMI (containing 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 1000u/ml and 0,1 mM non-essential amino acids) with 5% FBS at 37ºC with 7% $CO_2$. After 4h, non-adherent cells were removed by washing three times with warm PBS, and fresh complete RPMI was restored. Thus, trypomastigotes purified from supernatant of a Vero cell co-culture were added at the ratio of 5 parasites per adherent (mostly macrophages) cell. After 16h non-internalized parasites were removed and macrophages were cultured in complete RPMI medium at 37ºC with 7% $CO_2$ for 24 or 48h. For immunoblotting cells were washed twice with warm PBS and then detached from the plates using a cell scrapper (Falcon). Cells were processed for SDS-PAGE as previously described.

## XI. Isolation of CD11b+ cells from heart

10 to 12 BALB/c mice were infected i.p. with 2000 trypomastigotes Y strain. At 21 days post infection, mice were euthanized in a $CO_2$ chamber and hearts were aseptically removed, perfused with 10 ml PBS-heparin solution, and kept in cold HBSS. Then, hearts were pooled in a cell culture dish, washed thoroughly with HBSS and minced into small pieces with a sterile surgery blade. Heart pieces were then digested with an enzyme mixture as follows: 1x Trypsin (Gibco), 0,025 mg/ml collagenase type I, 0,2 u/ml DNAse I, 200 u/ml collagenase type IV in HBSS free (both collagenase and DNAse were purchased from Sigma). Digestion was repeated 5 consecutive times, during 15 minutes at 37°C. Cells released from digestions were added to an equal

volume of cold HBSS with $Ca^{2+}$ and $Mg^{2+}$ plus 10% fetal bovine serum (FBS) (in g/l: HBSS plus 0.14 $CaCl_2$, 0.047 $MgCl_2$, 0.049 $MgSO_4$, 0.35 NaHCO3, and 1.0 D-glucose, pH 7.4) and the resulting cell suspension was centrifuged at 200 g for 8 min. Supernatant was discarded and cell pellet was resuspended in complete DMEM supplemented with 10% FBS (vol/vol).

$CD11b^+$ cells were selected by magnetic cell sorting using CD11b Microbeads, MACS LS columns and MACS Separators (Miltenyi Biotec GmbH) following manufacturer instructions. Cell purity of $CD11b^+$ sorted population was analysed by FACS and results showed 95-99% of cell purity.

For $Ly6G^+$ cell sorting, anti Ly-6G MicroBead kit (Milteny Biotec GMbH) was used with the above mentioned separating system. $Ly6G^-$ fraction of the cell suspension was afterwards processed for $CD11b^+$ cell sorting.

## XII. Proliferative assays

$CD11b^+$ cells were obtained as mentioned above, counted, resuspended in complete RPMI and 100.000 cells per well were plated in flat 96 well plates. Spleen cell suspension was obtained as mentioned above and T cells were purified using Pan T cell kit (Milteny Biotec GMbH) and MACS LS columns and separators (Mylteny Biotec GmbH) following manufacturer instructions. Sorted T cells were counted and 100.000 cells per well were plated with or without $CD11b^+$ purified cells isolated from heart from infected mice, blood from infected mice, and blood from non-infected mice. Cells were stimulated with 10μg/ml purified anti-CD3 (clone 145-2C11, BD Pharmingen) and 2 μg/ml purified anti-CD28 (clone 37.51, BD Pharmingen) or with 5μg/ml concanavalin A and, where indicated, 24 μM $N^{\omega}$-Hydroxy-nor-L-arginine (nor-NOHA; Calbiochem), and/or 2mM $N^G$-monomethyl-L-arginine (L-NMMA; Calbiochem) were added. After incubation at 37°C and 5% $CO_2$ for 24 h, 1μCi [3H] thymidine (Amersham, Little Chalfont, UK) was added to each well. The cultures were harvested 18 h later and then processed for measurement of incorporated radioactivity in a liquid scintillation counter.

## XIII. Flow cytometry

Surface cell staining was performed in 96 well plates. Single cell suspensions were fixated with paraformaldehyde 1% in PBS for 1h at 4°C. Cells were washed with cold PBS throughout the whole staining procedure. Fc receptors on cells were blocked by incubating with purified anti-CD16/CD32 (Mouse BD Fc Block) (clone 2.4G2, BD Pharmingen) for 15 min. 4ºC. Cells were then stained with the following antibodies: Alexa 647-conjugated anti-CD11b (clone M1/70, BD Pharmingen), FITC-conjugated anti-F4/80 (clone BM8, e-Bioscience), Phycoerithrin (PE)-conjugated anti-CD11c (clone HL3, BD Pharmingen), FITC-conjugated anti-CD19 (clone MB19-1, e-Bioscience), PE-conjugated anti-MHC class II (I-A/I-E) (clone M5/114.15.2, e-Bioscience), FITC-conjugated anti-Ly-6C (clone AL-21, BD Pharmingen), PE-conjugated anti-Gr-1 (clone RB6-8C5, e-Bioscience). The antibodies Rat IgM (clone R4-22, BD Pharmingen), Rat IgG2a (clone R35-95, BD Pharmingen), Rat IgG2b (clone A95-1, BD Pharmingen) were used as isotype controls.
Cells were acquired on a FACSCalibur Cytometer (Becton Dickinson). Data were analysed with the FlowJo software.

## XIV. Statistical analysis

For *in vivo* experiments, data are shown as means ± s.e. or s.d. from triplicate determination of a representative experiment out of the 3 to 5 performed. Results shown from *in vitro* experiments are representative of at least two experiments performed by duplicate. Significance was evaluated by Student's *t*-test with Origin-Pro software; all differences mentioned were significant compared to controls ($p < 0,05$ or $p < 0,01$).

# RESULTS

# RESULTS

## I. Arginase I induction in hearts of mice infected with *T.cruzi*.

It is still unknown whether *T.cruzi* infection results in a different degree of pathology, but it is becoming accepted that it is likely to be related to host genetics. Infection of different strains of mice having distinct susceptibility or resistance to the parasite, in relation with their different genetic background, has been established as an experimental model for Chagas disease. Thus, we used BALB/c and C57BL/6 mice as susceptible and resistant experimental model of Chagas disease, respectively. Previous studies have already addressed the differences in parasitemia that exist between BALB/c and C57BL/6 mice, but to date no studies have reported parasite load in the heart, the most affected organ in Chagas disease. In order to investigate this, we infected BALB/c and C57BL/6 mice intraperitoneally with the Y strain of *T. cruzi* and parasites in heart were quantified at different d.p.i. by quantitative real time PCR. Figure 1 shows that maximum parasite load was detected in C57BL/6 heart at 14 d.p.i. and decreased thereafter. In contrast, maximal parasite burden in heart of BALB/c mice was somewhat delayed between 14-21 d.p.i. and remained elevated up to 42 d.p.i. Interestingly, burden was significantly higher in susceptible BALB/c than in resistant C57BL/6 mice from 14 to 42 d.p.i., and more than 3 orders of magnitude higher in BALB/c than C57BL/6 mice at 21 d.p.i.

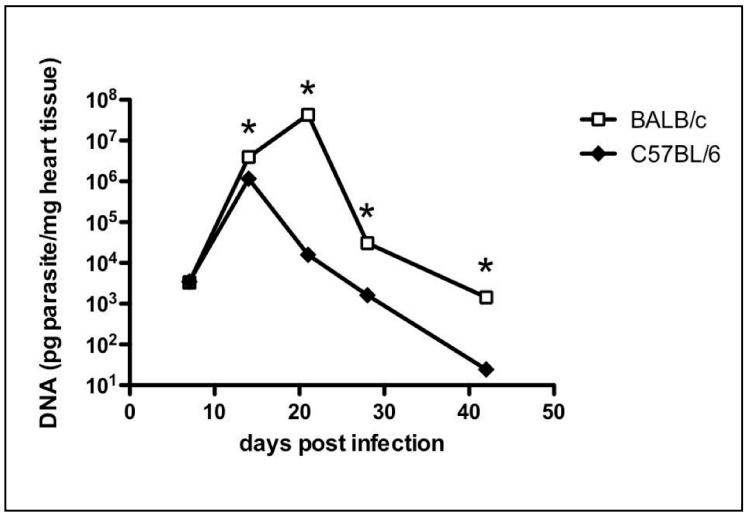


**Figure 1**. T. cruzi *DNA quantification in heart tissue of BALB/c and C57BL/6 infected mice.* DNA from heart tissue was isolated at indicated d.p.i. and qPCR was performed as previously described. *T. cruzi* DNA is expressed as pg of parasite DNA per mg of DNA from heart tissue sample. Results are represented as mean ± SD of triplicates of pooled DNA of 5 different mice. All differences between BALB/c and C57BL/6 mice parasite load were statistically significant except at 7 d.p.i. ($p < 0.05$). A representative experiment out of three performed is shown.

## I.1 Expression of L-arginine metabolic enzymes and transporters in heart tissue of mice infected with *T. cruzi*.

Next, we addressed the levels of enzymes individual in L-arginine metabolism. Quantification of **arginase I** and **arginase II** mRNAs showed significantly higher expression in BALB/c than C57BL/6 mice at all d.p.i. analyzed (Fig 2a-b). In contrast, **CAT-2** and **iNOS** mRNAs expression was higher in C57BL/6 than BALB/c mice at 14 d.p.i (Fig. 2d-e). However, CAT-2 and iNOS mRNAs expression was much higher in BALB/c mice at 21 d.p.i. and in the case of iNOS expression higher levels remained at 28 d.p.i. **CAT-1** showed in BALB/c mice at 7 and 21 d.p.i, but no differences were observed at 14 and 28 d.p.i. (Fig. 2c). The induction of ODC mRNA expression was not significantly different from non-infected controls (Fig. 2f).

Since arginase I mRNA showed major differences between C57BL/6 and BALB/c mice, further studies were performed to confirm that this was also occurring at the protein level. Arginase I protein induction in infected heart was much stronger in BALB/c than C57BL/6 mice and spanned from 14 to 42 d.p.i, in agreement with the mRNA data (Fig. 3a). Arginase I expression in C57BL/6 mice was observed only between 14 and 21 d.p.i. Moreover, arginase enzymatic activity nicely correlated with arginase I protein expression in heart extracts from both mice strains (Fig. 3b), being much higher again in BALB/c mice than in C57BL/6 mice peaking at 21 d.p.i. It is worth mentioning that, although arginase I protein was clearly detected by western blot technique in C57BL/6 mice, arginase activity levels, although higher than in non-infected mice only showed significant differences at 14 d.p.i. in this strain of mice. iNOS protein expression was higher in infected BALB/c than infected C57BL/6 mice, and coincident with the peaks of arginase I expression in each strain. Although we did not observe significant changes in ODC mRNA expression, ODC protein levels were greater, in hearts from infected BALB/c (maximum expression at 21 d.p.i) and much lower in infected C57BL/6 mice (peaking at 14 d.p.i.) (Fig. 3a). This result indicates that ODC is regulated at the post-translational level in heart tissue of mice infected with *T. cruzi*. This is not surprising, since ODC is one of the most highly regulated enzymes in eukaryotic cells at posttranscriptional levels.

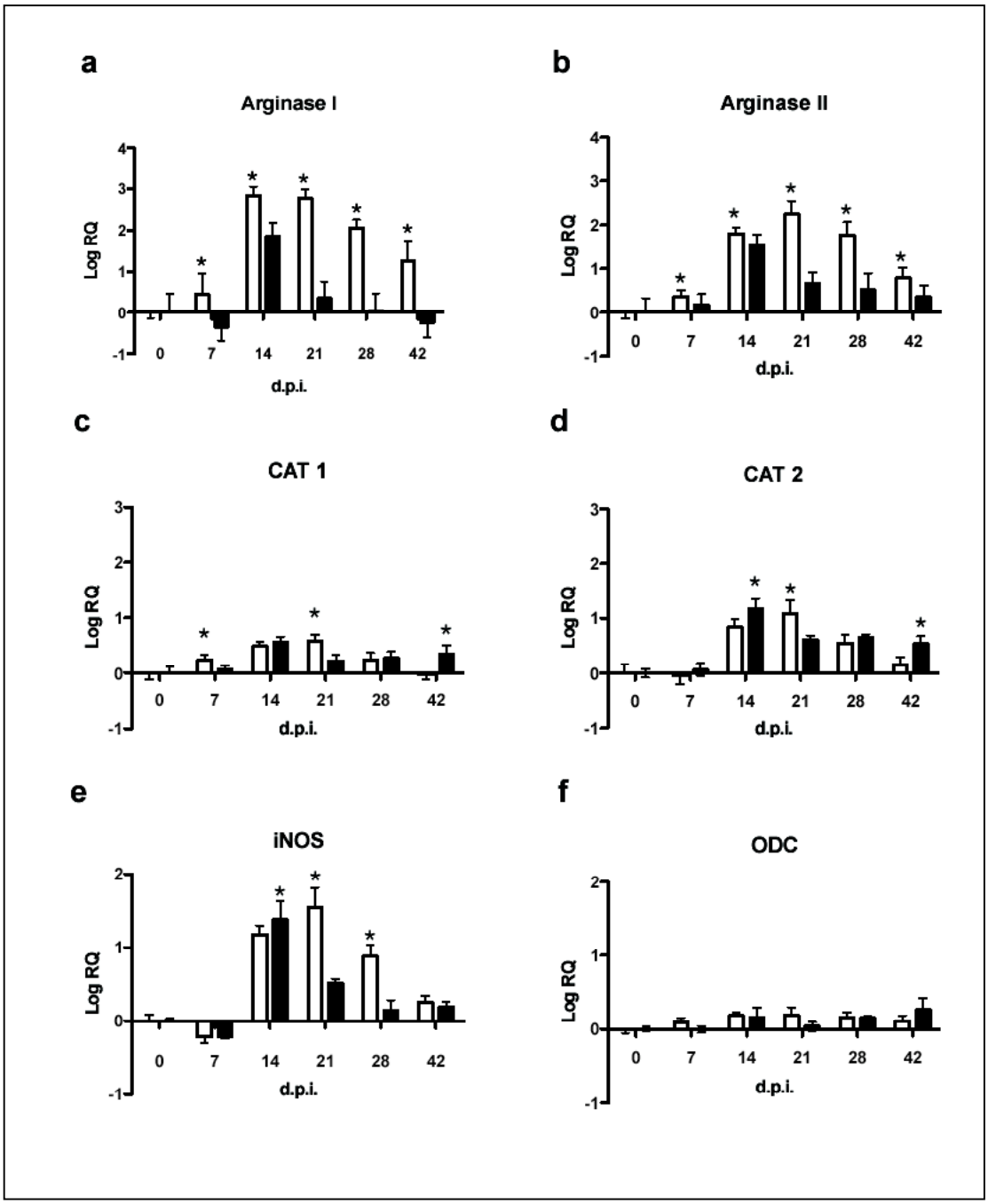


**Figure 2**. Q*uantification of mRNA leves of enzymes and transporters involved in L-arginine metabolism in heart tissue during* T. cruzi *infection.* RNA from heart tissue was isolated at different d.p.i. and qRT-PCR was performed. Results are expressed as the logarithm of RQ calculated from CT values as described in material and methods. mRNAs for: a) Arginase I; b) Arginase II; c) CAT-1; d) CAT-2; e) iNOS f) ODC. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. A representative experiment out of five is shown. Results are represented as mean ± SD of 3 different mice. All differences observed in infected mice respect to non-infected mice were statistically significant except for: a) C57BL/6 at 7, 21, 28 and 48 d.p.i.; b) C57BL/6 at 7 and 48 d.p.i.; c) C57BL/6 at 7 d.p.i.; d) BALB/c at 7 and 42 d.p.i. and C57BL/6 at 7 d.p.i.; f) BALB/c and C57BL/6 at all d.p.i. Significant differences observed between BALB/c and C57BL/6 mice are indicated by an asterisk. ($p < 0.05$).

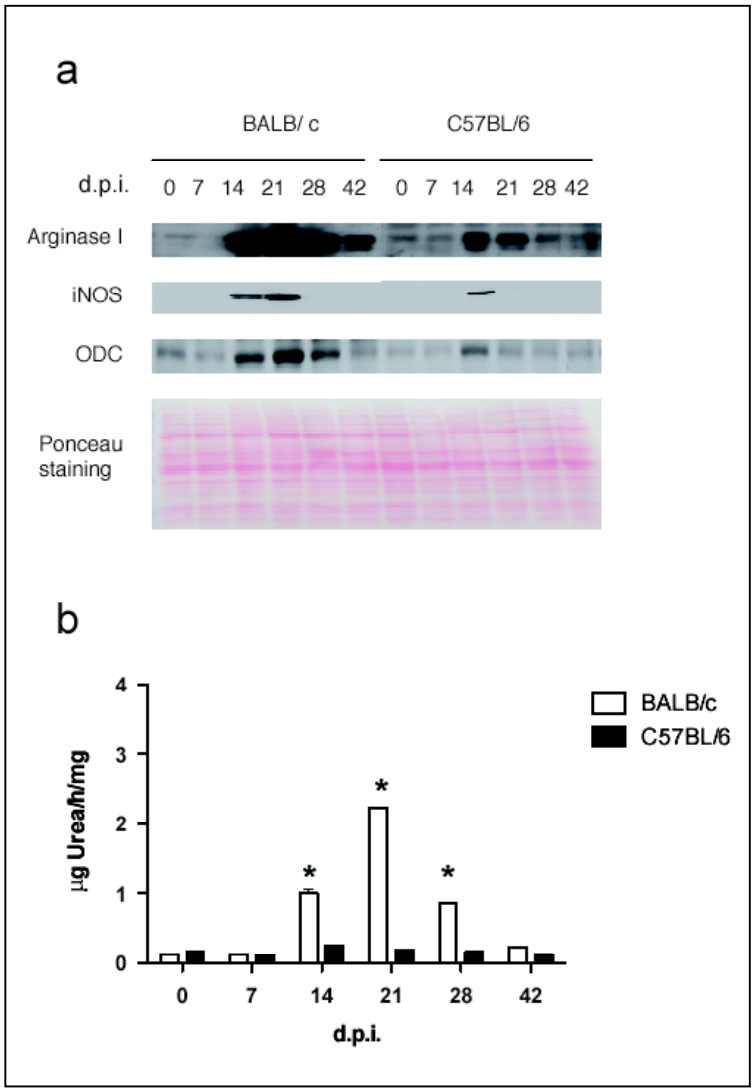


**Figure 3.** *iNOS, ODC and arginase I protein expression and arginase activity in heart during* T. cruzi *infection.* Protein extracts from heart tissue were isolated from 5 BALB/c and 5 C57BL/6 mice at different d.p.i. and pooled for western blot analysis: a) Arginase I, iNOS, ODC expression and Ponceau staining of the same membrane; numbers indicate d.p.i. b) Arginase activity of the same tissue extracts, expressed as µg of urea produced per hour per mg of protein sample (µg/h/mg), was assessed by urea colorimetric determination. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. All values for BALB/c mice were significantly higher compared to non-infected mice except at 7 d.p.i. and in C57BL/6 were only significantly higher at 14 d.p.i. Asterisks indicate significant differences between BALB/c and C57BL/6 mice ($p < 0.05$). A representative experiment out of 5 is shown.

## I.2 Arginase I expression in infected mice is restricted to heart tissue and peritoneal cells.

To determine whether arginase I induction upon *T.cruzi* infection was ubiquitous or restricted to heart tissue, we also studied the expression of arginase I and iNOS in peripheral blood mononuclear cells (PBMCs), spleen and lymph node from infected BALB/c mice at 21 d.p.i. In spleen and ganglia neither arginase I nor iNOS were induced. There was a slight induction of arginase I expression in PBMCs in comparison with the induction observed in heart tissue, but no iNOS was detected in PBMCs (Fig 4a).

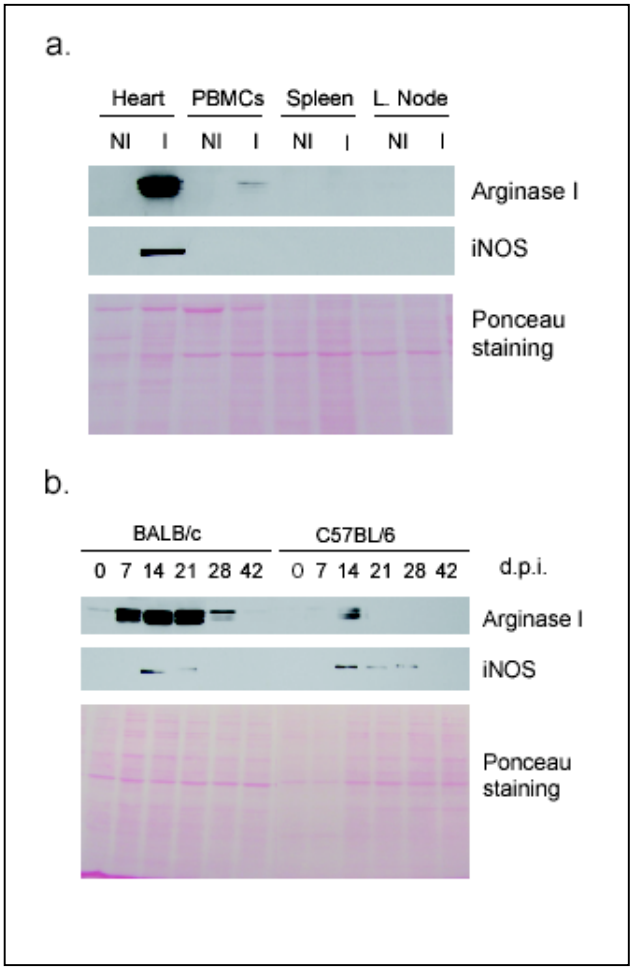


**Figure 4.** *iNOS and arginase I protein expression in different tissues.* Protein extracts were isolated and analysed by western blot using anti-arginase I or anti-iNOS antibodies and Ponceau staining. a) BALB/c heart tissue, peripheral blood mononuclear cells (PBMCs), spleen and lymph node were obtained from non-infected (NI) and infected (I) mice at day 21 post infection. Data are representative of at least two independent experiments. b) Peritoneal exudate cells (PECs) from 5 BALB/c or C57BL/6 mice of different d.p.i., as indicated, were collected and pooled as described in material and methods. A representative experiment out of three is shown.

Arginase I and iNOS were also detected in peritoneal exudate cells (PECs) from infected mice. Arginase I protein in BALB/c increased as early as 7 d.p.i. showing the highest levels at 14-21 d.p.i. and decreasing thereafter. In C57BL/6 much lower levels were observed. Only a faint band in 14 d.p.i. lane was seen indicating that arginase I expression in PECs from infected BALB/c mice is much higher and longlasting than in PECs from infected C57BL/6 mice. iNOS protein showed similar expression levels in PECs from both strains of mice, but in PECs from BALB/c mice was detected only at 14 and 21 d.p.i while in PECs from C57BL/6 mice iNOS expression was detected also at 28 d.p.i (Fig. 4b). This expression pattern, although slightly different from the one found in heart tissue, nonetheless points out to a higher expression of arginase I in BALB/c infected mice compared with C57BL/6 mice in a time-dependent manner. In PECs, arginase I expression is already seen at 7 d.p.i. faster than in heart, since mice are being infected intraperitoneally those are the first ones to encounter the parasite.

## I.3. Arginase I modulation in hearts of iNOS deficient mice

In order to test the contribution of the iNOS enzyme to heart expression and modulation of arginase I, iNOS deficient mice (iNOS$^{-/-}$) were infected with *T. cruzi* and compared with the infected wild type strain C57BL/6.

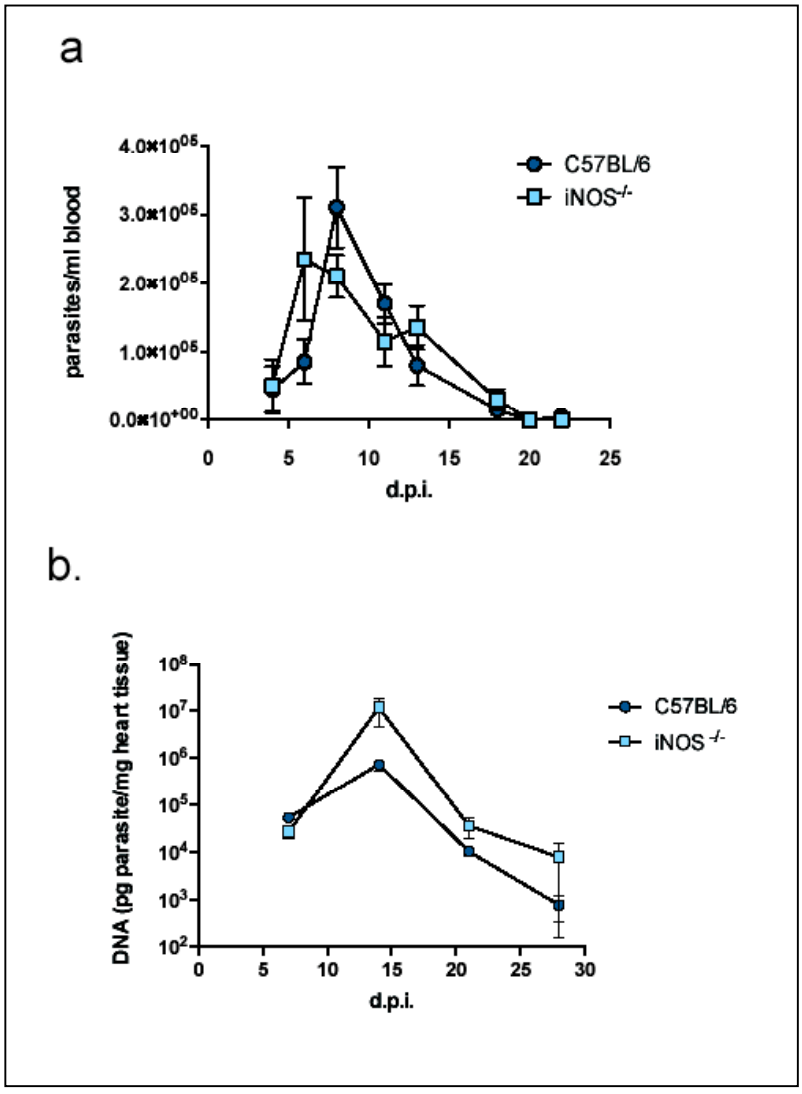


**Figure 5**. *Parasite load in iNOS$^{-/-}$ infected mice.* a) Parasitemia of infected wild type (C57BL/6) and iNOS$^{-/-}$ mice. Parasitemia was determined in blood from the tail as described in materials and methods. Results are presented as mean ± SE (n=6). A representative experiment out of three is shown. b) DNA from heart tissue was isolated at indicated d.p.i. and qPCR was performed as previously described. *T. cruzi* DNA is expressed as pg of parasite DNA per mg of DNA from heart tissue sample. Results are presented as mean ± SE (n=3).

Parasitemia in iNOS$^{-/-}$ mice did not significantly change respect to wild type mice (Fig. 5a); this fact agrees with some previous reports (Cummings and Tarleton, 2004). When parasite DNA was quantified in heart from infected iNOS deficient and C57BL/6 mice strains, parasite burden showed no significant differences between them (Fig. 5b). Despite the fact that neither parasite burden in blood or in heart tissue showed

any differences in iNOS deficient compared to wild type mice, heart arginase I expression (Fig. 6a) and arginase activity (Fig. 6b) were strongly increased in infected mice iNOS$^{-/-}$ compared to C57BL/6 mice. iNOS protein was induced heart homogenates from wild-type mice but, as expected, not in iNOS $^{-/-}$ mice (Fig. 6a). Thus, deletion of the iNOS gene triggered the increase in the induction of arginase I in heart tissue of mice infected with *T. cruzi.*

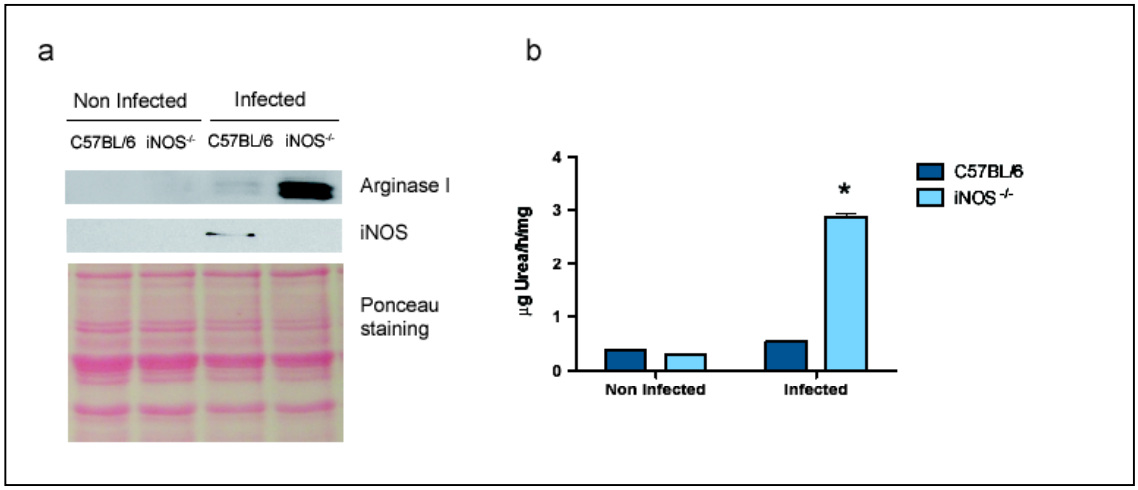


**Figure 6.** *Arginase I and iNOS expression in heart tissue from iNOS$^{-/-}$ infected mice.* a) Arginase I and iNOS expression in heart tissue extracts pooled from 3 wild-type and 3 iNOS $^{-/-}$ non-infected (NI) and infected (I) mice at 21 d.p.i. and Ponceau staining of the same membrane. b) Arginase activity of the same extracts expressed as µg of urea produced per hour per mg of protein sample. Results are presented as mean ± SE. Data are representative of at least two independent experiments.

## II. Th2 cytokines induce arginase I in heart tissue of mice infected with *T.cruzi.*

Cytokines are known to play an important role in the induction and generation early phase and the setting of the immune response during *T.cruzi* infection, but in previous studies with resistant and susceptible mice, no clear Th1 or Th2 pattern was shown (Powell et al., 1998; Zhang and Tarleton, 1996a, 1996b). Moreover, arginase I can be induced by TGFβ, IL-10, $PGE_2$, and Th2 cytokines (Boutard et al., 1995; Corraliza et al., 1995; Jost et al., 2003; Munder et al., 1998), while iNOS protein induction is mediated mostly by Th1 cytokines (MacMicking et al., 1997; Vila-del Sol et al., 2007). Thus, we next studied if Th1/Th2 cytokine levels in heart tissue were related with resistance and susceptibility to *T.cruzi*, and whether this related with arginase I expression.

## II.1. Th1 and Th2 cytokines are induced in heart during *T.cruzi* infection.

We evaluated the levels of several Th1 and Th2 cytokines in heart tissue from BALB/c and C57BL/6 mice infected with *T. cruzi* by quantitative mRNA RT-PCR. The results showed that **IFNγ** and **TNF** presented maximum expression at 14 and 21 d.p.i. in C57BL/6 and BALB/c mice, respectively (Fig. 7a and b). These cytokines were significantly higher in C57BL/6 than BALB/c mice at 14 d.p.i. but this situation was switched between 21 d.p.i and later times, being higher in BALB/c than C57BL/6 mice. On the other hand, **IL-4** and **IL-13** showed maximum expression at 14 d.p.i. in both mouse strains, being higher in BALB/c than C57BL/6 mice through infection. Interestingly, IL-4 and, especially, IL-13 expression in BALB/c was maintained at 21 d.p.i. (Fig. 7c and d). **IL-10** expression in the heart was significantly increased by infection and followed kinetics similar to TNF (Fig. 7e). The induction of **TGFβ** mRNA expression observed was not statistically different from non-infected controls (Fig. 7f). Thus, no pure Th1 or Th2 patterns are linked to strain susceptibility or infection stage. However, the Th1/Th2 balance was higher for C57BL/6 (resistant) than for BALB/c mice (susceptible) and Th2 cytokines were higher in BALB/c than in C57BL/6 mice during infection.

## II.2. TNF and IFNγ receptor deficient mice show a small increase of arginase I when infected with *T.cruzi*.

Despite the fact that Th2 cytokines have been reported to be responsible for arginase I induction in several *in vivo* and *in vitro* models, we also found high levels of Th1 cytokines in BALB/c mice expressing arginase I. In order to clarify the possible role of Th1 cytokines in arginase I induction, we infected mice deficient for TNF and mice deficient for the IFNγ receptor (IFNγR), two cytokines involved in Th1 response. It had previously been reported that mice deficient for TNF receptor (Castanos-Velez et al., 1998) and IFNγR$^{-/-}$ mice (Holscher et al., 1998) had increased susceptibility to *T.cruzi* infection and could not set up a proper immune response to eliminate the parasite and overcome the disease.

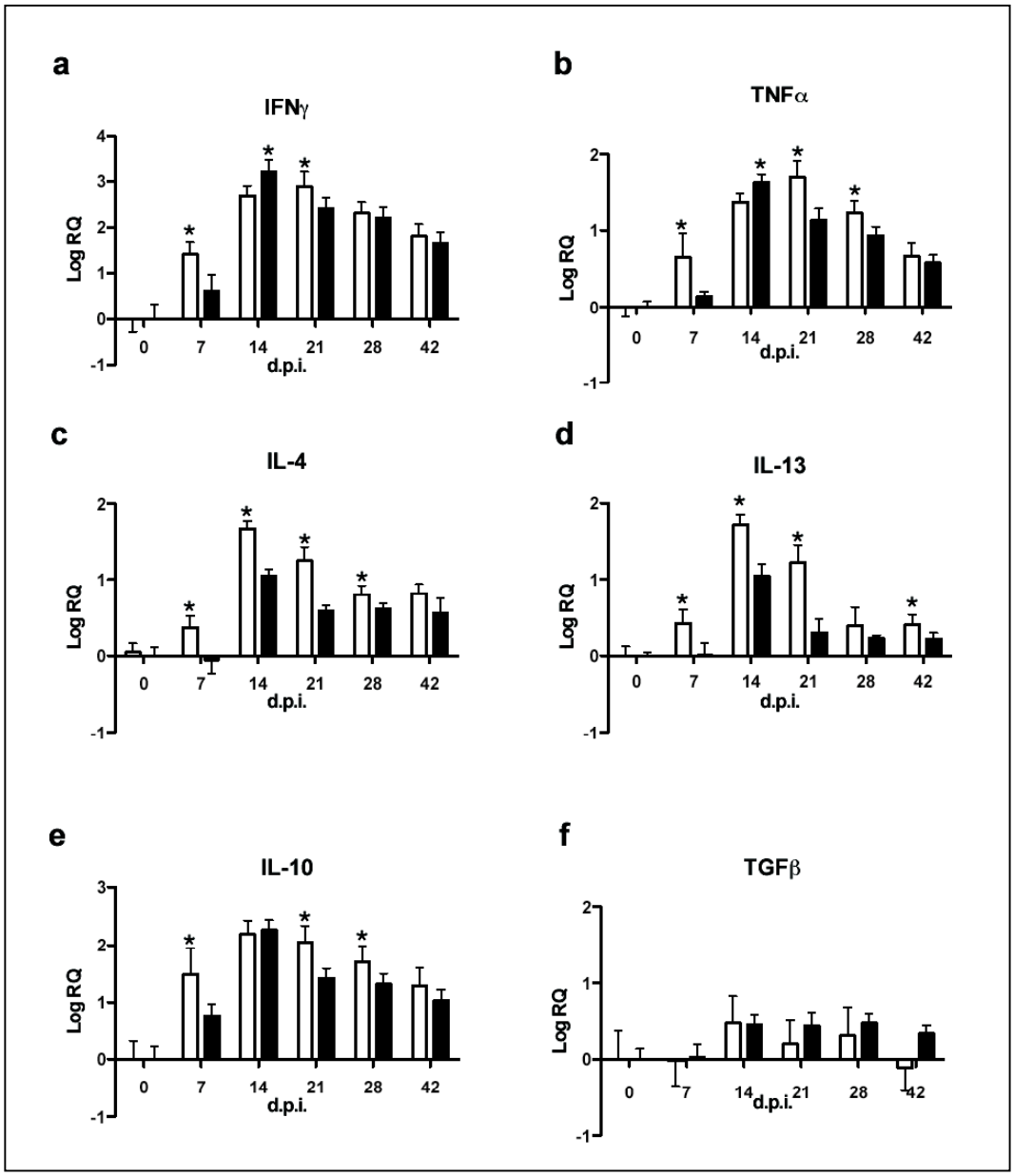


**Figure 7.** *Expression of cytokines in heart tissue during* T. cruzi *infection.* Total RNA from heart tissue was isolated at different d.p.i. from BALB/c and C57BL/6 mice and qRT-PCR was performed as described. Results are expressed as the logarithm of RQ calculated from CT values as described in materials and methods. mRNAs for: a) IFNγ; b) TNFα; c) IL-4; d) IL-13; e) IL-10 and f) TGFβ. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. A representative experiment out of three is shown. Results are represented as mean ± SD of 3 different mice. All differences observed in infected mice respect to non-infected mice were statistically significant except for: c) and d) C57BL/6 at 7 d.p.i.; f) BALB/c and C57BL/6 at all d.p.i.; Significant differences observed between BALB/c and C57BL/6 mice are indicated by an asterisk. ($p < 0.05$).

