Short-term therapy with simvastatin reduces inflammatory mediators and heart inflammation during the acute phase of experimental Chagas disease

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Trypanosoma cruzi infection induces progressive cardiac inflammation that leads to fibrosis and modifications in the heart architecture and functionality. Statins, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, have been studied due to their pleiotropic roles in modulating the inflammatory response. Our goal was to evaluate the effects of simvastatin on the cardiac inflammatory process using a cardiotropic strain of T. cruzi in a murine model of Chagas cardiomyopathy. C57BL/6 mice were infected with 500 trypomastigotes of the Colombian strain of T. cruzi and treated with an oral dose of simvastatin (20 mg/Kg/day) for one month and inflammatory and morphometric parameters were subsequently evaluated in the serum and in the heart, respectively. Simvastatin reduced the total cholesterol and inflammatory mediators (interferon-gamma, tumour necrosis factor-alpha, CCL2 and CCL5) in the serum and in the heart tissue at 30 days post-infection. Additionally, a proportional reduction in heart weight and inflammatory infiltration was observed. Simvastatin also reduced epimastigote proliferation in a dose-dependent manner in vitro and was able to reduce blood trypomastigotes and heart amastigote nests during the acute phase of Chagas disease in vivo. Based on these data, we conclude that simvastatin exerts a modulatory effect on the inflammatory mediators that are elicited by the Colombian strain of T. cruzi and ameliorates the heart damage that is observed in a murine model of Chagas disease.

Key words: Chagas cardiomyopathy - Trypanosoma cruzi - simvastatin - chemokines - inflammation

Chagas cardiomyopathy (CC) is a chronic clinical manifestation that is caused by the protozoan Trypanosoma cruzi. This protozoan induces persistent inflammation comprised of neutrophils, macrophages, CD4+ and CD8+ T cells (Reis et al. 1993, Brener & Gazzinelli 1997) that culminates in myocarditis, fibrosis and changes in the heart architecture and functionality. This illness, which affects approximately 10 million individuals in Latin America, is characterised by persistent inflammatory remodelling of the heart tissue and is becoming the most common form of progressive, non-ischaemic heart disease worldwide (Prata 2001, WHO 2010). Both systemically and at chronic inflammatory foci, inflammatory cells release inflammatory cytokines, such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) and various chemokines (e.g., CCL2, CCL3 and CCL5) that assist in the control of the parasite infection. However, these effectors also drastically affect heart tissue integrity and increase the cardiac inflammatory response, leading to a worsening of the disease phenotype (Talvani & Teixeira 2011).

The ability of the host to control the infection may dictate the balance that exists between the inflammatory disease course and the typical regulatory immune response. Newly developed cardiovascular therapeutics (e.g., ACE-inhibitors and beta-blockers) have been shown to reduce inflammatory infiltration, fibrosis, cardiac output ventricular function and circulating inflammatory cytokines and chemokines in experimental and human studies (Leon et al. 2003, Botoni et al. 2007, Paula-Costa et al. 2010, Coelho dos Santos et al. 2010). More recently, by blocking the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonate, HMG CoA reductase inhibitors (statins) have emerged as potent inhibitors of cholesterol and isoprenoid biosynthesis (Liao 2002). Furthermore, the pleiotropic effects of statins have been associated with the inhibition of inflammatory cytokine synthesis, a reduction in ventricular remodelling and an improvement in endothelial cell function through the increased production of endothelial nitric oxide and a decrease in the release of endothelin-1 and other inflammatory mediators (Elrod & Lefer 2005, Greer et al. 2006, Devaraj et al. 2007). Additionally, some studies have also suggested that the specific statin simvastatin may affect immune-mediated inflammation because of the documented ability of this statin to reduce the adhesion of inflammatory cells to the endothelium, inhibit leukocyte adhesion by direct interactions with the leukocyte-function antigen-1 and modulate the expression of the integrin dimer CD11b on monocytes. Simvastatin may also participate in the regulation of cy-

The anti-inflammatory properties of statins are independent of their effects on cholesterol levels, but despite a favourable safety profile for short treatment with statins (Node et al. 2003), few studies have described the role of statins during the inflammatory events triggered by T. cruzi infection. Our aim is to evaluate the effect of simvastatin on cardiac inflammation using a cardiotropic strain of T. cruzi (Colombian) in a murine model and to assess the modulation of immune parameters and epimastigote replication by statins in vitro.

