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Production and expression of inflammation and angiogenic parameters triggered by different genetic population of *Trypanosoma cruzi*

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On the fourteenth day of March 2014 at 02:00 p.m. in the Auditorium of the Centre of Distance Education (CEAD) at the Universidade Federal de Ouro Preto (UFOP), the committee met to evaluate the PhD thesis of the student **Deena Shrestha**. The defense of her thesis was initiated by oral presentation and then the committee members questioned the candidate. At the end, the members of the examining committee met in private, discussed the presentation and the work and decided to APPROVE the candidate. The obtaining title is conditioned to the compliance with all other requirements of the Post-Graduated Program in Biological Science, according its internal rules.

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DEDICATION

I dedicate this work to my parents and my husband, Bijay who always had faith on me and encouraged me to live up my dreams.

In biology, nothing is clear, everything is too complicated, everything is a mess, and just when you think you understand something, you peel off a layer and find deeper complications beneath. Nature is anything but simple.

-Richard Preston

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RESUMO

A cardiopatia induzida pela infecção pelo *Trypanosoma cruzi* aprensenta a inflamação como sua principal característica imunopatológica. Differente células inflamatórias contribuem para a produção de mediatores inflamatorios e regulatórios promotores diretos ou indiretos do processo denominado angiogênese inflamatória. As células cardíacas de mamíferos apresentam-se como importantes alvos do *T.cruzi* e, consequentemente, do próprio sistema imune do hospedeiro que objetiva a eliminação desses parasitos. Esse processo inflamatório culmina em uma destruição celular, em fibrose, hipóxia e alterações funcionais ao coração. A hipóxia e a produção de mediadores inflamatórios são estímulo bem definidos para o início da angiogênese. Assim, o objetivo desse estudo é estudar a participação do *T.cruzi* na produção de fatores inflamatórios e angiogênicos em animais C57BL/6 infectados com diferentes cepas desse parasito. Demonstrou-se a formação de novos vasos sanguíneos na matriz da esponja culminando no 14º dia pos-implante dorsal. Demonstrou-se, ainda, alta produção de mediadores inflamatórios no plasma de animais infectados com as cepas Y e Colombiana do *T.cruzi*. Esses animais também apresentaram elevados níveis plasmáticos de VEGF. Além disso, observou-se que a cepa Colombiana do *T.cruzi* foi capaz de induzir elevado infiltrado leucocitário e parasitos no tecido cardíaco. Possivelmente, devido ao alto parasitismo induzido pela cepa Colombiando do parasito, houve baixa produção (VEGF, ANG-1 e ANG-2) e expressão (VEGF) de fatores angiogênicos no tecido cardíaco. De forma interessante, animais infectados pela cepa VL-10 do *T.cruzi* demonstraram reduzida parasitemia, produção de citocinas inflamatórias e quimiocinas, bem como de infiltrado celular no tecido cardíaco. Além disso, o Benznidazol, a droga de escolha para o tratamento da doença de Chagas com alta citotoxicidade, foi capaz de manter os parâmetros angiogênicos similares aos animais não infectados. Também, o Enalapril e a Sinvastatina (fármacos utilizados para o tratamento de doenças cardioavasculares) foram capazes de reduzir citocinas inflamatóras em camundongos infectados, mas não a produção e a expressão de VEGF que mostrou-se similar aos animais infectados não tratados. Em suma, este estudo apresenta mediadores da angiogênese locais (coração) e sistêmicos durante a infecção pelo *T.cruzi*, sendo esses mediadores definidos pela genética e pela quantidade de parasitos presente no tecido cardíaco.

ABSTRACT

Inflammation is a key immune-pathophysiological characteristic of cardiomyopathy induced by *Trypanosoma cruzi*. Different inflammatory cells can contribute to the release of pro- and anti-inflammatory mediators which can directly or indirectly promote inflammatory angiogenesis. Mammalian cardiac cells are important targets to the protozoan *T. cruzi* driving an immune system that attempts to eliminate parasites. This inflammation culminates in cellular destruction, fibrosis, hypoxia and heart disturbances. Hypoxia and releasing of inflammatory mediators are well-defined stimuli to initiate angiogenesis. Therefore, we aimed to study the participation of *T. cruzi* in the production of inflammatory and angiogenesis mediators in C57BL/6 mice infected with different strains of this parasite. We found that in sponge matrix from infected mice, formation of new blood vessels were abrogated on day 14. Our data also demonstrated higher production of inflammatory mediators in plasma from mice infected with Y and Colombian strains of *T.cruzi*. These mice also demonstrated increase plasma VEGF. In addition, Colombian strain induced higher leukocyte infiltration and tissue parasites in cardiac tissue. However, possibly by the Colombian strain-associated higher tissue parasites, there were low production (VEGF, ANG-1 and ANG-2) and mRNA expression (VEGF) of angiogenic factors in cardiac tissue. It is very interesting that VL-10 infected C57BL/6 mice demonstrated decreased circulating parasite, production of inflammatory cytokines and chemokines, cellular infiltration in cardiac tissue. Furthermore, Benznidazole, a drug of choice for the treatment of Chagas disease, although exhibits numerous side effects, we observed that treament with this drug maintained angiogenic parameters similar to uninfected mice. Enalapril and Simvastatin (used for the treatment of cardiovascular diasease) treatment to infected mice diminished inflammatory cytokines but not VEGF expression and production which was similar to untreated infected mice. In summary, this study demonstrates angiogenesis mediators in local tissue (heart) and systemically during *T. cruzi* infection may be defined by the genetic and the load of tissue parasites.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

ACE	Angiotensin converting enzyme
ANG	Angiopoeitin
ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	Complementary deoxyribonucleic acid
CRT	Calreticulin
Ct	Threshold cycle
Cols	Colaboradores
DAB	Diaminobenzidine
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleeic acid
dTTP	Deoxythiosine triphosphate
DTU	Discrete typing unit
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
Ephrs	Ephrin receptors
GPI	Glycosylphosphatidylinositol
H & E	Hematoxyllin and Eosin
Hb	Hemoglobin
HGFH	Hepatocyte growth factor
HRP	Horse Radishperoxide
HTAB	Hexa-1,6-bis-decyltrimethylammonium bromide
IFN γ	Interferon gamma
IgG	Immunoglobulin
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-17	Interleukin-17
IL-4	Interleukin-4
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAPK	Mitogen- activated protein of kinase
MCP-1	Monocyte chemotactic protein-1
MIP 1	Macrophage Inflammatory protein 1
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MPC	Myeloid precursor cells

NF	Nuclear factor
NK	Natural killer cell
NOD	Nucleotide-binding oligomerization domain
OD	Optical Density
PAMPs	Pathogen associated membrane proteins
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
PlGF	Placental Growth Factor
PMN	Polymorphonuclear leukocytes
qPCR	Quantitative Polymerase Chain Reaction
RANTES	Regulated and normal T cell expressed and secreted
RNA	Ribonucleic acid
TGF-β	Transforming growth factor β
Th-1	Type I helper T cell
Th-17	T helper 17 cell
Th-2	Type II helper T cell
TIE	Tyrosine kinase
TIMP	Tissue inhibitors of metalloproteinase
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF alpha	Tumor necrosis factor alpha
TSP	Thrombospondin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organizations

1. INTRODUCTION

1. INTRODUCTION

1.1 Angiogenesis:

Angiogenesis, the formation of new capillary network from a preexisting vasculature, is a tightly regulated multistep process which maintains the balance between production and release of pro-angiogenic and anti-angiogenic factors (Sunderkotter et al., 1991). The process involves degradation of basement membrane, proliferation of endothelial cells, survival, and migration towards angiogenic stimulus, elongation and anastomosis (Costa et al., 2007). Judah Folkman (1933-2008) first stated that tumor growth was directly dependent on angiogenesis (Folkman, 1997). Angiogenesis occurs physiologically such as in embryo development, during wound healing and in several pathological conditions including atherosclerosis, proliferative retinopathies, tumor growth and chronic inflammation with abnormal proliferation of blood vessels (Carmeliet, 2000).

1.2 Process of angiogenesis:

Under normal conditions endothelial cells remain in the state of quiescence, however, when stimulated, the endothelial cells become active and initiate a cascade of events that culminate in the formation of new vessels (Laurent et al., 2007, Mariscalco et al., 2011).

The first step of the angiogenesis is the activation of endothelial cells, after stimuli, arising from pre-existing blood vessels. These activated cells release proteolytic enzymes that degrade the surrounding basement membrane. Endothelial cells migrate toward degraded extracellular matrix (Hood & Cheresh, 2002). The next step is the proliferation of endothelial cells and formation of capillary endothelial sprouts, which will be stimulated by a variety of growth factors, some of which are released by the degradation of their own extracellular matrix (Pettersson et al., 2000; Liekens et al., 2001, Dvorak, 2005, Okamoto et al., 2014). This step is followed by determining the polarity of endothelial cells which follows tip cell that moves according to gradient of angiogenic factors. Endothelial cells form capillary lumen through cell-cell and cell-matrix interactions finally forms capillary tubes (Bischoff, 1997, De Smet 2009). Newly formed blood vessel is stabilized after mesenchymal cell migration and their subsequent differentiation into pericytes or smooth muscle cells around the new vessels (Hirsch & D' Amore, 1997). These periendothelial cells are essential for maturation and stabilization of the vessels (Gabriele & Steven 2005, Veale & Fearon , 2006).

There are several pro- and anti-angiogenic components and signaling pathways that have been described in angiogenesis mechanism. Vascular endothelial growth factor (VEGF) and its receptors (VEGFRs), basic fibroblast growth factor (bFGF) Angiopoietin-Tie, Ephrin-EphRs, and Delta-Notch are major factors that play direct role in angiogenesis (Longatto et al., 2010). VEGF-A, the best characterized and important angiogenic factor, stimulates proliferation and migration of endothelial cells. VEGF-B and VEGF-C also play a key role in angiogenesis observed specially in extracellular matrix degraded area (Lohela et al., 2009). Placental growth factor (PIGF), a VEGF homolog, stimulates angiogenesis in a variety of conditions *in vivo* and is a key molecule in regulating angiogenic switch in pathological conditions (Luttun et al., 2002). Another signaling system, endothelium specific tyrosine kinase (Tie-2) receptor is a ligand for angiopoietins (Ang)-1 and -2, and presents an essential role in vessel growth, maturity, integrity, maintenance, and stabilization (Fagiani & Christofori, 2012). As a key regulator for vascular maintenance and stabilization, it plays critical role in tumor angiogenesis. Ang1 is constitutively expressed in many organs, whereas Ang-2 is predominantly expressed by activating endothelial cells at the site of vascular remodeling (Bach et al., 2007; Gale et al., 2002; Huang et al., 2011). Ang-2 is pro angiogenic molecule destabilizing the vessel to make it responsive to angiogenic growth factors such as VEGF (i.e., functions as a 'trigger' of remodeling) (Peters et al., 2004; Visconti et al., 2002) whereas Ang-1 promotes vascular stabilization and counteracts VEGF-induced angiogenesis (Fukuhara et al., 2009). Gene knockouts have shown that Ang-1 and Tie-2 play crucial roles in late vascular development. Therefore, vascular maturation appears to be controlled by the precise balance between Ang-1 versus Ang-2 (Thurston & Daly, 2012).

Thrombospondins (TSP)-1 and -2 are matricellular endogenous proteins that inhibit angiogenesis impairing endothelial migration, proliferation, survival and induction of endothelial cell apoptosis and suppressing the activity and availability of VEGF (Armstrong & Bornstein, 2003). TSP-1 and -2 antagonize the endothelial cell migration which is important to the formation of sprouting capillaries in CD-36 dependent or independent fashion (Dawson et al., 1997; Yamauchi et al., 2007; Oganesian et al., 2008).

During angiogenesis, proteolysis is one of the first steps involved in the formation of new blood vessels. Numerous proteases including matrix metalloproteases (MMPs) are involved during this step of angiogenesis (van Hinsbergh & Koolwijk, 2008). It has been shown during tumor growth, wound healing, inflammation and in activated endothelial cells, these MMPs are strongly induced in a paracrine and/or autocrine manner to stimulate

angiogenesis and increase VEGF release (Page-McCaw et al., 2007; Rundhaug, 2005). Opposing to MMPs, tissue inhibitors of metalloproteinases (TIMPs) abrogate angiogenic factor-induced endothelial cell proliferation and angiogenesis in MMP inhibition dependent or independent manner (Seo et al., 2003). Therefore, together with these circulating proteins, enzymes and receptors contribute to the equilibrium of angiogenesis process.