**TNF deficient mice** infected with the Y strain of *T.cruzi* showed a significant increase in parasitemia compared to their wild-type littermates (B6129Sv) at 16 d.p.i. (Fig. 8a). Longer times of infection could not be monitored since TNF deficient mice succumb to infection and most mice die at day 21 p.i. Arginase I expression in heart protein extracts from infected TNF deficient mice showed much reduced levels when compared with the infected wild-type mice at 18 d.p.i, the only time-point when this enzyme was detected (Fig. 8b). Surprisingly, no significant differences between these mice were observed when arginase activity was determined in the same protein lysates (Fig. 8c). iNOS protein was induced in wild-type mice at 18 d.p.i. but no protein was detected in TNF$^{-/-}$ mice (Fig. 8b), probably due to the fact that TNF is needed for a proper iNOS induction (Vila-del Sol et al., 2007).

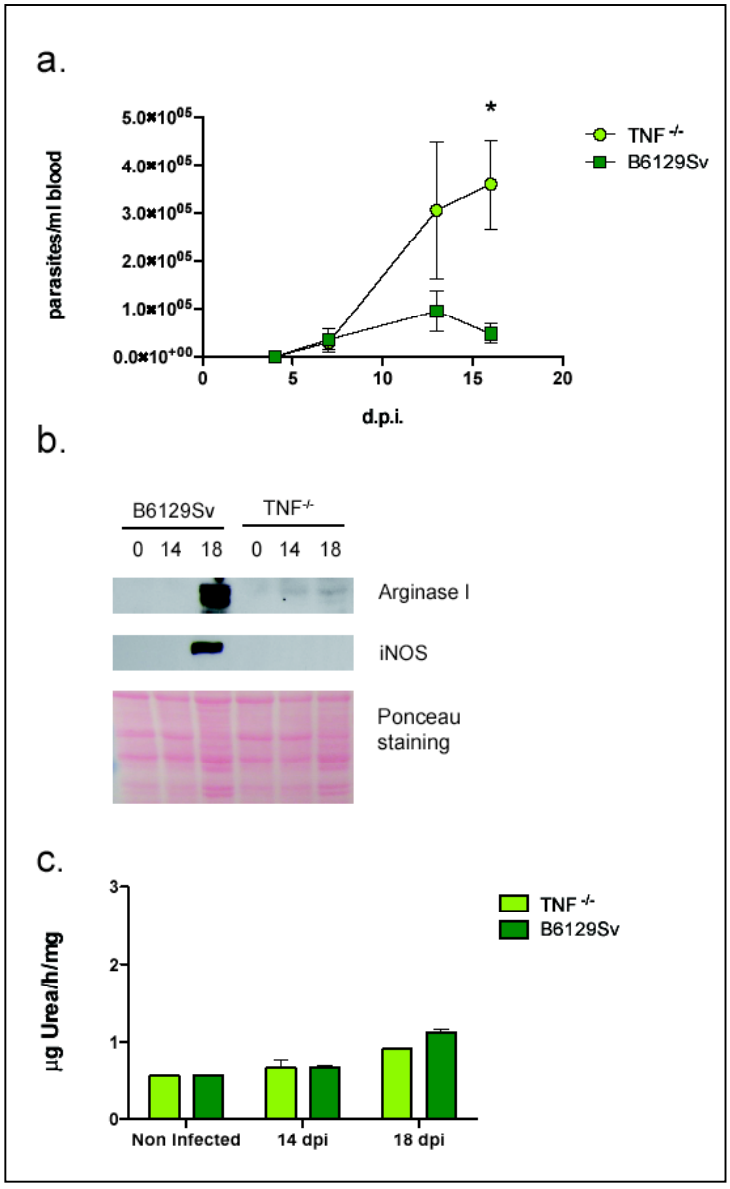


**Figure 8**. *Parasitemia, arginase I and iNOS expression and arginase activity in heart tissue of TNF$^{-/-}$ mice during* T. cruzi *infection.* a) Parasitemia was determined in blood from the tail as described in materials and methods. Results are presented as mean ± SE (n=4). Significant differences observed between TNF$^{-/-}$ and B6129Sv mice are indicated with an asterisk ($p< 0.05$). b) Arginase I and iNOS expression in heart tissue extracts pooled from 3 wild-type and 3 TNF $^{-/-}$ mice at indicated d.p.i and Ponceau staining of the same membrane. c) Arginase activity of the same extracts expressed as µg of urea produced per hour per mg of protein sample; Results are presented as mean ± SE (n=3). A representative experiment out of two is shown.

**INFγR deficient mice** also showed very high parasite numbers in blood when infected with a low inoculum of 500 parasites of the *T.cruzi* Y strain. Parasites in blood 13 d.p.i. were significantly much higher in IFNγR$^{-/-}$ mice when compared with their wild-type mice (129Sv) as seen in Fig. 9a. As in TNF deficient mice infection cannot be monitored longer than 16 d.p.i as mice die of infection.

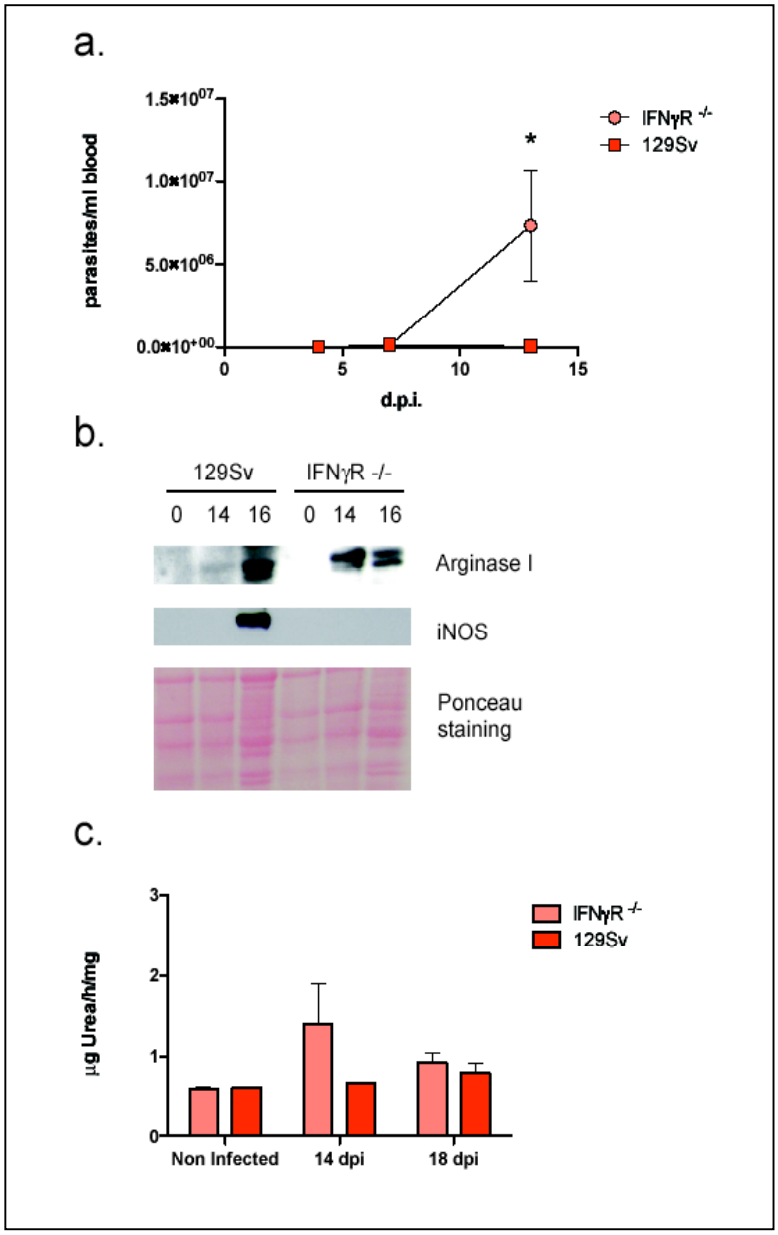


**Figure 9.** *Parasitemia, arginase I and iNOS expression, and arginase activity in heart tissue of IFNγR-/- mice during* T. cruzi *infection.* a) Parasitemia was determined in blood from the tail as described in materials and methods. Results are presented as mean ± SE (n=3). Significant differences observed between INFγR$^{-/-}$ and 129Sv mice are indicated with an asterisk ($p < 0.05$). b) Arginase I and iNOS expression in heart tissue extracts pooled from 3 wild-type and 3 INFγR$^{-/-}$ mice at the indicated d.p.i, and Ponceau staining of the same membrane. c) Arginase activity of the same extracts expressed as µg of urea produced per hour per mg of protein sample; Results are presented as mean ± SE (n=3). A representative experiment out of two is shown.

When arginase I expression levels were determined in heart lysates from infected mice, arginase I was detectable in IFNγR$^{-/-}$ mice already at 14 d.p.i and remained high at 16 d.p.i.; while the wild-type mice showed very small arginase expression at 14 d.p.i and only at 16 d.p.i it reached comparable expression level to those of mice (Fig. 9b).

Despite protein expression, analysis of the arginase activity of these heart lysates did not reveal any significant difference when comparing IFNγR deficient mice and the wild-type at any time-point analyzed and very low levels were detected (Fig. 9c). Again, iNOS expression was detected in the wild-type mice while no band corresponding to iNOS protein was seen in the IFNγR -/- mice, probably because IFNγ also is (as TNF) a very important factor for iNOS induction (Munoz-Fernandez et al., 1992).

## II.3. IL-10 deficient mice show a high susceptibility to *T.cruzi* infection and no arginase I or iNOS induction.

Although IL-10 is not considered a typical Th2 cytokine, it has been reported to be capable of inducing arginase in several biological models. This issue and the fact that mRNA IL-10 levels detected in hearts of infected BALB/c mice compared with C57BL/6 were remarkable, prompted us to study whether IL-10 had any role in arginase I induction in heart of mice when infected with *T.cruzi*. For this purpose, we infected IL-10 deficient mice and their wild-type strain (129Sv). When infected with Y strain trypomastigotes, IL-10 deficient mice showed extreme susceptibility to the infection, with most mice dying after 10 d.p.i.

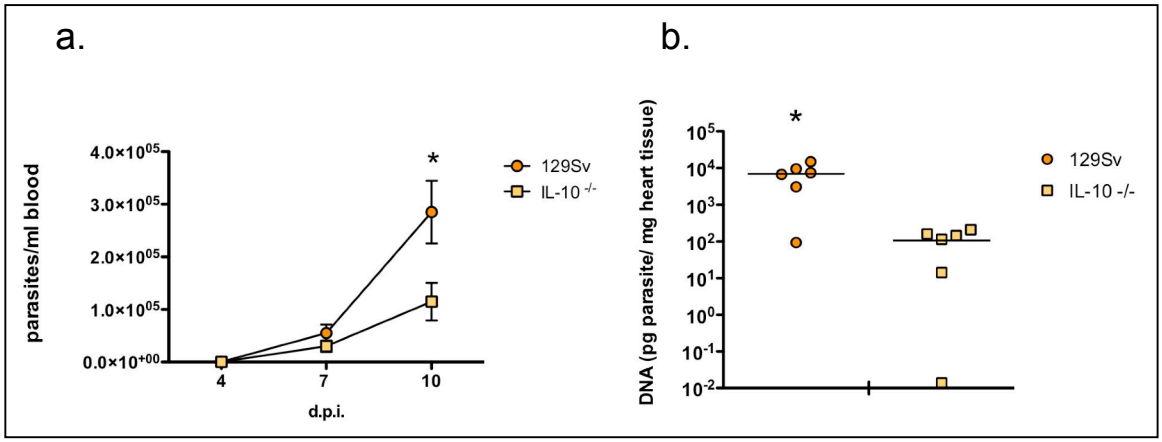


**Figure 10.** *Parasite load in IL-10$^{-/-}$ infected mice.* a) Parasitemia of infected wild type 129Sv and IL-10$^{-/-}$ mice. a) Parasitemia was determined in blood from the tail as described in materials and methods. Results are presented as mean ± SE of values from two independent experiments (n=6). b) DNA from heart tissue was isolated at indicated d.p.i. and qPCR was performed as previously described. *T. cruzi* DNA is expressed as pg of parasite DNA per mg of DNA from heart tissue sample. Results are shown in a scatter dot graph where values represented are from two independent experiments and the line indicates the mean of these values. Significant differences observed between IL-10$^{-/-}$ and 129Sv mice are indicated with an asterisk ($p < 0.05$).

However, in these mice both parasite number in blood at 10 d.p.i., and parasite burden in heart tissue, were significantly lower than the infected wild-type mice (Fig. 10). mRNA levels of arginase I, arginase II and iNOS in heart showed a small but significant increase in infected mice when compared to non infected. However, mRNA levels for Arginase I and II were significantly lower in infected IL-10$^{-/-}$ when compared to the wild-type (Fig. 11a and b). mRNA levels for iNOS in heart from infected IL-10 deficient mice at 11 d.p.i. did not show significant differences when compared to the wild-type (Fig 11c). Arginase I and iNOS protein expression were not detected in heart lysates of these infected mice, neither in IL-10 deficient or wild-type (Data not shown). In agreement with this, arginase activity in the same heart lysates showed no significant increase of arginase activity after *T.cruzi* infection and also no significant differences between IL-10 deficient and wild-type mice (Fig. 11d).

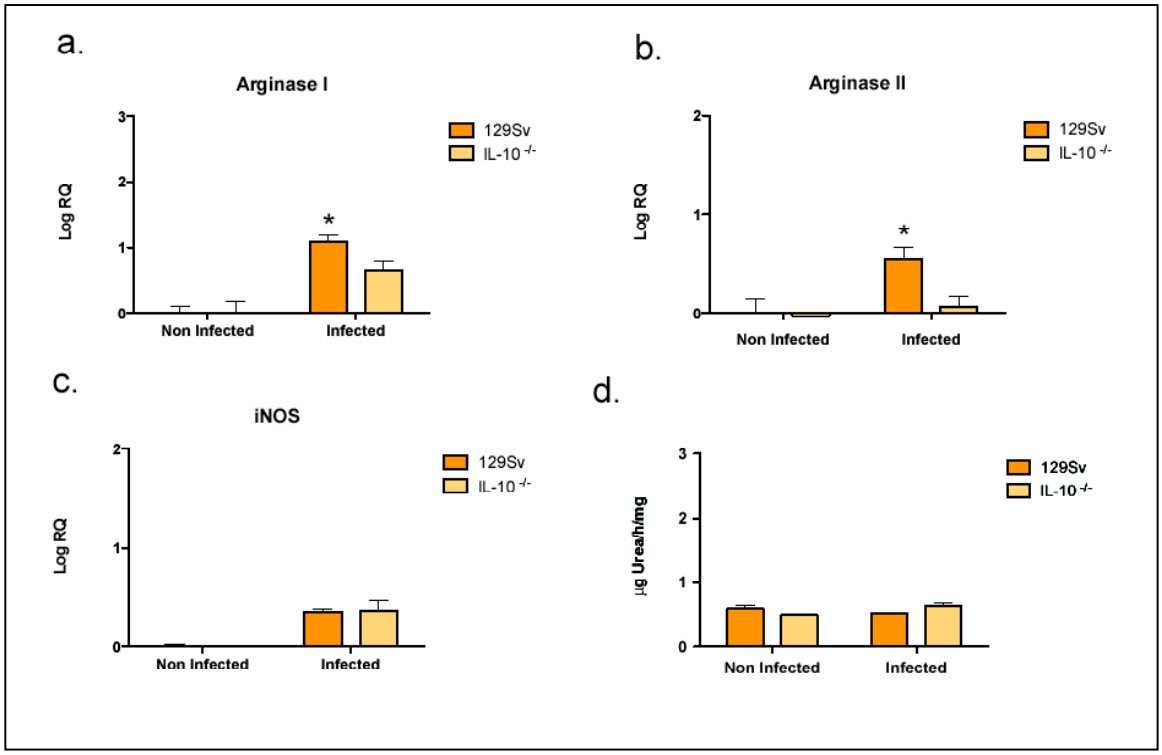


**Figure 11.** *Arginase I, arginase II and iNOS expression in heart tissue of IL-10$^{-/-}$ during* T. cruzi *infection.* Total RNA from heart tissue was isolated and qRT-PCR was performed as described for the following: a) Arginase I; b) Arginase II; c) iNOS. No arginase I or iNOS expression was detected in heart tissue extracts pooled from 3 wild-type and 3 IL-10$^{-/-}$ non-infected and infected mice at 11 d.p.i. d) Arginase activity of the same extracts expressed as µg of urea produced per hour per mg of protein sample (µg/h/mg). Results are presented as mean ± SE of values from two independent experiments (n=6).

Thus, no conclusion can be drawn from these experiments, probably due to the early mortality of IL-10$^{-/-}$ mice upon infection, which does not allow the ongoing of the immune response to trigger neither arginase I or iNOS protein expression. However, mRNA levels for arginase I and II were lower in hearts of IL-10$^{-/-}$ mice indicating that, in the development of a proper immune response, IL-10 might be contributing to arginase induction in hearts of infected mice.

## II.4. IL-13, alone or in cooperation with IL-4, triggers arginase I induction during *T.cruzi* infection.

IL-4 and IL-13 were the only cytokines whose mRNA levels were significantly higher in BALB/c mice than in C57BL/6 at all time points analyzed upon infection. This, together with the fact that Th2 cytokines, and specially IL-4, have been reported to induce arginase in several in vitro and in vivo biological models made them the best candidates for arginase I induction in heart tissue during *T.cruzi* infection. To test this hypothesis, we infected IL-4 and IL-4R deficient mice.

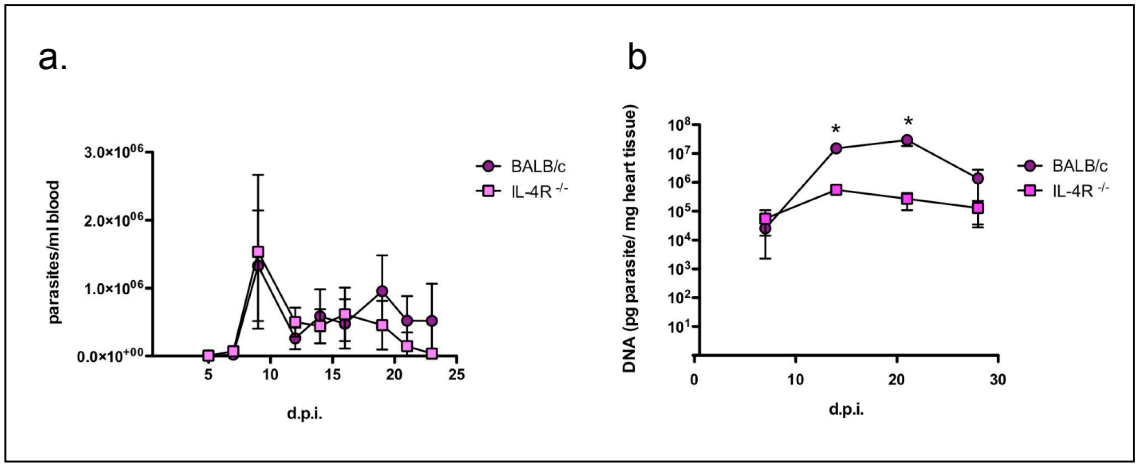


**Figure 12.** *Parasite load in IL-4R$^{-/-}$ infected mice.* a) Parasitemia of infected wild type BALB/c and IL-4R$^{-/-}$ mice. Parasitemia was determined in blood from the tail as described in materials and methods. Results are presented as mean ± SE (n=6). A representative experiment out of two is shown. b) DNA from heart tissue was isolated at indicated d.p.i. and qPCR was performed as previously described. *T. cruzi* DNA is expressed as pg of parasite DNA per mg of DNA from heart tissue sample. Results are presented as mean ± SE (n=3). Significant differences ($p< 0.05$) found between IL-4R$^{-/-}$ and BALB/c mice are indicated with an asterisk.

**IL-4R deficient mice**, which lack the common α chain of IL-4 and IL-13 receptors, have impaired signalling for both of these cytokines. When infected with Y strain of *T.cruzi*, no significant differences comparing with the wild-type mice (BALB/c) were observed in the parasitemia through the infection (Fig. 12a). However, when parasite burden was quantified in heart tissue, wild-type mice showed significantly higher around (100 fold) parasite burden than IL-4R deficient mice at 14 and 21 d.p.i. (Fig. 12b). As shown in figure 13a., arginase I expression in heart lysates was much higher in wild-type infected mice spanning from 7 to 28 d.p.i., than in IL-4R deficient, where a nice induction could only be detected at 14 and 21 d.p.i,, when it reached the highest level. Those arginase I expression levels correlated with arginase activity from the same heart homogenates (Fig. 13b). Arginase activity in hearts from IL-4R deficient mice showed significantly diminished activity at 14, 21 and 28 d.p.i when compared to the wild-type.

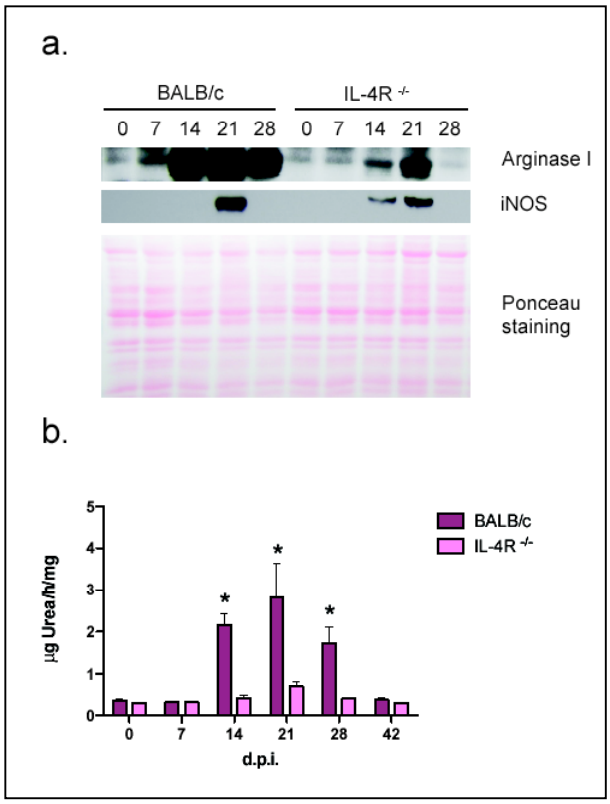


**Figure 13.** *iNOS, and arginase I protein expression and arginase activity during* T. cruzi *infection in heart tissue from IL-4R$^{-/-}$ mice.* Protein extracts from heart tissue were isolated from 3 IL-4R$^{-/-}$ and 3 BALB/c mice at different d.p.i. and pooled for western blot analysis. a) Arginase I, iNOS expression, and Ponceau staining of the same membrane; b) Arginase activity of the same tissue extracts, expressed as µg of urea produced per hour per mg of protein sample. Results are represented as mean ± SE (n=3). Asterisks indicate significant differences between BALB/c and IL-4R$^{-/-}$ mice ($p < 0.05$).

iNOS expression in heart homogenates from infected wild-type mice was higher compared to IL-4R deficient mice. However, expression of iNOS was restricted to 21 d.p.i in wild-type mice while its induction lasted longer in IL-4R deficient mice (from 14 to 21 d.p.i).

In **IL-4 deficient mice** *T.cruzi* infection, no significant differences were observed in parasitemia compared to the wild-type (Fig. 14a). Arginase I expression was induced in the heart of IL-4-/- infected mice but its expression did not differ from the one observed in hearts of infected wild-type mice (Fig. 14b). However, arginase activity in the same heart homogenates from infected mice was significantly higher in IL-4 deficient when compared to the wild-type mice (Fig. 14c). iNOS protein in heart lysates from infected IL-4 deficient were higher than in 129Sv mice (Fig. 14b).

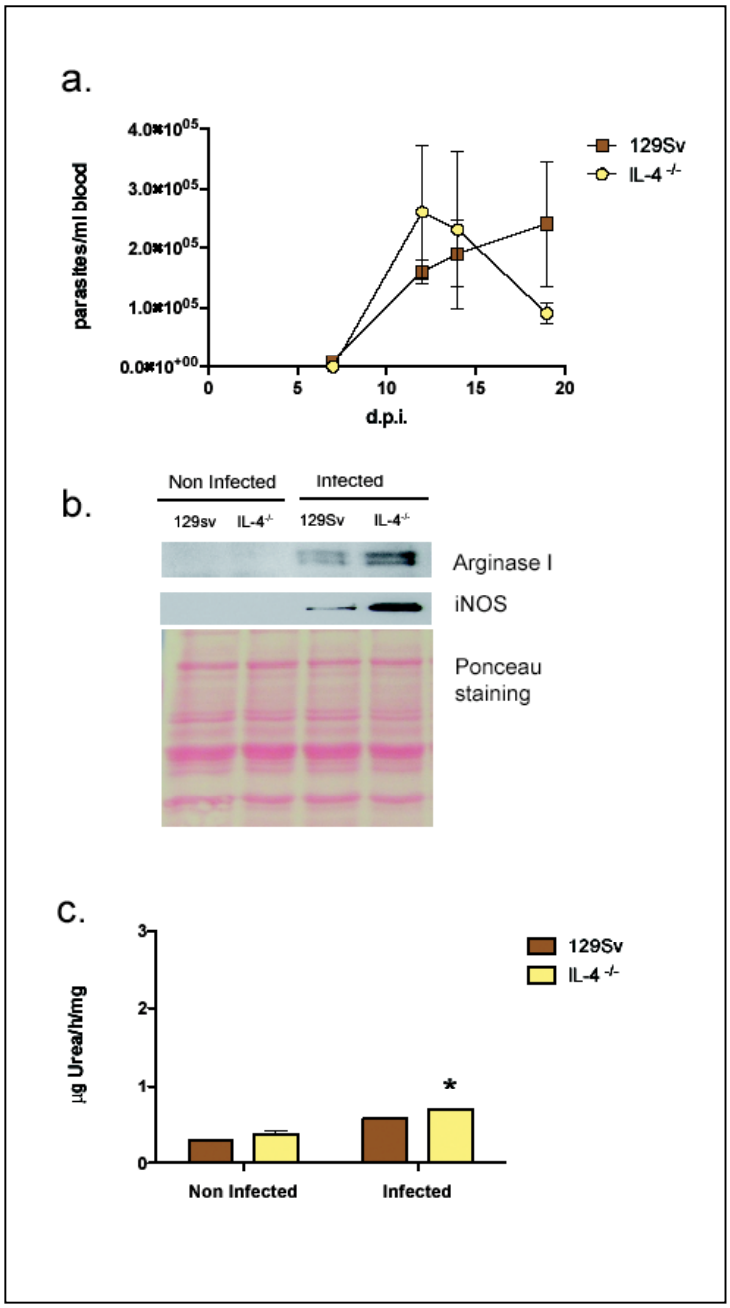


**Figure 14.** *Parasitemia, arginase I and iNOS expression, and arginase activity in heart tissue during* T. cruzi *infection of IL-4*$^{-/-}$ *mice.* a) Parasitemia was determined in blood from the tail as described in materials and methods. Results are presented as mean ± SE (n=3). b) Arginase I and iNOS expression in heart tissue extracts pooled from 3 wild-type (129Sv) and 3 IL-4$^{-/-}$ non-infected (NI) and infected (I) mice at 21 d.p.i. Ponceau staining of the membrane is shown. c) Arginase activity of the same extracts expressed as µg of urea produced per hour per mg of protein sample; Results are presented as mean ± SE (n=3). Significant differences observed between IL-4$^{-/-}$ and 129Sv mice are indicated with an asterisk ($p < 0.05$). A representative experiment out of two is shown.

Those results of *T. cruzi* infection of IL-4 and IL-4R deficient mice indicate that IL-4 itself is not responsible for arginase I induction in hearts from *T.cruzi* infected mice, although we can not exclude that it can cooperate with IL-13 in this purpose.

### II.5. Microsomal Prostaglandin $E_2$ synthase-1 (mPGES-1) is significantly induced in hearts of mice infected with *T. cruzi.*

The prostaglandin $E_2$ ($PGE_2$) has been shown to induce arginase I in bone marrow derived macrophages (BMM). Also, it has been reported that the uptake of apoptotic cells during *T. cruzi* infection causes release of $PGE_2$ (Freire-de-Lima et al., 2000). These facts lead us to analyze mRNA levels for the most common inducible isoform of prostaglandin I synthase: m-PGES-1 during *T.cruzi* infection in hearts of infected mice. Fig. 15 shows that mPGES-1 mRNA was significantly increased in heart of both BALB/c and C57BL/6 mice upon infection being significantly higher in BALB/c than C57BL/6 mice at 21 d.p.i. Thus, $PGE_2$ might be contributing to arginase I induction in heart tissue during *T.cruzi* infection. However, in C57BL/6 mice in PGES-1 levels remained elevated for longer periods after infections.

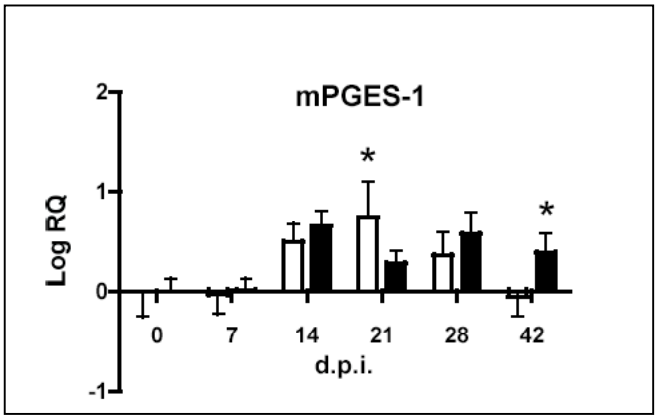


**Figure 15.** *mRNA quantification of microsomal prostaglandin $E_2$ synthase- 1 (mPGES-1) in heart tissue during* T. cruzi *infection.* RNA from heart tissue was isolated at different d.p.i. and qRT-PCR was performed. Results are expressed as the logarithm of RQ calculated from CT values as described in material and methods. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. Results are represented as mean ± SD of 3 different mice. All differences observed in infected mice respect to non-infected mice were statistically significant except for 7 d.p.i. Significant differences observed between BALB/c and C57BL/6 mice are indicated by an asterisk. ($p< 0.05$). A representative experiment out of three is shown.

## III. Cardiomyocytes express arginase II and iNOS while arginase I is expressed by CD68$^{+}$ cells infiltrating heart tissue of *T. cruzi* -infected mice.

### III.1. Arginase II and iNOS enzymes are expressed in cardiomyocytes in hearts of infected mice

To determine the cellular source of arginase and iNOS protein detected in the hearts of infected mice, we performed immunohistochemical staining. Fig 16 shows staining of a high percentage of cardiac fibres for both arginase II and iNOS in BALB/c infected mice (Fig. 16c and 16d) compared to non-infected mice (Fig. 16a and 16b). Non-specific staining was detected when slides from hearts of infected mice were incubated with the isotypic antibody (Fig. 16 e).

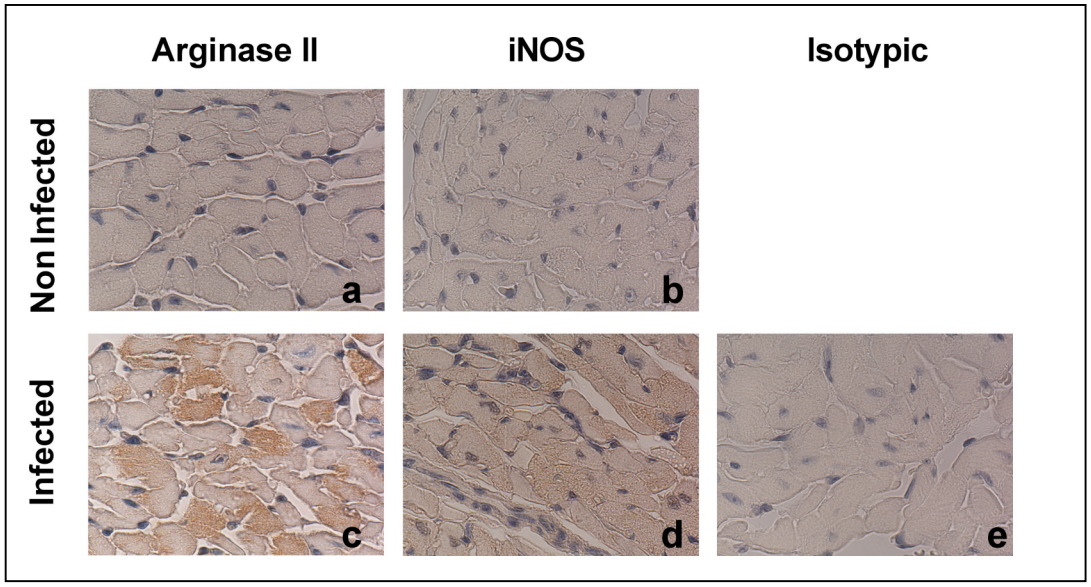


**Figure 16.** *Arginase II and iNOS expressing cells in heart tissue of BALB/C mice during acute* T. cruzi *infection.* Hearts from non infected mice and infected mice at 14 d.p.i. were analyzed by immunohistochemistry as described. a) Non-infected tissue stained with anti arginase II antibody, and b) with anti iNOS antibody. c) Infected tissue stained with anti arginase II antibody, and d) with anti iNOS antibody. e) Isotypic antibody control staining. Data are representative of several sections analyzed in at least 3 different mice. Magnification x1000.

### III.2. Arginase I does not colocalize with F4/80 or CD11c markers.

When processed and analyzed by confocal microscopy, hearts from infected BALB/c mice at 21 d.p.i. showed an infiltrate composed by arginase I positive cells (Fig. 17f); no staining for this enzyme was observed in cardiomyocytes or endothelial

cells. Arginase I-positive infiltrating cells showed a macrophage-like morphology (Fig 17b, 18b and 19f). **F4/80** and **CD11c** markers, as markers of macrophages and dendritic cells respectively, were tested in order to find out the arginase-1 expressing cell type; however, none of the antibodies stained tissue sections of hearts from *T.cruzi* infected mice (Fig 17a and 18a). As a control of the immunoreactivity of those antibodies, spleen sections from non-infected BALB/c mice were stained for these cell-markers (Fig 17e and 18e).