MATERIALS AND METHODS

Parasites - Blood trypomastigotes from the T. cruzi Colombian strain, which have a cardiac tropism and have been classified as discrete typing units I of T. cruzi (Zingales et al. 2009), were maintained through successive passages in Swiss mice at the Laboratory of Chagas disease, Federal University of Ouro Preto (UFOP), state of Minas Gerais, Brazil.

Experimental animals and infection - Male C57BL/6 mice weighing 18-20 g (50 days of age) from the Animal Facility at UFOP were inoculated intraperitoneally with 500 trypomastigotes from the T. cruzi Colombian strain. All procedures were performed in accordance with the guidelines issued by the Brazilian College of Animal Experimentation and these experiments were previously approved by the Ethic Committee in Animal Research at UFOP (CEUA) (protocol #2009/28). Animals were fed commercial food and water ad libitum. The mouse parasitaeemia was determined by collecting fresh blood from the tail vein on the 3rd day of infection and parasitaeemia was analysed until no parasites were detectable in the blood.

Statin and treatment scheme - A previous set of experiments was performed to determine the toxicity of simvastatin (Sanval, SP, Brazil) treatment in mice (n = 8/group) using a daily dose of 2, 20, 40 and 80 mg/kg. A dose of 20 mg/kg was selected for the current study and administered daily in the evening to account for the circadian rhythm of statins. A new set of mice were divided into the following groups: (i) 10 mice infected with T. cruzi receiving vehicle, (ii) 10 mice infected with T. cruzi and treated with simvastatin (20 mg/kg), (iii) 10 non-infected mice receiving vehicle only and (iv) 10 non-infected mice treated with simvastatin (20 mg/kg). This experiment was repeated twice and the survivors from the first and second experiments were grouped to obtain a total of 10 biological samples. Simvastatin was diluted in phosphate buffer (vehicle) for 15 min in an ultrasonic water bath and administered in the oropharyngeal cavity by gavage. This was repeated twice and the survivors from the first and second experiments were grouped to obtain a total of 10 biological samples. Simvastatin was diluted in phosphate buffer (vehicle) for 15 min in an ultrasonic water bath and administered in the oropharyngeal cavity by gavage. This therapy was initiated on the same day as T. cruzi inoculation and continued for 30 days post-infection. Treatment was typically administered in the morning. The animals were euthanized on the afternoon of the 30th day.

In vitro effect of simvastatin on Trypanosoma cruzi - To assess whether the HMG-CoA inhibitor simvastatin affects parasite survival in vitro, epimastigotes of the Y strain of T. cruzi were cultivated in liver infusion trip-
treated with simvastatin or untreated). This index corresponded to inflammatory leukocyte nuclei in addition to the background of cardiac cellular nuclei observed in data from non-infected mice, as previously shown (Caldas et al. 2008). Amastigote nests were also quantified by calculating the area occupied by parasites in the same sections used to quantify the inflammatory process.

**Heart mass measurement** - Hearts were carefully excised ex vivo and gently blotted on absorbent paper to remove blood from the ventricles before the wet weight measurements were calculated. The relative heart weight was calculated using the heart weight in mg/mouse body weight in grams (mg/g). This value was then used to evaluate the cardiac mass measurements obtained after 30 days of infection and after treatment with simvastatin in animals from two independent experiments (n = 10).

**Statistical analysis** - The results of the serological assays, immune assays and morphological and histopathology parameters were analysed by non-parametric Newman-Keuls Multiple Comparison and Tukey’s tests. A difference was considered significant if p was equal to or less than 0.05.

**RESULTS**

**The effect of simvastatin on circulating and in vitro parasite levels and on surviving C57BL/6 mice** - A dose-response for simvastatin was investigated using 2, 20, 40 and 80 mg/kg of animal weight during 30 days of *T. cruzi* infection. High doses of simvastatin (40 and 80 mg/kg) were observed to cause an elevated index of mortality on the 25th day of infection, which coincided with the day before peak parasitaemia was observed (Table). Among the animals treated with 2 mg and 20 mg of simvastatin, 60% and 40% mortality was observed, respectively, on the 25th day of infection, with no differences in the level of circulating parasites.