1.3 Inflammation and its association with angiogenesis:

Association between angiogenesis and inflammation can be evidenced by increased vascular permeability, monocyte/macrophage and neutrophil recruitment at angiogenic sites (Barcelos et al., 2004). Inflammation is the response of immune system triggered by the invading pathogens or damaged/injured tissue. It is a multifactorial process that regulates vascular responses, cellular (migration and activation of leucocytes) and systemic reactions aiming the homeostasis of the organism. The inflammatory response is eventually linked to the repair process which is accomplished by formation of new blood vessels. Therefore, angiogenesis is an essential process in the progression of many diseases aiding inflammatory response. Newly formed blood vessels are involved in the maintenance of the inflammatory state by transporting inflammatory cells to the site of inflammation and by supplying nutrients and oxygen to the inflamed tissue (Jackson et al., 1997).

The inflammatory reactions can be triggered by various stimuli, infections, trauma, physical and chemical agents, necrosis, foreign bodies and immune responses. Pathologists tend to classify acute and chronic inflammation in accordance with the morphological characteristics. The acute inflammatory process begins when cells sense the injury and release chemical mediators like histamines, proteases and cytokines promoting exudation of fluid, plasma proteins (edema) and migration of leukocytes, primarily the neutrophils (Rao et al., 2007). Chronic inflammation is associated with the infiltration of lymphocytes and macrophages, proliferation of blood vessels, tissue necrosis and fibrosis. Inflammation ends when the stimulus is removed and secreted mediators are destroyed or dispersed. There are also active anti-inflammatory mechanisms which modulate the response and prevent from tissue exacerbation. During the inflammatory process, chronic phase is usually observed with healing attempts by the replacement of damaged tissue by connective tissue leading to fibrosis of tissues (Wynn & Ramalingam, 2012).

During inflammation, inflammatory mediators promote endothelial activation, increasing their expression of adhesion molecules, favoring the recruitment of leukocytes

slowly rolling along the vascular endothelium and then transiently adhere to the endothelium. When recruited by chemokines, the leukocytes increase the avidity of its links with endothelial adhesion molecules and then pass between adjacent endothelial cells, a phenomenon called transmigration or diapedesis. These leukocytes in the interstitium migrate toward to harmful stimuli by chemotaxis (McIntyre et al., 2003, Kumar et al., 2010). Polymorphonuclear neutrophils are the predominant cells exuded during the first 24 hours after the initiation of the inflammatory process, with short half- life (Charo & Ransohoff, 2006). Another type of inflammatory cells such as monocytes migrate into the inflammatory site by chemotaxis, where they differentiate into dendritic cells and macrophages. Monocytes start migrating from the vessels 18 and 24 hours after initiation of diapedesis, these cells accumulate and becomes predominant cells after 48 hours (Visser et al., 2006; Rao et al., 2007). The macrophages produce a variety of cytokines and growth factors responsible for a wide variety of responses in many cell types including endothelial cells, epithelial cells and mesenchymal cells. Macrophages release biologically active substances that result in tissue remodeling and recruitment of additional leukocytes such as B, CD4+ and CD8+ lymphocytes, antigen-specific, that will amplify the immune response (Visser et al., 2006). Among these biological substances, reactive oxygen and nitrogen species, proteases, cytokines, chemokines, coagulation factors and arachidonic acid metabolites plus growth factors such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor (TGF)-beta, fibrinogenic cytokines and angiogenesis factors can be responsible for formation of new vessels (Maruotti et al., 2013). The inflammatory response is regulated by a balance between pro and anti-inflammatory factors that coexist in the injured site (Trace, 2002). The imbalance of these factors results in increased production of proteases, proteoglycans, mediators, prostaglandins lipid and that concomitantly, enhance inflammatory process (Mrowietz & Boehncke, 2006). As the inflammation involves the migration and extravasation of immune cells through the microcirculation, the endothelium plays a fundamental role in this process and as well as in angiogenesis too.

In the course of inflammation, there are large number of molecules known that can act as positive regulators of angiogenesis such as acidic and basic fibroblast growth factors (FGF-alpha and-beta, respectively), acidic and basic transforming growth factor (TGF-alpha and -beta, respectively), hepatocyte growth factor (HGF), TNF-alpha, PDGF, among many others (Karamysheva, 2008). Cytokines and chemokines involved in the inflammatory process and contribute directly or indirectly to angiogenesis pathway deserve consideration:

Interlukin-1 (IL-1). It has been shown to be a strong promoter of angiogenesis by modulating endothelial cells (Naldini & Carraro, 2005). The importance of IL-1 signaling in the host has been demonstrated by the dramatic reduction of inflammatory and angiogenic responses in matrigel plugs implanted in IL-1 receptor type I knockout mice (Carmi et al., 2009).

Interleukin-17 (IL-17): an inflammatory cytokine, expressed predominantly by Th17 cells, induces angiogenesis, cell migration, and cell invasion (Du et al., 2012; Moran et al., 2011).

Tumor Necrosis Factor alpha (TNF-alpha): It also enhances the expression of VEGF receptors and VEGF protein (Balkwill & Mantovani, 2001). Using subcutaneous implants of genetically deficient TNFR1, it was shown that there is decrease in angiogenesis (blood vessel formation) and VEGF levels (Barcelos et al., 2005).

Chemokines: Several studies have shown that the CXC chemokine carries important role in angiogenesis associated with inflammation, repair and tumor (Belperio et al., 2000; Moore et al., 1998; Szekanecz et al., 1998). It has been shown that CXC chemokines that contain the ELR motif (ELR +) are potent angiogenesis inducer *in vivo*. In contrast, the CXC without ELR motif (ELR-) are potent angiostatic factors (Strieter et al., 1995). It has been demonstrated that KC/CXCL1that binds to the CXCR receptor, monocyte chemotactic protein-1 (MCP-1)/CCL2, macrophage inflammatory protein (MIP)-1alpha /CCL3 and regulated on activation, normal T Cell Expressed and Secreted (RANTES)/CCL5 are involved in formation of new vessels (Addison et al., 2000; Stamatovic et al., 2006; Suffee et al., 2012).

IL-12 and IFN-gamma: Whereas pro-inflammatory and Th1 cytokines, such as IL-12 and IFN-γ behave as negative regulators of angiogenesis through direct and indirect effects on endothelial, tumor and immune cells (Naldini & Carraro, 2005).

Not only cytokines and chemokines, depending upon the type of phenotypes, inflammatory cells and its products may contribute to the fate either as anti- or pro-angiogenenic. For example, alternatively activated macrophages (M2), polymorphonuclear neutrophils phenotype (PMN) 2, decidual-like nature killer (NK) cell, alternatively activated dendritic cells (DC) possess angiogenesis properties, whereas classically activated macrophages (M1), PMN1 phenotype of neutrophil, activated NK cells and classically activated DC have anti-angiogenic properties (Noonan et al., 2008).

Although inflammation is an important defense mechanism present in the host system during infection, prolonged inflammation can result into adverse condition as described in various diseases. Inflammation induced angiogenesis, well referred as double edge sword, may either further exaggerate tissue damage due to excess recruitment of inflammatory leucocytes or may ameliorate the affected sites by providing necessary oxygen as well as nutrients along with the inflammatory cells to combat the injury leading to wound healing. During infection, angiogenesis can therefore be essential host mechanism to improve the disease conditions or may also be exploited by the parasites to guarantee their survival in the host (Ribatti et al., 2008).

1.4 *Trypanosoma cruzi* infection in human and experimental model:

Trypanosoma cruzi infection is responsible for the acute and chronic inflammation driving cardiomyocytes towards a progressive damage with consequent fibrosis and loss of functionality. Approximately 7-8 million of people in the world are infected with this parasite (WHO, 2013). *T. cruzi* is transmitted to humans through the bite wound of its triatomine insect host, the reduviid bug. Once within the body, *T. cruzi* is able to enter a variety of host cells where it differentiates into mammalian proliferative forms, the amastigotes. Within the cell, amastigotes divide and then differentiate into blood forms trypomastigotes. The parasitized cell ruptures, releasing trypomastigotes, which may infect adjacent cells or be disseminated through the blood and infect cells at other locations (de Souza et al., 2010)

Clinical symptoms of human and experimental *T. cruzi* infection in animal model consist of both acute and chronic phases. In the acute phase of the human infection, the signs and symptoms are either absent or mild and nonspecific. For these reasons, on the basis of clinical symptoms, most individuals are not diagnosed with *T. cruzi* infection until they are in the chronic phase of the disease. In human chagas disease, heart may be inflamed and exhibits mild enlargement during acute infection (Moncayo & Ortiz Yanine, 2006). Initially, cardiac inflammation is focal and lesions coalesce over time, followed by congestion, edema, and infiltration of the heart by mononuclear cells, mast cells and neutrophils are observed. Additionally, apart from cardiomyopathy, the disease may affect several other systems like digestive and neurologic (Py, 2011). It has been shown that parasitism occurs mostly in muscle fibers but may also be present in macrophages and ganglionic Schwann cells. Non-parasitized muscle fibers can also show distinct lesions (Wong et al., 1992). There are two possible outcomes after the acute phase of disease: death, in a very small number of

individuals, or entry into the chronic phase of infection. When present, the acute myocarditis resolves after several weeks and the heart regains its functions (Maya et al., 2010)

Over the next 10 to 30 years, only focal myocarditis of mild degree is observed in the majority of cases and most individuals remain in this state for all life. However, thirty percent of infected people develop a chronic, progressive, fibrosing cardiomyopathy of variable degree. The infiltrate in chronic Chagas heart disease consists of lymphocytes (T cells predominate and CD8+ lymphocytes are two to three times more abundant than are CD4+ cells) and to a lesser extent, macrophages, eosinophils, plasma cells, neutrophils and mast cells (Brener & Gazzinelli, 1997; dos Santos et al., 2001). The most part of this leukocyte recruitment into the heart tissue is driven by chemokine and chemokine receptors and, their over expression is clearly associated with heart dysfunction (Talvani et al., 2000; Talvani et al., 2004a; Talvani et al., 2004b). The perpetuation of parasites in leukocyte is rarely found in the heart but parasite DNA can be detected in some inflammatory lesions (Tarleton et al., 1997). Inflammatory cell infiltrates can lead to destruction of myocardium, epicardium and endocardium which is followed by replacement with connective tissue (fibrosis) and therefore responsible for decrease in heart contractility, reduced cardiac muscle mass, and loss of cardiac innervations (Dutra et al., 2005; Machado et al., 2005)

Trypanosoma cruzi possesses very fascinating mechanisms for its survival in the host cell and tissue. In *T. cruzi* infection, host immune system maintains to achieve balance between inflammatory and anti-inflammatory response In the acute phase, there is a huge inflammatory response to clear the infection whereas in the chronic phase, it is rather controlled in such a way that immune response is targeted to maintain the parasite load and inflammatory response low so as to keep infection under control and to prevent excessive tissue damage (Garcia et al., 2010; Maya et al., 2010). Absence or decrease in inflammatory response may lead to parasite multiplication and subsequently to the death of animals, in contrast to this, enormous inflammation may lead to tissue damage and again leading to death of the animals (Aliberti et al., 2001; Golgher & Gazzinelli, 2004; Talvani & Teixeira, 2011; Teixeira et al., 2002).

The different forms of the parasites have different membrane proteins or glycoproteins, stimulate production of inflammatory markers such as cytokine IFN-gamma, which activates various cells of the mononuclear phagocyte system that will act directly and indirectly against the parasite (by phagocytosis and nitric oxide production) in addition to

producing other cytokines (TNF-alpha, IL-12) and chemokines (CCL2/MCP1, RANTES/CCL5). These cytokines, chemokines and their receptors have a determinant role in the course of the inflammatory response during *T. cruzi* infection because the action of these mediators leads to leukocyte recruitment to infected tissues (Paiva et al., 2009). IL-12 stimulates the Natural Killer (NK) cells to produce more IFN-gamma and recruit lymphocytes, leading indirectly to produce more TNF-alpha, IL-12, chemokines and free radicals, a positive feedback system (Talvani & Teixeira 2011). This amplifying system can employ excessive amount of inflammatory cells and can be harmful for the host tissue. Hence, the increase of inflammatory markers is regulated with the production of cytokines such as IL -10 and IL-4 (Brenner & Gazzinelli, 1997, Aliberti et al., 2001; De Oliveira, 2007; Talvani et al., 2009).