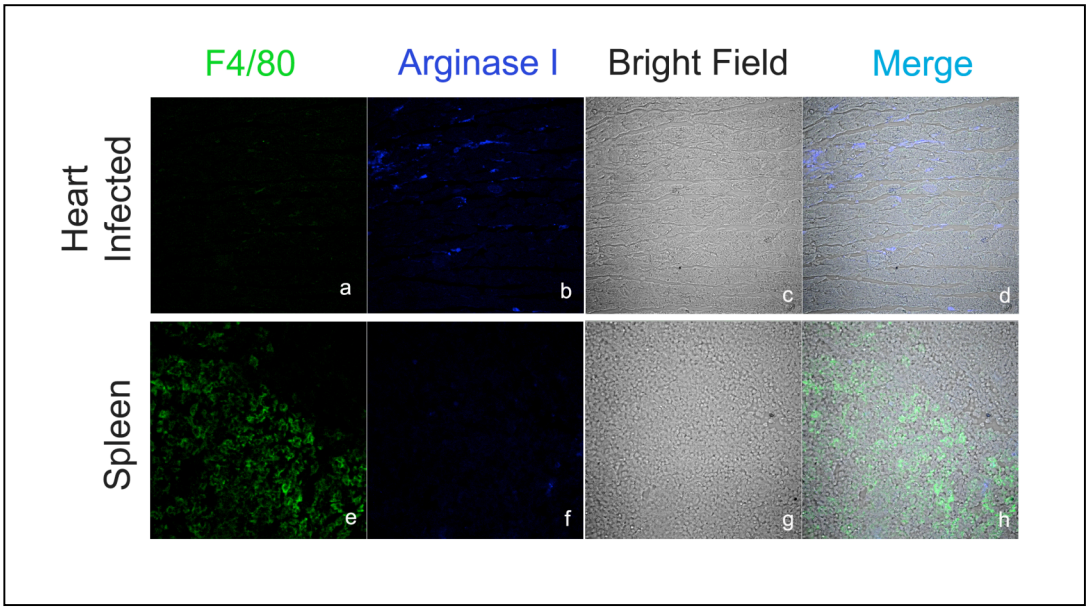


**Figure 17.** *Arginase I and F4/80 expressing cells in BALB/c mice heart tissue during acute* T. cruzi *infection.* Tissue sections obtained from hearts of BALB/c mice 21 d.p.i. and from spleen from non-infected mice were analysed by immunofluorescence confocal microscopy as described. Heart infected tissue stained with a) anti F4/80 antibody; b) with anti arginase I antibody; c) phase contrast; d) merge of a, b, and c. Spleen section stained with e) anti F4/80 antibody; f) anti-arginase I antibody; g) phase contrast; h) merge of e, f, and g. Magnification is x400. Data are representative of several sections analyzed in at least 3 different mice.

## III.3. $CD68^{+}$ infiltrating cells are responsible for arginase I expression.

Despite the negative result with macrophage markers above, we found that **CD68,** a tissue-macrophage marker with a predominantly intracellular expression, co-localized with arginase I (Fig. 19e to 19h) in tissue sections of hearts from *T. cruzi* infected mice. Heart tissue from non-infected mice showed a weak staining with anti-CD68 and anti-arginase I antibodies of some infiltrated resident cells (Fig. 19a to 19d). No specific staining was observed in sections stained with isotypic antibodies (Fig. 19e and 19f).

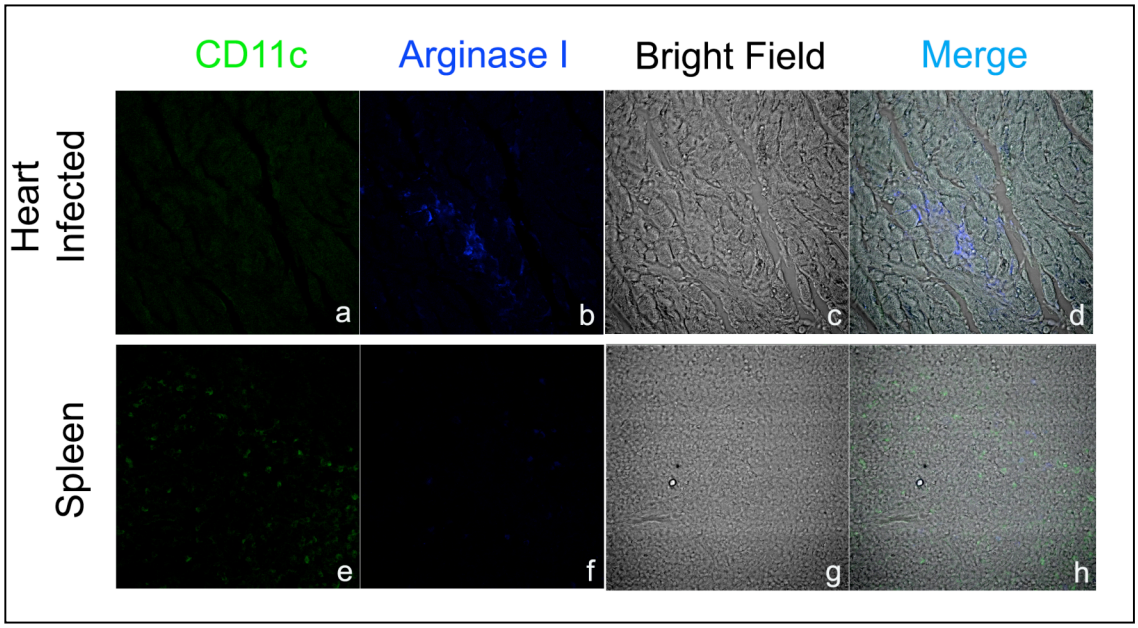


**Figure 18.** *Arginase I and CD11c expressing cells in BALB/c mice heart tissue during acute* T. cruzi *infection.* Tissue sections obtained from hearts of BALB/c mice 21 d.p.i. and from spleen from non-infected mice were analysed by immunofluorescence confocal microscopy as described. Heart infected tissue stained with a) anti CD11c antibody; b) with anti arginase I antibody; c) phase contrast; d) merge of a, b, and c. Spleen section stained with e) anti CD11c antibody; f) anti-arginase I antibody; g) phase contrast; h) merge of e, f, and g. Magnification is x400. Data are representative of several sections analyzed in at least 3 different mice.

In order to clarify whether the higher arginase I expression seen in BALB/c mice compared to C57BL/6 mice was due to differences in the amount of macrophages infiltrating heart tissue, we evaluated the extension of $CD68^+$ cell infiltration in BALB/c and C57BL/6 mice by quantification of CD68 mRNA. $CD68^+$ cell infiltration was induced upon infection, being maximal at 14 d.p.i. in both mouse strains and interestingly, no statistically significant differences were observed between them (Fig. 19k). This similarity in cell infiltration contrasted with the observed differences in arginase expression, suggesting that arginase I expression was higher in $CD68^+$ cells from infected BALB/c than C57BL/6 mice.

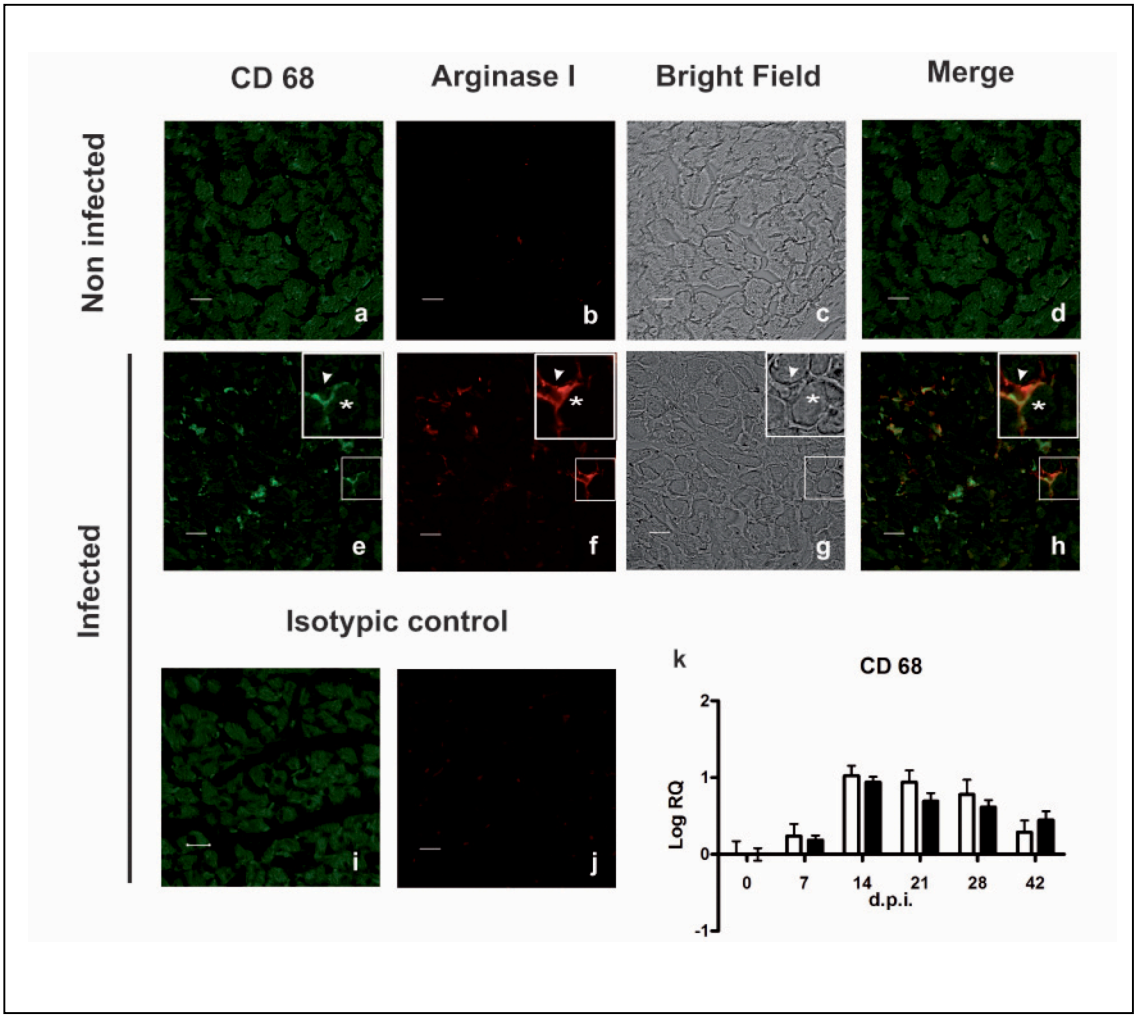


**Figure 19.** *Arginase I and CD68 expressing cells in BALB/c mice heart tissue during acute* T. cruzi *infection.* Tissue sections obtained from hearts of BALB/c mice at 21 d.p.i. were analysed by immunofluorescence confocal microscopy as described. Non-infected tissue stained with a) anti CD68 antibody; b) with anti arginase I antibody; c) phase contrast; d) merge of a and b. Infected tissue section stained with e) anti CD68 antibody; f) anti-arginase I antibody; g) phase contrast; h) merge of e and f. Boxed regions are shown at two-fold magnification of original (x400) in insets. The scale bar is 20μm. Data are representative of several sections analyzed in at least 3 different mice. k) Total RNA from heart tissue was isolated at 21 d.p.i. and CD68 quantitative RT-PCR was performed as described. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. Results are represented as mean ± SD of 3 different mice. All values for BALB/c and C57BL/6 mice were significantly higher compared to non infected mice. A representative experiment out of three is shown.

## III.4. Arginase I expression in primary cultures of cardiomyocytes and macrophages

iNOS and arginase II induction, but no arginase I, had been reported during *T. cruzi*-infection of cardiomyocytes *in vitro* (Aoki et al., 2004; Machado et al., 2000). Thus, we infected **cardiomyocytes** *in vitro* and analyzed arginase I and iNOS expression. iNOS but no arginase I protein was induced by infection in cardiomyocytes

(Fig. 20a). IL-4 triggered a strong arginase I induction in uninfected cardiomyocytes. This IL-4-dependent arginase I induction in non-infected cardiomyocytes was partially inhibited by *T. cruzi* infection. In contrast, the IFN-γ+LPS-dependent iNOS induction observed in non-infected cardiomyocytes was strongly potentiated by *T. cruzi* infection of those cells. Therefore, iNOS expression in cardiac cells can be directly triggered by the parasite and is probably responsible for the inhibition of IL-4- dependent arginase I expression in these cells (Fig 20a).

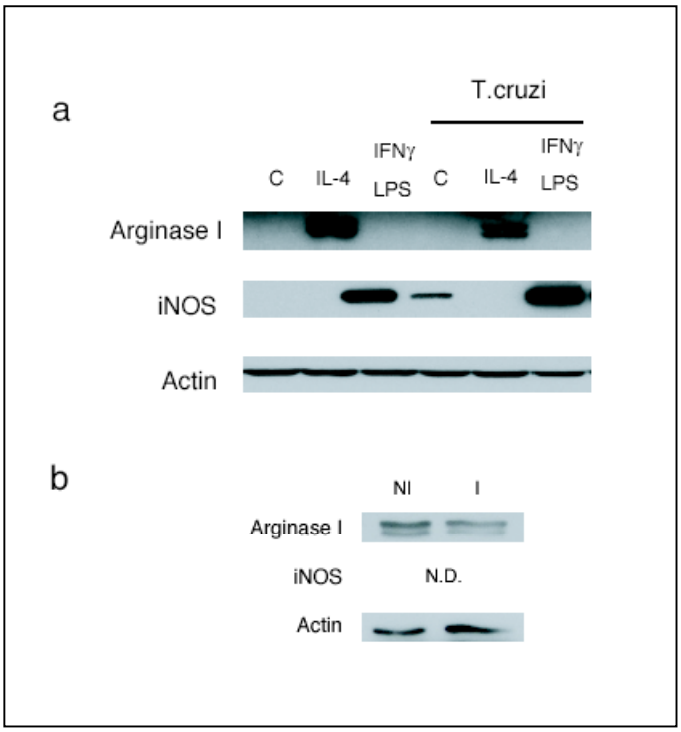


**Figure 20.** *Expression of arginase I and iNOS in BALB/c cardiomyocytes and peritoneal macrophages infected with* T.cruzi in vitro. a) Western blot of neonatal cardiomyocyte cultures non-infected (NI) and infected (I) *in vitro* with *T. cruzi* trypomastigotes cultured with medium alone (C), IL-4, LPS+ IFN-γ as indicated. Arginase I, iNOS and actin expression is shown. b) Arginase I, iNOS and actin expression in non-infected (NI) and infected (I) macrophage cultures; iNOS expression was not detected (N.D.). Results shown are representative of at least 3 experiments.

*T. cruzi* infection triggers NO production in IFN-γ activated macrophages but the parasite itself does not induce production of NO in this cell type (Bergeron and Olivier, 2006); we corroborated those results. Moreover, we found that *in vitro* infection of BALB/c mice resident peritoneal **macrophages** did not increase arginase I expression, in fact a decrease was observed over the basal levels (Fig. 20b). By extrapolating, those in vitro results to "in vivo" ones, we can suggest that arginase I induction seen in $CD68^+$ heart infiltrating cells is not likely to be triggered by the parasite but rather by the inflammatory environment in the heart set off during the immunological response against the parasite.

## IV. CD11b$^+$ cells purified from hearts of infected mice do not show a clear M1 or M2 pattern but exert NO mediated T cell suppression.

### IV.1. Purified CD11b$^+$ cells from hearts of infected mice do not show a polarized M1 or M2 signature.

In order to determine whether heart tissue infiltrating CD11b$^+$ cells belonged to the M2 type macrophages, we purified CD11b$^+$ cells from hearts of infected mice by magnetic sorting and performed qPCR for different genes already described to be transcriptionally induced in M2 macrophages *in vivo* by different *stimuli* or parasites (Ghassabeh et al., 2006). Results in Table 1 show that only 5 out of 10 genes of the described gene signature: Prosaposin, Triggering receptor expressed on myeloid cells 2 (Trem2), Chitinase 3-like 3 (Ym), Cadherin-1 and Folate Receptor-2 (Folr2) but not Resistin like alpha (Fizz1) Platelet-activating factor acetylhydrolase (Pafah), Selenoprotein P (Sep), Macrophage galactose N-acetyl-galactosamine specific lectin 2 (Mgl2), and Macrophage mannose receptor (Mmr), were transcribed in our cells purified from infected hearts indicating that these CD11b$^+$ cells had no clear M1 or M2 polarization.

| Gene | Fold |
|---|---|
| Fizz1 | n.d. |
| Pafah | 2,05 |
| Prosaposin | 16,71 * |
| Sep | 0,15 |
| Trem2 | 5,81 * |
| Mgl2 | 0,1 |
| Ym | 26075,97 * |
| Mmr | 5,33 |
| Cadherin-1 | 112,89 * |
| Folr2 | 6,81 * |

**Table 1.** *mRNA quantification of genes related to M2 activation in CD11b$^+$ heart infiltrating cells* . RNA was isolated from CD11b$^+$ population purified from hearts of BALB/c mice 21 d.p.i. and qRT-PCR was performed. Values are relative to those obtained from CD11b$^+$ population purified from peritoneal exudates from non-infected BALB/c mice. Results are expressed as RQ, calculated as described and normalized by S12 gene expression. Asterisks indicate that at least five-fold induction was detected in at least two of the three experiments performed.

### IV.2. Phenotypic characterisation of CD11b$^+$ purified cells: Myeloid suppressor cells.

Next, we approached characterization of our population of purified CD11b$^+$ cells from hearts from infected mice using flow cytometer analysis. For this, most common markers for CD11b$^+$ populations were used. These cells showed no staining for **F4/80** or

**CD11c,** markers for some subset of tissue macrophages and dendritic cells respectively corroborating immunohistochemical analysis (Fig. 21a and 21c); also, **CD 19** marker, present in B cells was not present in our isolated population (Fig. 21b). Purified cells did express **MHC class II**, which indicates that they represent mature cells capable of presenting antigen to T cells (Fig. 22c).

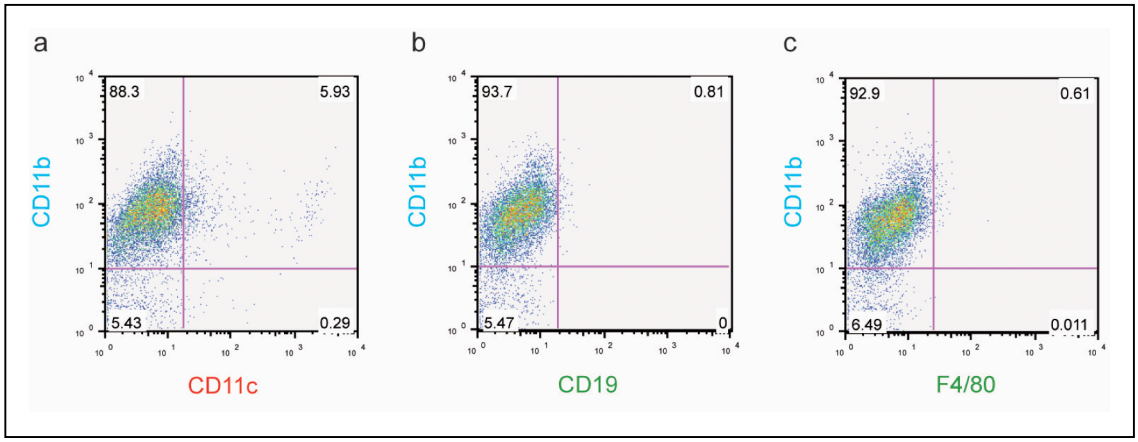


**Figure 21.** *Phenotypic characterization of CD11b$^+$ population purified from hearts of infected mice.* CD11b$^+$ population was purified from hearts of BALB/c infected mice as described in methods and materials. Cells were then stained for CD11b and for: a) CD11c, b) CD19 and c) F4/80. Numbers indicate inside panel % of positive cells of the gated populations. Data are representative of at least 3 experiments performed.

**Gr-1** marker is highly expressed on granulocytes, but has been also shown to be expressed in a subset of myelomonocytic cells named myeloid suppressor cells. Fig 22a shows that there are two different populations according to Gr-1 expression, CD11b$^+$ Gr1$^{high}$ cells and CD11b$^+$ Gr-1$^{int}$ cells. These two populations also vary on their expression of Ly-6C (Fig. 22e), mainly a monocytic cell marker, but also some expression is detected in neutrophils and dendritic cells. Fig 22 d shows that infiltrating cells are composed of two main populations: CD11b$^+$ Gr-1$^{high}$ Ly6C$^{int}$, which have been related to granulocyte CD11b$^+$ cells and CD11b$^+$ Gr-1$^{int}$ Ly6C$^{high}$, related to MSCs.

## IV.3. Monocytes (CD11b$^+$ Gr-1$^{int}$ Ly6C$^{high}$) but not granulocytes (CD11b$^+$ Gr-1$^{high}$Ly6C$^{int}$) are responsible for arginase I and iNOS expression.

Arginase I and iNOS expression were analysed in purified CD11b$^+$ population from hearts from *T.cruzi* infected mice. At 21 d.p.i. CD11b$^+$ cells were purified also from blood of the same infected mice or from non-infected mice. CD11b$^+$ cannot be

isolated from the hearts of uninfected mice since they do not infiltrate the heart. Fig 23 shows that CD11b$^{+}$ cells purified from heart of infected mice were the only population expressing both iNOS and arginase I enzymes.

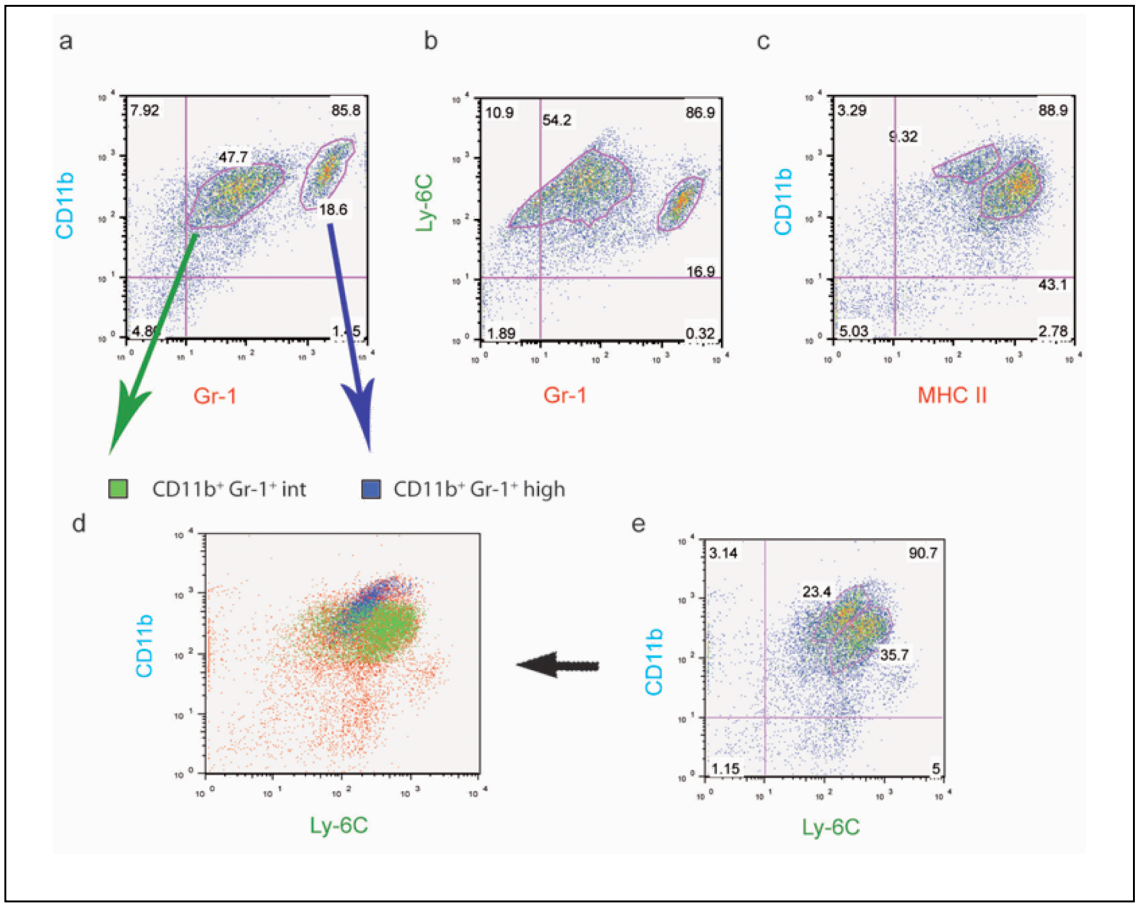


**Figure 22.** *Expression of Gr-1, Ly-6C and MHC class II in the CD11b$^{+}$ population purified from hearts of infected mice.* CD11b$^{+}$ population was purified from hearts of BALB/c infected mice as described in methods and materials, cells were then stained for CD11b and for: a) Gr-1, c) MHC class II, e) Ly-6C. Or for: b) Gr-1 and Ly-6C. d) Overlay in e from CD11b$^{+}$ Gr-1$^{+}$ populations in a. Numbers inside panels indicate % of positive cells of gated populations. Data are representative of at least 3 experiments performed.

In order to determine whether this arginase I and iNOS expression was expressed in CD11b$^{+}$ Gr-1$^{int}$ Ly-6C$^{high}$ or in the CD11b$^{+}$ Gr-1$^{high}$ Ly-6C$^{int}$ subset infiltrating the heart, we depleted Ly-6G$^{+}$ cells from the total heart and blood isolated population by positive selection eluting then heart and blood populations expressing low or no levels of Ly-6G on membrane. Eluted heart and blood Ly-6G$^{-}$ populations were then subject to CD11b$^{+}$ selection. Fig 24 shows how remaining CD11b$^{+}$ cells were the cell subset responsible for arginase I and iNOS expression in heart tissue and for the low arginase I expression found in PBMCs.

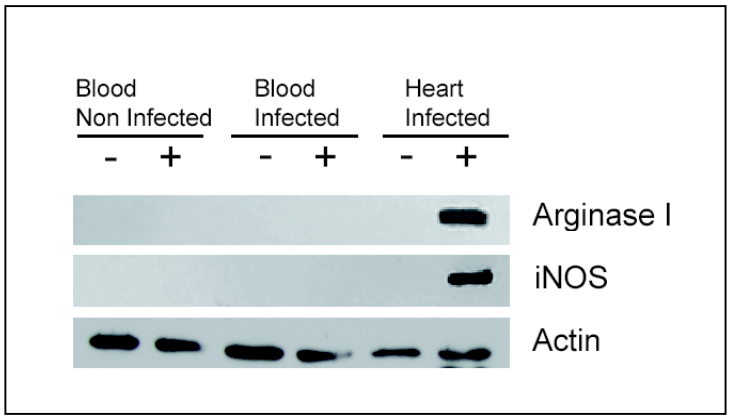


**Figure 23.** *Expression of arginase I, iNOS and actin in sorted CD11b populations from heart and blood of infected and non-infected mice.* CD11b$^{+}$ population was purified from hearts or blood of infected (21 d.p.i) or non-infected BALB/c mice as described in methods and materials; positive and negative fractions of sorted cells were then processed for western blot analysis using anti-arginase I, anti-iNOS, and anti-actin antibodies. + or – signs indicate positive or negative fraction of CD11b sorted population. Results shown are representative of at least three experiments performed.

Because anti Gr-1 antibodies recognize both Ly-6C and Ly-6G molecules, in this case, depleting high Ly-6G$^{+}$ expressing cells helped us distinguish between Gr-1$^{high}$ and Gr-1$^{int}$ populations; Gr-1$^{high}$ Ly-6C$^{int}$ expressing cells were depleted by Ly-6G positive depletion while Gr-1$^{int}$ Ly-6C$^{high}$ were present in the eluted population which was afterwards selected for CD11b. Altogether, this data indicate that CD11b$^{+}$ Gr-1$^{int}$ Ly-6C$^{high}$ cells corresponding to a MSC phenotype are the population subset responsible for arginase I and iNOS expression

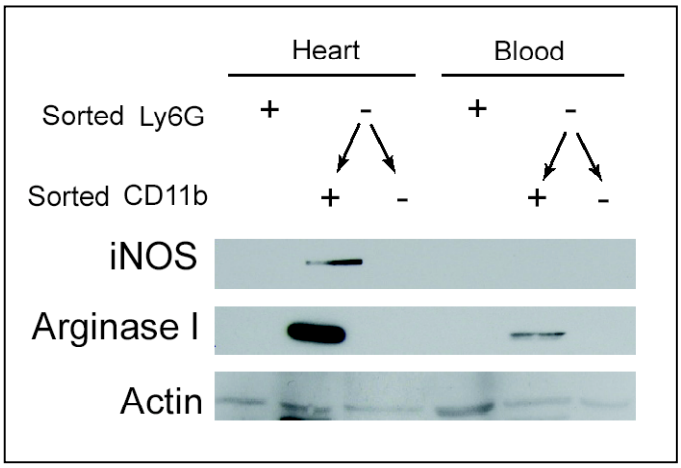


**Figure 24.** *Expression of arginase I, iNOS and actin in sorted Ly-6G and CD11b populations from heart and blood of infected mice.* Ly-6G$^{+}$ population and afterwards CD11b$^{+}$ population were purified from hearts or blood of infected BALB/c mice at 21 d.p.i as described; positive and negative fractions of sorted cells were then processed for western blot analysis using anti-arginase I, anti-iNOS, and anti-actin antibodies. + or – signs indicate positive or negative fraction of Ly6G and/or CD11b sorted population. Results shown are representative of at least three experiments performed.

## IV.4. Arginase modulates T cell suppression derived from iNOS activity.

In order to establish the capability of the CD11b$^{+}$ Ly-6C$^{high}$ Gr-1$^{int}$ subset of the cell population purified from hearts of infected mice to suppress T cell response, and act as MSCs, we performed proliferation assays. T cells purified from spleen from non-infected syngeneic mice were activated with concanavalin A and co-incubated with purified CD11b$^{+}$ cells from heart of infected mice or with CD11b$^{+}$ cells from blood of non-infected mice. iNOS and arginase inhibitors were added to assess the differential role of the putative inhibition of these enzymes.

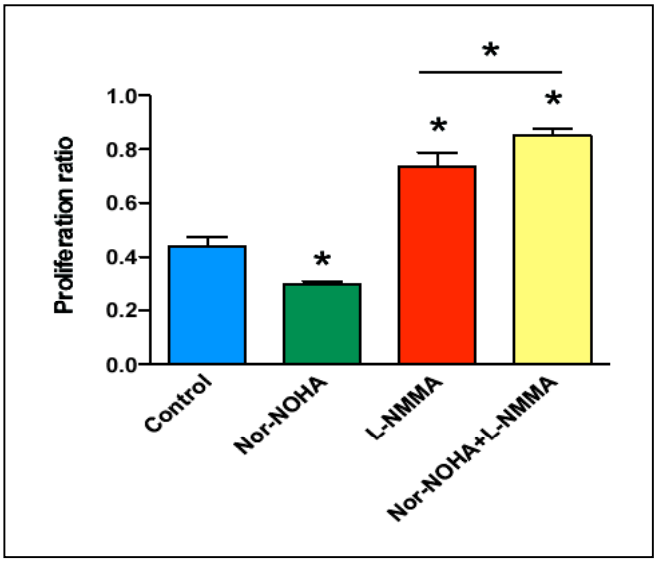


**Figure 25.** *T-cell suppressive activity of purified CD11b$^{+}$cells from hearts of infected mice.* CD11b$^{+}$ population was purified from hearts of infected BALB/c mice at 21 d.p.i and from blood of non-infected BALB/c mice as previously described. CD11b$^{+}$ cells were incubated with T cells isolated from spleen from a non-infected mouse with and, where indicated, with or without the following inhibitors: nor-NOHA 24μM and L-NMMA 2mM. Data are given as proliferation ratio: counts per minute (c.p.m.) of T cells incubated with CD11b$^{+}$ cells isolated from hearts of mice 21 d.p.i. / c.p.m. of T cells incubated with CD11b+ cells isolated from non-infected blood. One representative experiment out of four is shown. Results are represented as mean ± SE of triplicates. Asterisks indicate significant differences ($p< 0.05$) when compared with the control condition except when a line indicates the two conditions being compared.

Fig 25 shows proliferation expressed as a ratio of counts per minute (c.p.m.) of T cells incubated with CD11b$^{+}$ cells purified from hearts of infected mice divided by c.p.m. from T cells incubated with CD11b$^{+}$ cells purified from blood of non-infected mice. Impaired proliferation of T cells can be observed when incubated with CD11b$^{+}$ cells purified from hearts of infected mice in comparison with CD11b$^{+}$ cells from blood of non-infected mice. When arginase specific inhibitor **nor-NOHA** was added to the cell culture no prevention of the inhibition of cell proliferation was observed compared

to control non-treated cells, in fact, a further small decrease in proliferation was detected. However, when iNOS inhibitor **L-NMMA** was added, proliferation was significantly restored. Moreover, when both arginase and iNOS inhibitors were added to the culture, reestablishment of cell proliferation was even greater than with only L-NMMA treatment. These results indicate that NO released by iNOS or $H_2O_2$ released as a consequence of both iNOS and arginase activity are exerting a potent immunosuppressant activity and arginase is modulating this immunosuppresion.

# DISCUSSION

# DISCUSSION

Heart leukocyte infiltration is thought to play an important role in the myocarditis associated to *T. cruzi* infection both in the acute and the chronic phase. Up to date, most of the research in the field has focused in the characterization of T cells present in heart inflammatory infiltrate, while very few reports have addressed the role of monocytes/macrophages in the myocarditis of the chronic or acute phase. Furthermore, macrophages may become activated by different pathways, that play different and somehow opposite roles (Mantovani et al., 2004; Mills et al., 2000). This, prompted us to analyze the role of macrophages and its activation state in acute myocarditis associated to *T. cruzi* infection.

## I. Enzymes involved in L-arginine metabolism in the heart of mice infected with *T. cruzi*.

Our results show for the first time that arginase expression is strongly induced in heart tissue during acute *T. cruzi* infection in mice. However, this takes place together with an increased expression of iNOS. Besides, mRNA levels for CAT-1 and CAT-2 were induced in a time-dependent manner associated to iNOS and arginase I protein induction. The peak of parasite load correlated with peaks of arginase I and iNOS expression in heart tissue. This was observed in both susceptible BALB/c and resistant C57BL/6 mice, so no obvious relationship with susceptibility can be drawn. However, arginase I expression in susceptible BALB/c mice was higher and persisted for a longer period of time than in C57BL/6 mice, which controlled more efficiently the infection. Besides, ODC protein expression was also induced during the acute phase, likely resulting in an increment in polyamines synthesis needed for parasite replication.

Arginase I and iNOS expression were not equally induced in the different tissues or cells tested. Thus, these proteins were detected specifically in heart and in PECs, and, in the case of arginase, to a lesser extent in PBMCs. PECs showed an expression pattern, that although slightly different from the one found in heart tissue also points out to a time-dependent higher expression of arginase I in BALB/c infected mice compared with C57BL/6 mice. In PECs, arginase I expression is detected earlier in the infection

than in heart likely reflecting the fact that mice are being infected intraperitoneally and those cells are the first ones to encounter the parasite.

Although many reports describe an important role for iNOS mediated NO production to control *T. cruzi* infection *in vitro* (Gazzinelli et al., 1992; Metz et al., 1993; Munoz-Fernandez et al., 1992; Pakianathan and Kuhn, 1994; Plasman et al., 1994), infected iNOS deficient mice did not show any differences in parasite burden present in blood or in heart in comparison to wild-type infected mice. This suggests that *in vivo*, the toxic effect of iNOS derived products is not essential for parasite control or that it might be compensated by other NO synthases. Alternatively, NO may have opposite dual effect in vivo. Besides being cytotoxic for the parasite, iNOS derived NO may also contribute to parasite replication by inducing immunosuppresion of T. *cruzi* specific immune responses (Goni et al., 2002). Thus, it is possible that mice lacking iNOS, have less immunosuppresion *in vivo* leading to a better control of parasite replication despite having lower amount of the cytotoxic NO. Both mechanisms may be compensating each other, being the result the apparent lack of effect in replication.