Assuming 20 mg/kg/day as the ideal dose of simvastatin (due to the decreased mortality), we identified a significant reduction in the level of circulating parasites during peak parasitaemia on the 26th day and in the previous three days (Fig. 1A). Simvastatin did not cause an

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<tr>
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<td>5th day</td>
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*a: p < 0.05 when parasitemia was compared with untreated group; SEM: standard error of the mean.*
increased mortality in healthy animals and in those animals infected with *T. cruzi* this drug was able to protect 20% of the animals beginning on the 25th day after infection (Fig. 1B) compared with untreated, infected mice.

In parallel, we evaluated whether simvastatin could be acting directly on parasite proliferation. Therefore, to eliminate any interference with the host cells (e.g., macrophages, Vero cells etc.), epimastigotes of *T. cruzi* were utilised to perform an in vitro evaluation in an axenic culture. Interestingly, simvastatin was efficient in reducing the epimastigotes present in culture in a dose-dependent manner (Fig. 2).

**Cholesterol levels and cardiac weight** - High levels of circulating cholesterol were found in the serum of animals that were infected with *T. cruzi*. Low daily doses of simvastatin were able to modify this pattern in mice after 30 days of treatment (Fig. 3A). In contrast, because this study focused solely on the acute phase of the disease, simvastatin therapy was unable to prevent an increase in the relative weight of the heart of infected animals. This weight was evaluated by dividing the average of the mouse body weight by the heart weight (Fig. 3B).

**Pleiotropic effects of simvastatin on cytokine and chemokine profiles** - We investigated whether simvastatin therapy could shift the pattern of soluble inflammatory mediators upon murine *T. cruzi* infection. Serum homogenate levels of the inflammatory and regulatory cytokines IFN-γ, TNF-α and IL-10 (Fig. 4A, B, E) and chemokines CCL2 and CCL5 (Fig. 4C, D), which are essential for the control of parasite infection and leukocyte recruitment to inflammatory foci, were increased in animals infected with *T. cruzi* after 30 days of infection. Interestingly, daily treatment with simvastatin drastically reduced the levels of inflammatory cytokines (Fig. 4A, B) and chemokines (Fig. 4C, D). However, in these experiments, simvastatin did not statistically alter the level of the regulatory cytokine IL-10 in *T. cruzi*-infected mice or in uninfected animals (Fig. 4E). A similar investigation was performed on the supernatant from the cardiac tissue homogenate in which only inflammatory mediators were analysed. Again, simvastatin reduced the cardiac levels of TNF-α and IFN-γ (Fig. 5A, B) and the chemokines CCL2 and CCL5 (Fig. 5C, D) in animals infected with *T. cruzi*.

**Simvastatin alters amastigote nests and leukocyte infiltration into the heart** - Inflammatory infiltration into the heart is partially driven by chemokine patterns and by the presence of *T. cruzi* or parasitic antigens inside or in close proximity to the myocardium. Here, treatment with simvastatin for 30 days drastically reduced the inflammatory cell infiltration into the heart tissue (Fig. 6B) compared with simvastatin-infected (Fig. 6A) and uninfected animals (Fig. 6C). Quantification of the nuclei in the cardiac tissue reinforced these histopathological observations; a significant reduction in inflammatory leukocytes in the heart tissue (Fig. 6D) and a reduction in the number of amastigote nests of *T. cruzi* (Fig. 6E) in animals treated with simvastatin was observed.
DISCUSSION

The inflammatory process has been suggested to be the most important factor in acute and chronic Chagas disease, both in humans and experimental models (Lannes-Vieira et al. 2009, Talvani & Teixeira 2011). However, this hypothesis does not exclude the participation of *T. cruzi* or parasitic antigens as a trigger for the inflammatory process. Additionally, the parasitic and the host genetic diversity have been suggested to be responsible for driving and modulating aspects of the inflammatory response (Santos et al. 2009, Guedes et al. 2010). Several researchers have consolidated their efforts to clarify whether this inflammatory process dictates the clinical changes and the progression toward chronic heart failure that is observed in humans. Therefore, cardiovascular drugs that are routinely used (e.g., ACE inhibitors, beta-blockers and others) have been tested for the treatment of Chagas disease. With a new anti-inflammatory focus, studies have demonstrated a drastic reduction in leukocyte infiltration in the heart, fibrosis, circulating inflammatory cytokines/chemokines and improvements in cardiac output ventricular functions in Chagas disease (Morris et al. 1989, Leon et al. 2003, Botoni et al. 2007, Coelho dos Santos et al. 2010, Paula-Costa et al. 2010). Because of these results, new therapeutic targets based on statins, a lipid-modifying agent HMG-CoA, have emerged as a method to prevent cardiovascular diseases (Liao 2002, Cheng et al. 2005, Mizuno et al. 2011). The clinical benefits of statins are thought to be associated with their ability to reduce cholesterol synthesis, but the product of their enzymatic reaction, mevalonate, is also a precursor of cholesterol and many non-steroidal isoprenoid compounds. The post-translational modification of mevalonate promotes membrane and protein-protein interactions and could result in the modification of numerous inflammatory signalling pathways that affect and modulate the cellular function of immune mediators (Quist-Paulsen 2010).