It is known that membrane proteins of *T. cruzi* activate Toll like receptors (TLR)-1,2, 4 , 5, 6 or 9 present in macrophages and dendritic cells which in turn trigger the activation of nuclear factor kB (NF-kB) and mitogen- activated protein of kinase (MAPK) that lead to the synthesis of pro-inflammatory cytokines and chemokines (Campos and Gazzinelli, 2004; Bafica et al., 2006; Koga et al., 2006; McGettrinck & O'Neill, 2010; Nagajyothi et al.; 2012; Higashikuni et al., 2013) . Furthermore, other receptors, such as mannose receptors and nucleotide-binding oligomerization domain (NOD)-like receptor family and as retinoic acid-inducible gene I have also recently been shown to induce activation of inflammatory mediators. (Garrido et al., 2011, Silva et al., 2010). Different parasite proteins and glycoproteins like glycosylphosphatidylinositol (GPI), GPI-mucin, GPI transialidase, TC52, cruzipain and nucleic acid of *T. cruzi* bind with these receptors present on cells of the monocytic lineage and participate to activate these cells to produce nitric oxide (NO) and secrete inflammatory mediators (Fernandez-Gomez et al., 1998; Saavedra et al., 1999; Gazzinelli et al., 1999; Coelho et al., 2002, Gao & Pereira, 2001; Giordanengo et al., 2002; Shoda et al., 2001).

Hence, inflammation and angiogenesis are pivotal processes and interrelated in the progression of many diseases under inflammatory conditions, studying angiogenesis process in *T cruzi* infection can be a new and promising area of investigation. Because of prolong inflammation, there is impairment of cardiomyocytes leading to aggressive and progressive damage in the heart and is one of the main reasons for congestive heart failure in Chagas disease (Hidron et al., 2010). Recently, our group has found that *T. cruzi* antigens are responsible for inflammatory angiogenesis in the sponge model (Publication in process)

suggesting *T. cruzi* might induce inflammatory mediators leading to angiogenesis. The result of this experiment is represented by the following diagram:

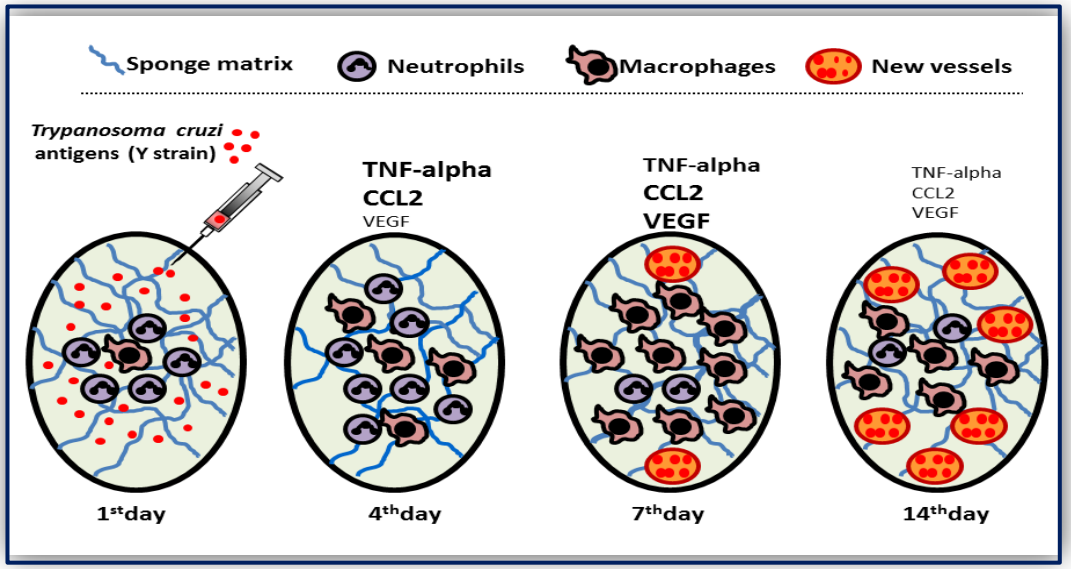

Figure I: Diagrammatic representation of *Typanosoma cruzi* antigen activated inflammation mediated angiogenesis in sponge model. After injecting *T. cruzi* antigen in sponge implant, there was increase in inflammatory cytokines (CCL2 and TNF-alpha) on day 4 and increase in VEGF as well as macrophage recruitment on day 7 consequently increasing the number of new blood vessels day 14. Increase in size of word denotes the increase production of soluble mediators and vice-versa.

However, studies have shown that calcium-binding proteins (Calreticulin - CRT) from *T. cruzi* can exert anti-angiogenesis and anti-tumor activity in the host and protect against ongoing neoplasic damage (Ferreira et al., 2004; Ferreira et al., 2005; Ramirez et al., 2011; Ramirez et al., 2012). Lopez and cols showed in *ex-vivo* angiogenesis assay, *T. cruzi* CRT completely abrogated capillary growth (Lopez et al., 2010). It has also been described that an augment of Thrombospondin-1 protein by calreticulin enhances cellular infection (Johnson et al., 2012). But, several infections have been reported to induce angiogenesis, known as infection induced angiogenesis through the regulation of TLRs (Grote et al., 2011).Interestingly, recent findings have shown link between Toll Like Receptor (TLR) stimulation and angiogenesis during inflammation (Cho et al., 2007; Grote et al., 2010; Jagavelu et al., 2010; Pollet et al., 2003; Varoga et al., 2006) and previous studies have demonstrated the role of several membrane proteins and glycoproteins such as GPI of *T. cruzi* bring about inflammation via TLR (Bafica et al., 2006; Campos & Gazzinelli, 2004; Koga et al., 2006; McGettrick & O'Neill, 2010). Similarly, different inflammatory chemokines and cytokines such as TNF-alpha, IFN-γ, MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, IL-10, TGF-β, IL-17 that play direct or indirect role in activating or regulating angiogenesis in *T.*

cruzi infection, are involved in immunopathology of Chagas disease (Paiva et al., 2009; Araujo-Jorge et al., 2008; Silva et al., 1991; Talvani et al., 2004b; Talvani & Teixeira, 2011). Therefore, it is plausible that there is presence of angiogenesis may be present during *T. cruzi* infection. Angiogenesis is further related with the MMPs (that stimulates angiogenesis) and increases VEGF release (Rundhaug, 2005; Zheng et al., 2006). It has been evidenced in *T. cruzi* infection, there is increased activities of cardiac matrix MMP-2 and MMP-9 (Gutierrez et al., 2008) that can reinforce angiogenesis in *T. cruzi* infection. Therefore, with all these studies and evidences, we hypothesize the possible role of *T. cruzi* in angiogenesis which is represented by following figure:

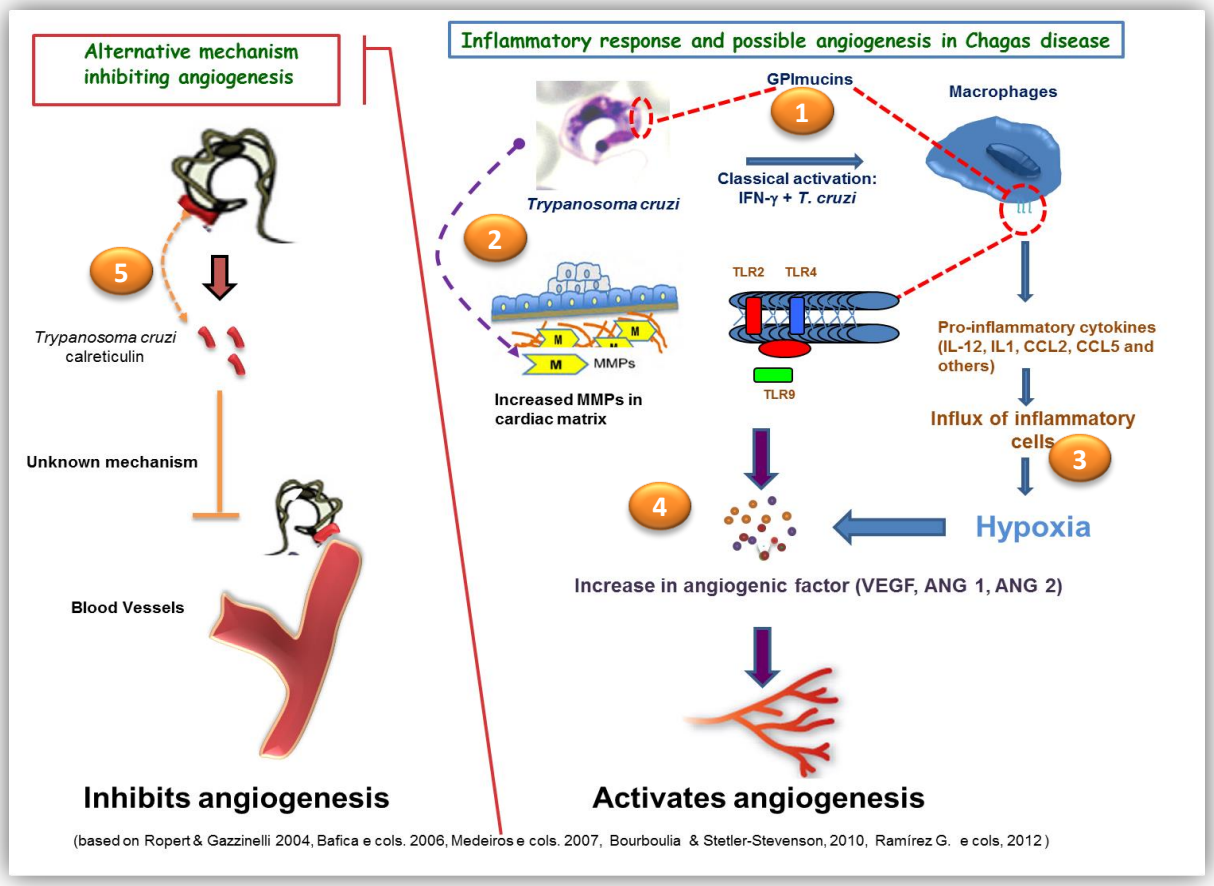

Figure II: The model of our proposed idea

1. Different antigens (PAMPs) of *T. cruzi* will be recognized by different inflammatory cells, for instance macrophages, through its TLRs releasing various proinflammatory chemokines and cytokines.
2. Parasites can also lead to production/release of MMPs in cardiac matrix.
3. Inflammatory mediators recruit more inflammatory cells at site of injury and cause hypoxic condition.

4. All these different factors can induce direct or indirect production of angiogenic proteins which can activate angiogenesis process, formation of new vessels.
5. On other hand, calreticulin can inhibit angiogenesis but the process of inhibition for the formation of new vessels is still unknown.

1.5 Pharmacologies:

Chemotherapies to treat *T. cruzi* infection are still a challenge even though various drugs have been discovered and are in use. Current standard chemotherapies are unsatisfactory because of their side effects and are inefficient to cure in chronic phase. Because of persistent parasite, in *T. cruzi* infection, inflammation is a characteristic feature which is responsible for the destruction of tissue and leading to the loss of tissue function. Therefore it is very important to develop chemotherapy strategies that not only control parasite but also inflammation so that inflammation related tissue remodeling (eg. angiogenesis) can be ignored. Another strategy that can help *T. cruzi* infected patient is to use drugs that improve functionality of the organ in combination with standard anti- *T. cruzi* drugs.

(i) *Benznidazole*, standard drug, is considered beneficial in controlling parasite load and disease progression. But during chronic Chagas cardiopathy in the symptomatic individuals, Benznidazole has nominal effect in heart and patient. Hence, treatment with Benznidazole along with combination with other anti-trypanosomal drugs and/or drugs used in cardiovascular diseases might be effective to improve heart condition in chronic symptomatic patients (Coura & Borges-Pereira, 2011). Our laboratory has observed that pharmacological therapies such as Enalapril and Simvastatin have benefited hearts in animals infected with *T. cruzi* (de Paula et al., 2010 Melo et al., 201; Silva et al., 2012).