Interestingly, Arginase I and iNOS are crossregulated by their products. In this regard iNOS deficient mice infected with *T. cruzi* showed very high arginase I expression and arginase activity in heart compared to wild-type mice. Despite this arginase activity increase, no significant differences were detected in parasite burden in the heart, prompting us to conclude that arginase induction during infection is not exclusively related to parasite proliferation, probably due to the fact that ODC, as the limiting enzyme of the polyamine synthesis pathway, is known to be strictly regulated. Thus, an increase in arginase is not directly translated into an increase in polyamines. In this regard, it is important to mention that other authors (Holscher et al., 1998) have reported extreme susceptibility to *T. cruzi* infection with a very high parasitemia in a different iNOS deficient mouse strain. Further studies that address the reasons of this different response, such as genetic backgrounds, or different *T. cruzi* strains are needed in order to assign a clear and reliable role for this enzyme in *T. cruzi* infection.

## II. Th1 and Th2 cytokines are expressed in heart during *T. cruzi* infection.

Arginase I and iNOS expression have been described to be regulated respectively by Th2 and Th1 cytokines. However, our analysis showed that both Th1 and Th2 cytokines were induced upon infection in both BALB/c and C57BL/6 hearts. It

is generally accepted that in many parasitic diseases, BALB/c susceptible mice mainly produce Th2 cytokines whereas C57BL/6 resistant mice Th1 cytokines (Fresno et al., 1997). However, we found that this Th1/Th2 dichotomy is not so strict in *T. cruzi* infection at least in the heart. Nonetheless, heart Th1/Th2 balance is higher in C57BL/6 than BALB/c mice, and the levels of Th2 cytokine expression were always higher and persisted longer in susceptible mice compared with resistant mice.

Very few studies have addressed the profile of Th1/Th2 cytokines in the heart and resistance/susceptibility to *T. cruzi* infection. In a previous report (Talvani et al., 2000), have also found that hearts of C57BL/6 mice have mRNA for both Th2 (IL-4, IL-10) and Th1 (IFNγ) cytokines. Other authors (Zhang and Tarleton, 1996a) were unable to detect Th2 cytokines in infected hearts of C57BL/6 mice by immunohistochemistry and showed very low levels of IFNγ producing cells. The reason of this apparent discrepancy may rely in the lower sensitivity of immunohistochemistry compared to our quantitative PCR.

Different mice lacking Th1 or Th2 cytokines were infected in order to establish a role for this cytokines in arginase I regulation and induction. IFNγR$^{-/-}$ and TNF$^{-/-}$ mice as representatives of proinflammatory/Th1 cytokines, had, as previously described, an increased susceptibility to *T. cruzi* infection presenting very high parasitemias and dying before 21 d.p.i. Both TNF and IFNγ are needed for iNOS induction (Munoz-Fernandez et al., 1992; Vila-del Sol et al., 2007), and our data corroborate this fact, since no iNOS was detected in hearts of neither TNF nor IFNγ deficient infected mice. This iNOS absence however, did not result in a high arginase I induction; this apparent contrast with data from iNOS$^{-/-}$ mice in which arginase I induction was increased. The differences between these models are not easy to explain but may reflect that genetic deletion completely eliminates iNOS function whereas in cytokine-deleted mice some background NOS may exist. Alternatively iNOS deficiency may require TNF or IFNγR signalling to induce arginase I.

IFNγR$^{-/-}$ mice infected with *T. cruzi* had similar levels of arginase I enzyme and showed a significant increase in arginase activity that did not differ statistically when compared to the wild-type. When infected with *T. cruzi*, TNF$^{-/-}$ mice showed lower levels of arginase I expressed in heart in comparison to the wild-type. However, when arginase activity from the same heart lysates was determined at 18 d.p.i., it was significantly increased in both TNF$^{-/-}$ and wild-type mice and statistical analysis did not

reveal significant differences between them, which might account for a compensating arginase II induction in this mice. The fact that in hearts of these mice arginase I was not highly induced with *T. cruzi* infection despite the absence of iNOS protein pointed to a regulating mechanism for arginase I other than the crossregulation with iNOS products.

In the onset of an immune response against *T. cruzi*, IL-10, TGFβ and Th2 cytokines as IL-4 and IL-13 counteract the excess of initially triggered pro-inflammatory response that, when excessive, can result in host damage. The lack of an appropriate Th1 mediated response in IFNγ and TNF deficient infected mice might not trigger a suitable compensating IL-10, TGFβ and Th2 cytokine mediated reaction that accounts for arginase I induction in heart. In this regard, IL-10 deficient mice infected with *T. cruzi* showed severe pathology and died in the early acute phase due to TNF mediated septic shock. However, IL-10 deficient mice presented lower parasitemia than wild-type mice in agreement with previous results (Holscher et al., 2000). Moreover, we found that the difference was seen also in parasite load in heart tissue. Arginase I and iNOS protein were not detected in heart tissue of these infected mice and arginase activity showed no significant increase in infected mice compared to the non-infected. However, when mRNA levels for arginase I, arginase II and iNOS were studied in hearts of infected IL-$10^{-/-}$ and wild-type mice, a small but significant induction was detected for these three genes. Moreover both arginases showed statistically lower levels in IL-10 deficient, when compared to the wild-type mice while no significant differences were seen in the same comparison for the iNOS gene.

When infected with *T. cruzi*, IL-$10^{-/-}$ mice die very early in the course of the infection and none of the enzymes subject of study were yet detected, although the data from mRNA suggest a possible role of IL-10 in arginase induction.

Analysis of mRNA levels in hearts from BALB/c and C57BL/6 mice infected with *T. cruzi* showed that IL-4 and IL-13 levels were had statistically higher in BALB/c mice than in C57BL/6 mice at all d.p.i. analyzed. IL-$4R^{-/-}$ mice, which have impaired signalling for both IL-4 and IL-13, showed no differences in parasitemia in comparison to the wild-type mice when infected with *T. cruzi*. However, parasite burden in heart tissue was significantly decreased in infected IL-4R deficient mice when compared to infected wild-type mice at 14 and 21 d.p.i. Moreover, arginase I expression in these hearts was higher in wild-type than in IL-4R deficient mice and also, arginase activity from the same lysates was much lower in IL-$4R^{-/-}$ when compared to the wild-type.

Thus, arginase I expression and arginase activity in hearts of mice infected with *T. cruzi* depends to a certain level on IL-13 and/or IL-4 signalling. iNOS expression is also slightly reduced in IL-4R deficient mice. In contrast, when IL-4 deficient mice were infected with *T. cruzi*, arginase I induction in heart tissue compared to the wild-type was not diminished but rather presented higher levels. Arginase activity was also significantly higher in hearts from IL-4 deficient mice when compared with wild-type. These results indicate that IL-4 is not implicated in arginase I induction in hearts of mice infected with *T. cruzi*. Since IL-4R seems to be involved, this suggests that this responsibility relies mainly in IL-13 or alternatively a cooperation of both IL-4 and IL-13 cytokines is needed.

However, other agents and inflammatory mediators have been described to be able to induce arginase I which might also be involved in its regulation. The work of (Freire-de-Lima et al., 2000) shows that the induction of ODC expression could be due to the uptake of apoptotic cells during *T. cruzi* infection, which causes release of $PGE_2$ and induction of TGF-β. Since the ODC substrate depends mainly on arginase, its induction could be mediated by TGF-β. However, a very weak induction of TGF-β mRNA was observed, as compared with other cytokines, suggesting that TGF-β is not responsible for the arginase I induction in our model. This is sharp contrast with original studies from (Zhang and Tarleton, 1996b) in which TGF-β expressing cells were highly abundant in the hearts of C57BL/6 by immunohistochemistry. Despite being much more sensitive the PCR technique to detect TGF-β mRNA, this cytokine was found in both strains of infected mice. The reason for the discrepancies are unknown, but may rely on the different strains of *T. cruzi* used to infect the mice or that unchecked cross-reaction of the TGF-β antibodies used in Zhang's studies. On the other hand, m-PGES-1 expression was significantly higher in BALB/c respect to C57BL/6 mice at 21 d.p.i., indicating that $PGE_2$ could partially contribute to the increased arginase I expression observed in BALB/c mice compared to C57BL/6 mice.

## III. Cell type expressing iNOS, Arginase II and Arginase I in heart tissue from *T. cruzi* infected mice.

We also analyzed the expression of these enzymes at the cellular level in heart tissue sections. We found that iNOS and arginase II were expressed in cardiomyocytes of infected BALB/c mice in agreement with previous reports that attributed a role for arginase II in cardiomyocyte survival (Aoki et al., 2004) and contractility (Steppan et al., 2006). Interestingly, arginase I expression was detected for the first time only in $CD68^+$ infiltrating macrophages, and according to protein expression, arginase I was very likely responsible of arginase enzymatic activity in heart tissue. Moreover, analysis of the number of infiltrating macrophages, by quantifying CD68 by qRT-PCR in heart tissue, showed that they were very similar in BALB/c and C57BL/6 mice. This indicates that the differences in cell number of infiltrating macrophages were not responsible for the increased arginase I expression and activity seen in BALB/c mice. Rather, heart infiltrating macrophages in infected BALB/c mice expressed more arginase I per cell than in C57BL/6.

We also found that *T. cruzi* infection triggered iNOS expression in cardiomyocytes, in agreement with previous observations (Machado et al., 2000). However, the parasite was unable to induce the expression of arginase I in cardiomyocytes and neither arginase I and iNOS in resident peritoneal macrophages.

Together, the *in vitro* and *in vivo* infection data suggest that induction of iNOS expression in cardiomyocytes could be triggered by the parasite, while arginase I expression in macrophages depends on Th2 cytokines, mainly IL-13. And additional contribution of IL-4, IL-10 and $PGE_2$ cannot be excluded. Interestingly, we found that cardiomyocytes expressed arginase I upon IL-4 stimulation *in vitro*, while the parasite alone was unable to induce its expression. However, arginase I was not observed in cardiac cells of infected BALB/c mice even though IL-4 was detected in cardiac tissue. Since infection partially inhibited IL-4-induced arginase I *in vitro,* it is likely that a similar mechanism is operating *in vivo*.

It is important to note that iNOS protein was not detected in circulating PBMCs in agreement with previous reports (Fabrino et al., 2004) rather in heart tissue, suggesting a specific important role of iNOS in this organ. A discrete induction of arginase I compared to the one detected in heart tissue was observed in circulating PBMCs but not in other lymphatic organs from acutely infected mice. This indicates

that arginase I is preferentially induced in macrophages infiltrating the heart and that its induction is mediated by the above-mentioned cytokines and also $PGE_2$ in an organ specific manner and not systemically.

## IV. Myeloid suppressor cells infiltrating hearts of mice infected with *T. cruzi*.

Isolation of $CD11b^+$ cells from heart inflammatory infiltrate of infected mice allowed us to study and functionally characterize these arginase I and iNOS expressing cells. In this regard, the population of infiltrating $CD11b^+$ cells did not show a clearly polarized M2 or M1 pattern when several genes, already described to be induced in several models of M2 populations (Ghassabeh et al., 2006), were analyzed. According to the surface markers expressed in sorted $CD11b^+$ population from hearts of mice infected with *T. cruzi*, these cells did not show staining for CD11c or F4/80 markers, which agrees with results encountered in the immunofluorescence staining of heart tissue sections, and neither for CD19 marker indicating that the numerous $CD11b^+$ population isolated from inflammatory infiltrate is not corresponding to dendritic cells, or B cells. Despite the fact that F4/80 marker of mature tissue macrophages was not detected, immunofluorescence of heart sections showed a $CD68^+$ population indicating that a high percentage of infiltrating cells are macrophages. Also, those $CD11b^+$ cells showed MHC class II expression suggesting a mature population capable of presenting antigen to T cells. Maybe the most important phenotypic characteristic of this $CD11b^+$ population was the expression of the Gr-1 molecule. Gr-1, also known as Ly6-G, is highly expressed in granulocytes, and to a lesser extent, in a subset of monocytes, which have been reported to have the ability of suppressing distinct T cell responses and are commonly grouped under the name: myeloid suppressor cells (MSCs) (Bronte et al., 2000; Bronte and Zanovello, 2005). The anti-Gr-1 antibody RB6-8C5 used in our analysis recognizes not only Ly6-G but also Ly6-C molecule, which is expressed in monocytes and in certain populations of neutrophils, dendritic cells and lymphocytes. According to the expression of Gr-1 and Ly6-C, $CD11b^+$ cells isolated from hearts of infected mice showed two distinct populations: $CD11b^+$ $Gr\text{-}1^{int}$ $Ly6\text{-}C^{high}$, corresponding with MSCs phenotype, and $CD11b^+$ $Gr\text{-}1^{high}$ $Ly6\text{-}C^{int}$ which corresponds with granulocyte phenotype population.

Arginase I and iNOS expression was found in infiltrating $CD11b^+$ cells from hearts from infected mice, and in much lower levels arginase was seen in $CD11b^+$ from blood from infected mice but none of the enzymes was detected in $CD11b^+$ from blood from non-infected mice. Depletion of Ly6-$G^+$ cells with magnetic beads using 1A8 clone specific for granulocyte cells (Daley et al., 2008) prompt us to the conclusion that $CD11b^+$ Gr-$1^{int}$ Ly-$6C^{high}$ $CD68^+$ $F4/80^-$ cells present in the heart inflammatory infiltrate of mice infected with *T. cruzi*, were responsible for arginase I expression observed in this organ and moreover, these cells also presented iNOS protein expression. No single report has analyzed the myeloid infiltrating cells in the hearts on *T. cruzi* infected mice. Nonetheless, in *Trypanosoma congolense*, infected BALB/c mice macrophages expressing both iNOS and arginase I were detected being this expression associated with pathology (Noel et al., 2002).

Besides, in acute *T. cruzi* strong suppression of T lymphocyte responses mediated by myeloid cells expressing NO has been described (Goni et al., 2002). Similarly, $CD11b^+$ cells from hearts of infected mice also showed a strong suppression of T cell proliferation when co-cultured with T cells isolated from spleen of non-infected mice. This impaired proliferation was even more pronounced in the presence of arginase inhibitor. However, only when iNOS enzyme was inhibited T cell proliferation was mostly restored, and an even greater recover of T cell proliferation was observed when both arginase and iNOS inhibitors were added simultaneously to the cell culture. Different mechanisms have been described to mediate T cell suppression for arginase, and/or iNOS enzymes. When both enzymes are being induced, competition for the common substrate L-arginine leads to the depletion of this amino acid from the extracelular milieu; with very low L-arginine concentrations, iNOS enzyme decreases NO formation in order to produce superoxide, which, due to its unstable nature, is converted to $H_2O_2$ responsible for toxicity and impaired T cell proliferation (Kusmartsev et al., 2004). If depletion of L-arginine occurs, arginase inhibition by nor-NOHA would result in higher availability of L-Arginine substrate for iNOS enzyme and production of NO and peroxinitrites that would aggravate even more the inhibition of the T cell response. But then, why both arginase and iNOS inhibitors reestablish on a greater way T cell proliferation than when iNOS inhibitor alone is present? This might be due to arginase-mediated depletion of L-arginine that might cause inhibition of T cell activation by decreasing CD3ζ expression (Rodriguez et al., 2003). Thus, arginase I has a complex way of modulating iNOS mediated impairment of T cell proliferation.

On one hand, few studies have addressed the role of arginase I in heart related to pathology. On the contrary, the role of NO in cardiac physiology has been widely studied (Shah and MacCarthy, 2000). For example, excessive NO production by iNOS may lead to myocarditis in many models including infectious models as Coxsackie virus induced myocarditis, which had some features similar to *T. cruzi* induced myocarditis (Girones and Fresno, 2003). Thus, arginase I induction in the heart, besides immunosupression, may be also a modulator of myocardial function (Post and Pieske, 2006) and attenuate an excessive NO signaling, thus protecting heart from NO mediated damage. In this regard, iNOS-derived NO plays an important role in ventricular dilation in acute murine chagasic myocarditis (Chandra et al., 2002)

In summary, we have analyzed the arginine metabolism enzymes in heart and showed for the first time that arginase I is expressed by infiltrated MSCs during acute experimental *T. cruzi* infection. Moreover, arginase I is induced by IL-13 probably in cooperation with IL-4, IL-10 and $PGE_2$ and has a complex dual role during *T. cruzi* infection by, on one side providing abundant substrate for ODC enzyme and the polyamine pathway, which might result in higher parasite proliferation, and on the other side by modulating iNOS mediated T cell suppression.

# CONCLUSIONS

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Results obtained in this thesis yielded the following conclusions:

1. Arginase I is induced in heart of mice infected with *T. cruzi* correlating with the parasite load in this organ.

2. Arginase I induction during *T. cruzi* infection is restricted to heart and PECs.

3. Arginase I induction in heart of mice infected with *T. cruzi* requires IL-4R signaling likely mediated by IL-13 with a possible cooperation with IL-4, IL-10 and $PGE_2$.

4. iNOS is expressed in cardiomyocytes and $CD11b^+$ $Gr\text{-}1^{int}$ $Ly\text{-}6C^{high}$ infiltrating cells in heart tissue from infected mice, while arginase I expression is restricted to $CD68^+$ $CD11b^+$ $Gr\text{-}1^{int}$ $Ly\text{-}6C^{high}$ infiltrating population.

5. $CD11b^+$ cells present in heart inflammatory infiltrate of mice infected with *T. cruzi* do not have a clear polarized M1 or M2 pattern of gene expression

6. $CD11b^+$ cells present in heart inflammatory infiltrate of mice infected with *T. cruzi* expressing iNOS and arginase I show a myeloid suppressor cell phenotype and modulate T cell proliferation *in vitro*.

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# APPENDIX 1

# RESUMEN

En la enfermedad de Chagas, causada por *Trypanosoma cruzi*, los macrófagos y los cardiomiocitos son las dianas principales de la infección. Por otro lado, los macrófagos de pueden activar en diferentes vías por citoquinas Th1 o Th2 dando lugar a activación clásica o alternativa, lo que resulta en una respuesta distinta. Estas vías diferentes se distinguen a menudo por la expresión de varias enzimas implicadas en el metabolismo de la L-arginina en tejido cardiaco durante la infección *in vivo* de ratones BALB/c y C57BL/6. Encontramos que la expresión de la óxido nítrico sintasa inducible (iNOS), arginasa I y arginasa II, así como la ornitina decarboxilasa, eran mucho mayores en ratones BALB/c comparada con C57BL/6 y relacionada con la carga parasitaria en tejido cardiaco. Citoquinas de tipo Th1 y Th2 se expresan en tejido cardiaco de las dos cepas infectadas, pero el balance Th1/Th2 era predominantemente Th1 en ratones C57BL/6 y Th2 en ratones BALB/c en el pico de carga parasitaria durante la infección. Usando ratones genéticamente deficientes en varias citoquinas o en sus receptores, encontramos que la IL-13, probablemente cooperando con IL-4, IL-10 y prostaglandina $E_2$ están induciendo la expresión de arginasa I. iNOS y arginasa II se expresan en cardiomiocitos. Interesantemente, macrófagos $CD68^+$ infiltrados en corazón fueron el principal tipo celular expresando arginasa I. Cuando fueron purificadas, las células $CD11b^+$ infiltradas en corazón que expresan ambas arginasa I e iNOS no presentaron un patrón definido de activación M1/M2, pero sí mostraron un fenotipo característico de células mieloides supresoras. Además, estas células $CD11b^+$ modularon la proliferación de células T *in vitro*. Por tanto, la arginasa I puede influenciar la supervivencia del parásito, y podría estar también regulando la respuesta inflamatoria de las células T durante la infección.

# INTRODUCCIÓN

## I. Enfermedad de Chagas

### I.1. Aspectos generales y ciclo de vida.

La tripanosomiasis americana, o enfermedad de Chagas, es un desorden multisistémico que, de acuerdo con el último informe de la OMS, afecta aproximadamente a 18 millones de personas con 120 millones en riesgo. Se trata de una enfermedad endémica en América Latina donde se distribuye a través de 18 países desde el cono sur del continente (Fig. 1), hasta Méjico (WHO, 2002)

La transmisión de esta enfermedad a los humanos ocurre fundamentalmente de tres maneras distintas: Vía insecto vector, transfusión de sangre infectada, y transmisión vertical.

***Trypanosoma cruzi,*** un protozoo flagelado de la familia *Kinetoplastidae*, fue identificado por primera vez por por Carlos Chagas en 1909 como agente causal de la enfermedad que se llamó así en su honor. Este parásito presenta un ciclo de vida complejo que implica varias etapas tanto en vertebrados como en el insecto vector. *T. cruzi* tiene tres morfologías distintas: **epimastigote**, que se replica en el insecto vector triatomino, **tripomastigote**, que infecta las células del hospedador vertebrado, y **amastigote**, que se replica intracelularmente dentro de las células del hospedador (Burleigh and Andrews, 1998; Tanowitz et al., 1992).

La trasmisión de *T.cruzi* a humanos ocurre cuando las heces liberadas, mientras succiona sangre del hospedador vertebrado, por el insecto triatomino que contienen tripomastigotes metacíclicos infectivos, penetran a través de la piel en el torrente sanguíneo. Es allí donde las formas flageladas del parásito infectan una amplia variedad de células del hospedador (fundamentalmente macrófagos y cardiomiocitos). Una vez en el interior de las células, las formas tripomastigote se transforman a amastigote, los cuales de multiplican intracelularmente. Después de ésto, los amastigotes salen de la célula y dan lugar a trypomastigotes, que son liberados al torrente sanguíneo donde se diseminan e infectan nuevas células otra vez. Es en esta etapa cuando los parásitos pueden ser succionados del torrente sanguíneo por la chinche triatomina mientras se alimenta de sangre. Una vez alcanzan la tripa del insecto, los tripomastigotes se

trasforman en epimastigotes, y se multiplican a través del tracto digestivo hasta que alcanzan los intestinos donde se transforman en tripomastigotes metacíclicos y son liberados junto con las heces cuando el insecto se alimenta de sangre del hospedador vertebrado.

Los individuos que residen en las áreas rurales de América Latina son los que se encuentran en un mayor riesgo de infección, porque los insectos viven en las cabañas y se alimentan constantemente picando a sus habitantes durante la noche. La OMS ha dirigido varios programas para la eliminación del insecto vector, y para la educación en la comprensión de la salud con un fuerte éxito en el cono sur donde la incidencia ha bajado del orden de un 70% desde 1985. También se necesita realizar un examen de la sangre para prevenir la transmisión a través de transfusión sanguínea para poder prevenir y controlar la enfermedad. Dos fármacos (benznidazol y nifurtimox) están disponibles para el tratamiento de la fase aguda de esta enfermedad pero, además de tener un porcentaje muy alto de efectos secundarios, no son útiles para la fase crónica de la enfermedad. Además, hasta la fecha, sigue faltando una inmunoterapia efectiva o una vacuna.

### I.2. Clínica de la enfermedad.

Se pueden distinguir dos fases, aguda y crónica, en la enfermedad de Chagas (Kirchhoff, 1993; Prata, 2001; Tanowitz et al., 1992). En la fase aguda, pocos días después de la infección, aparece una lesión inflamatoria local en el sitio de infección donde los tripomastigotes metacíclicos infectan y experimentan las primeras rondas de multiplicación. Entonces, ocurre la diseminación del parásito por el cuerpo y los tripomastigotes sanguíneos se pueden observar fácilmente (parasitemia); un pequeño número de pacientes desarrollan síntomas de afección cardiaca que refleja una miocarditis severa subyacente. Esto puede conducir a un fallo cardiaco, responsable de las pocas muertes que ocurren en la fase aguda de la enfermedad de Chagas. También puede presentarse meningoencefalitis, especialmente en algunos pacientes inmunosuprimidos (Hoff et al., 1978). No obstante, la fase aguda de la enfermedad permanece fundamentalmente no diagnosticada sin síntomas graves. En cambio, las manifestaciones más comunes y la patología más severa de la enfermedad aparece muchos años (de 10 a 30) después de la infección por *T. cruzi* en aproximadamente 30% de las personas infectadas, en lo que se conoce como la fase crónica de la enfermedad.

Durante esta fase, los parásitos circulantes no se pueden detectar en sangre, pero se produce daño progresivo que implica al esófago, colon y corazón. En la fase crónica, el corazón es es el órgano más afectado; frecuentemente se desarrolla cardiomiopatía siendo el fallo congestivo de corazón la causa más común de muerte de éstos pacientes.

### I.3. Modelo experimental para la enfermedad de Chagas: el ratón.

El ratón, por su tamaño pequeño y su fácil mantenimiento, ha sido el animal preferido para un gran número de experimentos. Los ratones son susceptibles a la infección por *T cruzi* y desarrollan fase aguda y crónica en cierto modo similar a la patología descrita en humanos.

Sin embargo, tanto las cepas de ratón como de parásito, al igual que el procedimiento de inoculación, determinan la susceptibilidad y la patogénesis de la enfermedad de Chagas en el modelo de ratón, pero, a pesar de las diferencias, los ratones muestran una fase aguda donde la forma sanguínea del parásito puede ser detectada en sangre mediante técnicas microscópicas abarcando desde los 7 a los 30 días. Después de este periodo, la fase crónica comienza a desarrollarse; no se pueden detectar parásitos en sangre, y se puede observar cardiomiopatía chagásica en estudios histopatológicos de ratones infectados.

## II. Respuesta inmune en la enfermedad de Chagas.

La infección por *T. cruzi* desencadena una respuesta inmune compleja. Como patógeno intracelular, implica un intricado, y todavía no bien entendido mecanismo de interacción de muchos mecanismos humorales y celulares de la inmunidad innata y adaptativa.

### II.1. Inmunidad innata

Como parte de la primera barrera que el parásito encuentra después de entrar en el hospedador, moléculas y células efectoras de la inmunidad innata juegan un papel muy importante en la respuesta inmunológica contra *T. cruzi.*

Después de romper la barrera de la piel, el parásito entra en la sangre y tejidos, y desata la respuesta temprana del complemento y las proteínas de fase aguda (Proteína C

reactiva, componente amiloide P del suero, $\alpha_2$Macroglobulina). Estas moléculas se unen a *T. cruzi* y media su **endocitosis mediada por receptor**. También, macrófagos y células dendríticas expresan en su superficie el receptor de manosa, que reconoce carbohidratos con patrones moleculares que no se encuentran en las células del hospedador.

*T. cruzi* también es capaz de entrar activamente en una gran variedad de tipos celulares, especialmente macrófagos, desencadenando una diversidad de interacciones moleculares que movilizan la respuesta de la inmunidad innata del hospedador. Los macrófagos secretan **IL-12** que activa a las células **NK** para producir **IFNγ** (Aliberti et al., 1996), esta citoquina actúa recíprocamente en macrófagos los cuales liberan **TNF** para actuar sinérgicamente junto con la IL-12 e IFNγ e inducir la producción de NO es estas células (Abrahamsohn and Coffman, 1996; Munoz-Fernandez et al., 1992). Sin embargo, hay varias evidencias que apoyan el hecho de que la respuesta inflamatoria está autorregulada y que la activación del macrófago y la producción de NO está reducida por las llamadas citoquinas anti-inflamatorias. Se ha encontrado que la **IL-10** se requiere para prevenir una respuesta excesiva pro-inflamatoria durante la infección por *T. cruzi* (Gazzinelli et al., 1992; Hunter et al., 1997; Reed et al., 1994), y algo similar ocurre con **TGFβ** (Silva et al., 1991) aunque esta citoquina puede tener otras funciones además de la regulación inmune (Hall and Pereira, 2000; Ming et al., 1995). Algunos autores reivindican que la IL-4 está también regulando a la baja el IFNγ y la inflamación cuando se encuentra cooperando con la IL-10 (Abrahamsohn et al., 2000), pero otros informes muestran que ésta citoquina tendría efectos parecidos a aquellos del IFNγ con actividad tripanocida (Golden and Tarleton, 1991; Wirth et al., 1989). Aunque el papel de la **IL-13** no esta claro de momento, se ha descrito que esta citoquina pudiera estar implicada en la regulación de la liberación de IFNγ (Antunez and Cardoni, 2001).

Los macrófagos expuestos al IFNγ se vuelven muy efectivos como células presentadoras de antígenos (APCs) e interaccionan y presentan péptidos a los linfocitos T. Además, el TNF es capaz de inducir la maduración de células dendríticas y su migración desde la piel y mucosa a los órganos linfoides para empezar a activar la expasión de linfocitos específicos para antígenos microbianos (Fearon and Locksley, 1996). Pero no sólo las APCs profesionales son capaces de presentar péptidos del parásito, la habilidad de *T. cruzi* para invadir un alto número de tipos celulares de mamíferos los implica en la respuesta inflamatoria e inmunológica. Se ha demostrado

que los cardiomiocitos responden a la infección liberando NO, citoquinas y quimioquinas (Fichera et al., 2004; Machado et al., 2000).

## II.2. Inmunidad adaptativa

La respuesta inmunológica frente a *T. cruzi* también implica activación de los componentes de la respuesta adaptativa humoral y celular. La infección aguda experimental por *T. cruzi* en el modelo murino desencadena una activación intensa y policlonal de linfocitos B, que implica una superproducción de **inmunoglobulinas (Ig)**. La respuesta frente a los antígenos en esta fase representa sólo una pequeña fracción de la Ig total producida (Minoprio et al., 1989). Esta respuesta policlonal es ampliamente dependiente de las células $CD4^+$ T cooperadoras (Minoprio et al., 1989).

La importancia de las dos subpoblaciones principales de linfocitos T, $CD4^+$ y $CD8^+$, a la hora de controlar la infección está ampliamente establecido. *T. cruzi* es capaz de infectar prácticamente cualquier tipo celular en los tejidos del hospedador (Lenzi et al., 1996); después de escapar de la vacuola parasitófora en el citoplasma (Nogueira and Cohn, 1976), los antígenos del parásito serán procesados y presentados en el contexto de la molécula de MHC de clase I, la cual será reconocida por las células T **$CD8^+$** efectoras. Por otro lado, los parásitos muertos, antígenos solubles del parásito, o parásitos que no pudieron escapar de la vacuola parasitófora (McCabe et al., 1984), serán presentados sólo por APCs profesionales a través de MHC de clase II a las células T **$CD4^+$**, que cooperarán con las células B para inducir anticuerpos. Además, a pesar de que los ratones deficientes en células B sucumben a la infección (Kumar and Tarleton, 1998), la respuesta inmunológica protectiva parece depender en su mayoría de las células $CD8^+$ que producen IFNγ. Las células T $CD8^+$ citotóxicas son capaces de controlar la infección a través de un mecanismo de perforina/granzyma de muerte para células infectadas y/o apoptosis mediada por FAS (Kumar and Tarleton, 1998). Sin embargo, hay trabajos indicando que las células T $CD8^+$ no pueden controlar completamente la infección porque se vuelven carentes de respuesta (Martin and Tarleton, 2004).

Las **citoquinas** juegan un papel clave en la regulación tanto de la inducción, como del tipo de respuesta inmunológica, pero no se ha visto una polarización clara hacia Th1 *versus* Th2 estudiando ratones susceptibles o resistentes a la infección por *T.*

*cruzi* (Powell et al., 1998; Zhang and Tarleton, 1996a, 1996b). Por lo tanto, el papel de las citoquinas Th1/Th2 durante la infección no ha sido establecido por el momento.

Además, se han sugerido una variedad de quimioquinas que juegan un papel clave en el influjo de células inmunológicas a los tejidos diana durante la infección, revisado en (Silva et al., 1991; Teixeira et al., 2002). CCR5 y sus ligandos, juegan un papel central en la migración de células T al corazón (Machado et al., 2005) y en la proliferación del parásito (Hardison et al., 2006), CXCL9 y CXCL19, no afectan a la migración de los leucocitos, pero si se bloquean con anticuerpos neutralizantes, dan lugar a una reducción en la carga parasitaria en corazón (Hardison et al., 2006). Además, quimioquinas como CXCL12 y CCL4, han sido implicadas en la migración y el desarrollo de timocitos (Mendes-da-Cruz et al., 2006). En conjunto, hay una red compleja, en la que quimioquinas específicas juegan distintos papeles en la patofisiología de la enfermedad.

### II.3. Autoinmunidad e inmunomodulación.

Entre todos los posibles mecanismos responsables de la patogénesis de la enfermedad crónica de Chagas, la **autoinmunidad** es una de las que ha recibido más apoyo experimental pero también ha creado más controversia (Girones et al., 2005; Girones and Fresno, 2003; Kierszenbaum, 1986, 1999; Levin, 1996; Soares et al., 2001; Tarleton, 2001, 2003). Por otro lado, hay estudios que sugieren que la **persistencia del parásito** en los tejidos del hospedador es la clave de la patogenia de la forma crónica de la enfermedad puesto que el tratamiento disminuye la severidad de la enfermedad (Tarleton, 2001).

Varios mecanismos pueden explicar cómo un patógeno infeccioso puede romper la auto-tolerancia inmunológica. Entre ellos, mimetismo molecular, "bystander activation", y activación policlonal de linfocitos son mecanismos que han sido ampliamente documentados en la enfermedad de Chagas (Girones et al., 2005; Kierszenbaum, 2003). Sin embargo, ninguno de estos tres mecanismos es exclusivo y, cualquier combinación de los mismos o los tres pueden estar teniendo lugar.

Un aspecto importante, y normalmente no considerado, es el papel de las células T reguladores (Tregs), de las cuales se ha descrito que suprimen respuestas autoinmunes. Además de las clásicas Tregs que salen del timo, hay otras, Tr1 y Th3, las cuales adquieren capacidad reguladora en la periferia (Battaglia et al., 2002). Por lo

tanto, una definición del papel de las Tregs durante las infección por *T. cruzi,* puede contribuir a iluminar los mecanismos que controlan la patogénesis observada en la enfermedad de Chagas crónica.

## III. Regulación de las respuestas inmunológicas por el metabolismo de la L-arginina.

La L-arginina puede ser metabolizada dentro de las células por dos enzimas principales, la arginasa y la óxido nítrico sintasa inducible (iNOS) para dar lugar a urea y L-ornitina, y L-citrulina y óxido nítrico (NO) respectivamente.

La L-arginina también puede ser metabolizada a través de una vía menos conocida por la **Arginina Decarboxilasa** (ADC) que produce Agmatina, la cual puede regular tanto la producción de óxido nítrico como la síntesis de poliaminas (Satriano, 2004).

Los transportadores de aminoácidos catiónicos CAT-1, CAT-2, y CAT-3 son una familia de proteínas transmembrana que transfieren L-arginina (entre otros aminoácidos catiónicos) desde el exterior al compartimento intracelular para compensar la degradación producida por la arginasa y la iNOS. La expresión de CAT-3 está restringida al cerebro (Verrey et al., 2004), pero CAT-1 y CAT-2 están presentes en macrófagos y en algunos casos, se ha descrito que juegan un papel regulando la activación del macrófago en respuesta a parásitos (Wanasen et al., 2007; Yeramian et al., 2006).

### III.1. Arginasa

Hasta la fecha, se han descrito en mamíferos dos isoformas de arginasa. Estas isoformas está codificadas por dos genes distintos y tienen distinta localización subcelular (Jenkinson et al., 1996).

La **Arginasa I**, también conocida como arginasa hepática, está localizada en el citoplasma, y se expresa altamente en el hígado, pero también, de manera más restringida en otros tipos celulares, como los macrófagos. Esta isoforma se induce principalmente por citoquinas de tipo Th2 (IL-4, IL-13) (Corraliza et al., 1995; Munder et al., 1998), y también IL-10, TGFβ, factor estimulador de colonias de monocitos y

granulocitos (GM-CSF) y prostaglandina $E_2$ ($PGE_2$) (Boutard et al., 1995; Corraliza et al., 1995; Jost et al., 2003).

La **arginasa II**, está localizada en la mitocondria; se expresa en una amplia variedad de tejidos y de tipos celulares, fundamentalmente en el riñón (Levillain et al., 2005), pero también en próstata, intestino delgado (Gotoh et al., 1997), y cardiomiocitos (Aoki et al., 2004). Esta isozima está inducida fundamentalmente por LPS y dibutiril AMPcíclico (Gotoh et al., 1996).

Las dos isoformas de la arginasa catalizan la misma reacción y no se ha descrito todavía una clara distinción en funciones para cada una de ellas, se trata de un aspecto actualmente en debate (Cederbaum et al., 2004).