**Fig. 4:** simvastatin reduces serum levels of CCL2, CCL5 and proinflammatory cytokines. Treatment with 20 mg of simvastatin/kg/mouse daily decreased levels of proinflammatory cytokines interferon-gamma (IFN-γ) (A), tumour necrosis factor-alpha (TNF-α) (B) and chemokines CCL2 (C) and CCL5 (D) while regulatory cytokine interleukin (IL-)10 (E) was not alternate in mice infected with *Trypanosoma cruzi*. Analysis was done between groups of animals treated with vehicle (white) and those treated with simvastatin (black) using animals from two independent experiments (total n = 10) and data were shown as mean/standard error of the mean.
The pleiotropic effects of statins have previously been studied in the context of *T. cruzi* infection. Inhibition of the growth of epimastigotes of *T. cruzi* has been observed upon treatment with 10-0 mg/mL of statin (lovastatin) that may be a result of the inhibition of C14 acetate incorporation into parasite sterols (Florin-Christensen et al. 1990). *T. cruzi*, similar to many fungi and yeasts, requires specific sterols for its survival during different stages of its life cycle (Urbina 2009). In our in vitro investigation, the growth of cultured epimastigotes was reduced by the presence of simvastatin in a dose-dependent fashion (1.9 mM-2.5 mM). HMG-CoA reductase is already known to catalyse the NADPH-dependent reduction of HMG-CoA to mevalonate, which is primarily located in mitochondria. Therefore, simvastatin could potentially represent a competitive inhibitor of this enzyme in epimastigotes (in vitro) and in trypomastigotes (in vivo) (Hurtado-Guerrero et al. 2002).

The reduction in parasitaemia in mice using simvastatin (20 mg/kg/daily) coincided with decreased mortality in animals after the 25th day of infection. Even with a significant reduction in the levels of inflammatory mediators, such as IFN-γ and TNF-α, 30-40% mortality was expected in mice during peak parasitaemia based on the biological characteristics of the Colombian strain in isogenic C57BL/6 mice (Talvani et al. 2000, Paula-Costa et al. 2010). Unfortunately, mice do not survive well due to the alterations in the metabolic pathways that are a result of a persistent inflammatory response induced by specific strains of *T. cruzi* (Sánchez-Guillén et al. 2006), which is a limitation of this experimental model. However, mortality in our experimental model of Chagas disease could be delayed if we initiated a statin plus an anti-*T. cruzi* drug chemotherapeutic strategy, as previously demonstrated by Urbina et al. (1993). In this previous study, the authors indicated that lovastatin was able to potentiate the therapeutic effects of ketoconazole, an azolic anti-fungal drug. Additionally, combination therapy with both drugs at doses that offered only limited protection against *T. cruzi* was able to eliminate the presence of circulating parasites and prevent mortality (Urbina et al. 1993).

Investigations that have analysed the effect of statins on *T. cruzi* infection were only associated with chemotherapy or with the capacity of the drugs to block sterol (ergosterol) formation in parasites, thereby affecting parasitic growth or the capacity of the parasite to infect mammalian cells (Florin-Christensen et al. 1990, Urbina et al. 1993, Hankins et al. 2005, Priotto et al. 2009). Here, inflammatory cardiac disease in mice was analysed to measure the ability of simvastatin to reduce cholesterol in infected animals, to modulate the levels of systemic and cardiac pro-inflammatory cytokines (IFN-γ, TNF-α) and chemokines (CCL2/MCP-1 and CCL5/RANTES) and to alter the heart inflammatory process at the end of the acute phase of the disease.