(ii) *Simvastatins*, inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, are statins that reduce lipid and present cardiovascular protective properties (Kapur & Musunuru, 2008). Simvastatin has pleiotropic properties with anti-inflammatory effects and can stimulate angiogenesis (Zhu et al., 2008). It has been demonstrated that Simvastatin therapy has ameliorated cardiac remodeling in animals infected with *T. cruzi* (Melo et al., 2011; Silva et al., 2012)

(iii) *Enalapril*, an inhibitor of angiotensin converting enzyme (ACE), is generally used in the treatment of cardiovascular diseases (e.g. heart failure and coronary artery diseases) (Bowling et al., 2012; Tani et al., 2009). Previous study from our laboratory demonstrates that Enalapril

can have anti-*T. cruzi* and anti-inflammatory activities reducing inflammatory cells during acute phase of experimental *T. cruzi* infection in mice model (de Paula et al., 2010).

It is generally accepted, that inflammatory angiogenesis is ongoing process during development of Chagas disease. In experimental cardiomyopathy induced by *T. cruzi* parasites, little is known about the expression and the role of many different pro- and anti-angiogenesis stimuli. Understanding angiogenesis and their mediators in experimental *T. cruzi* may help to better understand host parasite interaction and as well as focusing on substitution or regeneration of damaged cardiac tissues and structures angiogenesis may represent a paradigm shift in Chagas heart disease therapeutic approach.

2. JUSTIFICATION

2. JUSTIFICATION

Angiogenesis and inflammation are always cross-linked as both processes are well interconnected. *T. cruzi* infection is characterized by intense inflammation in both acute and chronic stages of the infection. Therefore it is likely possible that persistence of inflammation in infected individuals may promote angiogenesis. New blood vessel formation helps functioning of tissues and organs. It can, however, also augment inflammation, as increased formation of new blood vessels may further induce recruitment of inflammatory cells which may follow with increased production of cytokines and chemokines. Most of the fatalities of the disease seen in *T. cruzi* infection are mainly by organ failure, especially cardiac malfunctioning. Likewise in experimental model for example, in mice, the severity and/or mortality of the disease is extremely high if not treated. The death is usually caused by high inflammatory response and high parasitemia.

Recently, there are few evidence highlighted in the literature that calreticulin may exhibit anti-angiogenic and anti-tumor effects. But the mechanism of this protein for anti-angiogenesis is still not known in *T. cruzi* infection although there are few papers that have shown increased thrombospondin 1, an anti-angiogenic factor, by this protein. Based on our previous studies using sponge model, there is an indication of increase in angiogenesis in the sponge implanted with total antigen of *T. cruzi* (Y strain) in mice.

It is yet not clear whether angiogenesis is friend or foe in *T. cruzi* infection. The process may be one of the mechanisms that parasite may induce in the host in order to sustain within it or on the other hand, a possible host response against infection whilst it may improve cardiac functioning in infected host. Angiogenesis is ongoing process and plays significant role in wound healing and chronic inflammation; our study on an inflammation induced angiogenesis in *T. cruzi* infection would be of great value with regards to the understanding pathophysiological characteristics of the disease condition. There are very few information available on whether *T.cruzi* induces or inhibits angiogenesis *in vivo*. Therefore, our study will be helpful to evaluate unique properties of different *T. cruzi* strains to evolve *T. cruzi* infection associated with inflammation and angiogenesis. Furthermore, study of these intertwined processes will help to improve understanding of mechanisms related to host-*Trypanosoma* interactions.

3. OBJECTIVES

3. OBJECTIVES

3.1 General objective:

To determine inflammation and angiogenesis parameters in *Trypanosoma cruzi* infection locally and systemically using three different strains of the parasite and analyze possible interference of pharmacological drugs in inflammation and angiogenic mediators.

3.2 Specific objectives:

(i) Evaluate inflammatory angiogenesis in a polyester-polyurethane sponge discs in *T. cruzi* (Y strain) infected mice.

(ii) Quantify inflammatory mediators and VEGF in plasma from C57BL/6 mice infected with Y, VL-10 and Colombian strain of *T. cruzi* in acute phase of infection

(iii) Determine inflammatory cell infiltration and area of amastigote nest in heart from C57Bl/6 mice infected with Y, VL-10 and Colombian strain of *T. cruzi* in acute phase of infection.

(iv) Determine mRNA levels of pro- and anti-angiogenic factors by real time PCR in heart from C57BL/6 mice infected with Y, VL-10 and Colombian strain of *T. cruzi* in acute of infection.

(v) Monitor blood vessels using anti-CD31 antibody by immunohistochemistry in heart during acute experimental *T. cruzi* infection.

(vi) Analyse the interference of anti-*T. cruzi* therapy (Benznidazole) and heart disease pharmacologies (ACE inhibitor and statins) in inflammation and angiogenic parameters in acute phase of infection.

4. MATERIALS AND METHODS

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4.1 Strains of *Trypanosoma cruzi*

VL-10 strain is considered as a resistant to Benznidazole (Caldas et al., 2008; Filardi & Brener, 1987) and according to the new nomenclature (Zingales et al., 2009) it is classified as discrete typing unit II (DTU II) (Moreno et al., 2010)

Y strain is considered partially sensitive to Benznidazole and was classified as *T. cruzi* II (Zingales et al., 2009)

Colombian strain, resistant to Benznidazole, was isolated from Colombia and was typed as Biodeme Type III and recently classified *T. cruzi* I, as referred by Momem (Camandaroba et al., 2001; Zingales et al., 2009)

All these strains were maintained by successive passages in Swiss mice in the Animal Care Facility of Universidade Federal de Ouro Preto (UFOP), Ouro Preto, Minas Gerais, Brazil.

4.2 Animals

C57BL/6 male mice of age 8-10 weeks were used for the purpose of our study. Mice were housed and maintained at the animal central facility in the (UFOP). All animal experiments and procedures were approved by the Institution's Committee on the ethical handling of laboratory animals (CEUA Protocol No 043/2010).

4.3 *Trypanosoma cruzi* infection and drug administration in experimental models

In the first step in order to show the process of inflammatory angiogenesis after infection, polyester polyurethane sponge discs were implanted subcutaneously on neck pouch of animals (n=5) followed by intraperitoneal infection with Y strain (100 trypomastigotes) of *T. cruzi*. In second step, animals (n=10) were infected with 100 trypomastigotes forms of *T. cruzi* using following strains: (i) Y (ii) VL-10 and (iii) Colombian. In final stage, to verify the interference of drugs, new groups of mice were infected with Colombian strain, and then the following drugs were orally by gavage for 20 days for infected and uninfected groups; - (i) Simvastatin (20mg/kg/day), (ii) ACE inhibitor Enalapril (25mg/Kg/day), (iii) Benznidazole (100mg/kg/day) and (iv) vehicle phosphate buffer (for untreated group).

Parasitemia was determined daily by analysing tail bleed samples (5 µl) on the optic microscopy (Brener., 1962) and mortality was evaluated during the experiment. Animals were euthanized in acute phase (12 days of infection for Y strain and 24 days of infection for Colombian and VL-10 strain). For sponge implanted groups, animals were euthanized on day 1, day 4, day 7 and day 14 according to the protocol described by (Andrade et al., 1987). Blood was collected for immune assay. Half part of hearts were fixed with 10% formalin to perform immunohistochemistry and histology and another half part of hearts were designated to study immunological, morphological and angiogenic parameters. Sponges were collected for biochemical, immunological and histological analysis.

4.4 Heart mass measurement

Ex vivo hearts were carefully excised and gently blotted on absorbent paper to remove blood before wet weight measurement. The relative heart weight was calculated using heart weight / mouse body weight and used to evaluate cardiac mass measurement at the time of euthanasia with each group infected with different strains of *T. cruzi* (n=10/group).

4.5 Enzyme-linked immunosorbent assay (ELISA)

Plasma from all experimental groups of animals and supernatants from polyester polyurethane sponge disc (described latter) was collected and maintained at -80^{o}C. In parallel, fragment of 10mg of cardiac tissue from each animal was homogenized in cold RIPA buffer with protease inhibitor and supernatant was collected and also stored at -80^{o}C. Later, these biological samples were defrosted and were used to measure soluble biological markers (TNF-alpha, CCL2, CCL5, VEGF, IL-10, IL-17 and MIP-1α). Sandwich ELISA was performed following manual from the manufacturer (Peprotech and R&D systems). 96 well plates were first coated adding 100ul of monoclonal antibodies anti TNF-alpha (Peprotech), anti CCL2 (R & D Systems), anti CCL5 (Peprotech), anti VEGF (Peprotech), anti CCL3 (Peprotech), anti IL-10 (Peprotech) and anti IL-17 (Peprotech) and were incubated overnight at room temperature. Then, plates were washed four times with wash buffer (PBS and 0.05% TWEEN) followed by blocking step to avoid nonspecific binding. Blocking step was performed by adding block buffer (PBS and 1% BSA; pH 7.4) and incubated at room temperature for 1 hour. It was then washed once again as before. Standards (100µl/well) in mentioned dilutions and samples were added (25 µL/well), followed by incubation at room temperature for 2 hours. Plates were then washed, and 100µL/well of the appropriate

biotinylated detection antibody diluted in reagent diluent (PBS, 1% BSA and 0.05% TWEEN for R&D system, PBS, 0.1% BSA and 0.05% TWEEN for Peprotech; pH 7.4) was added and incubated for 2 hours at room temperature. Plates were then washed once again. For the R & D system, streptavidin-horseradish peroxidase in 1:200 dilution was added (100 µL/well), and the plates were incubated for 20 min at room temperature. Plates were again washed and then 100 µL/well of the substrate solution (1:1 mixtue of hydrogen peroxide and tetramethylbenzidine- Sigma Chemical Co.) was added, and plates were incubated in dark for 30 min at room temperature. Reaction was terminated adding 50 µL/well 1 M H2SO4 solution. Plates were read at 450nm in ELISA plate reader (SoftMaxPR040). For Peprotech, after detection antibody step, avidin horseradish peroxidase in1:2000 dilution was added (100µl/ml) and plate was incubated for 30 min. Plates were washed and then 100µl of the substrate solution (ABTS liquid substrate Solution, Sigma Cat#A3219) was added. Plates were incubated at room temperature for color development and the color development was read at 405nm wavelength in ELISA plate reader. The plates were monitored at 5 min intervals for approximately 30 min and then, reading at 20 min were taken for our study.

4.6 Histopathology analysis

To analyze and quantify inflammatory infiltrates and amastigote nest areas, fragments of cardiac tissue were stained by hematoxylin and eosin (HE) staining. Hearts and polyester polyurethane sponge discs were fixed for 24 hours in 10% buffered formalin (conventional histology). Tissues were embedded in paraffin/paraplast, sectioned, stained with Hematoxylin-Eosin staining and examined by light microscopy. Two sections were taken from each heart including both atria and ventricle. Each section was examined for evidence of mononuclear and polynuclear cellular infiltration and *T. cruzi* nests. Nucleus of each cell in the section was quantified from 20 images whereas amastigote nest area was quantified from 35 images taken at an objective lens of 40X by Microcamera Leica DM 5000 B (Leica Application Suite, version 2.4.0R1). Quantification of infiltration was analyzed by using Leica Qwin V3 program and amastigote nest area was quantified by Image J 1.45s, NIH, USA (www.imagej.nih.gov/ij)

4.7 mRNA expressions of angiogenic factors by quantitative real time PCR

4.7.1 Extraction of RNA

Extraction of RNA was done according to instructions provided by RNA-SV total RNA isolation system kit-promega. Fragment of heart approximately 40 mg from each of infected and control mice (studied under different conditions) was macerated and homogenized in 175µl of RNA lysis buffer. This lysate was transferred into new tube and 350µl of RNA dilution buffer was added. This mixture was homogenized, incubated at 70^{o}C for 3 min and then centrifuged for 10 min at 13,000×g for selective precipitation of cell proteins. Supernatant that contained RNA was transferred carefully into a new tube. 200µl of 95% ethanol was added to precipitate RNA. This solution was transferred into a spin column assembly and centrifuged at 12,000 x g for 1 min. In this step, RNA will be attached on silica membrane layer on the spin basket. The liquid obtained in the collection tube was discarded and RNA was washed using 600µl of RNA wash solution. After centrifugation, 50ul of DNase mix (40µl of yellow core buffer, 5 µl of 0.09M $MnCl_2$ and 5 µl of DNAse I enzyme) was added in column to digest contaminated genomic DNA. After incubation of 15 min at 20-25^{o}C, reaction was stopped using 200µl of DNA stop solution and centrifuged at 14,000 x g for 1 min. It was then followed by washing process using RNA wash solution to purify RNA, eliminating contaminants, proteins and cellular impurities. Finally, total RNA was eluted through membrane with the addition of nuclease free water.