La arginasa está implicada en el ciclo de la urea catalizando la conversión de L-arginina a L-ornitina y urea. Después, la L-ornitina puede ser metabolizada por la Ornitina Aminotrasferasa para aumentar la L-prolina, la cual es responsable de la síntesis de colágeno. Por otro lado, la Ornitina Decarboxilasa (ODC), es la enzima clave de la ruta de las poliaminas; aumenta la producción de poliaminas que son necesarias para la proliferación de todas las células eucariotas. ODC es una enzima de eucariotas altamente regulada con una de las vidas medias más cortas descrita en mamíferos, fundamentalmente por la unión de la antizima (AZ) que dirige la degradación de la ODC por el proteasoma 26 S (Pegg, 2006).

### III.2 iNOS

Junto con otras isoformas de la NOS (nNOS en tejido neuronal, y eNOS en células endoteliales), iNOS cataliza la oxidación de L-arginina a L-citrulina y NO, con la formación del intermediario $N^{\omega}$-hydroxy-L-Arginine (NOHA).

La isoforma iNOS se encuentra en una gran diversidad de tipos celulares en el sistema inmune (Wu and Morris, 1998) y también en cardiomiocitos (Tsujino et al., 1994). El inductor más común de la iNOS es IFNγ combinado con LPS, pero también otras citoquinas como la IL-12, IL-1, y TNF.

La iNOS genera tanto NO como ión superóxido ($O_2^-$) que conduce a la formación de peroxinitritos ($ONOO^-$) (Xia and Zweier, 1997). Sin embargo, concentraciones muy bajas de L-arginina pueden dar lugar a baja formación de NO y alta generación de superóxido, que es muy inestable y es convertido a peróxido de hidrógeno ($H_2O_2$) y oxígeno (Kusmartsev et al., 2004).

**NO**, especies reactivas del nitrógeno (**RNOS**) como $ONOO^-$, y especies reactivas del oxígenos (**ROS**) como el $O_2^-$ y $H_2O_2$, producidas por la iNOS, se sabe que son mecanismos muy eficientes a la hora de luchar contra patógenos, pero también tienen una alta autotoxicidad y, cuando se encuentran en cantidades excesivas, pueden causar patología.

### III.3. Balance de arginasa e iNOS.

Hay una regulación cruzada muy importante entre los productos de las reacciones de la iNOS y de la arginasa. Se ha descrito que $N^{\omega}$-hidroxi-L-arginina (NOHA), una forma intermedia en la síntesis de NO, inhibe arginasa (Daghigh et al., 1994) y se ha mostrado que las poliaminas pueden estar afectando a la expresión de iNOS (Mossner et al., 2001; Sonoki et al., 1997); además, la arginasa se ha implicado en la regulación a la baja de la iNOS vía depleción de L-arginina, puesto que el descenso en la disponibilidad de este aminoácido bloquea la síntesis de la iNOS y su estabilidad (El-Gayar et al., 2003; Lee et al., 2003). Es importante destacar que arginasa e iNOS comparten L-arginina como sustrato y que, a pesar de que la constante de afinidad de la iNOS para la L-arginina sea 1000 veces mayor que la de la arginasa, la Vmax de ésta es mas de mil veces más rápida que la de la iNOS, lo que resulta en una utilización similar del sustrato por parte de estas dos enzimas (Wu and Morris, 1998).

Debido a el hecho de que comparten sustrato, estas enzimas se han implicado en la regulación del balance Th1/Th2 durante los procesos inmunológicos, y han sido usadas como marcadores de activación M1/M2 (Mills et al., 2000).

### III.4. Metabolismo de la L-arginina en enfermedades parasitarias.

Varias infecciones por bacterias intracelulares pueden presentar una evolución modificada por las rutas arriba mencionadas; éste es el caso, por ejemplo, de Helicobacter pylori (Gobert et al., 2002; Gobert et al., 2000) y *Chlamidia sp.* (Huang et al., 2002).

El balance arginasa-iNOS también modifica muchas infecciones de protozoos; Se ha demostrado que la arginasa de los macrófagos durante la infección por *Trypanosoma brucei* disminuye las concentraciones de L-arginina, y de esta manera evita la eliminación del parásito dependiente de NO (Gobert et al., 2000); también, en

la infección por *Trypanosoma congolense*, la arginasa y la activación alternativa de macrófagos juegan un papel crucial en el desarrollo de la enfermedad crónica (Noel et al., 2002). Durante la infección de *Leishmania sp. in vitro* (Iniesta et al., 2001; Iniesta et al., 2002) está claro que los inhibidores de la arginasa reducen la patología mediante reducción de la replicación del parásito dentro de los macrófagos.

*T. cruzi* desencandena la producción de citoquinas y quimioquinas en cardiomiocitos (Machado et al., 2000) y en macrófagos (Bergeron and Olivier, 2006) infectados que inducen una actividad tripanocida muy potente dependiente de óxido nítrico (Gazzinelli et al., 1992; Metz et al., 1993; Munoz-Fernandez et al., 1992; Pakianathan and Kuhn, 1994), pero los resultados en ratones infectados todavía no están claros (Cummings and Tarleton, 2004; Girones et al., 2006; Holscher et al., 1998). Además, en exceso, el NO puede tener también un efecto citotóxico en el hospedador y llevar a la supresión de células T (Goni et al., 2002).

Por otro lado, monocitos de sangre periférica mostraron niveles disminuidos de óxido nítrico durante la enfermedad de Chagas en ratas, debido al aumento de la actividad arginasa (Fabrino et al., 2004). Además, macrófagos infectados *in vitro* (y macrófagos de ratones infectados), a través de la interacción con el receptor de vitronectina, aumentan la liberación de TGFβ y $PGE_2$, lo que aumenta la actividad ODC en macrófagos promoviendo así la proliferación de los parásitos (Freire-de-Lima et al., 2000; Lopes et al., 2000). Así mismo, la cruzipaína, un antígenos de *T. cruzi*, regula a la alza la actividad arginasa en macrófagos y promueve el crecimiento intracelular del parásito (Giordanengo et al., 2002; Stempin et al., 2002; Stempin et al., 2004). Además, este antígeno promueve la inducción de arginasa II en cardiomiocitos actuando así como factor de supervivencia, porque es capaz de rescatarlos de apoptosis (Aoki et al., 2004).

Es importante destacar que en otros tripanosomátidos como *Trypanosoma brucei y Leishmania donovani*, la replicación es controlada por el inhibidor de la síntesis de poliaminas α-difluorometilornitina (DFMO) a través de la inactivación de la ODC del parásito. Sin embargo, *T.cruzi* carece de arginasa (Camargo et al., 1978) y de ODC, y el tratamiento con DFMO ha resultado ser inefectivo. El parásito depende de una fuente externa de poliaminas para su crecimiento obteniéndolas probablemente del hospedador. Como alternativa, *T. cruzi* puede sintetizar poliaminas de la L-arginina vía agmatina (Persson, 2007). Además, durante la infección por *T. cruzi* en macrófagos de ratón y mioblastos de rata, la inhibición farmacológica de la ODC, redujo la invasión y la

proliferación intracelular y su efecto era revertido por la adición exógena de agmatina o putrescina (Kierszenbaum et al., 1987).

## IV. Metabolismo de la L-arginina en células mononucleares fagocíticas. Regulación de la respuesta inmunológica.

### IV.1 Activación del monocito-macrófago: M1 & M2

Las células del linaje monocito-macrofágico son consideradas un tipo celular muy heterogéneo dependiendo en el estado de diferenciación y activación, lugar y distribución en el tejido.

Tradicionalmente, la activación de los macrófagos se consideraba cuando desarrollaban una respuesta citotóxica contra patógenos intracelulares y también cuando liberaban mediadores solubles de la respuesta inmunológica. Hoy en día, la capacidad de los macrófagos para deshacerse de los patógenos intracelulares, se conoce que está mediada por NO (Hibbs, 2002), RNOS y ROS (Kusmartsev et al., 2004; Xia and Zweier, 1997) y que está inducida por citoquinas del tipo Th1 (como IFNγ y TNF) solos, o junto a productos microbianos. Estos macrófagos, también muestran capacidad para producir altos niveles de IL-12 (en humanos también IL-23), IL-1, TNF, IL-6, y bajos niveles de IL-10 (revisado en (Mosser, 2003)). Este estado de activación lleva a la comunidad científica a llamar a estas células **macrófagos activados clásicamente** o macrófagos **M1** (por analogía a Th1).

En oposición al tipo de macrófagos mencionados arriba, los fagocitos mononucleares que no muestran propiedades citotóxicas y que liberan grandes cantidades de IL-10 en lugar de IL-12, se denominan **macrófagos activados altenativamente**. Esta terminología se concibe para aquellos macrófagos que son activados por las citoquinas IL-4/IL-13 y que expresan altos niveles de arginasa I, a los que algunos autores denominaron **M2** , incluyendo también a aquellos que muestran un estado de "desactivación" en presencia de IL-10. Otros autores también distinguen entre la activación alternativa los que ellos llaman macrófagos activados de tipo II (Anderson et al., 2002), fagocitos mononucleados que están expuestos a complejos inmunológicos y LPS; estas células secretan niveles altos de IL-10 y bajos de IL-12 y desencadenan respuestas de tipo Th2 en linfocitos T (Anderson and Mosser, 2002).

Debido al hecho de que se necesita un consenso para establecer una nomenclatura común para estas células fagocíticas mononucleadas, Mantovani y colaboradores (Mantovani et al., 2004) proponen lo siguiente:

M1 se corresponde a la activación clásica arriba descrita. El término genérico para M2 se utiliza para definir una activación distinta de la M1 basado en propiedades comunes funcionales (baja secreción de IL-12 e implicación en respuestas Th2, inmunoregulación y remodelación del tejido). Además, las tres formas bien definidas de M2 se denominarán como M2a, inducidos por IL-4/IL-13; M2b, inducidos por complejos inmunológicos y agonistas de receptores toll-like (TLRs), o M2c inducidos por IL-10 y glucocorticoides.

Ghassabeh y colaboradores (Ghassabeh et al., 2006) identificaron una firma de genes comunes para una respuesta genérica M2 desarrollada en diferentes condiciones patológicas analizando varios genes agrupados en grupos funcionales (anti-inflamación, cicatrización, angiogénesis, regulación del ciclo celular, y otros).

## IV. Células supresoras mieloides.

En los años 70 y principios de los 80, los investigadores describieron lo que llamaron células supresoras naturales; eran células no linfoides capaces de suprimir la respuesta de los linfocitos a inmunógenos o mitógenos de una manera independiente de MHC (Strober, 1984). Hoy por hoy, se conoce que estas células son un subtipo de células mielomonocíticas denominadas **células mieloides supresoras (MSCs),** las cuales se acumulan durante la respuesta inmunológica aguda y crónica frente a patógenos, estrés inmunológico (después de exposición a superantígenos y su consiguiente activación extensiva de células T), crecimiento de tumores y desarrollo... y otros casos.

Las MSCs están equipadas con mecanismos extremadamente eficientes para destruir los patógenos invasores, y además son muy eficientes a la hora de suprimir la activación de células T, revisado en (Bronte and Zanovello, 2005).

Estas células se identifican comunmente en ratones por la expresión de los marcadores de membrana CD11b y Gr-1; junto con estos marcadores, las MSCs, pueden expresar una gran variedad de marcadores diferenciadores de células mieloides como Ly6-C, CD31 (Bronte et al., 2000); también hay importantes diferencias en el fenotipo dependiendo del lugar anatómico en que se localicen o de las condiciones

patológicas. Una clasificación rígida para las MSCs no está disponible en la actualidad; se necesitaría separar el continuo que es la diferenciación mieloide a pasos muy definidos (Bronte and Zanovello, 2005).

## Supresión de las células T y el metabolismo de la L-arginina

Arginasa e iNOS, tanto en combinación como por separado, son usadas por las MSCs para inhibir las respuestas T (fig)

**Arginasa**: La pérdida de la cadena CD3ζ, es hasta la fecha, el único mecanismo que se ha descrito y demostrado que tenga un efecto en la función de la célula T. Cuando las MSCs se aislaron de tumores, la actividad arginasa causó la depleción del aminoácido L-arginina en el microambiente extracelular; ésto inhibió la re-expresión de la cadena ζ del CD3 despues de su internalización inducida por señalización mediada por el TCR y debida a la estimulación por antígeno de las células T, impidiendo de este modo la funcion de estas células (Baniyash, 2004; Rodriguez et al., 2004).

**iNOS**: El NO no impide los eventos de activación temprana desencadenados por el TCR, actúa en cambio al nivel de la señalización mediada por el receptor de la IL-2, bloqueando la fosforilación y la activación de las moléculas señalizadoras (Bingisser et al., 1998; Mazzoni et al., 2002).

**Arginasa e iNOS**: Cuando las dos enzimas están inducidas, se produce una depleción extracelular de la L-arginina. Esto conduce a la iNOS a cambiar su función de producción de NO a producir principalmente ión superóxido, y como consecuencia ROS y RNOS. Estas especies pueden tener múltiples efectos inhibitorios en las células T. Se ha demostrado que la combinación de la actividad de la arginasa y de la iNOS es importante en MSCs de ratones con tumores, pero también en infecciones crónicas con helmintos (Bronte et al., 2003; Brys et al., 2005). Esto indica que la co-expresión de iNOS y arginasa podría ser considerada un marcador de MSCs (Bronte and Zanovello, 2005). Es importante considerar en este aspecto que algunos autores describen un mecanismo de regulación para el RNA mensajero de la iNOS cuando los niveles de L-arginina son bajos, lo que resultaría en un descenso de la estabilidad de la iNOS (El-Gayar et al., 2003).

## MSCs en infecciones por parásitos

Existen varias evidencias del papel de las MSCs en enfermedades parasitológicas. Células T humanas estimuladas en presencia de un extracto crudo *de H. pylori* muestran proliferación reducida y este hecho se correlacionaba con una descenso en las expresión de CD3ζ (Zabaleta et al., 2004). Además, resultados de infecciones experimentales con *Schistosoma spp.* (Terrazas et al., 2001), *Candida albicans* (Mencacci et al., 2002), y *T.cruzi* (Abrahamsohn and Coffman, 1995; Goni et al., 2002) ilustran la existencia de un mecanismo de inmunosupresión inducido por IFNγ, dependiente de NO y mediado por las células MSCs que colonizan el bazo durante la fase aguda de la enfermedad.

# CONCLUSIONES

Los resultados obtenidos en este trabajo han dado lugar a las siguientes conclusiones:

1. La arginasa I se induce en corazones de ratones infectados con *T. cruzi* correlacionándose con la carga parasitaria en este órgano.

2. La inducción de arginasa I durante la infección por *T. cruzi* está restingida a corazón y a células del peritoneo.

3. La inducción de arginasa I en corazones de ratones infectados con *T. cruzi* requiere de señalización por el receptor de IL-4 presumiblemente mediada por IL-13 con una posible cooperación con IL-4, IL-10 y $PGE_2$.

4. La iNOS se expresa en cardiomiocitos y en células $CD11b^+$ $Gr\text{-}1^{int}$ $Ly\text{-}6C^{high}$ infiltradas en tejido cardiaco de ratones infectados, mientras que la expresión de arginasa I está restringida a la población $CD68^+$ $CD11b^+$ $Gr\text{-}1^{int}$ $Ly\text{-}6C^{high}$ infiltrante.

5. Las células $CD11b^+$ presentes en el infiltrado inflamatorio de corazón de ratones infectados con *T. cruzi* no presentan una polarización clara hacia un patrón de expresión génica M1 o M2.

6. Las células $CD11b^+$ presentes en el infiltrado inflamatorio de corazón de ratones infectados con *T. cruzi* que expresan iNOS y arginasa I, presentan un fenotipo de célula mieloide supresora y modulan la proliferación de células T *in vitro*.

# APPENDIX 2

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Abstract: In Chagas disease, caused by Trypanosoma cruzi, macrophages and cardiomyocytes are the main targets of infection. Classical activation of macrophages during infection is protective while alternative activation of macrophages is involved in survival of host cells and parasites. We studied the expression of inducible nitric oxide synthase and arginase, as markers of classical and alternative activation, respectively, in heart tissue during in vivo infection of BALB/c and C57BL/6 mice. We found that expression of arginase I and II as well as ornithine decarboxylase, were much higher in BALB/c compared to C57BL/6 mice and related to parasite burden in heart tissue. Inducible nitric oxide synthase and arginase II were expressed by cardiomyocytes. Interestingly, heart infiltrated CD68̣ macrophages were the major cell type expressing

arginase I. Th1 and Th2 cytokines were expressed in heart tissue in both infected mouse strains, but the Th1/Th2 balance was predominantly Th1 in C57BL/6 mice and Th2 in BALB/c mice at the peak of parasite infection. Our results suggest that Th2 cytokines induce arginase expression which may influence host and parasite cell survival, but might be also down-regulating the counter productive effects triggered by inducible nitric oxide synthase in heart during infection.

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Full title: **iNOS and arginase expression in heart tissue during mouse acute *Trypanosoma cruzi* infection: arginase I is expressed in CD68$^+$ infiltrating macrophages.**

Short title: **Arginase expression and *T. cruzi*.**

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**ABSTRACT**

In Chagas disease, caused by *Trypanosoma cruzi*, macrophages and cardiomyocytes are the main targets of infection. Classical activation of macrophages during infection is protective while alternative activation of macrophages is involved in survival of host cells and parasites. We studied the expression of inducible nitric oxide synthase and arginase, as markers of classical and alternative activation, respectively, in heart tissue during *in vivo* infection of BALB/c and C57BL/6 mice. We found that expression of arginase I and II as well as ornithine decarboxylase, were much higher in BALB/c compared to C57BL/6 mice and related to parasite burden in heart tissue. Inducible nitric oxide synthase and arginase II were expressed by cardiomyocytes. Interestingly, heart infiltrated $CD68^+$ macrophages were the major cell type expressing arginase I. Th1 and Th2 cytokines were expressed in heart tissue in both infected mouse strains, but the Th1/Th2 balance was predominantly Th1 in C57BL/6 mice and Th2 in BALB/c mice at the peak of parasite infection. Our results suggest that Th2 cytokines induce arginase expression which may influence host and parasite cell survival, but might be also down-regulating the counter productive effects triggered by inducible nitric oxide synthase in heart during infection.

Key words: *Trypanosoma cruzi;* arginase; iNOS; cardiomyocyte; alternative activation; macrophage; Th1/Th2 balance; heart tissue; BALB/c; C57BL/6.

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## INTRODUCTION

*Trypanosoma cruzi*, a flagellated protozoan parasite, is the causative agent of Chagas disease, an illness affecting several million people in Latin America. During *T. cruzi* infection, macrophages are one of the most important cells to fight the parasite. These cells metabolize L-arginine by two main pathways. One involves inducible nitric oxide synthase (iNOS), responsible for nitric oxide (NO) production. This pathway is controlled by T helper (Th)1 cytokines known to induce iNOS expression and classical activation of macrophages (M1), and is essential for the early control of mouse [1], macrophage [2, 3] and cardiomyocyte [4] infections. However, excessive NO can also have cytotoxic effect in the host and lead to immune suppression of T cells [5] and pathology [6].

The second pathway involves arginase, which is induced in alternative activation of macrophages (M2), mainly by Th2 cytokines [7] . Arginase catalyzes conversion of L-arginine to urea and L-ornithine, required for polyamine synthesis which is essential for the growth of cells and all trypanosomatidae. The limiting enzyme in this pathway is ornithine decarboxylase (ODC) which has a low half life [8]. Two arginase isoforms have been described; arginase I, located in the cytoplasm, has restricted expression, and is mainly induced by Th2 cytokines, but also interleukin (IL)-10 [9] and transforming growth factor (TGF)-β [10]. Arginase II, located in the mitochondria, is induced in many cell types by lipopolysacharide (LPS) and cyclic adenosine monophosphate [11]. N-Hydroxy-L-arginine, an intermediary in NO synthesis, inhibits arginase [12] whereas polyamines can decrease iNOS expression [13, 14]. Moreover, arginase has been implicated in downregulating iNOS protein synthesis

and stability by decreasing L-arginine availability [15, 16], that depends on the cationic amino-acid transporter (CAT)-1 and CAT-2 [17, 18].

*Helicobacter pilori* [19, 20] and *Chlamydia sp.* [21] can modulate the metabolic routes mentioned above. The arginase/iNOS balance also modifies the outcome of several protozoan infections. Induction of macrophage arginase during *Trypanosoma brucei* infection depletes L-arginine, and may avoid NO parasite dependent killing [22]. Moreover, arginase and alternative activation play a crucial role in the development of a *Trypanosoma congolense* chronic infection [23]. During *Leishmania sp. in vitro* [24, 25] and *L. major in vivo* infection [26, 27] arginase inhibitors reduce pathology by lowering parasite replication.

On the other hand, *T. cruzi* triggers the synthesis of cytokines and chemokines in infected cardiomyocytes [4] and macrophages [28] that induce potent NO dependent trypanocidal activity [3, 29-31]. However, peripheral blood monocytes showed decreased NO production during *T. cruzi* infection in rats, due to the increase of arginase activity [32]. Cruzipain, a major *T. cruzi* antigen, up-regulates arginase activity in macrophages promoting intracellular growth of the parasite [33-35] and induces arginase II in cardiomyocytes promoting their survival [36].

Taken all these previous evidences together, the aim of this work was to study the role of classical and alternative activation pathways during experimental *T. cruzi* infection in mice with different susceptibility to the disease focusing in the most important and affected organ in Chagas disease: the heart.

## MATERIALS AND METHODS

### Parasites and mice

Y and Tulahuén *T. cruzi* strains were obtained from Dr. John David (Harvard Medical School Department, Boston, Massachusetts, U.S.A.). Blood trypomastigotes were maintained in infected mice. 6 to 8-week-old BALB/c and C57BL/6 mice (Harlan, Interfauna Iberica) and iNOS$^{-/-}$ mice (NOS2$^{tm1\ Lau}$, Jackson Laboratories) were maintained under pathogen-free conditions in compliance with normative [37]. All experiments were performed in groups of 3-5 mice non-infected or infected with $2x10^3$ Y trypomastigotes, except when stated different, by intraperitoneal injection.

### Quantitative real time PCR and RT-PCR

Heart DNA was isolated with High Pure PCR Template preparation Kit (Roche), Heart tissue PCR reactions contained 100 ng genomic DNA and *T. cruzi* detection was performed by quantitative real time (q)PCR as described [38].

Heart RNA was extracted with TRIzol reagent (Invitrogen). Quantitative real time (q)RT-PCR analysis was done with High Capacity cDNA Archive Kit (Applied Biosystems) and amplification of different genes (arginase I, arginase II, iNOS, CAT-1, CAT-2, ODC, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-4, IL-10, IL-13, TGF-β, prostaglandin E2 synthase (PGE2s) and Ribosomal 18S) was performed in triplicate using TaqMan MGB probes and the TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900 HT instrument (Applied Biosystems). Quantification of gene expression was calculated by the comparative threshold cycle ($C_T$) method, normalized to the ribosomal 18S control and efficiency of the reverse transcription reaction ($RQ= 2^{-\Delta\Delta C_T}$).

### Protein expression and activity

Heart protein extracts were prepared utilizing a PT 1300 D homogenizer (Polytron). For western blot analyses 15 μg of tissue extract were fractionated on SDS-10% polyacrylamide gel and transferred to a nitrocellulose membrane. Primary antibodies were diluted as follows: anti-mouse iNOS (BD Transduction) 1:5000, goat anti-mouse Arginase I and Actin 1:1000 (Santa Cruz, Biotechnology, Inc.) and anti-ODC 1:100 (Sigma-Aldrich). Membranes were incubated with horseradish peroxidase (HPO) rabbit anti-goat antibody (Sigma-Aldrich) 1:10000, or goat anti-mouse (Pierce) 1:1000 as secondary antibody. Proteins were detected with Supersignal reagent (Pierce). Ganglia, spleen, peripheral blood mononuclear (PBMCs) and cultured cells were treated as above except for the mechanical disruption. Arginase activity was measured as previously described [39].

**Immunohistochemistry**

Hearts from mice infected with $10^4$ Tulahuén tripomastigotes were removed, fixed in 10% neutral buffered paraformaldehyde and embedded in paraffin. Four micrometer-thick sections were mounted on gelatine coated glass slides, deparaffinized and rehydrated, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in PBS, permeabilized with 1% Triton X-100 in PBS, washed and non-specific binding blocked with 2% bovine serum albumine (BSA). Samples were incubated with anti-arginase II, anti-iNOS and isotypic antibodies (Santa Cruz Biotechnology, Inc.) diluted 1:100, washed with PBS and incubated with anti-rabbit HPO (Sigma-Aldrich) diluted 1:250, incubated with diaminobenzidine/hydrogen peroxide and counterstained with hematoxylin.

**Confocal immunofluorescence**

Hearts were fixed in 4% paraformaldehyde in PBS solution, incubated in 30% sucrose solution, embedded in Tissue-Tek O.C.T. compound (Sakura) and frozen. 10-15 μm thick sections were fixed in acetone. Incubation with antibodies was done at 4ºC as follows: 10 μg/ml goat anti-mouse arginase I, rat anti-mouse CD68 (Serotec), goat IgG (Jackson ImmunoResearch) and rat IgG (BD Transduction) antibodies. 1:50 $F(ab')_2$ Goat anti-rat IgG fluorescein isothiocyanate (Serotec), 1:50 Cy5-conjugated Donkey anti-goat IgG (Jackson Immunoresearch) antibodies. Pictures were taken on a LSM510 META confocal laser coupled to an Axiovert 200 (Zeiss) microscope.

**Cell cultures**

BALB/c neonatal cardiomyocyte culture was obtained as described [40]. More than 90% of cells were cardiomyocytes as detected by immunostaining with antibody to mAchR M2 as described [36]. BALB/c mice peritoneal cells were collected with 0.34 M sucrose and plated in RPMI with 5% FBS; after 4h, non-adherent cells were removed by washing three times with warm PBS, and fresh RPMI was restored. Trypomastigotes were obtained in co-cultures with Vero cells (CCL-81, ATCC), and 5 Y trypomastigotes were added per cardiomyocyte or macrophage. 16h later non-internalized parasites were removed and cells were cultured in complete RPMI medium for 48h. In addition, cardiomyocytes were incubated with: IL-4 (20 ng/ml, BioSource International), IFN-γ (25U/ml, BioSource International), LPS (1μg/ml, *Escherichia coli* serotype O26:B6 Sigma-Aldrich), as indicated.

**Statistical analysis**

For *in vivo* experiments, data reported are mean ± SD of 3 individual mice. Significance was evaluated by Student's two-tailed *t*-test.

## RESULTS

### Expression of L-arginine metabolic enzymes and transporters in heart tissue of mice infected with *T. cruzi.*

BALB/c and C57BL/6 mice were infected and parasites in heart tissue were quantified at different days post infection (d.p.i.) by qPCR. Figure 1 shows that maximum parasite load was detected in C57BL/6 heart at 14 d.p.i. In contrast, parasite burden in heart of BALB/c mice was maximal at 21 d.p.i. and decreased thereafter. Parasite equivalents were significantly higher (about 2 logs after parasite load peak) in susceptible BALB/c than in resistant C57BL/6 mice.

Quantification of arginase I and II mRNAs showed significantly higher expression in BALB/c than C57BL/6 mice at all d.p.i. analyzed (Fig 2a-b). In contrast, CAT-1, CAT-2 and iNOS mRNAs expression was higher in C57BL/6 than BALB/c mice at 14 d.p.i (Fig. 2c-e). However, CAT-1, CAT-2 and iNOS mRNAs expression was higher in BALB/c mice at later times p.i. The induction of ODC mRNA expression was not significantly different from non-infected controls (Fig. 2f).

Since arginase I mRNA showed major differences between C57BL/6 and BALB/c mice, we wanted to confirm this at the protein level. Arginase I protein induction in infected heart tissue was much stronger in BALB/c than C57BL/6 mice and spanned from 14 to 42 d.p.i, in agreement with the mRNA data (Fig. 3a). Arginase I expression in C57BL/6 mice was observed only between 14 and 21 d.p.i. Moreover, arginase enzymatic activity correlated nicely with arginase I protein expression in heart extracts from both mice strains (Fig. 3b), being much higher again in BALB/c mice than in C57BL/6 mice peaking at 21 d.p.i. In addition, iNOS protein expression was higher in infected BALB/c than infected C57BL/6 mice.

Although we did not observe significant changes on ODC mRNA expression, ODC protein was induced in BALB/c hearts (maximum expression at 21 d.p.i) and in much lower extent in C57BL/6 mice (peaking at 14 d.p.i.) (Fig. 3a). This indicates that ODC is regulated at the post-translational level in heart tissue of mice infected with *T. cruzi*.

We also studied the expression of arginase I and iNOS in PBMCs, spleen and ganglia from infected BALB/c mice at 21 d.p.i. In spleen and ganglia neither arginase I nor iNOS were induced (Fig 3c). There was a slight induction of arginase I expression in PBMCs in comparison with the induction observed in heart tissue, but no iNOS was detected in PBMCs, indicating that arginase I and iNOS are preferentially expressed in heart tissue during acute *T.cruzi* infection.

**Cardiomyocytes express arginase II and iNOS while arginase I is expressed by CD68$^+$ cells infiltrating heart tissue of *T. cruzi* -infected mice**

To determine the cellular source of arginase and iNOS protein detected in the infected hearts, we performed immunohistochemical staining. Fig 4 shows staining of a high percentage of cardiac fibres for both arginase II and iNOS in BALB/c infected mice (Fig. 4c and 4d) compared to non-infected mice (Fig. 4a and 4b).

Arginase I expression in hearts was analyzed by confocal microscopy. Hearts from infected BALB/c mice at 21 d.p.i. showed an infiltrate composed by arginase I positive cells (Fig. 5f); no staining was observed in cardiomyocytes or endothelial cells. Arginase I-positive infiltrating cells showed a macrophage-like morphology. The CD68 macrophage marker, but not the dendritic cell marker CD11c (data not shown), was found to co-localize with arginase I (Fig. 5e to 5h) in tissue sections of hearts from *T. cruzi* infected mice. Non infected heart tissue showed a weak staining with anti CD68 and anti-arginase I antibodies of some infiltrated resident cells (Fig. 5a to 5d). No

specific staining was observed in sections with isotypic antibodies (Fig. 5e and 5f). Next, we evaluated the extension of $CD68^+$ cell infiltration in BALB/c and C57BL/6 mice by quantification of CD68 mRNA. $CD68^+$ cell infiltration was induced upon infection, being maximal at 14 d.p.i. in both mouse strains and interestingly, no statistically significant differences were observed between them (Fig 5k). This similarity in cell infiltration contrasted with the observed differences in arginase expression, suggesting that arginase I expression was higher in $CD68^+$ cells from infected BALB/c than C57BL/6 mice.

**Arginase I expression in primary cultures of cardiomyocytes and macrophages**

iNOS and arginase II induction, but no arginase I, had been reported during *T. cruzi*-infection of cardiomyocytes *in vitro* [4, 36]. Thus, we infected cardiomyocytes in vitro and analyzed arginase I and iNOS expression. iNOS but no arginase I protein was induced by infection in cardiomyocytes (Fig. 6a). Besides, the IL-4-dependent arginase I induction in non-infected cardiomyocytes was partially inhibited by *T. cruzi* infection. In contrast, the IFN-γ+LPS-dependent iNOS induction observed in non-infected cardiomyocytes was strongly potentiated by *T. cruzi* infection of those cells. Therefore, iNOS expression in cardiac cells can be directly triggered by the parasite and is probably responsible for the inhibition of IL-4- dependent arginase I expression (Fig 6a).

*T. cruzi* infection triggers NO production in IFN-γ activated macrophages but the parasite itself does not induce production of NO in this cell type [28]. We found that *in vitro* infection of BALB/c mice resident peritoneal macrophages did not increase arginase I expression (Fig. 6b). Therefore, arginase I induction seen in $CD68^+$ heart infiltrating cells is not likely triggered by the parasite but rather by the inflammatory environment in the heart set off during the immunological response against the parasite.

In order to test the contribution of the iNOS gene to heart expression of arginase I we infected iNOS$^{-/-}$ mice. Parasitemia in iNOS$^{-/-}$ did not significantly change respect to wild type (W.T.) mice (figure 6c), in agreement with previous reports [41]. However, heart arginase I expression (Fig. 6d) and activity (Fig. 6e) were strongly increased in iNOS$^{-/-}$ compared to W.T. mice. Thus, deletion of the iNOS gene triggered the increase in the induction of arginase I in heart tissue of mice infected with *T. cruzi.*

**Both Th1 and Th2 cytokines and PGE2s are induced in heart tissue of mice infected with *T. cruzi***

We tested the levels of several cytokines in heart tissue from mice infected with *T. cruzi* by quantitative mRNA RT-PCR. The results showed that IFN-γ and TNF-α, presented maximum expression at 14 and 21 d.p.i. in C57BL/6 and BALB/c mice, respectively (Fig. 7a-b). These cytokines were significantly higher in C57BL/6 than BALB/c mice at 14 d.p.i. but this situation was switched between 21 and later times., being higher in BALB/c than C57BL/6 mice. On the other hand, IL-4 and IL-13 showed maximum expression at 14 d.p.i. in both mouse strains, being higher in BALB/c than C57BL/6 mice through infection. Interestingly, IL-4 and, specially, IL-13 expression in BALB/c was maintained at 21 d.p.i. (Fig. 7c-d). IL-10 expression in the heart was significantly increased by infection and followed kinetics similar to TNF-α (Fig. 7e). The induction of TGF-β mRNA expression observed was not statistically different from non-infected controls (Fig. 7f). PGE2s mRNA was significantly increased in heart of both BALB/c and C57BL/6 mice upon infection being significantly higher in BALB/c than C57BL/6 mice at 21 d.p.i. (Fig. 7g). Thus, no pure Th1 or Th2 patterns are linked to strain susceptibility or infection stage. However, the Th1/Th2 balance was higher for C57BL/6 (resistant) than for BALB/c mice (susceptible) and Th2 cytokines and PGE2s were higher in BALB/c than in C57BL/6 mice.

## DISCUSSION

Heart leukocyte infiltration is thought to play an important role in the myocarditis associated to *T. cruzi* infection. However, up to date, most of the research in the field has focused in the characterization of T cells present in heart inflammatory infiltrate, while very little effort has been directed to the study of macrophages. Macrophages may become activated by different pathways, that play different and somehow opposite roles. Our results show for the first time that arginase expression and activity are strongly induced in heart tissue during acute *T. cruzi* infection in mice, together with an increased expression of iNOS. This was observed in both susceptible BALB/c and resistant C57BL/6 mice. The peak of parasite load correlated with peaks of arginase I and iNOS expression in heart tissue. Moreover, arginase I expression in BALB/c mice was higher and persisted for a longer period of time than in C57BL/6 mice, which controlled more efficiently the infection. Besides, ODC protein expression was also induced during the acute phase, suggesting an increment in polyamines synthesis needed for mammalian cell proliferation, but more importantly, for parasite replication.

We also analyzed the cellular source of these enzymes. We found that iNOS and arginase II were expressed in cardiomyocytes of infected BALB/c mice in agreement with previous reports that attributed a role for arginase II in cardiomyocyte survival [36] and contractility [42]. Interestingly, arginase I expression was detected for the first time only in $CD68^+$ infiltrating macrophages, and according to protein expression, arginase I was very likely responsible of arginase enzymatic activity in heart tissue. In this regard, previous work reported a role for cruzipain in promoting parasite replication by triggering arginase I expression in macrophages [35]. On the other hand, quantification of $CD68^+$ cells by mRNA in heart tissue showed that their numbers were very similar in

BALB/c and C57BL/6 mice, indicating that the amount of infiltrating macrophages was not responsible for the increased arginase I expression and activity seen in BALB/c mice. Rather, infiltrating macrophages in infected BALB/c expressed more arginase I per cell than in C57BL/6.

We found that *T. cruzi* infection triggered iNOS expression in cardiomyocytes, in agreement with previous observations [4]. However, the parasite was unable to induce the expression of arginase I in cardiomyocytes and neither arginase I and iNOS in resident peritoneal macrophages.