Based on our previous experience, an early anti-inflammatory intervention with simvastatin could be essential for the treatment of long-term cardiac inflammation and to reduce heart architectural and functional changes in experimental Chagas disease (Melo et al. 2011). A daily dose of simvastatin (20 mg) was capable of reducing pro-inflammatory IFN-γ and TNF-α, but not regulatory IL-10 and did not ameliorate the clinical parameters (left ventricle ejection fraction and diastolic diameter of left ventricle) of dogs infected with *T. cruzi* during the acute phase of the disease.

Fig. 5: heart tissue concentrations of interferon-gamma (IFN-γ) (A), tumour necrosis factor-alpha (TNF-α) and cytokines CCL2 (C) and CCL5 (D) in *Trypanozoma cruzi*-infected mice treated or not with simvastatin. Homogenate of 100 mg of cardiac tissue from 10 animals were processed and inflammatory mediators (TNF-α, IFN-γ, CCL2 and CCL5) were evaluated on the 30th day post-infection. Each dot represents the result in a single animal and the bar the mean value of the group.
chronic phase of disease. We investigated the pleiotropic effects of simvastatin in mice because they are the most widely used species to investigate the immunology of the acute events of Chagas disease. In experimental Chagas disease, dogs have been useful in the study of cardiac parameters because of their similarities with human cardiac disease, but an increased understanding of inflammatory events (e.g., the chemokine network and cellular activation/recruitment) during the acute phase of Chagas disease has been well described in the murine model (Talvani & Teixeira 2011). In addition, the reproducibility of these data in *T. cruzi* infection using two different mammalian models and different genetic populations of *T. cruzi* would reinforce the application of simvastatin as an immunomodulatory therapeutic agent.

In the murine model, some authors have suggested that some of the observed cardiac destruction is due to the presence of parasites in the heart tissue and an increase in leukocyte infiltration that leads to necrosis and fibrosis (Andrade 1983, Brener & Gazzinelli 1997). The chemokines CCL2/MCP-1 and CCL5/RANTES have been largely associated with leukocyte (monocytes and lymphocytes) recruitment to inflammatory foci to combat parasite infection, but this infiltration inevitably results in damage to the host tissues (Aliberti et al. 1999, Talvani et al. 2000, Teixeira et al. 2002, Lannes-Vieira et al. 2009, Paiva et al. 2009). In fact, the role of CCL5 in the recruitment of CCR5+ leukocytes has been reinforced by experiments that indicate that CCR5-deficient mice are more susceptible to *T. cruzi* infection after the reduction of macrophages and T-cell migration into the heart, especially during the early stages of infection (Machado et al. 2005, Hardison et al. 2006). Other evidence of this phenomenon is based on the partial blockade of the CC-chemokine receptor inhibitor (Met-RANTES), which induces a reduction in the leukocyte influx (modulated by *T. cruzi*), followed by a reduction in parasitaemia and a reduction in fibronectin deposition in the heart tissue (Marino et al. 2004, Medeiros et al. 2009). Simvastatin was able to drastically reduce CCL2/MCP-1 and CCL5/RANTES, thereby culminating in a reduction in the migration of inflammatory cells into the cardiac organ; this effect could be associated with a good cardiac prognosis in experimental models. Few studies involving statins and *T. cruzi* infection have focused on cardiac host inflammation, but simvastatin has previously been shown to be capable of reducing the expression and serum levels of CCL2/MCP-1 and CCL5/RANTES and CCR2 and CCR5 receptors in both in vitro and in vivo models of human immunodeficiency virus, vasculopathy and diabetes/metabolic diseases (Nabatov et al. 2007, Tsuchiya et al. 2007, Yin et al. 2007, Lin et al. 2009).

In conclusion, we have demonstrated for the first time that simvastatin has pleiotropic effects in modulating the systemic and cardiac levels of CCL2/MCP-1 and CCL5/RANTES in isogenic mice infected with the cardiotropic strain of *T. cruzi* (Colombian). This therapeutic strategy also reduced the levels of circulating/tissue parasites, soluble inflammatory mediators (TNF-α and IFN-γ) and cardiac leukocyte infiltration during the acute phase of experimental disease. Therefore, given the key role of inflammation in the pathogenesis of Chagas disease, additional trials are needed in different experimental models to further support the use of statins as pharmacological agents to reduce cardiac inflammation in humans.

![Fig. 6: inflammatory infiltration is reduced by treatment with simvastatin.](image-url)
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