4.7.2 Quantification of RNA-determination of yield and quality

Quantification of RNA was performed in NanoVue Plustm (GE Healthcare). Pure RNA will exhibit an A260/A280 ratio of 2.0. However, it should be noted that, due to the variations between individual starting materials and in performing the procedure, the expected range of A260/A280 ratios for RNA will be 1.7–2.1 and for A260/A230 ratio will be 1.8–2.2.

4.7.3 Reverse transcriptase

Reverse transcription was done according to the instruction of GeneAmp RNA PCR kit- Applied Biosystems. Mastermix was prepared containing 2µl of 10X RT buffer, 0.8µl of 25X deoxynucleotides mix (dATP, dGTP, dCTP and dTTP), 1 µl of RNase inhibitor, 1µl of multiScribe reverse transcriptase, 2µl of 10X random primers, sample in concentration of 0.035µg/ µl and DEPC water in sufficient quantity to make the final volume 20µl. Then, the tubes were submitted in thermocycler with following temperatures: 25^{o}C 10 min, 37^{o}C 120

min, 85^{o}C 5 min and rest at 4^{o}C. After this process cDNA was ready for PCR and maintained at -80^{o}C.

4.7.4 Amplification

Relative quantitative gene expression for Real time PCR was done in ABI 7300 (applied Biosystem), utilizing SYBER® Green Master mix for detection of amplicons. Messenger-RNA expression of CCL2, CCL5 and their receptors CCR2, CCR5, TNF-alpha, IL -10, VEGF, ANG-1, ANG-2, TSP-1 and TSP-2 was analyzed by quantitative real time PCR using the primers as shown in table 1. For each reaction, mix containing 5µl of SYBER®Green Master Mix, 0.7µl of each sense and anti-sense primer (10pmol/µl), 2µl of cDNA and ultra-pure water in sufficient quantity to make a volume of 10 µl were used. Conditions for thermocycler was as following: 50^{o}C for 20 min, 95^{o}C for 10 min, 40 cycles of 94^{o}C for 30 sec, 59.6^{o}C for 30 sec and 72^{o}C for 1 min. PCR amplification was performed in duplicate wells using standardized conditions. Samples containing the highest amount of each target will be used to create standard curves of the transcribed cDNA. In the same run, the threshold cycle (Ct) values of the samples (20-fold diluted cDNA) will be measured and relative expression levels were determined. PCR amplifications will be performed in duplicate wells using standardized conditions. Ct values obtained from duplicate were used to calculate the expression of target gene after normalizing by reference gene (β actin gene). Then, was verified difference of Ct observed between test samples and Ct obtained from the amplification of pool of cDNA from mice which were not infected and same pool was used in all experiments. Then it was checked how many times the test samples expressed mRNA expression in relation to that normally present in uninfected mice using formula $2^{-\Delta\Delta Ct}$.

Table 1: List of primers (sense and antisense). Primer sequences were determined based on nucleotide obtained from GeneBank Database.

Primer	Primer sequences
Angiopoeitin 1	5’ GGGGGAGGTTGGACAGTAA 3’ 5’ CATCAGCTCAATCCTCAGC 3’
Angiopoeitin 2	5’ GATCTTCCTCCAGCCCCTAC 3’ 5’ TTTGTGCTGCTGTCTGGTTC 3’
Vascular Endothelial Growth factor A	5’ AAAAACGAAAGCGCAAGAAA 3’ 5’ TTTCTCCGCTCTGAACAAGG 3’
Thrombospondin 1	5’ GAAGCAACAAGTGGTGTCAGT 3’ 5’ ACAGTCTATGTAGAGTTGAGCCC 3’
Thrombospondin 2	5’ CCTCAACTACTGGGTAGAAGGC 3’ 5’ TGACACTGTCGATAAGATCGCA 3’
CCL2	5’ AACTGCATCTGGCTGAGC 3’ 5’ CAGCACCAGCCAACTCTC 3’
TNF alpha	5’ TGAGTGACCAAGGGACAGAACC 3’ 5’ AGCCAGGAGGGAGAACAG 3’
CCL5	5’ ACCCTCTATCCTAGCTCATC 3’ 5’ CGTGTTTGTCACTCGAAG 3’
CCR2	5’ CCTGTCCACTAATGCGTTTC 3’ 5’ GCAAAGCCAGACCACAATG 3’
CCR5	5’ CCCTGTCATCTATGCCTTTG 3’ 5’ GCTTGCACGATCAGGATTG 3’
IL-10	5’ ACTACCAAAGCCACAAGG 3’ 5’ AAGAGCAGGCAGCATAG 3’

4.8 Preparation of sponge disc and implantation

Polyester-polyurethane sponge discs 5mm thick and 8mm diameter (Vitafoam Ltd, Manchester, UK) were used as the matrix for fibrovascular tissue growth. The sponge discs were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min before the implantation surgery as previously described (Andrade et al., 1987; Barcelos et al., 2004). Animals were anesthetized injecting intraperitoneally with Xilazine and Ketamine (8mg/kg and 60mg/kg respectively). Dorsal hair of mice was then shaved and skin was wiped with 70% ethanol. Sponge discs were aseptically implanted into a subcutaneous neck pouch. Animals were then infected with Y strain (100 parasites) of *T. cruzi*. Animals not infected were used as control groups.

4.9 Quantification of angiogenesis by hemoglobin measurement

The extent of the vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin method. In Days 1, 4, 7 and 14 post-implantation, animals were euthanized and sponge implants were carefully removed. The sponges were dissected from adherent tissue, weighed, homogenized (Tekmar TR-10, OH) in 2ml of Drabkin reagent (Labtest, Sao Paulo, Brazil) and centrifuged at 4^{o}C at

13000rpm for 40 min. The supernatant was filtered through a 0.22mm Millipore filter. Hemoglobin concentration of samples was determined by measuring absorbance at 540nm using an ELISA plate reader and was compared against a standard curve of hemoglobin (Barcelos et al., 2004).

4.10 Immunohistochemistry analysis

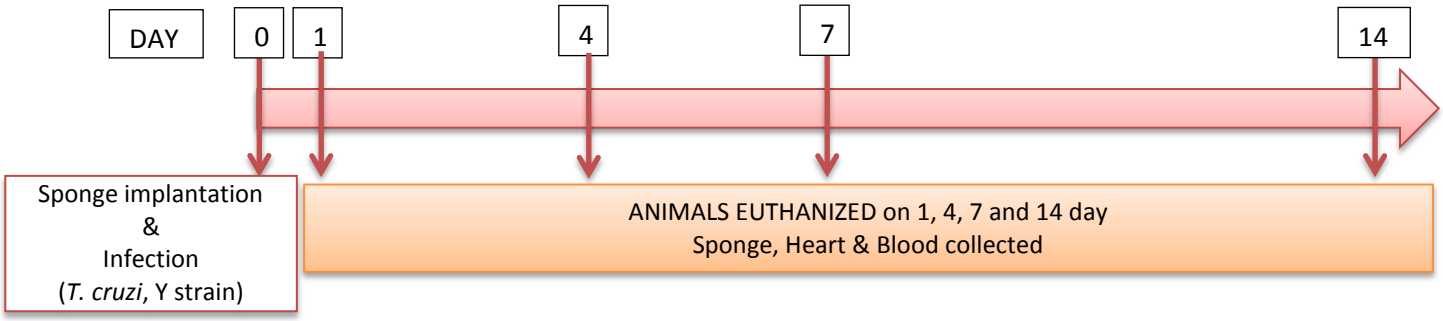
Consecutive 4μm sections were cut and mounted on gelatinized slides. Tissue sections were deparaffinized and rehydrated in graded ethanol. The CD-31 antigens were retrieved using solution retrieval (DAKO) at pH 6.0 and heated for 20 minutes in water bath at 98°C. The slides were immersed in 3% hydrogen peroxide for 15 minutes to ensure endogenous peroxidase blocking. Then Slides were incubated in 2% bovine serum albumin (Sigma-Aldrich) for 30 mins at room temperature to reduce non-specific protein binding. The histological sections were incubated in a humid chamber for 1 hour at room temperature with a primary rat monoclonal IgG2a antibody for PECAM 1 (Santa cruz Biotechnology) in 1:50 dilution. Antigen amplification was done with the Advance HRP link (DAKO) for 30 minutes at room temperature. For all slides, 3, 3′-diaminobenzidine (DAKO) was used as a chromogen. Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Negative controls were obtained by omitting the primary antibody.Numbers of blood vessels were counted from 10 photos taken from each heart. A grid with 100 intersections was used and blood vessels at the intersection were counted using program Corel DRAW version 11.633 windows.

4.11 Statistics

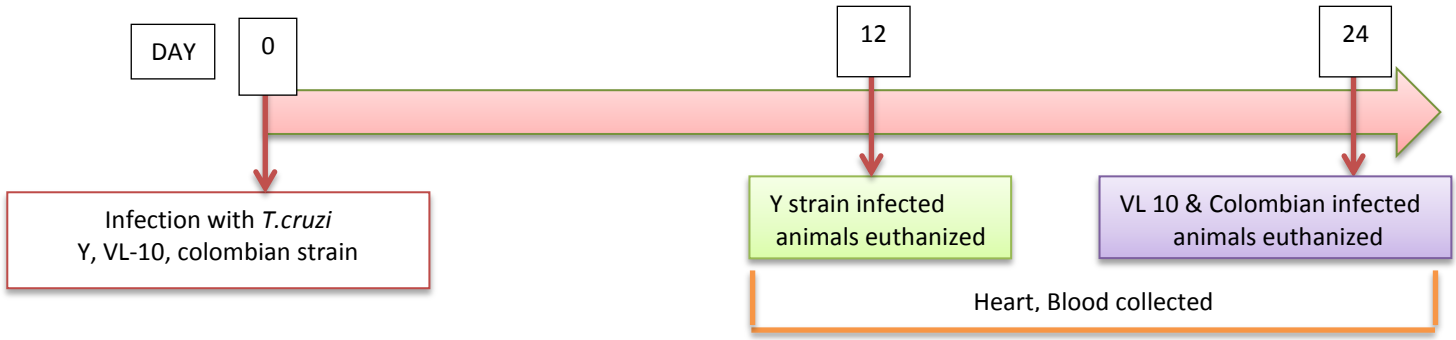
Data were expressed as mean ± SEM. Data were analyzed by One-Way analysis of variance (ANOVA) followed by a post test Bonferroni test or students's t-test for unpaired data as appropriate. For all analyses, GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA) was used. A P-value <0.05 was considered statistically significant.

4.12 Experimental Design

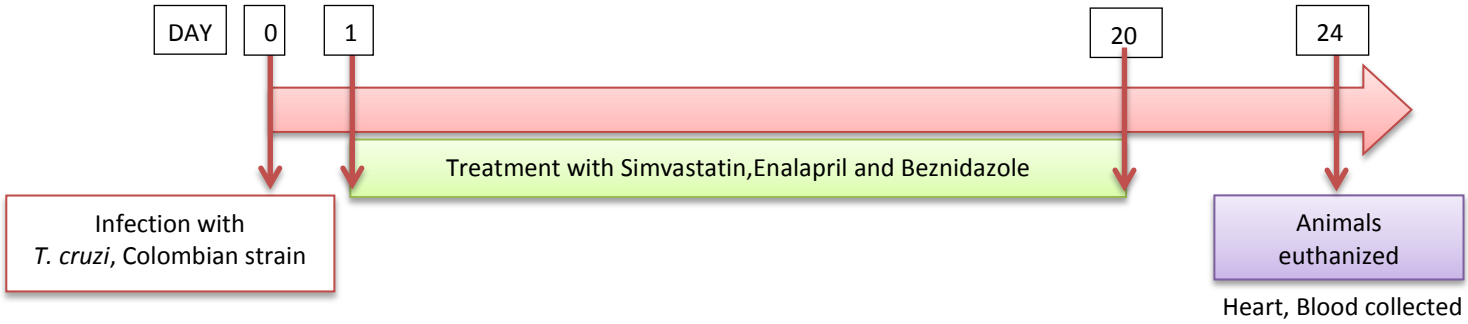
Experiment 1: Sponge implantation



Experiment 2: Different strains *of T. cruzi* infection



Experiment 3: Pharmacological therapies intervention



Post euthanasia techniques

1. **Immunoaasay**
2. **Biochemical analysis**
3. **Morphometric analysis**
4. **qPCR**
5. **Immunohistochemistry**

5. RESULTS

5. RESULTS

Previously, our group has experiment on sponge implant using antigen from *Trypanosoma cruzi* (Y strain) in Swiss mice. We found that sponge implant of 14 days from mice which were injected with the antigen, demonstrated increased number of blood vessels than sponge with the PBS as described in figure I (da Silva et al unpublished). Therefore, our first step was to use live parasite and observe the response in sponge implant. Sponges were implanted subcutaneously in C57BL/6 mice and 100 trypomastigote forms of *T. cruzi* (Y strain) were inoculated in peritoneum. Inflammatory and angiogenic parameters were assessed on day 1, 4, 7 and 14 after sponge implantation in infected and control groups. In the second step, we focused our study in heart in C57BL/6 mice infected with different strains (Colombian, Y and VL-10 strains) of *T. cruzi* to study inflammation and angiogenesis in the acute phase of infection. In the final step, we used different pharmacological drugs (Simvastatin, Enalapril and Benznidazole) to study the interferences of these drugs in inflammation and angiogenesis in mice infected with Colombian strain of *T. cruzi*.