Together, the *in vitro* and *in vivo* infection data suggest that induction of iNOS expression in cardiomyocytes could be triggered by the parasite, while arginase I expression in macrophages depends on other factors, very likely Th2 cytokines such as IL-4 and IL-13 which in fact were highly elevated in the heart during infection. Interestingly, we found that cardiomyocytes expressed arginase I upon IL-4 stimulation, while the parasite alone was unable to induce its expression in vitro. However, arginase I was not observed in cardiac cells of infected BALB/c mice even though IL-4 is found in cardiac tissue. Since infection partially inhibits IL-4-induced arginase I *in vitro* it is likely that a similar mechanism is operating *in vivo*.

It is important to note that a discrete induction of arginase I compared to the one detected in heart tissue was observed in circulating PBMCs but not in other lymphatic organs from acutely infected mice. This indicates that arginase I is preferentially induced in macrophages infiltrating the heart, likely by the elevated IL-4 and IL-13 levels present in this organ. iNOS protein was not detected in PBMCs in agreement with previous reports [32] rather in heart tissue, suggesting a specific important role of iNOS in this organ.

Our analysis showed that both Th1 and Th2 cytokines were induced upon infection in both BALB/c and C57BL/6 hearts. It is generally accepted that in many parasitic diseases, BALB/c susceptible mice mainly produce Th2 cytokines whereas C57BL/6 resistant mice Th1 cytokines [43]. However, we found that this Th1/Th2 dichotomy is not so strict in *T. cruzi* infection at least in the heart. Nonetheless, heart Th1/Th2 balance is higher in C57BL/6 than BALB/c mice, and the levels of Th2 cytokine expression were always higher and persisted longer in susceptible mice compared with resistant mice.

The induction of ODC expression could be due to the uptake of apoptotic cells during *T. cruzi* infection, which causes release of $PGE_2$ and induction of TGF-β [44]. Since the ODC substrate depends on arginase, its induction could be mediated by TGF-β. However, a very weak induction of TGF-β mRNA was observed, as compared with other cytokines, suggesting that TGF-β is not the main responsible of arginase I induction in our model. On the other hand, PGE2s expression was significantly higher in BALB/c respect to C57BL/6 mice at 21 d.p.i., indicating that $PGE_2$ could partially contribute to the increased arginase I expression observed in BALB/c mice compared to C57BL/6 mice.

In the heart, NO has been described to affect cardiac vasodilation and muscle contractility [45]. Our experiments demonstrated elevated iNOS expression in heart tissue during acute *T. cruzi* infection likely generating high NO levels, which could negatively affect heart function. In contrast, arginase expressed by infiltrated macrophages and cardiomyocytes, could control excessive NO production. We found an even higher induction of arginase I upon *T. cruzi* infection in the hearts of $iNOS^{-/-}$ mice which presented less severe myocardial inflammation than wild type mice in previous

reports [46]. This suggests that arginase I could be preventing the development of iNOS-dependent deleterious effects.

During acute *T. cruzi* infection there is also a strong suppression of T lymphocyte responses mediated by myeloid cells expressing NO [5]. Besides, L-arginine depletion could cause inhibition of T cell activation by decreasing CD3ζ expression [47]. Since arginase is a marker of myeloid suppressor cells (MSC) involved in immune suppression [48], it is possible that the induction of iNOS and arginase seen in infected heart suppress T cell activation in the heart allowing parasite replication. In this direction, it is tempting to speculate that arginase expressing infiltrated macrophages are either M2 macrophages or MSC, although further characterization needs to be performed.

In summary, we have analyzed arginine metabolism enzymes in heart and showed for the first time that arginase I is expressed by infiltrated macrophages during acute experimental *T. cruzi* infection. This suggests that arginase I could have a role in regulating the immune response in heart tissue and the development of cardiac pathology. Arginase I may play a role in host cell and parasite growth, counteracting NO dependent vasodilation and cardiac muscle contractility, questions that will be addressed in the future. However, our immediate work will focus in the characterization of heart infiltrated macrophages and the elucidation of the role of IL-4 and IL-13 in the induction of arginase in heart tissue during acute mice infection.

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**Figure legends**

**Figure 1**. T. cruzi *DNA quantification in heart tissue of BALB/c and C57BL/6 infected mice.* DNA from heart tissue was isolated at indicated d.p.i. and qPCR was performed as previously described. *T. cruzi* DNA is expressed as pg of parasite DNA per mg of DNA from heart tissue sample (pg parasite DNA/mg heart tissue). Empty boxes indicate the values for BALB/c mice and filled rhombus the values for C57BL/6 mice. Results are represented as mean ± SD of triplicates of pooled DNA of 5 different mice. All differences between BALB/c and C57BL/6 mice parasite load were statistically significant except at 7 d.p.i. ($p < 0.05$). A representative experiment out of three is shown.

**Figure 2**. *mRNA quantification of enzymes and transporters involved in L-arginine metabolism in heart tissue during* T. cruzi *infection.* RNA from heart tissue was isolated at different d.p.i. and qRT-PCR was performed. Results are expressed as the logarithm of RQ calculated from CT values as described in material and methods. mRNAs for: a) Arginase I; b) Arginase II; c) CAT-1; d) CAT-2; e) iNOS f) ODC. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. Results are represented as mean ± SD of 3 different mice. All differences observed in infected mice respect to non-infected mice were statistically significant except for: a) C57BL/6 at 7, 21, 28 and 48 d.p.i.; b) C57BL/6 at 7 and 48 d.p.i.;c) C57BL/6 at 7 d.p.i.; d) BALB/c at 7 and 42 d.p.i. and C57BL/6 at 7 d.p.i.; f) BALB/c and C57BL/6 at all d.p.i. Significant differences observed between BALB/c and C57BL/6 mice are indicated by an asterisk. ($p < 0.05$).A representative experiment out of five is shown.

**Figure 3.** *iNOS and arginase I protein expression and activity during* T. cruzi *infection in heart and other tissues.* Protein extracts from heart tissue were isolated from 5 BALB/c and 5 C57BL/6 mice at different d.p.i. and pooled for western blot analysis a)

Arginase I, iNOS, ODC expression and Ponceau staining of the same membrane; numbers indicate d.p.i. b) Arginase activity of the same tissue extracts, expressed as μmol of urea produced per hour per mg of protein sample (μmol/h/mg), was assessed by urea colorimetric determination. Empty bars indicate the values for BALB/c mice and filled bars the values for C57BL/6 mice. All values for BALB/c mice were significantly higher compared to non infected mice except at 7 d.p.i. and in C57BL/6 were only significantly higher at 14 d.p.i. Asterisks indicate significant differences between BALB/c and C57BL/6 mice. c) BALB/c heart tissue, peripheral blood mononuclear cells (PBMCs), spleen and ganglia were obtained from non-infected (NI) and infected (I) mice at day 21 post infection. Protein extracts were isolated and analysed by western blot using anti-arginase I or anti-iNOS antibodies and Ponceau staining. Data are representative of at least two independent experiments.

**Figure 4.** *Arginase II and iNOS expression of BALB/c mice heart tissue during acute* T. cruzi *infection.* Hearts from non infected mice and infected mice at 14 d.p.i. were analyzed by immunohistochemistry as described. a) Non-infected tissue stained with anti arginase II antibody, and b) with anti iNOS antibody. c) Infected tissue stained with anti arginase II antibody, and d) with anti iNOS antibody. e) Isotypic antibody. Data are representative of several sections analyzed in at least 3 different mice. Magnification x1000.

**Figure 5.** *Arginase I and CD68 expression in BALB/c mice heart tissue during acute* T. cruzi *infection.* Tissue sections obtained from BALB/c mice hearts at 21 d.p.i. were analysed by immunofluorescence confocal microscopy as described. Non-infected tissue stained with a) anti CD68 antibody; b) with anti arginase I antibody; c) phase contrast; d) merge of a and b. Infected tissue section stained with e) anti CD68 antibody; f) anti-arginase I antibody; g) phase contrast; h) merge of e and f. Boxed

regions are shown at two-fold magnification of original (x400) in insets. The scale bar is 20μm. Data are representative of several sections analyzed in at least 3 different mice. k) Total RNA from heart tissue was isolated at 21 d.p.i. and CD68 quantitative RT-PCR was performed as described. Results are represented as mean ± SD of 3 different mice. All values for BALB/c and C57BL/6 mice were significantly higher compared to non infected mice. A representative experiment out of three is shown.

**Figure 6.** *Expression of arginase I and iNOS in BALB/c cardiomyocytes, peritoneal macrophages and heart tissue of iNOS $^{-/-}$ mice*. a) Western blot of neonatal cardiomyocyte cultures non-infected (NI) and infected (I) *in vitro* with *T. cruzi* trypomastigotes cultured with media alone (C), IL-4, LPS+ IFN-γ as indicated. Arginase I, iNOS and actin expression is shown. b) Arginase I, iNOS and actin expression in non-infected (NI) and infected (I) macrophage cultures; iNOS expression was not detected (N.D.). e) Parasitemia of infected wild type (W.T.) (filled circles) and iNOS$^{-/-}$ (filled squares) mice. d) Arginase I expression of heart tissue extracts pooled from 3 W.T. and 3 iNOS $^{-/-}$ non-infected (NI) and infected (I) mice at 21 d.p.i. and Ponceau staining of the same membrane. e) Arginase activity of the same extracts expressed as μmol of urea produced per hour per mg of protein sample (μmol/h/mg). Filled bars indicate the values for W.T. non-infected (NI) and infected (I) mice and dashed bars the values for NI and I iNOS$^{-/-}$ mice. Data are representative of at least two independent experiments.

**Figure 7** *Expression of cytokines and PGE2s in mouse heart tissue during* T. cruzi *infection.* Total RNA from heart tissue was isolated at different d.p.i. from BALB/c and C57BL/6 mice and qRT-PCR was performed as described. Results are expressed as the logarithm of RQ calculated from CT values as described in material and methods. mRNAs for: a) IFN-γ; b) TNF-α; c) IL-4; d) IL-13; e) IL-10 and f) TGF-β and g) PGE2s. Empty bars indicate the values for BALB/c mice and filled bars the values for

C57BL/6 mice. Results are represented as mean ± SD of 3 different mice. All differences observed in infected mice respect to non-infected mice were statistically significant except for: c) and d) C57BL/6 at 7 d.p.i.; f) BALB/c and C57BL/6 at all d.p.i.; Significant differences observed between BALB/c and C57BL/6 mice are indicated by an asterisk. ($p < 0.05$). A representative experiment out of three is shown.

Figure 1

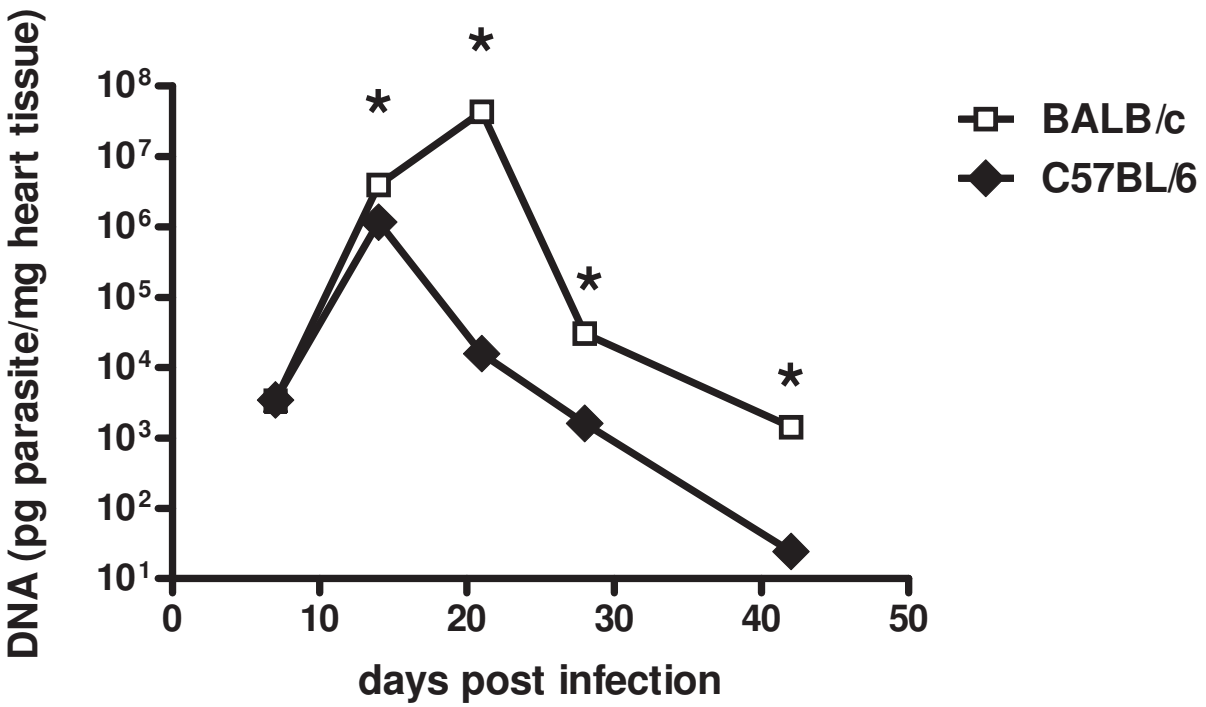

DNA (pg parasite/mg heart tissue)
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
0
10
20
30
40
50
days post infection
*
*
*
*
BALB/c
C57BL/6

Figure 2

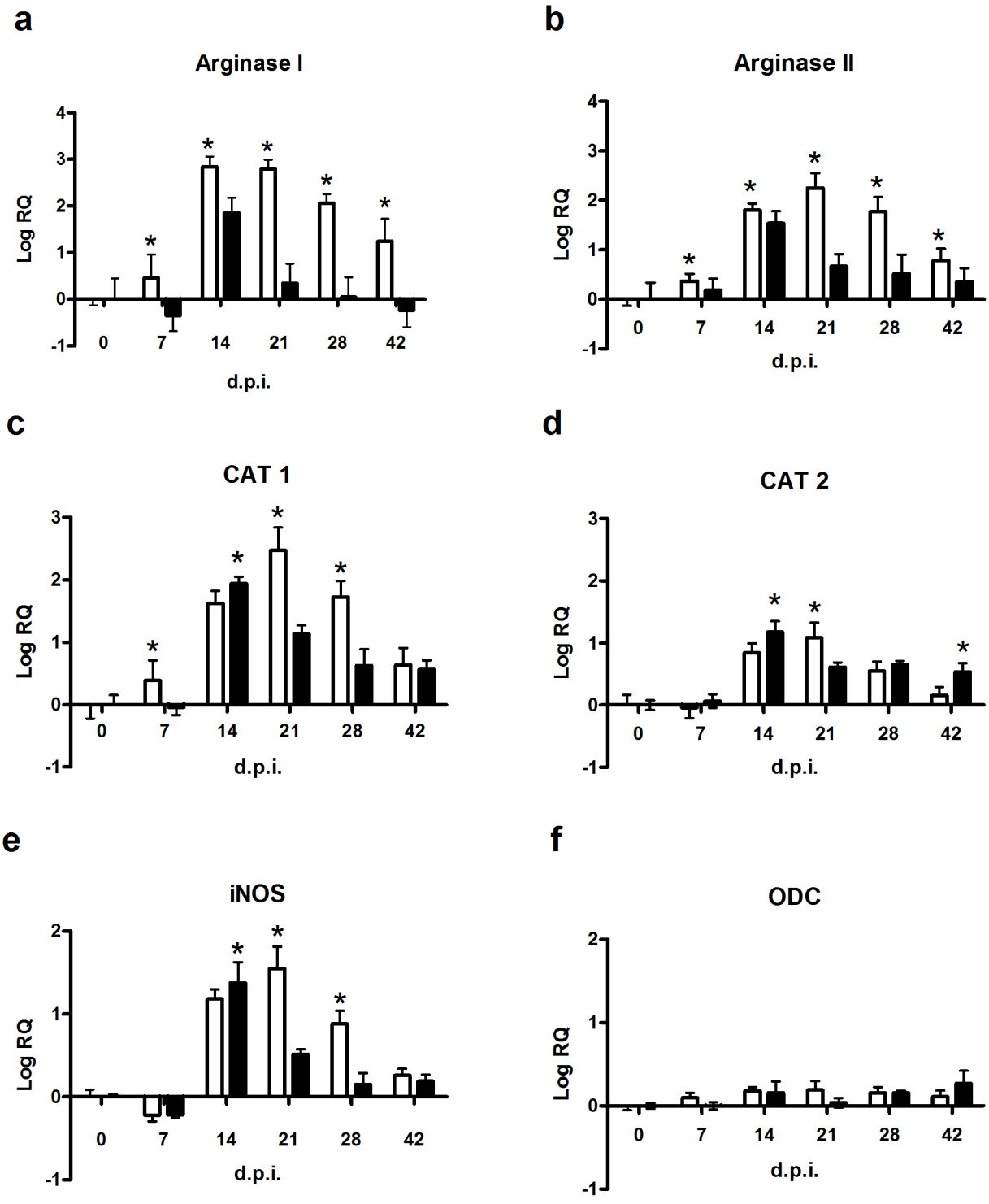
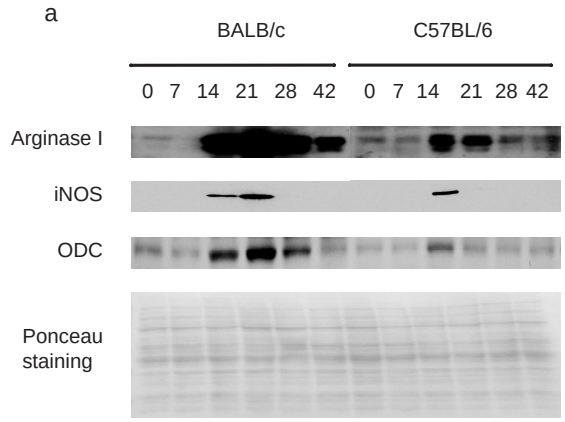
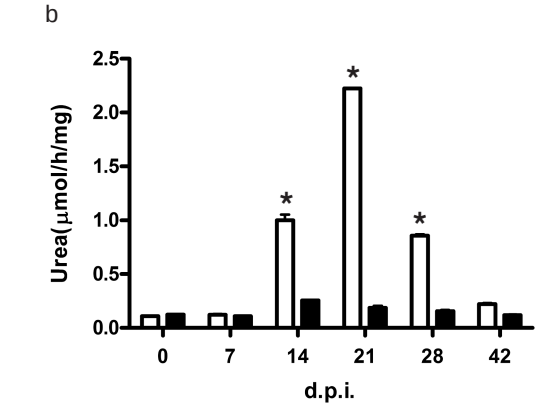

a
Arginase I
Log RQ
d.p.i.
b
Arginase II
Log RQ
d.p.i.
c
CAT 1
Log RQ
d.p.i.
d
CAT 2
Log RQ
d.p.i.
e
iNOS
Log RQ
d.p.i.
f
ODC
Log RQ
d.p.i.

**Figure 3**


a
BALB/c
C57BL/6
0 7 14 21 28 42 0 7 14 21 28 42
Arginase I
iNOS
ODC
Ponceau staining


b
Urea(μmol/h/mg)
2.5
2.0
1.5
1.0
0.5
0.0
0 7 14 21 28 42
d.p.i.

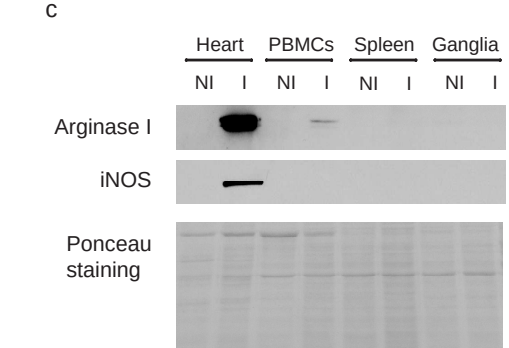

c
Heart PBMCs Spleen Ganglia
NI I NI I NI I NI I
Arginase I
iNOS
Ponceau staining

Figure 4

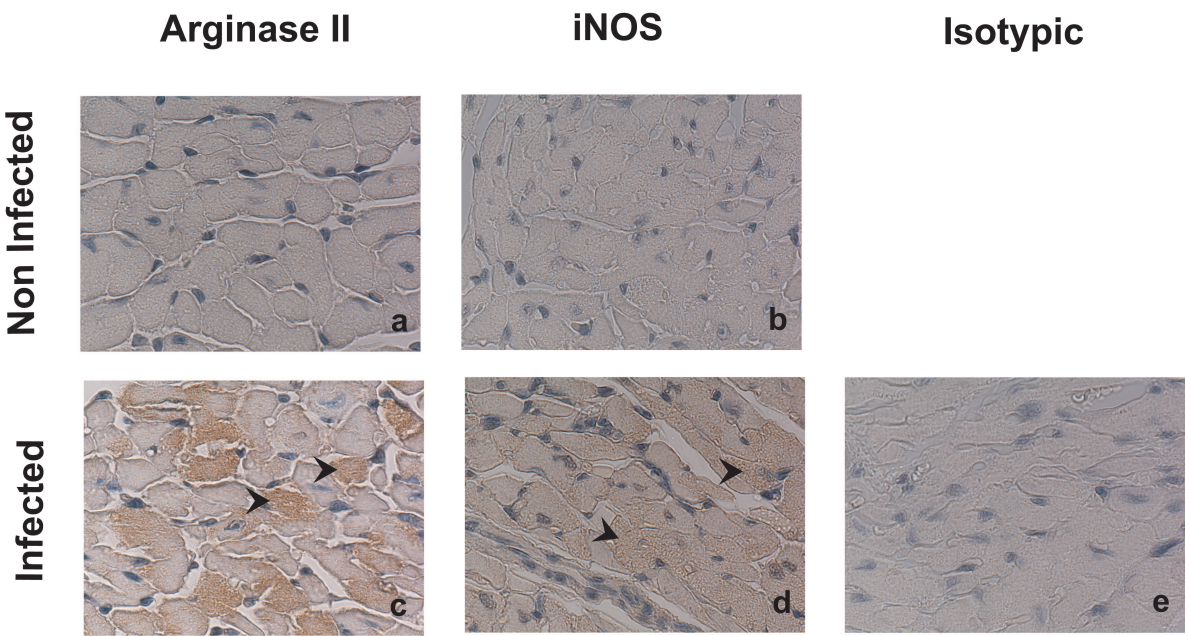


Figure 5

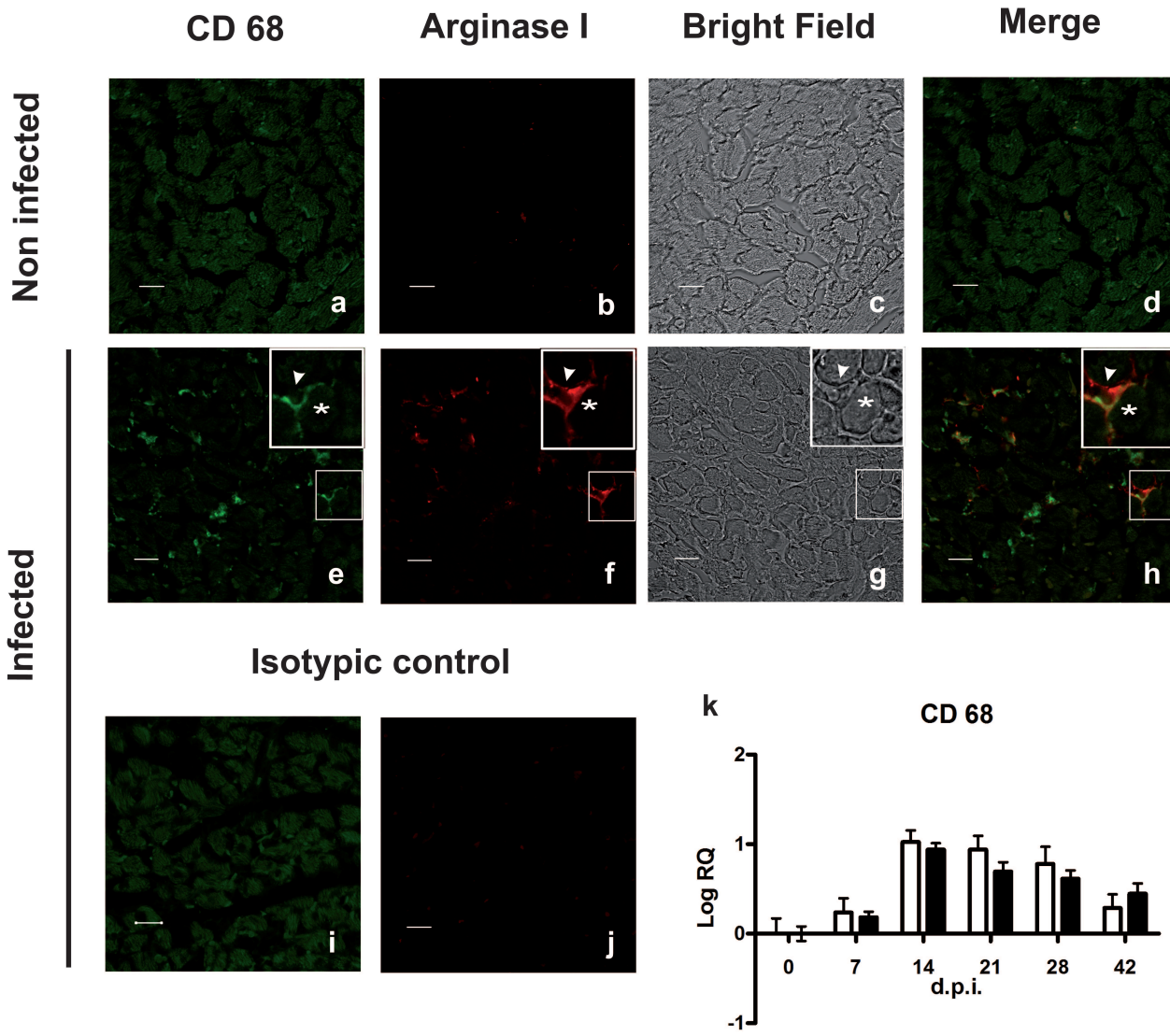
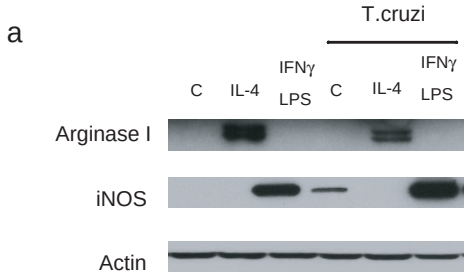
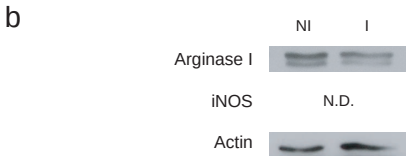
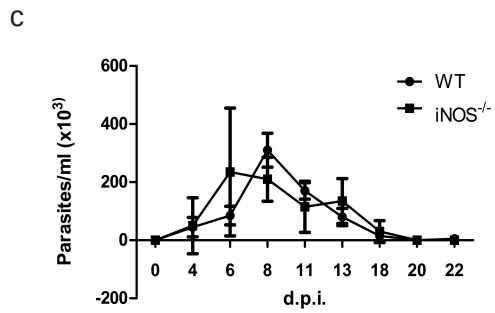
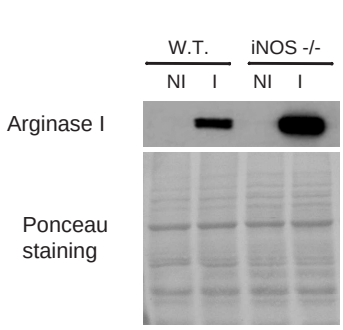

CD 68
Arginase I
Bright Field
Merge
Non infected
Infected
a
b
c
d
e
f
g
h
Isotypic control
i
j
k
CD 68
Log RQ
2
1
0
-1
0
7
14
21
28
42
d.p.i.

**Figure 6**


a
T.cruzi
C
IL-4
IFNγ LPS
C
IL-4
IFNγ LPS
Arginase I
iNOS
Actin


b
NI
I
Arginase I
iNOS
N.D.
Actin


c
WT
iNOS-/-
Parasites/ml (x10³)
600
400
200
0
-200
0
4
6
8
11
13
18
20
22
d.p.i.


d
W.T.
iNOS -/-
NI
I
NI
I
Arginase I
Ponceau staining

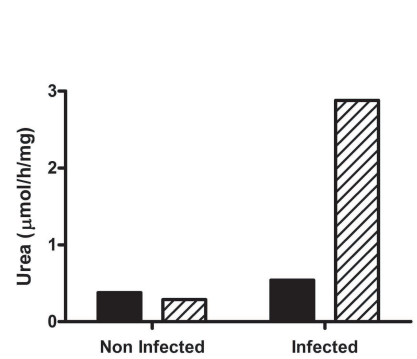
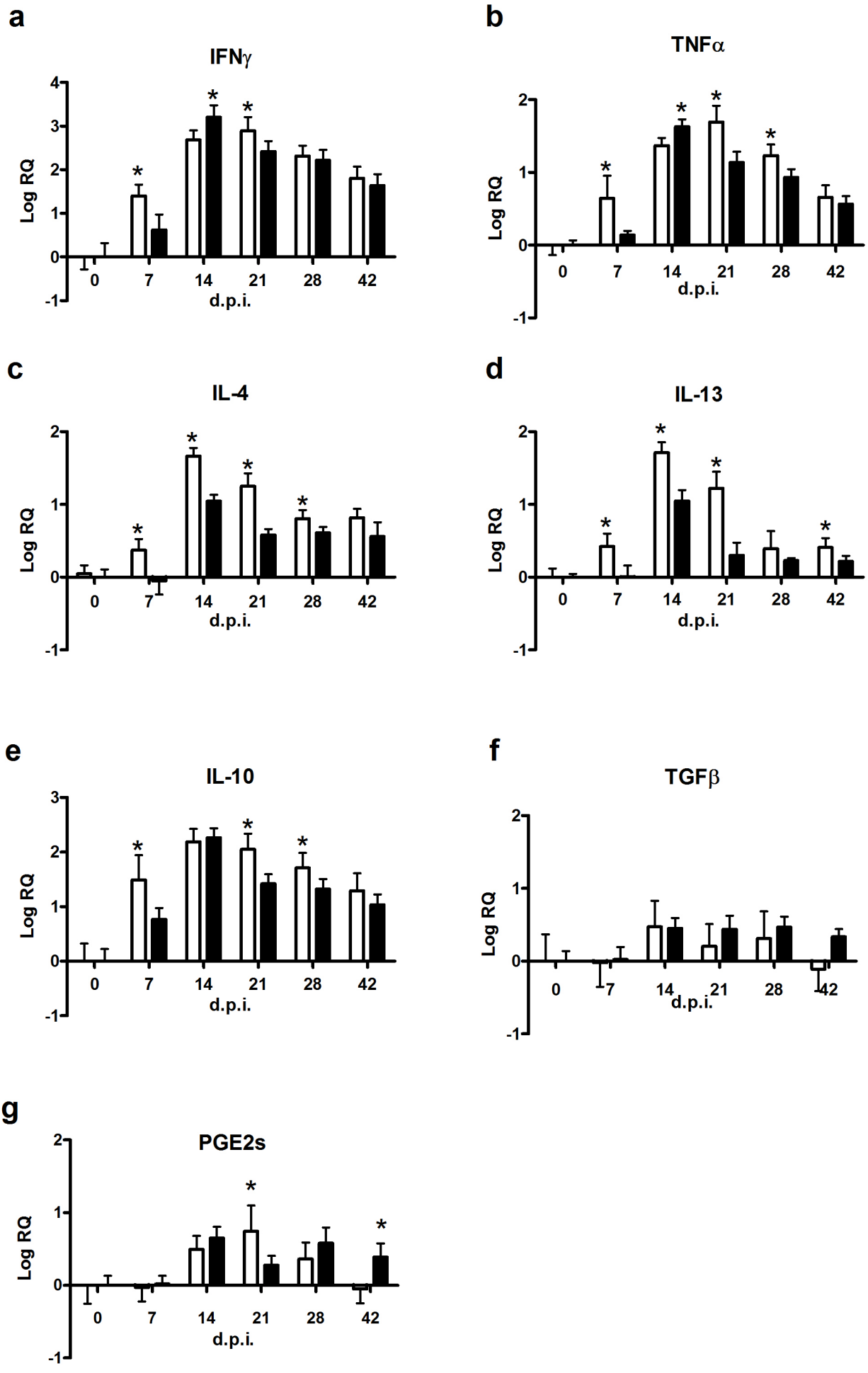

e
Urea (μmol/h/mg)
3
2
1
0
Non Infected
Infected

Figure 7


a
IFNγ
Log RQ
d.p.i.
b
TNFα
c
IL-4
d
IL-13
e
IL-10
f
TGFβ
g
PGE2s
0
7
14
21
28
42

# Role of *Trypanosoma cruzi* Autoreactive T Cells in the Generation of Cardiac Pathology

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**ABSTRACT: Chagas disease, caused by *Trypanosoma cruzi*, affects several million people in Central and South America. About 30% of chronic patients develop cardiomyopathy probably caused by parasite persistence and/or autoimmunity. While several cross-reactive antibodies generated during mammal *T. cruzi* infection have been described, very few cross-reactive T cells have been identified. We performed adoptive transfer experiments of T cells isolated from chronically infected mice. The results showed the generation of cardiac pathology in the absence of parasites. We also transferred cross-reactive SAPA-specific T cells and observed unspecific alterations in heart repolarization, cardiac inflammatory infiltration, and tissue damage.**

**KEYWORDS: *T. cruzi*; autoreactive T cells; autoimmunity; Chagas disease**

## INTRODUCTION

Chagas disease is a debilitating multisystemic disorder that affects several million people (approximately 18 million individuals are infected with

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**doi: 10.1196/annals.1381.046**

*Trypanosoma cruzi* (*T. cruzi*), with 120 million at risk) in Central and South America.[1–4] It is caused by the flagellated protozoan parasite *Trypanosoma cruzi*, with a complex life cycle involving several stages in both vertebrates and insect vector.[2,5] Individuals residing in rural areas of Latin America are at highest risk of infection. The World Health Organization has conducted several programs for the elimination of the insect vector, with high impact in reducing the incidence of new infections.[4] On the other hand, transfusion-acquired Chagas disease is becoming a significant health problem in countries other than Central and South America, especially those receiving high numbers of immigrants from that region.[6–8]

Two phases, acute and chronic, can be differentiated in Chagas disease.[2,3,9] In the acute phase, encompassing few weeks after infection, a local inflammatory lesion appears at the site of infection where the parasite undergoes the first rounds of replication. After parasite dissemination through the body, circulating blood trypomastigotes are easily observed in blood and a small number of patients develop symptoms of severe myocarditis, leading, in some instances, to heart failure responsible for the few deaths in acute Chagas disease.[10,11] However, the acute phase mostly remains undiagnosed without severe clinical symptoms. On the contrary, the severe pathology and the most common manifestations of this disease develop many years (10–30) after the initial infection with *T. cruzi* in the so-called chronic phase, in 30–40% of the infected people.[2,3,9] During the chronic phase, circulating parasites cannot be observed by inspection of blood but progressive tissue damage occurs involving the esophagus, colon, and heart.[2,3] Treatment with benznidazol or nifurtimox is effective during the acute phase of infection, specially in newborns,[12,13] but usually no treatment is prescribed for the chronic phase.[2,3] Several treatment[14–18] and vaccination[19–23] trials have been conducted, but to date an effective therapy or vaccine is still lacking.

Pathology during chronic *T. cruzi* infection is postulated to be caused by parasite persistence or autoimmunity or by a combination of both mechanisms.[24–26] Understanding T cell responses during infection is crucial in the development of efficient vaccines to avoid unwanted secondary effects. To date there is no systematic assay to study immunodominant T cell responses in infection. Some peptide candidates are being tested for protection and/or pathology. Here we review some of the literature about T cell responses during *T. cruzi* infection and study the contribution of T cells generated during *T. cruzi* infection to cardiac pathology focused, in particular, in T cells specific for the C-terminal repeats of the *T. cruzi* shed acute phase antigen (SAPA) cross-reactive with the Cha autoantigen.