5.1. Study of inflammation and angiogenic parameters in sponge implanted animals

5.1.1 Evaluation of inflammatory chemokine and cytokine levels in sponge supernatant and plasma of infected and uninfected mice:

C57BL/6 mice were implanted with sponge and then infected with Y strain of *T. cruzi.* The implants were well tolerated by all animals. No signs of infection at the implant site or rejection by the animals were observed during the 14-days of implantation. Inflammatory cytokines such as CCL2, CCL5, TNF-alpha and regulatory cytokine IL-10 were first measured in the sponge implant supernatant and in plasma from both control and test groups. We observed that the production of CCL2, TNF-alpha and IL-10 (Fig 1 A, C and D) was similar in infected and uninfected animals when measured in supernatant from day 14 sponge. But, we observed an increased production of CCL5 in sponge supernatant from day 7 onwards in infected mice. However, in plasma, there was increased CCL5 and TNF-alpha production (Fig 2 A and B) in infected mice on day 14 whereas IL-10 was increased in plasma from uninfected mice on day 14 (Fig 2 C).

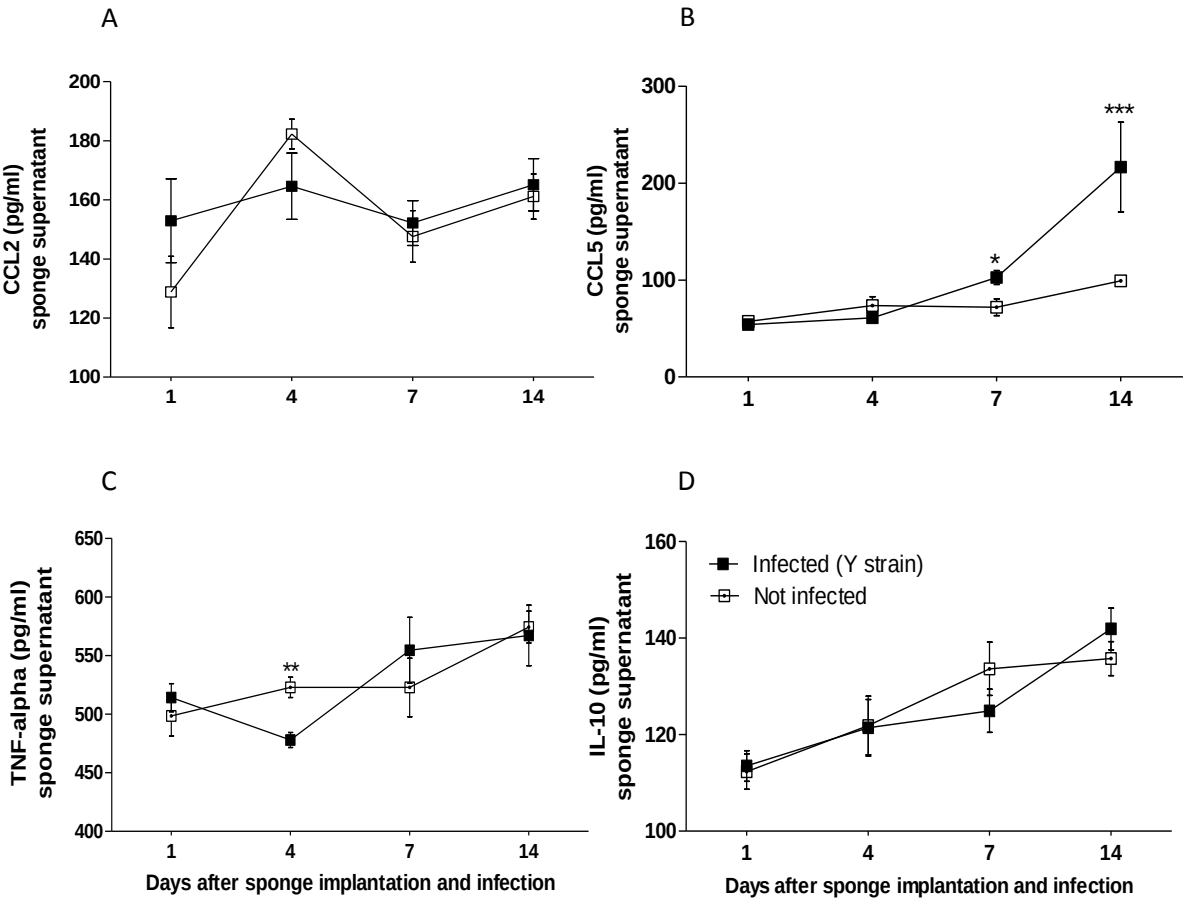


Figure 1. Evaluation of inflammatory mediators in sponge implants obtained from infected and uninfected C57BL/6 mice. Implanted sponges were excised from C57BL/6 mice on day 1, 4, 7 and 14. CCL2, (A), CCL5 (B), TNF-alpha (C) and IL-10 (D) were measured in sponge supernatant by ELISA. Data are shown as a mean of 5 animals. *,** &*** denote that difference in the production is significant at $P<0.05$.

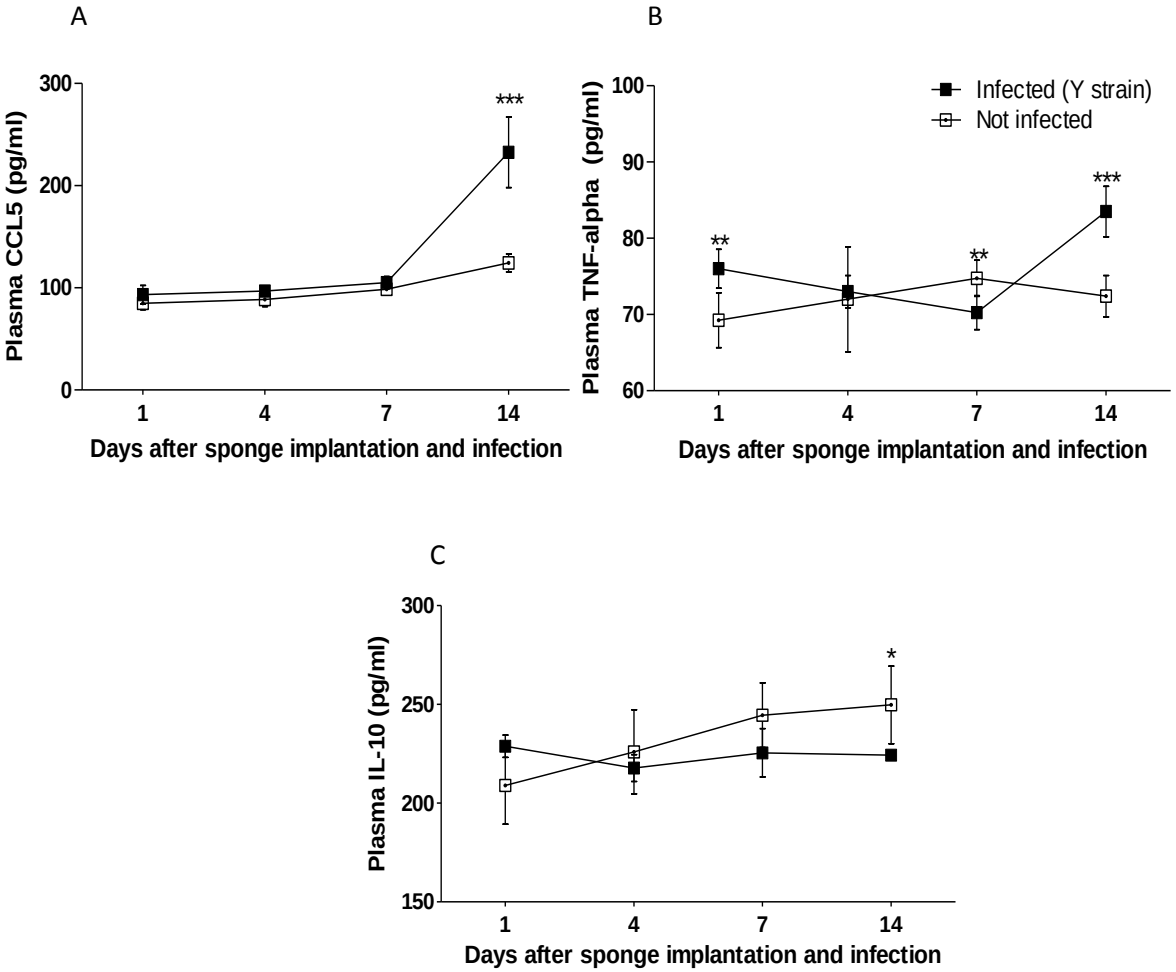


Figure 2. High chemokine and cytokines production in plasma from infected C57BL/6 mice. Blood was collected from control and infected C57BL/6 mice on day 1, 4, 7 and 14 after sponge implantation. CCL5 (A), TNF-alpha (B) and IL-10 (C) were measured in plasma by ELISA. Data are shown as a mean of 5 animals. *,** &*** denote that difference in the expression is significant at $P<0.05$.

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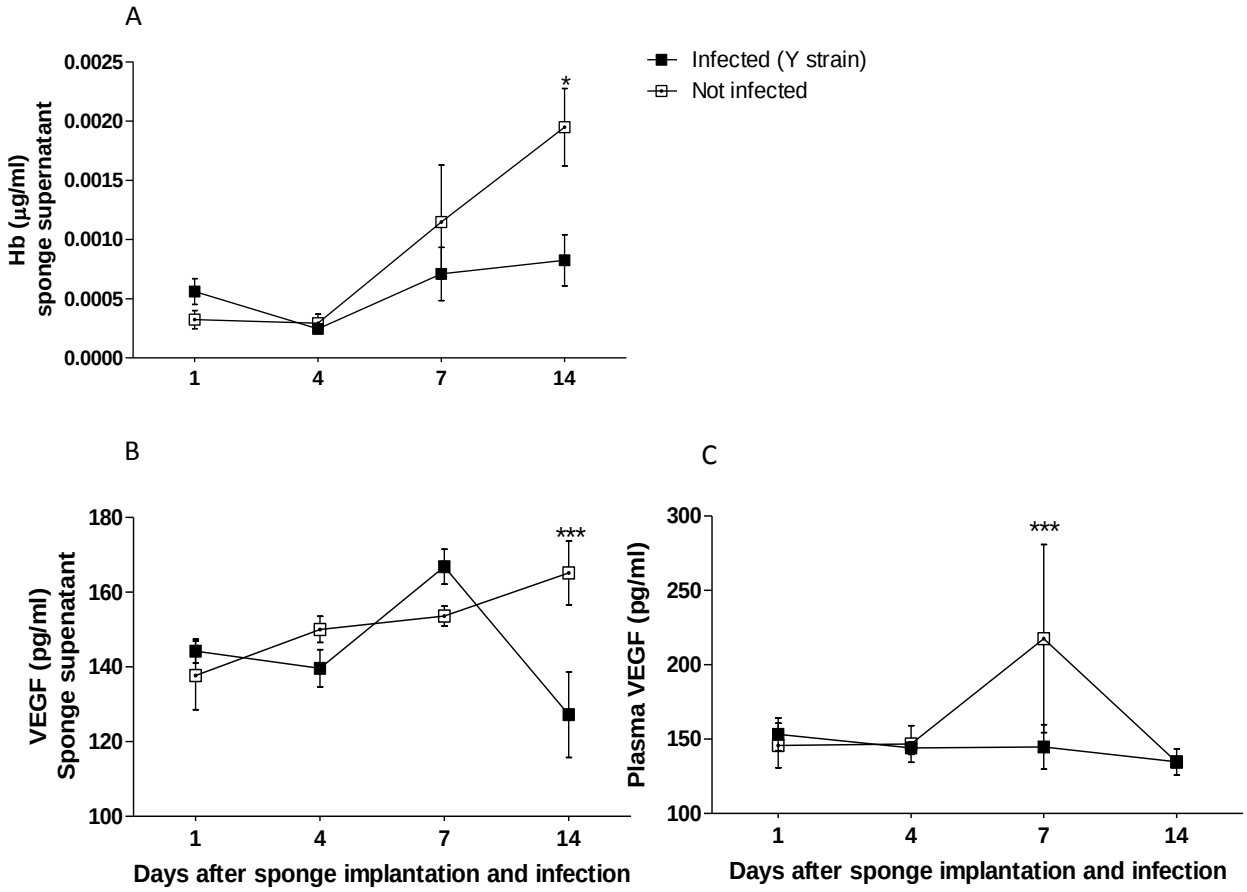


Figure 3. Hemoglobin and VEGF content was reduced in sponge from *Trypanosoma cruzi* infected C57BL/6 mice. Biochemical analysis of hemoglobin (Hb) content in supernatant (A) and the level of vascular endothelial growth factor (VEGF) measured by ELISA in sponge supernatant and plasma (B) and (C), respectively. Data are shown as a mean of 5 animals. * and *** denote that difference in the expression is significant at $P<0.05$.

Once we confirmed the presence of inflammatory mediators in both plasma and supernatant, we further analyzed angiogenic parameters in sponges and plasma from infected and uninfected mice. In sponge implant, angiogenesis is biochemically measured by quantifying hemoglobin and by histology. We also measured VEGF by ELISA in supernatant and plasma. We found that mice infected with Y strain of *T. cruzi* showed reduced vascularization in sponge implant as revealed by decrease content of the hemoglobin (Hb), VEGF and blood vessels. The figure 3 A shows that, in supernatant from the sponge implant, Hb content was significantly reduced on day 14 in infected than uninfected mice. In uninfected mice, we observed that Hb content increased gradually during the experiment from day 4. Similarly, in the plasma on day 7 (as shown in Fig 3C), and in the supernatant on

day 14 (Fig 3B) VEGF level was higher in uninfected animals. This result is further confirmed by the histological studies of sponge of day 14 (Fig 4) where we observed increased number of blood vessels in sponge from uninfected mice.

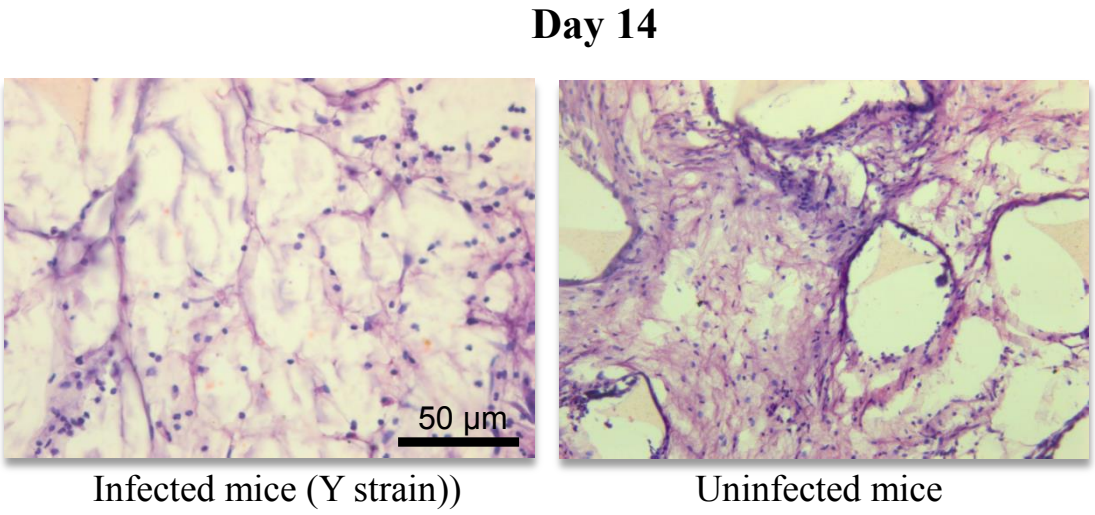


Figure 4. H & E staining of sponge sections of 14 days from infected (Y strain of *T. cruzi*) and uninfected mice. Sponge sections were stained using haematoxylin and eosin staining method and photo was taken at the 10X magnification. The sponge implant was filled by various inflammatory cell types, blood vessels, fibroblasts and collagen (connective tissue). Bar = 50 um.

Given below (Fig 5) is an illustration of an image of sponge matrix taken from day 1, 4, 7 and 14 infected and uninfected mice. We observed that until day 7, there was no difference in vascularization in sponge matrix in both infected and uninfected animals while on day 14, sponges from the infected animals showed diminished vascularization as compared to uninfected animals. Our observation from these sponge photo coincided with the Hb content and histology of the sponge.

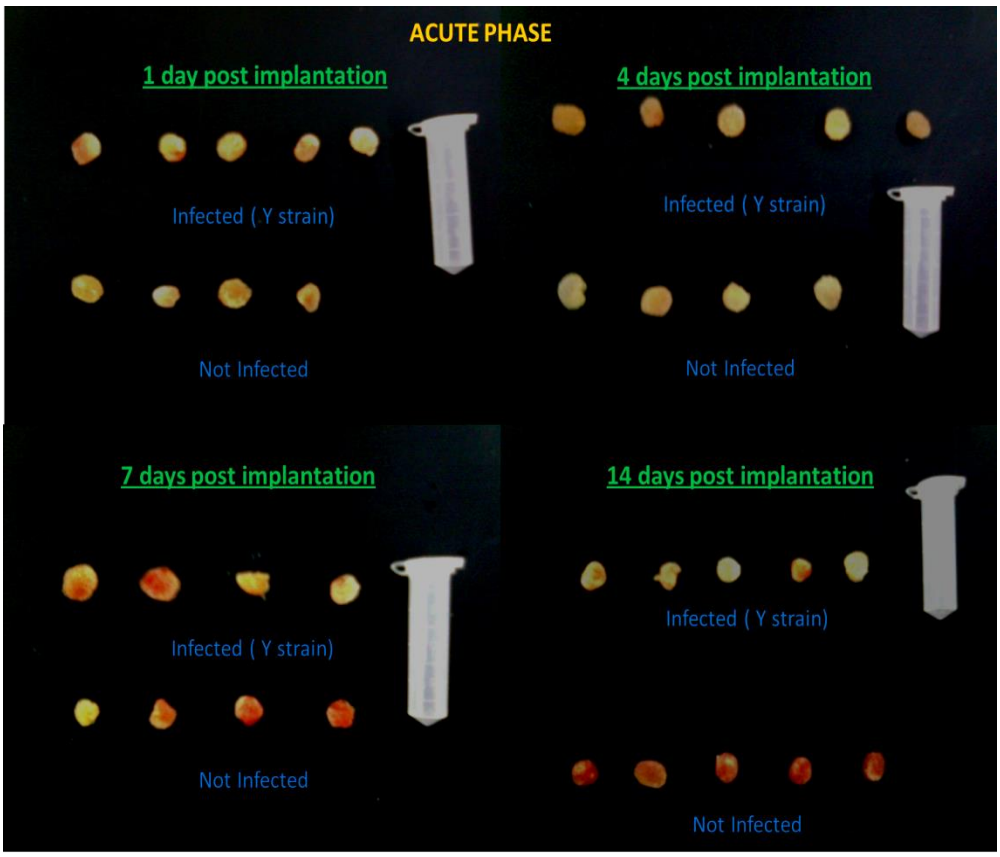


Figure 5. Decreased vascularization in sponge implants in infected C57BL/6 mice on day 14. This figure demonstrates that sponge from animals infected with Y strain parasites showed diminished vascularization (clear sponge) on day 14 than in sponges obtained from the uninfected animals (evident by dense red colour).

5.2 Inflammation and angiogenesis by different strains of *Trypanosoma cruzi.*

Studying angiogenesis using sponge implant in *T. cruzi* infection does not reflect the actual scenario of angiogenesis in tissue. Outcome of the disease progression in *T. cruzi* infection depends on number of factors, genetics of the parasite is one of them. Therefore, we selected three eminent strains of *T. cruzi* parasite with their unique characteristics to evolve disease. We compared inflammatory and angiogenic parameters among C57BL/6 mice infected with Y, VL-10 and Colombian (100 parasites) strains of *T. cruzi*.

5.2.1 Parasitemia curve and relative weight of heart to body ratio.

The parasitemia curves were obtained by examining fresh blood from C56BL/6 mice inoculated with Y, VL-10 and Colombian strains of *T. cruzi*. The profile of each curve is represented in figure 6 A and represents the biological characteristic of each strain used in this study. The pre-patent period varied from 6 for Y strain, 9 for Colombian and 13 days for VL-10 strain of *T. cruzi*. During this study, mice infected with Y and Colombian strains showed high parasite load while the pattern of those infected with VL-10 was comparatively low. In the acute phase, at time of euthanasia for groups infected with different strains of *T. cruzi*, it was also demonstrated that those infected with Colombian strain of *T. cruzi* presented significant increase in the weight of heart to weight of body while animals infected with Y and VL-10 strains showed ratio similar to not infected mice as in figure 6B.

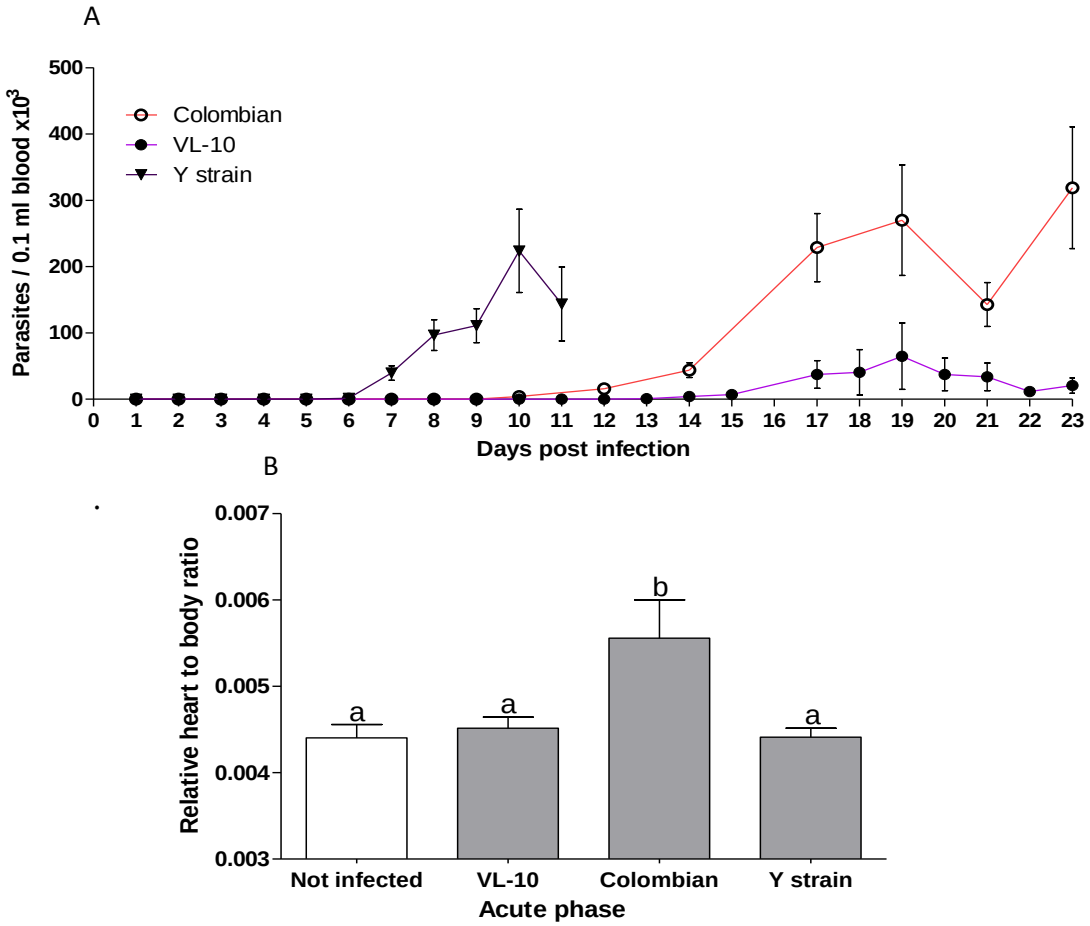


Figure 6. Parasitemia curve and relative weight of heart to body ratio from C57BL⁄6 mice infected with different strains of *T. cruzi*. C57BL⁄6 mice were infected with Colombian, Y and VL-10 strain (100 trypomastigotes/mouse) of *T. cruzi*. Parasitemia was evaluated until 11 days for Y, 23 days for Colombian and VL-10 strain (A). Weight of animals was taken before euthanasia and hearts were excised and weighed (B). Data are shown as a mean of 10 animals and different letters denote that difference in the expression is significant at $P<0.05$

5.2.2 Profile of inflammatory mediators in plasma in C57BL/6 mice infected with different strains of parasite in acute phase.