## AUTOIMMUNITY AND *T. CRUZI* INFECTION

The finding of a T cell rich inflammatory mononuclear cell infiltrate and the scarcity of parasites in heart lesions[27,28] questioned the direct participation of

*T. cruzi* in Chronic chagasic cardiomopathy (CCC) and suggested the possible involvement of autoimmunity.[24,29–36] Autoimmune responses can be induced through molecular mimicry, a mechanism by which infectious agents may trigger an antigen-specific B and/or T cell-specific immune response against self. In addition, cytokine mediated bystander activation may directly stimulate surrounding cytotoxic T cells in the absence of direct triggering of the T cell receptor. Both mechanisms may contribute to tissue damage. Studies about autoimmunity in Chagas disease were reported many years ago,[37–44] and they are supported by a large body of circumstantial evidence thereafter and had been extensively and sequentially reviewed.[24,30,31,33,45,46]

On the other hand, recent evidence is challenging this view. Thus, with the use of more sensitive techniques, parasite antigens or DNA are detected during the chronic phase. This has led to the suggestion that damage is more likely caused either by an inflammatory response against the parasite or to the parasite replication itself than by autoimmunity.[29,32,47] However, it should be emphasized that to date there is no unequivocal demonstration that either autoimmunity or parasite-specific immunity is the actual cause of pathogenesis.

To consider that an infectious disease has an autoimmune etiology the following postulates have to be demonstrated: (1) association of the disease with a particular microorganism; (2) identification of the culprit microorganism epitope(s) that elicits the cross-reactive response; (3) T cell or B-cell populations against that epitope(s) should be expanded in the infection; (4) elimination of the cross-reactive epitope(s) from the microorganism should result in nonpathogenic infection; (5) autoreactive T cells should be able to transfer the disease.[48] According to the literature, in Chagas disease, postulates 1–3 have been described, the 4th is very difficult to demonstrate due to high gene copy number for many *T. cruzi* genes and several putative epitopes, and the 5th is still debated.

## MOLECULAR MIMICRY

In *T. cruzi* infection many examples of molecular mimicry at the level of antibodies have been described.[49] Thus, anti-*T. cruzi* circulating antibodies that cross-react with host heart and neural cells is a common finding in chagasic humans and animal models of infection,[24,33,35] but with few exceptions none of the autoantibodies seem to be the leading cause of autoimmune pathogenesis. However, descriptions are scarce of cross-reactive T cells in this infection (TABLE 1). Mainly, cross-reactive T cell epitopes had been studied for a given antigen after the B-cell epitope was identified. Parasite and self-peptide antigen epitopes recognized by antibodies during *T. cruzi* infection had been identified by different methods (affinity chromatography, peptide sequencing, cDNA library immunoscreening). Contrarily, for T cell epitopes there is no systematic assay to identify them, so very few have been described.

**TABLE 1. T cell reactivity against host antigens: defined cross-reactive epitopes**

| | Cross-reactive antigens | Species | Molecular definition | Cross-reactive epitopes | Reference |
|---|---|---|---|---|---|
| Host | Cardiac myosin heavy chain | Human | T cells | AAALDK<br>::: :: | (72,73) |
| *T. cruzi* | B13 | | | AAAGDK | |
| Host | Cha | Mouse | T cells | SLVTCPAQGSLQSSPSMEI<br>: . :: : :.:: . | (68) |
| *T. cruzi* | Shed acute phase antigen (SAPA) | | | STPSTPADSSAHSTPSTPV | |
| Host | Cardiac myosin heavy chain | Mouse | DTH | | (66,67) |

Studies of immunodominant T cell responses during infection would be very useful in vaccine design and also to avoid secondary effects as potentially pathogenic epitopes. This observation can be made extensive to other infectious diseases.

## AUTOREACTIVE T CELLS

A great effort has been made in characterizing the nature of the inflammatory infiltrate in chronic and acute phases of *T. cruzi* infection both in humans and mouse,[50–57] but there are only a few reports addressing the role of T cells in the generation of pathology (TABLE 1).[49] Evidence supporting the role for autoantigen-specific autoimmunity in disease pathogenesis derives from studies on T cell-mediated immunity in mice. Ribeiro-Dos-Santos *et al*. have described that $CD4^+$ T cell line obtained from a chronic chagasic mouse proliferated in response to either a crude *T. cruzi* Ag preparation or heart tissue extracts from different animal species.[58] In culture, this cell line arrests the beating of fetal heart cells, and more importantly, induces myocarditis in immunized mice and promotes rejection of transplanted normal hearts in the absence of *T. cruzi*.[58] The requirement of the parasite to cause this rejection in mice transferred with T cells from infected mice has also been widely debated.[58–61] Thus, there are opposite results indicating that rejection of syngeneic-transplanted hearts in chronically infected mice takes place in the presence[60] or in the absence[61] of the parasite.

Cunha-Neto *et al*. have also proposed that myosin cross-reactive T lymphocytes infiltrating heart tissue lesions are involved in the generation of chronic chagasic pathology.[59,62] These T cells are activated by cardiac myosin heavy chain cross-reactive *T. cruzi* antigen (Ag; TABLE 1).[49,62] Moreover, T cells from chagasic patients with overt heart disease or asymptomatic patients responded to *in vitro* stimulation with B13 with increased interferon (IFN)-γ

and reduced interleukin (IL)-4 production suggesting a type 1 helper T cells (Th1)-type cytokine profile.[63,64] Those authors proposed that heart damage in CCC could be due to a delayed-type hypersensitivity (DTH) process initiated by B13 inducing the release of inflammatory cytokines.

Noteworthy, immunological tolerance to heart Ag prior to their infection by *T. cruzi* resulted in less intense cardiomyopathy than control nontolerized animals,[65] which would be in favor of the autoimmune pathology hypothesis. This treatment only affects $CD4^+$ responses and not the production of anti-myosin IgG discarding B-cell autoreactivity as the cause of pathology. Although this suggests that the regime to make the mice tolerant was not as effective as expected, at least regarding the humoral response (Th2 mediated), it supports that the response involved in heart damage is Th1 mediated. Leon *et al*. have described in the acute phase that myosin autoimmunity, while potentially important inflammatory mechanism in acute and chronic infection, is not essential for cardiac inflammation,[66] although immunization with a *T. cruzi* extract induced a DTH response against myosin.[67]

The Cha autoantigen was isolated by immunoscreening of a human Jurkat cDNA expression library using a pool of sera from chagasic patients, and is expressed also in mice with a high percentage of homology. In addition, Cha presented high homology with *T. cruzi* antigens (SAPA and Tenu 2845/36 kDa antigen), being recognized by high percentage of human and mouse chagasic sera.[68] When tested in enzyme-linked immunosorbent assay (ELISA) Cha showed high specificity and sensitivity in the diagnosis of chagasic sera.[69] The SAPA antigen is involved in evasion of the immune system and the C-terminal repeats behaved as a strong T cell epitope during experimental infection. It belongs to the transialidase family, the most abundantly represented in the *T. cruzi* genome.[70] Moreover, it is characterized for presenting several copies of each gene. In particular the cross-reactive epitope is present in several repeats in the C-terminal of SAPA and other members of the family as Neuraminidase. For this reason, the demonstration of the 5th autoimmune postulate is unaffordable, because engineering a parasite devoid of this sequence is not possible. On the other hand, the high representation of SAPA cross-reactive repeats and the inmunodominant response observed during infection are in agreement with a possible pathogenic role of autoreactive T cells.

## ADOPTIVE TRANSFER OF T CELLS AND AUTOIMMUNITY

Transfer of T cells from chronically infected mice to naive syngeneic mice lead to heart infiltration, detectable 60 days later.[68] These experiments were criticized thereafter for the lack of proper controls as purity of the transferred cells and for the detection of putative residual parasites present in the purified T cells.[29,36,49,71] We should emphasize that fluorescent staining with anti-CD4 antibody showed that 99% of the transferred cells were positive for this

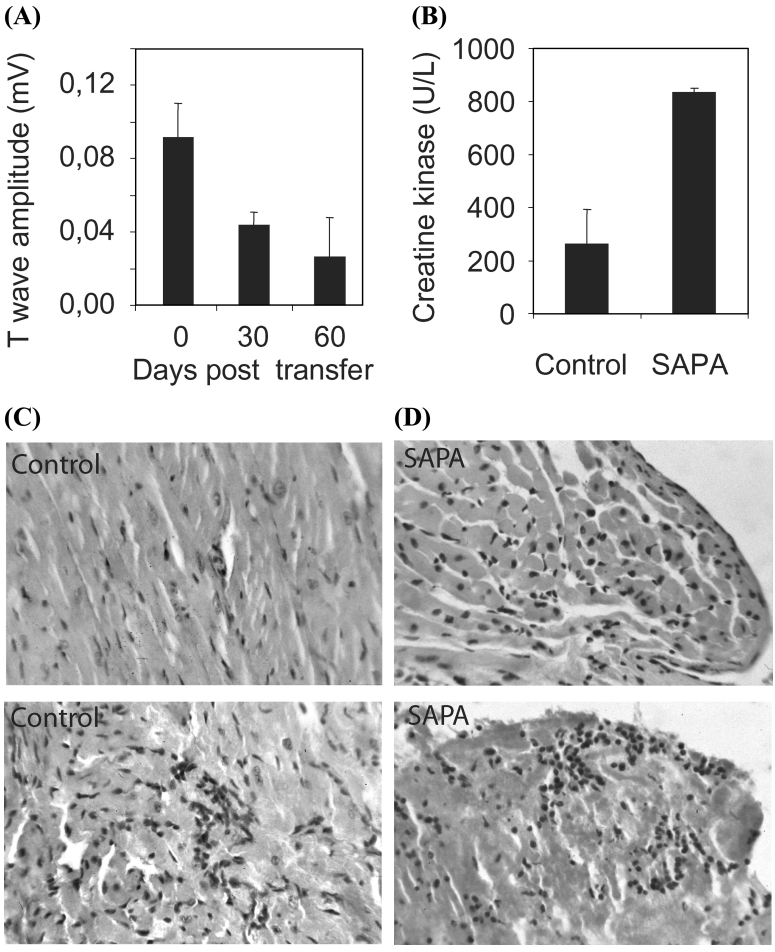


**FIGURE 1.** Cardiac pathology observed after adoptive transfer of SAPA-specific cross-reactive T cells in recipient mice. The following parameters indicative of cardiac damage were observed: (**A**) a gradual decrease in electrocardiographic T wave amplitude was observed at 30 and 60 days after adoptive transfer of SAPA-specific cross-reactive T cells in comparison with T wave amplitude prior to transfer; (**B**) an increased creatine kinase activity in serum of SAPA-transferred mice respect to control mice; (**C, D**) an inflammatory infiltrate was observed in H&E stains of myocardial and endocardial tissue, respectively, in mice transferred with SAPA-specific cross-reactive T cells in comparison with control mice.

T cell marker, and recipient mice were devoid of *T. cruzi* evaluated by sensitive polymerase chain reaction (PCR). No parasite DNA was detected in blood at 10, 20, and 60 days, and neither in cardiac tissue at 60 days post-transfer, suggesting that T cell purification got rid of any residual parasite (unpublished

results). To study in more detail the possible pathogenic role of a specific cross-reactive T cell epitope as SAPA, we performed adoptive transfer experiments of a CD4+ T cell clone generated from spleen cells of mice immunized with the SAPA cross-reactive peptide. We recorded temperature, activity, heart beat, and electrocardiographic data, by telemetry in conscious mice before transfer and 30 and 60 days post-transfer. Mice in control and SAPA groups presented normal basal temperature values, conscious activity, and heart beat rate (data not shown). Interestingly, electrocardiogram results showed a significant alteration in heart function measured as T wave amplitude or repolarization at 30 and 60 days post-transfer, being more severe at 60 days post-transfer (FIG. 1A). This alteration is characteristic of inflamed and edematous hearts and is known as unspecific alterations in heart repolarization. In addition, cardiac damage measured by creatine kinase activity in serum was increased at 60 days post-transfer (FIG. 1B). The hematoxylin and eosin (H&E) stain of heart tissue showed that different structures, such as myocardium and endocardium, presented inflammatory infiltrate in mice transferred with SAPA CD4+-specific T cells in comparison with recipient mice transferred with control CD4+ cells (FIG. 1C, D, respectively). These results may explain the unspecific repolarization alterations previously observed.

Future experiments will focus on determining the immune mechanism(s) responsible for the pathology observed after *T. cruzi*-specific T cell adoptive transfer.

## CONCLUSIONS

A systematic study of the specificity of the T cell response during *T. cruzi* infection is not possible to date. However, the study of the known antigens that produce strong T cell cross-reactive responses can give some hints for vaccine design. Our results showed that T cells purified from chronically infected mice and T cells specific for the SAPA cross-reactive antigen were able to induce alterations in heart repolarization, cardiac inflammation, and tissue damage in recipient mice. This indicates that an autoimmune component exists that should be considered for future preventive and therapeutical strategies.

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# *Trypanosoma cruzi*-Induced Molecular Mimicry and Chagas' Disease

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**Abstract** Chagas' disease, caused by *Trypanosoma cruzi,* has been considered a paradigm of infection-induced autoimmune disease. Thus, the scarcity of parasites in the chronic phase of the disease contrasts with the severe cardiac pathology observed in approximately 30% of chronic patients and suggested a role for autoimmunity as

the origin of the pathology. Antigen-specific and antigen-non-specific mechanisms have been described by which *T. cruzi* infection might activate T and B cells, leading to autoimmunity. Among the first mechanisms, molecular mimicry has been claimed as the most important mechanism leading to autoimmunity and pathology in the chronic phase of this disease. In this regard, various *T. cruzi* antigens, such as B13, cruzipain and Cha, cross-react with host antigens at the B or T cell level and their role in pathogenesis has been widely studied. Immunization with those antigens and/or passive transfer of autoreactive T lymphocytes in mice lead to clinical disturbances similar to those found in Chagas' disease patients. On the other hand, the parasite is becoming increasingly detected in chronically infected hosts and may also be the cause of pathology either directly or through parasite-specific mediated inflammatory responses. Thus, the issue of autoimmunity versus parasite persistence as the cause of Chagas' disease pathology is hotly debated among many researchers in the field. We critically review here the evidence in favor of and against autoimmunity through molecular mimicry as responsible for Chagas' disease pathology from clinical, pathological and immunological perspectives.

**Abbreviations**

| | |
|---|---|
| Ag(s) | Antigen(s) |
| IFN | Interferon |
| CTL | Cytotoxic T lymphocyte |
| IL | Interleukin |
| TNF | Tumor necrosis factor |
| mAb | Monoclonal antibody |
| iNOS | Inducible nitric oxide synthase |
| DTH | Delayed-type hypersensitivity |
| TCR | T cell receptor |
| ECM | Extracellular matrix |
| MHC | Major histocompatibility complex |
| Mhc | Myosin heavy chain |
| CMhc | Cardiac myosin heavy chain |
| SMhc | Skeletal myosin heavy chain |
| APC(s) | Antigen-presenting cell(s) |
| ICAM | Intercellular adhesion molecule |
| CCC | Chronic chagasic cardiompathy |
| VCAM | Vascular cell adhesion molecule |

## 1 Chagas' Disease

### 1.1 General Aspects and Life Cycle

Chagas' disease (Chagas 1909) is a debilitating multisystemic disorder which affects several million people (approximately 18 million individuals are in-

fected with *Trypanosoma. cruzi*, with 120 million at risk) in Central and South America (Moncayo 1999; Prata 2001; Tanowitz et al. 1992) and is considered a paradigm of infection-mediated autoimmune disease. It is caused by the flagellated protozoan parasite *Trypanosoma cruzi*, with a complex life cycle involving several stages in both vertebrates and insect vectors. *T. cruzi* has three main different morphologies: epimastigote, which replicates in the blood-sucking triatomine insect vector; trypomastigote, which infects the vertebrate host's cells; and amastigote, which replicates intracellularly in the host's cells (Burleigh and Andrews 1998; Tanowitz et al. 1992).

Transmission of *T. cruzi* to humans occurs when feces released by the bug while it takes a blood meal, containing infective metacyclic trypomastigote forms of the parasite penetrate, into the bloodstream, where the metacyclic forms infect a wide variety of host phagocytic and non-phagocytic cells. Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm, where they transform into amastigotes, which multiply intracellularly (see Fig. 1 for details).

Individuals residing in rural areas of Latin America are at highest risk of infection, because the bugs live in these dwellings and feed on the inhabitants at night. The World Health Organisation has conducted several programs for the elimination of the insect vector, with great results on the incidence of new infections (Moncayo 1999). On the other hand, transfusion-acquired Chagas' disease is becoming a significant health problem in countries other than Central and South America, especially those receiving high numbers of immigrants from that region (Kirchhoff 1989; Wendel 1998).

## 1.2 Clinical Findings

Two phases, acute and chronic, can be differentiated in Chagas' disease (Kirchhoff 1993; Prata 2001; Tanowitz et al. 1992). In the acute phase, encompassing a few weeks after infection, a local inflammatory lesion appears at the site of infection, where the metacyclic trypomastigotes infect and undergo their first rounds of multiplication. After parasite dissemination through the body, circulating blood trypomastigotes are easily observed in blood (parasitemia) and a small number of patients develop symptoms of cardiac insufficiency, reflecting an underlying severe myocarditis, leading, in some instances, to heart failure responsible for the few deaths in acute Chagas' disease (Dias et al. 1956; Prata 1994). Meningoencephalitis may also occur, especially in some immunosuppressed patients (Hoff et al. 1978). However, the acute phase mostly remains undiagnosed without severe clinical symptoms. In contrast, the severe pathology and the most common manifestations of this disease develop

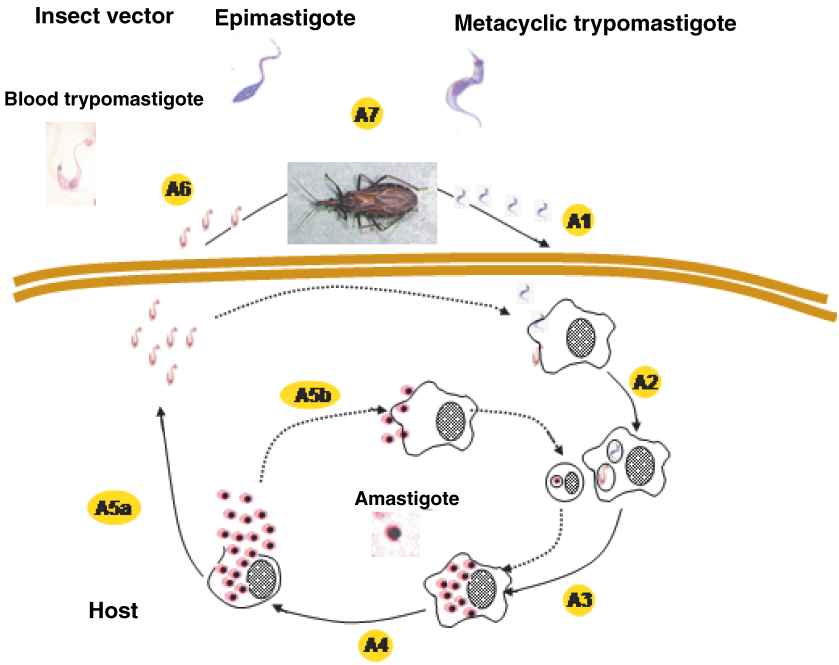


**Fig. 1** Infective cycle of *Trypanosoma cruzi*. Transmission of *T. cruzi* to humans occurs when feces released by the bug while it takes a blood meal and containing infective metacyclic trypomastigote forms of the parasite (*A1*) penetrate into the bloodstream, where they infect a wide variety of host phagocytic and non-phagocytic cells (*A2*). Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm, where they transform into amastigotes, which multiply intracellularly (*A3*). At some point, the amastigotes break off from the cell (*A4*) and differentiate into non-replicative flagellated blood trypomastigotes which in turn can penetrate and infect adjacent susceptible cells or spread to infect cells and tissues at distant locations of the body (*A5a*). Amastigotes can also directly infect phagocytic cells (*A5b*). Muscle cells, including those of the heart, are amongst the most heavily infected. Circulating trypomastigotes may be taken up by a new triatomine bug during a blood meal (*A6*). Inside the vector's intestine, ingested blood trypomastigotes differentiate into replicative epimastigotes, which, as they move to the mid and lower gut, transform into non-replicative but infective metacyclic trypomastigotes

many years (10 to 30) after the initial infection with *T. cruzi* in the so-called chronic phase, although only in 30%–40% of the infected people (Kirchhoff 1993; Prata 2001; Tanowitz et al. 1992). During the chronic phase, circulating parasites cannot be observed by inspection of blood but progressive tissue damage occurs involving the esophagus, colon and heart (Prata 2001; Tanowitz

et al. 1992). Treatment with benznidazol or nifurtimox is effective during the acute phase of infection, but no treatment exists for the chronic phase (Prata 2001; Tanowitz et al. 1992). To date, an effective immunotherapy or vaccine is still lacking.

### 1.3 Immune Response

The immune response against this parasite is complex and far from being clearly established. Both humoral and cellular immune responses are involved in controlling *T. cruzi*, which is not surprising because of the complexity of the parasite's life cycle. Thus, although B cell-deficient mice succumb to infection (Kumar and Tarleton 1998), the protective immune response seems to depend on $CD8^+$ T cells that produce interferon (IFN)-γ. $CD8^+$ T cells can control the infection through cytotoxic T lymphocyte (CTL)-induced perforin/granzyme-mediated killing of infected cells and/or FAS-mediated apoptosis (Kumar and Tarleton 1998). However, there are reports indicating that $CD8^+$ T cells cannot completely control infection because they become unresponsive (Martin and Tarleton 2004). Cytokines play a key role in regulating both the induction and type of immune response as well as parasite replication in infected hosts (Fresno et al. 1997). Macrophages, which can be infected by *T. cruzi*, also play a crucial role in the elimination of this parasite. Activation of monocytes by cytokines released by Th1 cells seems to be a key process in controlling infection in vitro as well as in vivo. Thus, Interleukin (IL)-12 produced by macrophages in response to infection mediates resistance to *T. cruzi* (Aliberti et al. 1996). Tumor necrosis factor (TNF)-α and IFN have been identified as the most important cytokines involved in the killing of intracellular *T. cruzi* through an NO-mediated-L-arginine dependent killing mechanism (Gazzinelli et al. 1992; Muñoz-Fernandez et al. 1992). This was corroborated in vivo, because anti-IFN-γ monoclonal antibody (mAb) administration results in a drastic increase in parasitemia and mortality (Silva et al. 1992; Torrico et al. 1991). Moreover, mice deficient for IFN-γ receptor and inducible nitric oxide synthase (iNOS) had an increased susceptibility to infection and parasitemia (Holscher et al. 1998; Goni et al. 2002), although the role of NO has been recently disputed because some iNOS-deficient mice do not seem to be more susceptible to infection (Laucella et al. 2004). TNF-R1-$FcIgG_3$ transgenic mice are also more susceptible to *T. cruzi* infection, clearly indicating a protective role for TNF-α (Castanos-Velez et al. 1998).

# 2
# Chronic Chagasic Cardiopathy

## 2.1
## Pathological Findings

The most important pathology of Chagas' disease develops 10–30 years after primary infection and affects several internal organs, mainly, heart, esophagus and colon, as well as the peripheral nervous system. The heart is the organ most commonly involved; cardiopathy frequently develops, congestive heart failure being a common cause of death in these patients. Megaesophagus and/or megacolon may also develop in chronic chagasic patients, which in the most severe form can cause life-threatening malnutrition and intractable constipation. Chronic chagasic cardiopathy (CCC) is thus the most devastating manifestation of Chagas' disease. However, despite affecting about a third of the infected people the pathogenesis of CCC is still poorly understood.

CCC may be considered a progressive disease, in which myocardial inflammation and fibrosis plays a pivotal role (Carrasco Guerra et al. 1987; Higuchi et al. 1987; Pereira Barretto et al. 1986). Higher percentages of severe myocarditis, fibrosis and myocardial hypertrophy are found in CCC patients with heart failure compared to patients in the indeterminate phase and with cardiac arrhythmia. Examination of the hearts of CCC patients who have died of heart failure shows biventricular enlargement with occasional apical aneurysms. In addition, individuals with CCC often develop mural thrombi, which may cause cerebrovascular accidents. Histological examination of the heart reveals diffuse interstitial fibrosis, lymphoid infiltration and damaged myocytes, all occurring in the apparent absence of parasites. Fibrosis and chronic inflammation are also detected in the conduction system of the heart, which may account for the high incidence of arrhythmias.

## 2.2
## Mechanisms of Pathogenesis

Despite intensive research, the etiology of Chagas' heart disease, both in humans and in experimental animal models of the disease, is not clearly understood. Although the acute and chronic phases of the disease share some similar pathological findings, it is still unclear whether similar pathogenic mechanisms operate. In this regard, infiltration by $CD4^+$ T cells seems to take place in the acute phase of the disease, whereas $CD8^+$ T cells predominate in the chronic phase (Henriques-Pons et al. 2002). Moreover, it is plausible that the pathology of the acute phase may affect the final outcome of the chronic phase.

To date, many pathogenic mechanisms have been described to explain how cardiac pathology develops. They can be mediated directly by the parasite or caused by an inflammatory/immune/autoimmune mechanism or a combination of these. These mechanisms are summarized below:

- *Primary neuronal damage* resulting in denervation of the parasympathetic autonomous system in the heart. This was one of the first pathogenic mechanisms described during the acute phase (Koberle 1961, 1970). However, subsequent studies only show slight neuronal damage in the heart, suggesting that neuronal lesions are an epiphenomenon, secondary to inflammation and fibrosis (Davila et al. 1991, 2002; Rossi 1996).
- *T. cruzi-induced damage to cardiomyocytes,* due to the cytopathic effect caused by intracellular infection with amastigotes or by the release of secreted *T. cruzi* product(s), which can be toxic for host cells and tissues (Koberle and Nador 1955). This is an obvious mechanism, but may have only some relevance in the acute phase and in heavily parasitized or immunosuppressed patients.
- *Parasite-induced microvascular changes* may lead to cardiac hypoperfusion and finally to myocyte degeneration and chronic inflammation (Factor et al. 1985; Morris et al. 1990; Petkova et al. 2001).
- *Persisting T. cruzi antigens* may act as trigger for specific $CD4^+$ or $CD8^+$ T-cell mediated responses of either the delayed-hypersensitivity (DTH) type or cytotoxic $CD8^+$ cells that lead to damage to infected cells or to bystander cells in the host tissues (Ben Younes-Chennoufi et al. 1988; Tarleton 2001; Tarleton and Zhang 1999). This mechanism may take place in both the acute and the chronic phase.
- *Autoimmunity* may occur by a variety of mechanisms (listed in Table 1). Those could be due to *T. cruzi* antigen (Ag)-specific mechanisms (molecular mimicry) or non-parasite Ag-specific effector mechanisms and are discussed in detail below.

An important point which is often ignored in this debated field is that none of the mechanisms listed above is mutually exclusive. Moreover, it seems unlikely that heart damage can be attributed to only one of these mechanisms.

## 2.3 Immunological Findings

In CCC, 50% macrophages, 40% T cells with a predominance of $CD8^+$ over $CD4^+$ T cells and 10% B cells comprise the inflammatory infiltrate

**Table 1** Mechanisms for activation of T and B cells in autoimmune diseases

| |
|---|
| a. Microbial antigen specific: |
| – Molecular mimicry between parasite and host antigens triggers autoimmunity |
| – Bystander activation (TCR dependent) |
| b. Microbial antigen non-specific: |
| – Release of autoantigen(s) during an infection |
| – Bystander activation (TCR independent) |
| – Cryptic epitopes |
| – Superantigens |

(Cunha-Neto et al. 2004). T cell receptor (TCR) Vβ transcripts are heterogeneous in heart biopsies from CCC patients (Cunha-Neto et al. 1994) which is a characteristic of other well-defined autoimmune diseases. The number of $CD4^+$ T cells increased in parallel to the number of $CD8^+$ T cells in acute-phase but not in chronic-phase patients with heart failure, suggesting an immunological imbalance.

Cytokines and chemokines produced in response to the parasite may upregulate vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, increased on endothelial cells of patients, which recruit $VLA\text{-}4^+LFA\text{-}1^+CD8^+$ T lymphocytes (dos Santos et al. 2001). In this regard, a role for cell adhesion molecules and integrin receptors, extracellular matrix (ECM) components , matrix metalloproteinases and chemokines has been proposed in the differential recruitment and migration in infected hosts of *T. cruzi*-elicited $CD8^+$ and inflammatory cells into the heart and other susceptible host tissues (Marino et al. 2003a, 2003b). It is worth noting that ECM components may absorb parasite Ags and cytokines which could contribute to the establishment and perpetuation of inflammation. Moreover, we have found that *T. cruzi* requires β1 integrins to gain access to the cell (Fernandez et al. 1993). The inflammatory response, which is probably recurrent, undergoing periods of more accentuated exacerbation, is most likely responsible for progressive neuronal damage, microcirculation alterations, heart matrix deformations and consequent organ failure.

CCC patients have increased expression of major histocompatibility complex (MHC) molecules. Thus, class I MHC are upregulated in the sarcolemma of myocytes in the myocardium (Higuchi Mde et al. 2003) and there is also evidence for an over-expression of class II MHC in endothelial cells (Benvenuti et al. 2000; Laucella et al. 1999; Reis et al. 1993). This may favor the presentation of cryptic epitopes to infiltrating T cells.

## 3
## Autoimmunity and Infection

Two main classes of mechanisms have been described by which infectious agents might activate T and B cells, leading to autoimmunity: Ag-specific and Ag-non-specific (Table 1). The Ag-specific mechanisms mostly state that sequence similarity between infectious agents and self-proteins (molecular mimicry or epitope mimicry) is responsible for the triggering of the autoimmune response (Oldstone 1989; Penninger and Bachmaier 2000; Rose 2001; Rose and Mackay 2000; Wucherpfennig 2001). Autoreactive B and/or T cells, in response to foreign Ags originated by molecular mimicry, can arise from a T/B cell cooperation mechanism, but experimental direct evidence is still scarce (Oldstone 1989; Rose and Mackay 2000). However, there are as yet no absolute formal proofs demonstrating that molecular mimicry is the initiating event of human autoimmune disease and responsible for the pathology, as noted recently (Benoist and Mathis 2001; Fourneau et al. 2004). Probably, Chagas' disease is close to that paradigm. There is some consensus that in order to prove the involvement of epitope mimicry in a disease of suspected autoimmune etiology five criteria must be demonstrated experimentally (Benoist and Mathis 2001; Kierszenbaum 1986) (see Table 2).

The microbial Ag-non-specific theory has several variations. The common characteristic is that no particular microbial determinant is implicated, although the infection may be the initial event which triggers the autoimmune reaction. For example, infection might cause host cell destruction, which results in the release of large quantities of normally sequestered Ags. Those cryptic epitopes found in intracellular proteins are not normally presented in the context of Class I MHC and are therefore not normally encountered by host lymphocytes. These Ags could then be captured by dendritic cells that migrate to T cell areas of the lymphoid organs, where they trigger naïve T cells, or presented at the invasion site, leading to activation of autoreactive cells (but

**Table 2** Criteria required for demonstration of the involvement of molecular mimicry in a disease of suspected autoimmune etiology

| |
|---|
| 1. Association of the disease with a particular microorganism |
| 2. Identification of the culprit microorganism epitope that elicits the cross-reactive response |
| 3. T or B cell populations against that epitope should be expanded in the infection |
| 4. Elimination of the cross-reactive epitope from the microorganism should result in non-pathogenic infection |
| 5. Autoreactive T cells should be able to transfer the disease |

not against the infecting microorganism). In addition, cryptic epitopes may initiate and maintain autoimmunity through various non-mutually exclusive mechanisms (Lanzavecchia 1995). Those cryptic epitopes can be presented by non-professional Ag-presenting cells (APCs, such as B cells) and induce T cell activation. Autoreactive B cells initiate autoimmunity in the absence of T cells specific for the self-Ag. Alternatively, autoreactive B cells may take up a foreign Ag that cross-reacts with a self-Ag at the B cell level but contains different T cell epitopes. Finally, activated B cells, which efficiently take up and present self-Ag, may prime autoreactive T cells. All these mechanisms may result in a self-sustained autoimmune response.

Microbial infection may result in bystander activation, which may take place in the setting of a proinflammatory milieu. Thus microbial infection induces the release of proinflammatory cytokines such as TNF and chemokines which could be able to activate autoreactive T cells by lowering the threshold of activation (Kim and Teh 2001; Vakkila et al. 2001). These T cells may then proliferate in response to self-Ags presented on host APCs. Inflammation could also alter lymphocyte migration patterns and activate APCs, rendering them more effective as APCs by enhancing Ag uptake and processing, cell surface expression of major MHC molecules, or costimulatory molecules. Finally, infection might provoke polyclonal lymphocyte activation via either a mitogen or a super-Ag effect (Stauffer et al. 2001).

## 4 Autoimmunity in *T. cruzi* Infection

The finding of a T cell-rich inflammatory mononuclear cell infiltrate and the scarcity of parasites in heart lesions questioned the direct participation of *T. cruzi* in CCC and suggested the possible involvement of autoimmunity, although this remains a hotly debated issue (Engman and Leon 2002; Kierszenbaum 1986, 1999; Levin 1996; Soares et al. 2001; Tarleton 2001, 2003). Several early studies on Chagas' disease already emphasized the scarcity of parasites in histological sections in the chronic phase of the disease (Andrade and Andrade 1955; Mazza 1949). Since then, much research in the field has focused on the possibility that autoimmune responses set off by molecular mimicry and/or bystander activation contribute to tissue damage. Those mechanisms were initially reported many years ago (Acosta and Santos-Buch 1985; Cossio et al. 1984, 1974a, 1974b; McCormick and Rowland 1989; Santos-Buch and Teixeira 1974; Takle and Hudson 1989; Wood et al. 1982) and they were supported by a large body of circumstantial evidence thereafter and have been extensively and sequentially reviewed (Eisen and Kahn 1991; Engman

and Leon 2002; Kierszenbaum 1986, 1999; Leon and Engman 2001; Soares et al. 2001). Although the presence of "anti-self" immune responses in *T. cruzi* infections has been unquestionably demonstrated, the case of the mediation of cross-reactive antibodies or T cells in pathology is still far from settled. Taking into account the variety of the mechanisms of induction of autoimmunity shown in Table 1 the relevant question is, Which mechanisms can be applied to *T. cruzi* infection?

On the other hand, mounting evidence is challenging this view. Thus, with the use of more sensitive techniques, parasite Ags or parasite DNA has been detected during the chronic phase, attributing all the damage either to an inflammatory response against the parasite or to the parasite replication itself (reviewed in Tarleton 2001, 2003; Tarleton and Zhang 1999). It should be emphasized that to date there is no unequivocal demonstration that either autoimmunity or parasite-specific immunity is pathogenic.

## 5
## Molecular Mimicry

The detection of circulating anti-*T. cruzi* antibodies that cross-react with host heart and neural Ags is a common finding in chagasic humans and animal models of infection (reviewed in Engman and Leon 2002; Kierszenbaum 1999, 2003) but, with few exceptions, none of the autoantibodies seems to be the leading cause of autoimmune pathogenesis. In *T. cruzi* infection many examples of molecular mimicry at the level of T cells or antibodies have been described (recently reviewed in Cunha-Neto et al. 2004). However, few of these have been extensively studied and/or defined at the molecular level (see Table 3). We will focus our review only on those examples.

### 5.1
### Mimetic B Cell Epitopes

#### 5.1.1
#### Myosin

Probably the most studied cross-reactive autoantigen in Chagas' disease is myosin. Several *T. cruzi* Ags have been shown to cross-react with myosin (cardiac or skeletal muscle) and have been implicated in pathogenesis through molecular mimicry. Cunha-Neto and collaborators have described cardiac myosin heavy chain (CMhc) as a major Ag of heart-specific autoimmunity and suggested the possible relevance of myosin recognition in human CCC (Cunha-Neto et al. 1995; Kalil and Cunha-Neto 1996). Antibodies to CMhc

**Table 3** Molecular mimicry described during *T. cruzi* infection. Defined and partially defined cross-reactive epitopes

| | Cross-reactive antigens | Cross-reactive epitopes | Molecular definition | Reference |
|---|---|---|---|---|
| Host<br>*T. cruzi* | Cardiac myosin heavy chain (CMhc)<br>B13 | AAALDK<br>::: ::<br>AAAGDK | Ab<br>T cells | Abel et al. 1997 |
| Host<br>*T. cruzi* | 23-kDa ribosomal protein<br>Ribosomal protein (R13) | EESD(D/E)DMGFGLFD<br>:: : : ::::::::<br>EEED D DMGFGLFD | Ab | Levitus et al. 1991 |
| Host<br>*T. cruzi* | β1 Adrenergic receptor<br>Ribosomal protein PO | AESDE<br>::: :<br>AESEE | Ab | Ferrari et al. 1995 |
| Host<br>*T. cruzi* | β1 Adrenergic receptor[a]<br>M2 muscarinic receptor[a]<br>Ribosomal protein (R13) | --ED-D-GF-LFD[a]<br>--EDDDMGF-LFD[a]<br>EEEDDDMGFGLFD | Ab | Mahler et al. 2004 |
| Host<br>*T. cruzi* | 47-kDa neuron protein<br>FL-160 | TPQRKTTEDRPQ | Ab | Van Voorhis et al. 1991 |
| Host<br>*T. cruzi* | Cha antigen<br>Shed acute-phase antigen (SAPA) | SLVTCPAQGSLQSSPSMEI<br>: . :: : :.:: .<br>STPSTPADSSAHSTPSTPV | T cells | Girones et al. 2001b |
| Host<br>*T. cruzi* | Cha antigen<br>TENU2845/36 kDa | MRQLDTNVER<br>.:::: ::.<br>LRQLDF-VEE | Ab | Girones et al. 2001a, 2001b |

[a]Residues of the R13 epitope that when interacting with purified antibodies trigger stimulation of the denoted receptor.

were found in symptomatic as well as asymptomatic patients. Affinity-purified anti-CMhc antibodies specifically recognized two polypeptides of 140,000 and 116,000 Da in *T. cruzi* trypomastigotes. At the molecular level, cross-reactivity was shown to exist between the amino acid sequence AAALDK of CMhc and the AAAGDK sequence from a recombinant *T. cruzi* peptide named B13 (Table 3). All sera from patients with CCC disease, but only 14% of sera from asymptomatic chagasic patients, recognized B13 (Gruber and Zingales 1993). These results have been often disputed, although no contradictory results have been published (see Kierszenbaum 2003). However, there were some discrepancies between the 100% reactivity of the sera from chronic patients

with overt heart disease with CMhc and only 61% reactivity in those with *T. cruzi,* a finding difficult to reconcile with molecular mimicry.

Cruzipain, a well-defined and highly abundant *T. cruzi* Ag, is involved in CCC pathogenesis by various direct and indirect mechanisms. The latter are also related to cross-reactivity with myosin, although not with CMhc but with skeletal muscle myosin heavy chain (SMhc). Thus purified anti-cruzipain antibodies raised in cruzipain-immunized mice cross-react with SMhc (Giordanengo et al. 2000a, 2000b) and, more importantly were associated with heart conduction disturbances in those animals. Moreover, ultrastructural findings revealed severe alterations of cardiomyocytes and IgG deposit on heart tissue of immunized mice. Giordanengo et al. investigated whether antibodies induced by cruzipain transferred from immunized mothers to their offspring could alter the heart function in the pups. All IgG isotypes against cruzipain derived from transplacental crossing were detected in pups' sera. Electrocardiographic studies performed in the offspring born to immunized mothers revealed conduction abnormalities (Giordanengo et al. 2000b). These results provide strong evidence for a pathogenic role of the humoral autoimmune response induced by a purified *T. cruzi* Ag in the development of experimental Chagas' disease. More recently, Sterin-Borda et al. have reported that immunization with cruzipain also induces autoantibodies against muscarinic acetylcholine receptors which can be implicated in pathology (Sterin-Borda et al. 2003). However, in both cases described above the molecular identification of cross-reactive epitopes of cruzipain and host proteins is still lacking.

On the other hand, *T. cruzi*- infected A/J mice (a strain of mice highly susceptible to *T. cruzi* infection) generated anti-myosin IgG, both in the acute phase and the chronic phase of infection (Leon et al. 2001). Moreover, heart lesions resembling those seen in *T. cruzi*-infected mice can be induced by immunization with purified myosin. However, not all mouse strains are equally susceptible to myocytolysis after *T. cruzi* infection (Leon and Engman 2001). Interestingly, in C57BL/6 mice, the levels of anti-myosin IgG found after *T. cruzi* infection were small or undetectable and no myocarditis was observed in the acute phase (Leon et al. 2001). Moreover, the C57BL/6 mouse strain has been claimed not to develop cardiac autoimmunity after immunization with myosin (Neu et al. 1987). These results suggest that generation of anti-myosin antibodies by *T. cruzi* infection or myosin immunization depends on the genetic background of the host and that there is a clear relationship between anti-myosin IgG and heart damage. However, from these results it is unclear whether anti-myosin IgG is the cause or the effect of heart damage. Accordingly, we have seen that C57BL/6 mice infected with *T. cruzi* did not develop clinically relevant myocarditis in the acute phase. However, at 120 days after infection, C57BL/6 mice developed a milder myocarditis compared to mice

deficient for iNOS gene with the same genetic background (Girones et al. 2004). Because C57BL/6 developed lower parasitemias than iNOS knockout mice in the acute phase, this may be taken as an indication that the presence or absence of myocarditis may depend on the initial level of control of parasite replication rather than on the genetic background of the host.

In contrast to the above, other reports suggested that anti-myosin antibodies are not involved in the pathogenesis. For example, immunization with myosin in immunosuppressed mice did not induce autoantibodies but still caused myocarditis (Neu et al. 1990). How myosin can trigger myocarditis in these immunosuppressed mice is difficult to envisage. Moreover, passive transfer of a high-titer anti-myosin antibody preparation failed to induce myocarditis (Neu et al. 1990). Because the fine specificity of the different anti-myosin Igs has not been addressed in most of those studies, they are difficult to compare. Myosin is a very large molecule and it is possible that the myosin determinant(s) recognized by the different sera are not identical.

Some authors believe that other mechanisms than molecular mimicry can explain myosin autoreactivity (see Benoist and Mathis 2001; Engman and Leon 2002; Kierszenbaum 2003). They feel that mimicry is less likely to be occurring than Ag release due to myocardial damage leading to expansion of normally tolerant myosin-reactive T cells, particularly because myosin autoimmunity is seen in myocarditis associated with other insults. Thus anti-myosin antibodies are induced in patients with heart disease unrelated to *T. cruzi* infection such as viral myocarditis, myocardial infarction, coronary artery bypass and heart valve surgery, among others (de Scheerder et al. 1989; Fedoseyeva et al. 1999; Nomura et al. 1994). B cell anti-myosin response seems to be mainly responsible for pathology in other heart infections, induced by Coxsackie B3 viral infection (Rose and Hill 1996) or by bacteria (Cunningham 2004). In this regard, it is worth mentioning that peptides of CMhc, a cytoplasmic protein, are associated with MHC class II molecules on APCs even in normal mouse myocardium (Smith and Allen 1992) and MHC class II molecules are increased in the heart of *T. cruzi*-infected patients and animals. Cardiomyocyte damage caused either by parasite replication in the heart or by inflammation may release self-Ags, leading to the induction of anti-heart antibodies rather than anti-cross-reactive *T. cruzi* Ags. Thus it could be likely that the initial heart tissue destruction resulting from infection could induce anti-myosin immunity in Chagas' heart disease, being thus the effect and not the cause of the pathology. Alternatively, is possible that although several pathogens may share the ability to destroy the heart they may have different cross-reactive epitopes with heart proteins (myosin). Thus it is possible that the trigger is the combination of pathogen and damage together, although the fine specificity of the autoreactive response against myosin will be different for each heart pathogen.

In summary, before suggesting a possible role for anti-myosin immunity in Chagas' heart disease, some questions need to be fully addressed: (1) Is the damage during *T. cruzi* infection different from heart tissue injury of a different etiology? (2) Do anti-myosin antibodies truly contribute to chagasic pathology? (3) If anti-myosin antibodies appeared after the occurrence of tissue damage, would they aggravate the pathology by mediating the destruction of intact cardiomyocytes?

### 5.1.2 Ribosomal Proteins

Another set of autoantigens which have been involved in CCC pathology are ribosomal proteins. Anti-ribosomal P protein antibodies were detected in the serum of chagasic patients and their titer associated with the degree of myocarditis, suggesting a correlation between the appearance of these antibodies and heart pathology (Levin et al. 1990, 1989; Skeiky et al. 1992). By screening a *T. cruzi* expression cDNA library with such sera, some DNA clones were identified. One of the clones, termed JL5, codified for a *T. cruzi* ribosomal protein, TcP2L, and showed sequence homology with human P ribosomal proteins. The homology was between the EDDDMGFGLFD region of Tc2PL and the SD(D/E)DMGFGLFD sequence present in the C-terminal region of human P ribosomal protein (R13 epitope) which was responsible for the cross-reactivity in chagasic serum (Table 3). However, reactivity with ribosomal proteins is also found in some patients with systemic lupus erythematosus (SLE); approximately 15% of SLE patients have autoantibodies to a shared epitope (H13) located in the C-terminal regions of the ribosomal proteins, P0, P1, and P2 (Elkon et al. 1986). However, antibodies against ribosomal proteins from CCC and SLE patients show differential recognition. Thus sera from patients with chronic Chagas' heart disease have been shown to contain relatively high levels of anti-R13 but low levels of anti-H13 antibody (Lopez Bergami et al. 1997), whereas both titers are comparable in SLE sera (Kaplan et al. 1997, 1993). Despite this positive correlation between molecular mimicry and pathology, some discrepancies exist. First, attempts to link anti-R13 reactivity by ELISA in the sera with the symptomatology in chronic or asymptomatic patients failed to find a significant correlation. Thus 60% and 49%, of chronic and asymptomatic sera, respectively, displayed reactivity with R13 but varied significantly depending on the geographical origin of the patients (Aznar et al. 1995). Moreover, no correlation between anti-R13 reactivity and cardiomyopathy was found in a group of 14 patients from whom endomyocardial biopsies and blood samples were taken at the same time. Furthermore, mice immunized with TcP2L developed antibodies against

the cross-reactive epitope, as well as many others, in contrast with the fine specificity of antibodies obtained from infected mice (Sepulveda et al. 2000).

On the other hand, some evidence indicates that those anti-P ribosomal antibodies could be pathogenic. Thus purified IgG, reactive with the C-terminus epitope of *T. cruzi* ribosomal P protein, caused a chronotropic alteration in primary rat cardiomyocytes through selective stimulation of β1-adrenergic receptors (Elies et al. 1996; Ferrari et al. 1995). However, in this case, the relevant cross-reactive epitope included the AESDE amino acid sequence from the second extracellular loop of the human β1-adrenergic receptor, which is homologous to the internal AESEE sequence of TcP0 (Table 3) (Ferrari et al. 1995). Moreover, passive transfer of a mAb against R13, which cross-reacts with the human β1-adrenergic receptor, had a chronotropic effect on cultured rat cardiomyocytes (Mahler et al. 2001). Mice immunized with P0 *T. cruzi* ribosomal protein develop electrocardiographic alterations late after immunization, when the titer of antibodies is extremely high, similar to those in chagasic animals but not identical to the complex response of chronic *T. cruzi* infection (Lopez Bergami et al. 2001). In contrast, those hyperimmunized with TcP2L died at an earlier time and did not show heart inflammation.

Sera from chagasic patients also contain IgG antibodies which immunoprecipitated human M2 muscarinic cholinergic receptor molecules and which were able to activate them, having an agonist effect on cardiomyocytes and causing partial desensitization (Leiros et al. 1997). The original stimulus for the formation of these antibodies was not ascertained and whether they could cause heart dysfunctions of the types seen in chagasic patients remains an open question.

### 5.1.3 Cha

We have described an autoantigen, Cha, a mammalian transcription factor which is recognized by almost all chagasic sera and by sera from *T. cruzi*-infected mice (Girones et al. 2001b). This Ag was isolated by screening of a library with seven CCC sera and has two regions of homology with *T. cruzi*, one with in an expressed sequence tag of the parasite (TENU2845) and another with SAPA, the Ag shed in the acute phase of *T. cruzi* infections (Table 3) (Cazzulo and Frasch 1992; Pollevick et al. 1993). Interestingly, we found that the two epitopes, named R1 and R3, are recognized by T and B cells, respectively, both having significant sequence homology. Very interestingly, there is a strong association of anti-Cha (R3) antibodies and pathology. Thus the titer of the sera from chagasic patients against R3 increases with symptomatology and decreases with treatment (Girones et al. 2001a). However, we have not

determined yet whether anti-R3 antibodies have any effect on pathology of the disease. Future experiments will focus on this. Our hypothesis is that these antibodies arise during infection by cooperation of Cha-specific B cells with T cells of different specificity (Fig. 2).

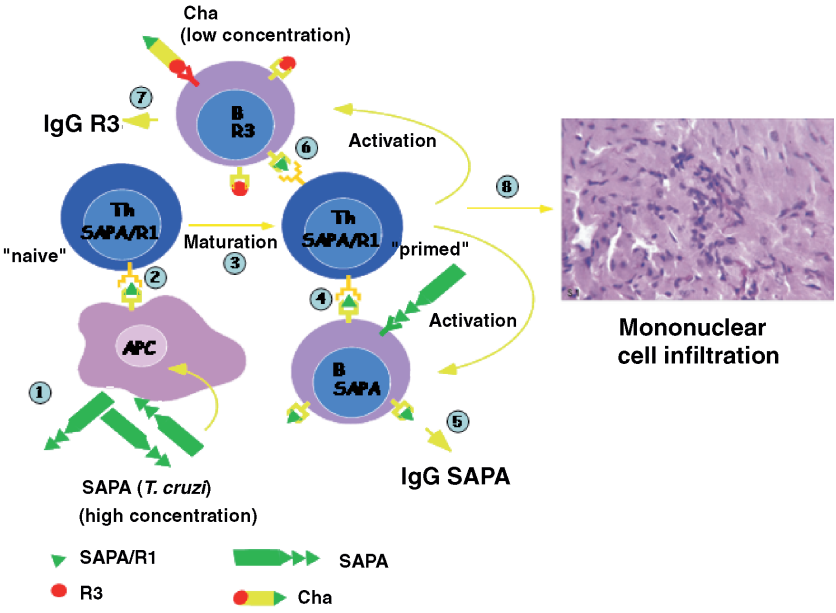


**Fig. 2** Generation of anti Cha T/B cell responses. *T. cruzi* infection causes the secretion of parasite Ags to the medium and lyses host's cells, triggering release of self Ags. In particular, during *T. cruzi* infection SAPA Ag is released to the extracellular environment and is taken up by macrophages (*1*). There SAPA is processed intracellularly and presented to naïve T cells through MHC Class II molecules (*2*). These T cells undergo maturation and develop into primed effector T cells specific for SAPA and the cross-reactive epitope R1 of Cha (*3*). Interaction of SAPA/R1 T cells with B cells of the same specificity (*4*) triggers anti-SAPA antibody production (*5*), which is observed during infection. However, the Cha Ag epitopes can be presented on the surface of B cells by MHC Class II molecules by two possible mechanisms: (a) The Cha epitopes can be naturally presented on B cells and (b) the Cha epitopes can be released during infection due to lysis of infected cells. Then, SAPA/R1 T cells can interact with B cells that present the R1 cross-reactive epitope of Cha (*6*) and trigger anti-Cha(R3) antibodies of different specificity (*7*). On the other hand, SAPA/R1 T cells are able to induce inflammatory infiltrates and damage in hearts of recipient mice through cytokines and/or activation of CD8 T cells (*8*)

## 5.2 Autoreactive T Cells

Perhaps the best evidence supporting a role for autoantigen-specific autoimmunity in disease pathogenesis derives from studies on T cell-mediated immunity in mice. Ribeiro-Dos-Santos et al. have reported that a $CD4^+$ T cell line obtained from a chronic chagasic mouse consisting of approximately 95% $CD4^+$ T cells proliferated in response to either a crude *T. cruzi* Ag preparation or heart tissue extracts from different animal species (Ribeiro-Dos-Santos et al. 2001). In culture, this cell line arrests the beating of fetal heart cells and, more importantly, induces myocarditis in immunized mice and promotes rejection of transplanted normal hearts in the absence of *T. cruzi* (Ribeiro-Dos-Santos et al. 2001). The requirement of the parasite to cause rejection in mice transplanted with T cells from infected mice has been also widely debated (Cunha-Neto et al. 1995; dos Santos et al. 1992; Ribeiro-Dos-Santos et al. 2001; Tarleton et al. 1997). Thus rejection of syngeneic transplanted hearts in chronically infected mice has been shown to take place either in the absence (dos Santos et al. 1992) or in the presence (Tarleton et al., 1997) of the parasite. These differences may be due to the different mice and parasite strain combinations used, and when the presence of the parasite is required for rejection, inflammation and not *T. cruzi* replication may be necessary to provide the necessary adjuvant effect to trigger autoreactivity and could be the rejection-inducing agent in the implanted hearts.

Besides proposing that B cell cross-reactivity against myosin is involved in pathogenesis, Cunha-Neto et al. have also proposed that myosin cross-reactive T lymphocytes infiltrating heart tissue lesions are also involved in chronic chagasic heart tissue lesions (Cunha-Neto et al. 1996, 1995). These T cells are also activated by CMhc cross-reactive *T. cruzi* Ag B13 as in B cells (Cuhna-Neto 2000; Cunha-Neto et al. 1996) (Table 3). Thus T cells from chagasic patients with overt heart disease or asymptomatic patients responded to in vitro stimulation with B13 with increased IFN-ɣ and reduced IL-4 production, suggesting a Th1-type cytokine profile (Cunha-Neto and Kalil 2001; Cunha-Neto et al. 1998). Those authors proposed that heart damage in CCC could be secondary to the release of inflammatory cytokines and a DTH process initiated by B13. However, the assumption that pathology arises from molecular mimicry between B13 *T. cruzi* and CMhc has been challenged by other authors because T cell autoreactivity against B13 was shown to exist not only in CCC but also in asymptomatic patients and in other cardiopathies (Kierszenbaum 2003). Moreover, both the level of the response to B13 and the cytokine production profile of lymphocytes from asymptomatic chagasic

patients were similar to those of T cells from patients with overt heart disease (Cunha-Neto and Kalil 2001).

It is noteworthy that immunological tolerance to heart Ags induced in mice by heart Ag administration and anti-CD4 antibody before their infection by *T. cruzi* resulted in less intense cardiopathy than that in control non-tolerized animals (Pontes-de-Carvalho et al. 2002), which is in favor of an autoimmune pathology. This treatment affects $CD4^+$ responses and not the production of anti-myosin IgG. Although this suggests that the regime to make the mice tolerant was not as effective as expected, at least regarding the humoral response (Th2 mediated), it is becoming increasingly evident that the response involved in heart damage is Th1 mediated. Recently, Leon et al. have described (although in the acute phase) that myosin autoimmunity, while a potentially important inflammatory mechanism in acute and chronic infection, is not essential for cardiac inflammation (Leon et al. 2003), although immunization with a *T. cruzi* extract induced a DTH response against myosin (Leon et al. 2004).

We also studied the T cell response to Cha autoantigen during *T. cruzi* infection. T lymphocytes from *T. cruzi*-infected mice also proliferated to recombinant Cha. More interestingly, transfer of T cells from chronically infected mice to naïve syngeneic mice led to heart infiltration and to production of anti-Cha antibodies, detectable 60 days later (when chronic pathology arises in mice after *T. cruzi* infection) (Girones et al. 2001b). Transferred T cells were almost pure CD3 cells (99%). Consistently, transfer of T cell clones specific for SAPA/R1 cross-reactive epitopes results in heart infiltration in the absence of anti-Cha antibody production (Girones et al., in preparation). Therefore, in some cases the presence of the parasite is not necessary to produce pathology if one transfers activated autoreactive T cells. How this takes place and whether the Cha autoantigen (normally an intracytoplasmic protein) comes to be presented to T cells are under investigation in our laboratory (see Fig. 2 for a hypothetical model). The observed anti-Cha response is likely due to a cooperation of R1-Cha-specific T cells with naïve anti-R3 autoreactive B cells. We believe that Cha autoreactive T cells are responsible for the heart damage, and that Cha autoantibodies are an epiphenomenon secondary to heart tissue destruction. Our results suggest that T cells cooperate with naïve B cells in the animal after heart damage because transfer of T cell clones induces heart infiltration but no anti-Cha antibodies. Although our results suggest that Cha may be involved in pathology, this by no means indicates that Cha would be the only autoantigen involved in the pathology of Chagas' disease.

## 6
## Bystander Activation

As reviewed recently by von Herrath et al. (2003), bystander activation is defined as the activation of autoreactive lymphocytes that do not recognize microbial Ags. This can be mediated through cytokines and/or APCs (TCR-independent bystander activation). However, bystander activation might also require concurrent exposure to the cognate Ag. Ag-specific cells induced by molecular mimicry can be activated by a non-specific stimulus such as other

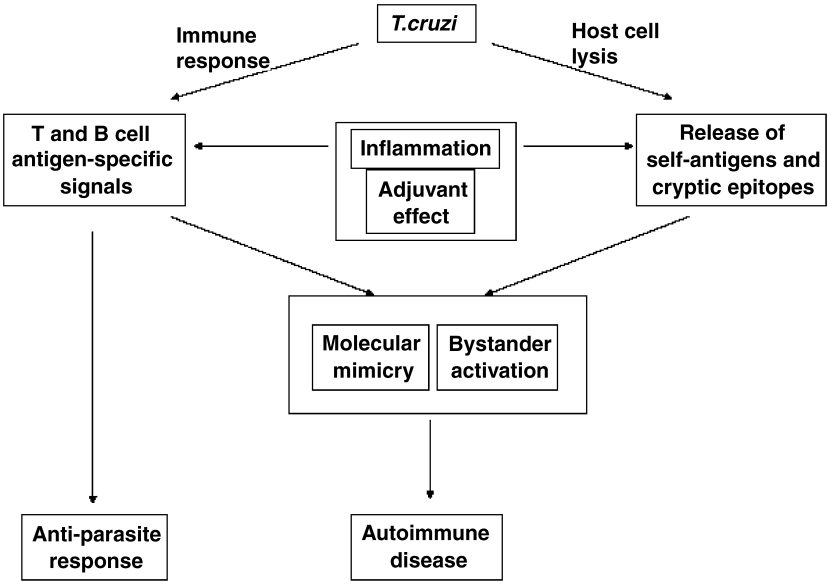


**Fig. 3** Diagram of the different mechanisms of induction of pathogenicity by *T. cruzi*. *T. cruzi* induces T and B cell anti-parasite responses which, through molecular mimicry with extracellular Ags or epitopes in Ags normally presented by APCs, can lead to autoimmune disease. *T. cruzi* can lead to secretion of cytokines which mediate some cardiac damage, liberating autoantigens reconized by autoreactive T cells and autoantibodies that further damage the cardiac tissue via bystander activation. Simultaneously parasite replication can induce release of self-antigens, usually intracellular, which contain cryptic epitopes that can be presented by APCs. Also, over-expression of intracellular Ags induced by *T. cruzi* can result in presentation of cryptic epitopes by APCs. If cryptic epitopes are cross-reactive with *T. cruzi* epitopes, then autoimmune disease can arise. *T. cruzi* contains several molecules capable of stimulating the immune system in a non-Ag-specific manner, known as the adjuvant effect, which together with the release of self-antigens and exposure of cryptic epitopes can contribute to sustain a local immune activation known as bystander activation

infections or heart damage, or by adjuvants in experimental settings, to induce autoimmune disease. Regarding *T. cruzi* Ags, both types of bystander activation have been described. *T. cruzi* parasitization of host tissue induces the release of autoantigens (Talvani et al. 2000) and a proinflammatory environment rich in cytokines, nitric oxide and chemokines sufficient to activate autoreactive T cells by lowering the threshold of activation (Fedoseyeva et al. 1999) (see Fig. 3). These cells may then proliferate in response to self-Ag presented on host APC. If this were the case for myosin, aberrant Ag presentation per se would not be necessary, because peptides of cardiac myosin, a cytoplasmic protein, are found complexed with class II MHC molecules on APCs even in normal mouse myocardium (Smith and Allen 1992) and are increased in the heart of infected patients and animals. The anti-self response is initiated and tissue damage may ensue if the response is of sufficient intensity.

## 7
## Parasite Persistence

Despite all the facts mentioned above, several researchers in this field defend the idea that *T. cruzi* persistence in the infected host is solely responsible for the damage in the chronic phase. Tarleton has reviewed all the arguments in favor of the parasite persistence hypothesis to explain the pathogenesis of chronic Chagas' disease in general and of CCC in particular (Tarleton 2001, 2003; Tarleton and Zhang, 1999). Arguments in favor of the idea that disease is linked to parasite presence are supported by the fact that treatments which decrease the parasite burden in the acute phase are associated with a decrease in clinical symptoms (Viotti et al. 1994). Enhancing the efficiency of the anti-parasite response by immunotherapy, gene deletion, or vaccination results in decreased severity of the chronic phase, not exacerbation of disease as predicted by the autoimmune hypothesis (Tarleton 2003). However, this argument cannot be used against autoimmunity because the fine specificity (cross-reactivity?) of those anti-parasite responses was not studied. Effective chemotherapy could also enhance anti-*T. cruzi* immunity in mice (Olivieri et al. 2002). In humans, the link between persistence of *T. cruzi* and clinical disease is also supported by the tissue-specific detection of parasite DNA in the hearts, but not in the esophageal tissue, of individuals with cardiac disease, and vice versa (Jones et al. 1993; Vago et al. 1996). Very recent data in humans show a higher frequency of parasite-specific IFN-γ-producing $CD8^+$ T cells among chronic Chagas' disease patients with mild clinical disease than in 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 chronic-

stage disease (Laucella et al. 2004). Apparently this supports the parasite persistence hypothesis in opposition to autoimmunity, arguing strongly in favor of the participation of an effective anti-parasite response in preventing disease (Tarleton 2003). Conversely, it has been observed that immunosuppressive treatments correlate with exacerbation of the infection and disease (Ferreira and Borges 2002) although this is not always the case. Thus the use of cyclosporin A has been shown to reactivate parasitemia in several but not all of the heart-transplanted patients. In general, chagasic heart transplants are not rejected or suffer from myocarditis despite the use of immunosuppressive drugs (see Kierszenbaum 2003). These data have been taken as an argument against an autoimmune-based pathology in CCC because transplanted hearts given to patients with the most severe cases of Chagas' heart disease remained essentially undamaged for so many years. However, we need to be cautious because few studies have gone more than 10 years when in a normal infection the pathology of CCC sometimes appears 15–30 years after primary infection. Moreover, if proven true, this mostly discards autoantibodies as the main pathological cause of CCC, but not autoreactive T cells. The same treatment that suppressed alloantigen T cell reactivity may have suppressed autoreactive T cells. So a role for T cells cannot be discarded.

In addition, we have found that autoreactivity in the chronic phase is also linked to parasitemia because the antibody titer and number of reactive T cells against the Cha autoantigen are lower in C57BL/6 (non-susceptible) than in BALB/c (susceptible) mice (Girones et al. 2001b). Moreover, potentially pathogenic anti-Cha autoantibodies also decreased with chemotherapeutic treatment of Chagas' patients. The titer of anti-Cha antibodies, as well as anti-*T. cruzi* antibodies, decreased in parallel with treatment and increased with symptomatology (Girones et al. 2001a) (Table 4). Thus anti-parasite response, some anti-self responses and pathology seem to go together. This poses a word

**Table 4** Myocarditis and antibody responses in chagasic patients increase with symptomatology and decrease with treatment

| Chagasic patients | Anti-Cha antibodies | Anti-*T. cruzi* antibodies | Myocarditis |
|---|---|---|---|
| Symptomatic | +++ | +++ | +++ |
| Asymptomatic untreated | ++ | ++ | − |
| Asymptomatic treated | + | + | − |

The presence or absence of myocarditis was given by clinical histories of patients. Antibody response was taken from Girones et al. 2001a (OD 450 nm<0.3, $^{+}$; OD 450 nm between 0.3 and 1.0, $^{++}$; OD 450 nm<1.0, $^{+++}$).

of caution in interpreting some clinical data when not all aspects of the problem are measured. Those results may be interpreted in very different ways: (a) the parasite is the only cause of the disease and anti-parasite and anti-self responses are direct consequences of parasite replication, (b) pathology may be caused by the anti-*T. cruzi* response or (c) the parasite is the trigger of autoimmune response which is the effector mechanism.

The presence of *T. cruzi* in the chronic phase of the disease was already observed in early descriptions (Vianna 1911) and was documented subsequently by other authors (Almeida et al. 1984; Teixeira Vde et al. 1993). With more sensitive techniques such as polymerase chain reaction (PCR), the parasite (more properly parasite DNA) is commonly detected in chronic patients (reviewed in Higuchi Mde et al. 2003). Recent immunohistochemistry studies have demonstrated higher frequencies of *T. cruzi* Ags, reaching 100% of hearts from chronic chagasic patients who died due to heart failure when several samples of the myocardium were analyzed (Higuchi 1993; Palomino 2000). Many previous failures to detect parasite Ags in biopsy material from patients in the chronic phase have been attributed to the fact that it seems necessary to examine several different sections of the heart to detect the parasite in this phase of the disease (Higuchi Mde et al. 2003). Using a mouse strain which develops chagasic cardiomyopathy when infected with a highly virulent *T. cruzi* strain, amastigotes were detected in myocytes through the chronic phase, although their numbers were low and much lower than in the acute phase (Guarner et al. 2001). A general finding not always acknowledged by the supporters of the parasite persistence hypothesis is that there is no direct correlation between the sites of parasite detection and heart damage, and also no correlation between the levels of parasites (for example, as detected by PCR) and clinical findings (Monteon-Padilla et al. 2001). However, a significant association between the presence of *T. cruzi* Ags in the heart and severe or moderate inflammation was observed both in humans (Higuchi Mde et al., 2003) and in animal models of the disease (Buckner et al. 1999). However, the number of parasites was low in relation to the intensity of the myocarditis and whole myocardial fibers containing parasites did not elicit inflammation (Higuchi Mde et al. 2003). This suggests two possibilities: exuberant host reactions to the few remaining parasites, either immune mediated or not, or autoimmune-induced inflammation. Parasite Ags probably work as a trigger response against the myocardial fibers. In addition, it is plausible that some lesions lack parasites or parasite Ags because of the effective clearance of parasites from the site by an effective anti-parasite immune response, thus preventing observation of an exact correlation. However, this is difficult to reconcile with the fact that a strong anti-parasite immune response results in decreased symptoms (Laucella et al. 2004).

Thus parasites are somehow present in the chronic phase, but what one ought to know is whether relevant parasite Ags persist and are presented by APCs to T cells. No matter the Ag recognized, Ag-specific T cells must be stimulated to become effector cells (helper, cytotoxic or other). For this, the Ag needs to be presented. Although some APCs could be very efficient in presenting Ags, it is rather unlikely that there are enough parasite Ags to continuously support chronic T cell stimulation.

Recently, it has been shown that *T. cruzi* kinetoplast DNA is able to integrate into human and other mammalian cell genomes and was transmitted to the descendants (Nitz et al. 2004). This has important implications not only for the detection of parasite mentioned above (some based in kinetoplast DNA) but also for pathology, because *T. cruzi* kinetoplast Ag could be continuously presented and may continuously trigger a response to those Ags of the parasite, thus killing normal cells.

## 8
## Coexistence of Parasite Persistence and Autoimmunity

We think that because cardiac myosin autoimmunity develops in the acute phase, when there is lysis of cardiac myocytes and easily detectable parasites, it is very likely that the two processes, bystander damage and molecular mimicry, co-exist until the chronic phase, where damage is produced via effector cells recognizing cross-reactive *T. cruzi*/autoantigen through molecular mimicry.

Thus we propose that the parasite is the trigger which activates some T cells (autoantigen/cross-reactive parasite Ag). Once they are activated, they secrete inflammatory cytokines which mediate some cardiac damage. This liberates autoantigen which is also recognized by some other autoreactive T cells and autoantibodies which further damage the cardiac tissue via bystander activation. This is like a vicious cycle triggered by parasite Ags but fueled by cross-reactive autoantigens and implies that purely parasite-specific T cells may cause very little cardiac damage. This also involves two of the proposed pathogenic mechanisms: bystander damage and molecular mimicry. *T. cruzi* might also function as an adjuvant for an immunological cross-reaction between common parasitic and myocardial fiber Ags, resulting in severe lymphocytic myocarditis (see Fig. 3).

Thus parasites are necessary to trigger autoantibodies and autoreactive T cells and may be necessary to maintain them in the chronic phase. Altogether, we believe that active *T. cruzi* infection is necessary to trigger the autoimmune

process, most likely through autoreactive T cells, which once induced can produce the cardiac pathology.

Obviously, the elucidation of the mechanisms of pathogenesis in Chagas' disease may have implications for vaccination and therapy. If autoimmunity by molecular mimicry is responsible, anti-*T. cruzi* chemotherapy would not necessarily suppress pathogenic autoimmune responses initially elicited by parasite Ags and subsequently boosted by host tissue Ags. Also, in the search for protective vaccine we should discard *T. cruzi* Ags that elicit pathogenic anti-self responses.

## 9 Final Remarks

As mentioned above, several criteria, put originally forth in the *T. cruzi* field by Kierszenbaum 1986 and more broadly by Benoist et al. (Benoist and Mathis, 2001) must be met to consider a disease as caused by molecular mimicry (see Table 2). In *T. cruzi* infection, the first three conditions have been clearly demonstrated, and this has allowed the identification of several candidate autoantigens. If there were a unique cross-reactive Ag, infection with genetically deficient parasites lacking the inducing Ag, or infection of knockout mice lacking the cross-reactive autoantigen, would prevent the disease. However, as multiple autoantigens seem to be involved in the pathology of Chagas' disease, such experiments are very difficult to perform, and therefore the fourth criterion has not been demonstrated yet.

The fifth criterion is considered to be the decisive test of the concept of autoimmunity.

In most publications about autoimmunity in Chagas' disease the putative causes are either autoantibodies or autoreactive T cells originated by molecular mimicry between parasite and host Ags. Nevertheless, evidence for the mediation of cross-reactive antibodies or T cells in pathology is still far from settled. Moreover, most of the data come from experimental *T. cruzi* infection, and an additional problem is the extrapolation of the results to the human model which is more difficult to study.

One way to determine the pathological effect of autoreactive T or B cells would be to immunize mice with cross-reactive Ags to see whether this induces pathology. However, immunization with an autoantigen (injected together with adjuvants and via different routes than natural infection) may not reflect the way the autoantigen is presented during natural infection and may elicit hyperimmune responses, tolerance or regulatory T cells which may suppress

autoimmunity. An alternative approach is the transfer of putative autoreactive T cells from chronically infected mice specific for a given autoantigen. Either immunization with or transfer of T cells specific for autoantigens may answer some of these questions and determine which of the candidates are really relevant for pathology. In this respect, the presence of autoreactive T cells against Cha proteins shown in our experiment with Cha autoantigen is the closest to this. Recently, an interesting observation was made by von Herrath et al. (2003), proposed that autoimmune diseases could be induced and exacerbated by many different microbial infections. Their hypothesis is that after infection there is exposure to self, foreign and environmental agents. After clearance of infection, the inflammatory response drops, but when there are additional infections the threshold for autoimmunity is reached and autoaggressive T cells expand and develop.

On the other hand, the parasite persistence hypothesis is based on the fact that *T. cruzi* persists in the chronic phase of Chagas' disease and that treatment against the parasite results in a decrease of the severity of the disease. There are also some questions that need to be fully addressed: (1) Why do lesions develop primarily in the heart and not at other sites of parasite persistence? (2) Why does parasite burden not always correlate with disease severity? A demonstration of these hypotheses is also difficult to perform, because one ought to separate the components of the immune response, self and anti-self during infection. However, we think that things are not so easy, because co-existence of self and non-self Ags would enhance the immune response against both, being always triggered by the parasite.

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