Grade of inflammation is, in part, determined by the strain of parasite. We evaluated circulating inflammatory mediators in plasma from mice infected with Y, VL-10 and Colombian strain by performing ELISA for CCL2, CCL3, CCL5, IL-17, IL-10 and TNF-alpha. Animals infected with Colombian strain showed significant increase and high level of all chemokines (CCL2, CCL3 and CCL5), followed by Y strain with significant increase in CCL2 and CCL5 and, finally, in VL-10 infected mice only CCL5 was increased significantly (Fig 7 A, B & C). In addition, we also detected elevated inflammatory cytokines IL-17 and TNF-alpha and the regulatory one IL-10 in mice infected with Colombian and Y strains of *T. cruzi* (Fig 7 D, E & F) but only Il-17 was elevated in VL-10 infected mice.

In parallel, with measuring of inflammatory mediators, the main circulating angiogenesis mediator, vascular endothelial growth factor (VEGF), was elevated in mice infected with Colombian and Y strain as shown in figure 8, presenting consistency with the increased levels of other soluble inflammatory mediators.

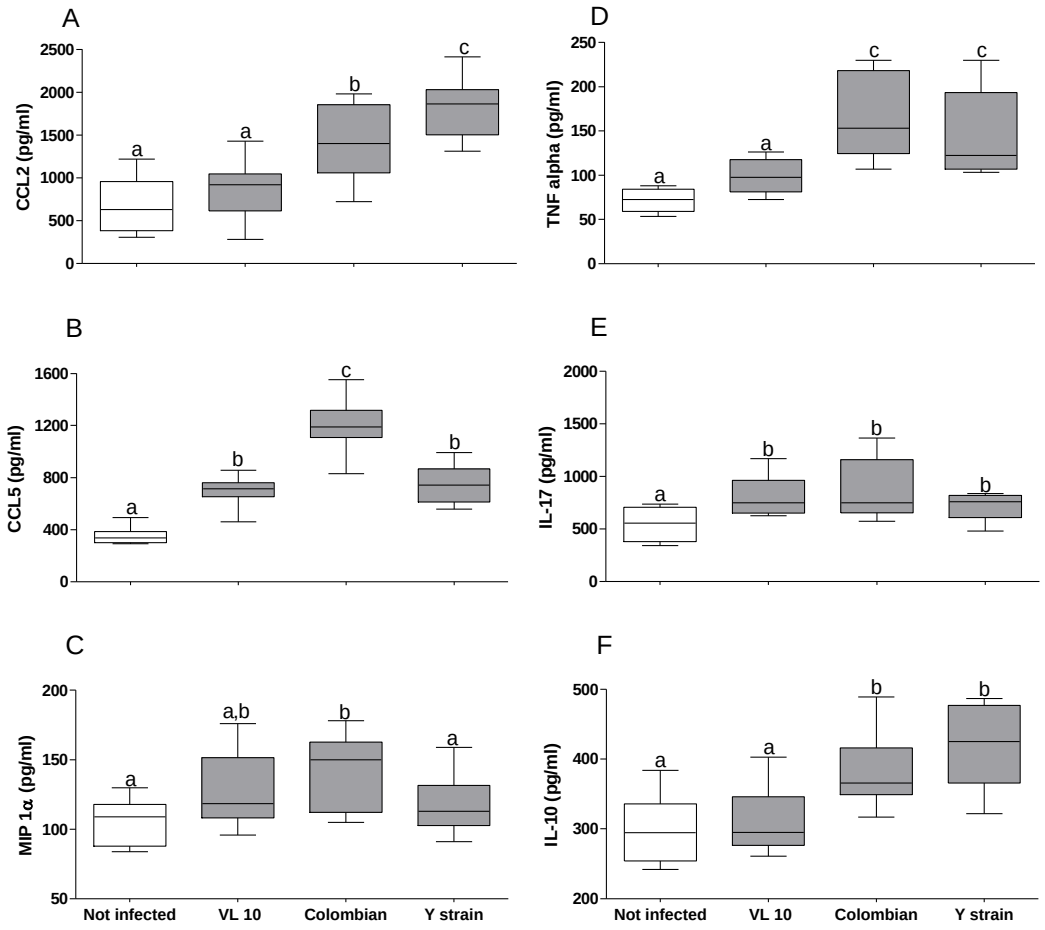


Figure 7. Increased production of inflammatory mediators in Colombian strain infected C57BL/6 mice in acute phase of infection. CCL2/MCP-1 (A), CCL5/RANTES (B), CCL3/ MIP 1α (C), TNF-alpha (D), IL-17 (E) and IL-10 (F) were measured in plasma by ELISA in all C57BL/6 mice infected with different strains of *T. cruzi*. Data are shown as a mean of 10 animals and different letters denote that difference in the production is significant at $p<0.05$.

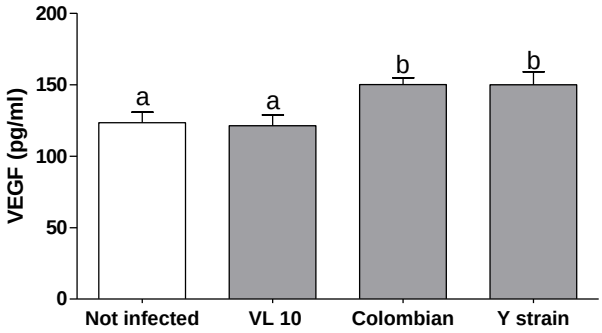


Figure 8. Vascular endothelial growth factor (VEGF) in plasma from C57BL⁄6 mice infected with different strain of *Trypanosoma cruzi* in acute phase of infection. VEGF was measured in plasma by ELISA in C57BL/6 mice infected with Y, VL-10 and Colombian of *T. cruzi*. Data are shown as a mean of 10 animals and different letters denote that significance difference at $p<0.05$.

5.2.3 Inflammatory infiltrate and parasitism in cardiac tissue

Considering heart as the main target of experimental *T. cruzi* infection and the foci for the inflammatory process against parasite, cardiac tissue was here investigated aiming the inflammation-dependent angiogenesis. Although all mice were inoculated with same number of parasite, evaluation of heart tissue showed the influx of inflammatory cells into the heart in a *T. cruzi*-strain dependence. Colombian strain demonstrated dense influx of inflammatory cells followed by Y strain and VL-10 respectively (Fig 10A, B). The expression of inflammatory cytokines (TNF-alpha and IL-10) (9C, D), chemokines (CCL2 and CCL5) (9A, B), and their respective receptors (CCR2 and CCR5) (9E, F) in heart section was elevated in all infected mice. It was very interesting to find that animals infected with Colombian strain expressed more CCL2, TNF-alpha and expression of CCL5 was 100X, 6X and 4X more than not infected, Y and VL-10 strain-infected mice, respectively. Amastigote nests were also quantified in heart through the occupied area by the amastigote forms of parasites (Fig 10 A, C). The Colombian strain presented higher area of amastigote nest in the heart tissue and very few amastigotes were found in the heart from mice infected with *T. cruzi* VL-10 strain. In Y strain infected mice amastigote nests were not found in this study.

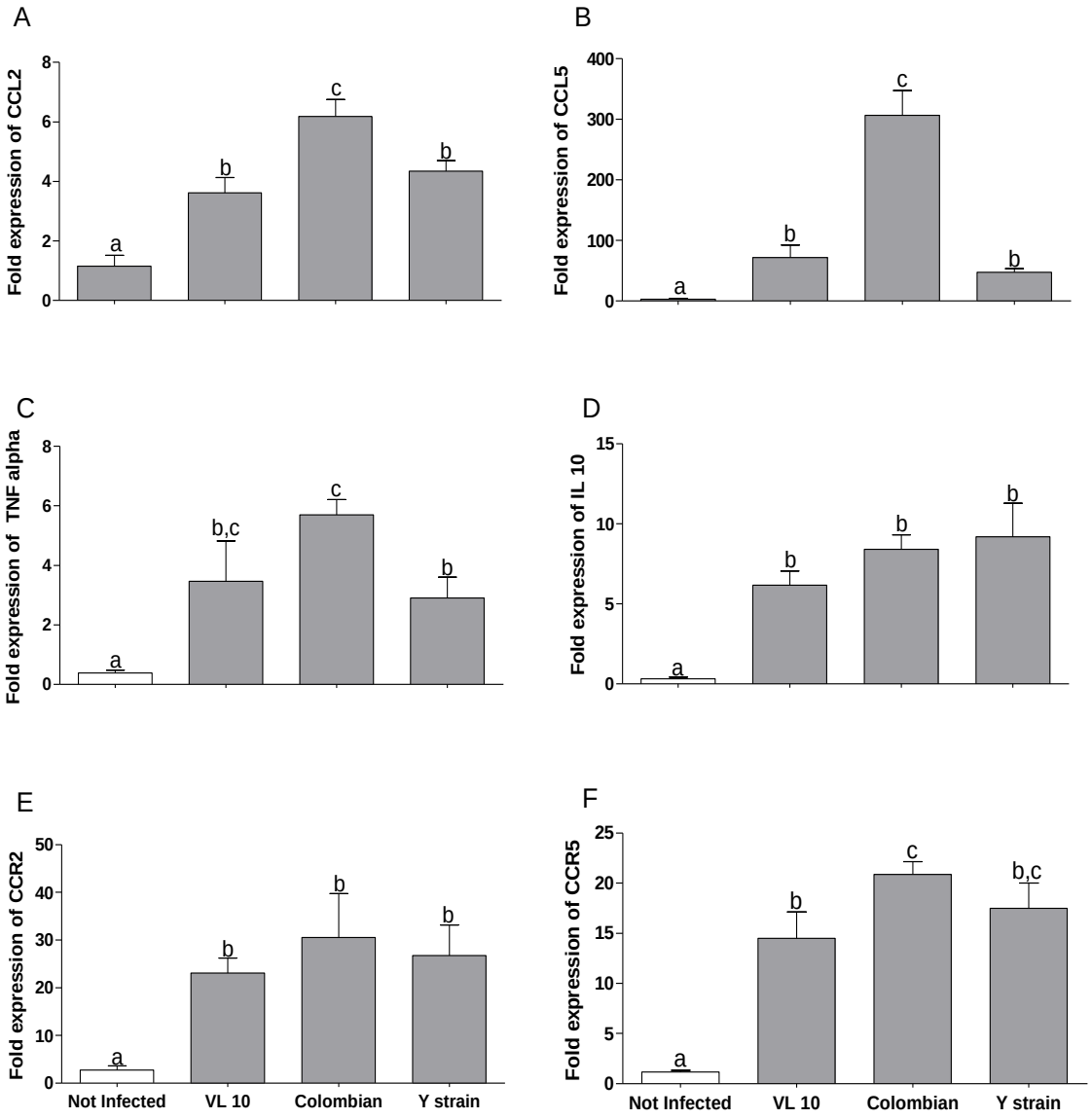


Figure 9. Relative expression of CCL2, CCL5, TNF-alpha, IL-10, CCR2 and CCR5 in heart tissue from C7BL/6 mice in acute phase of infection. mRNA expression of CCL2(A), CCL5(B), TNF-alpha (C), IL-10 (D), CCR2(E) and CCR5 (F) was measured by quantitative PCR in heart from animals infected or not with different strains of parasites. Data are shown as a mean of 10 animals. Different letters denote that difference in the expression is significant at P<0. 05.

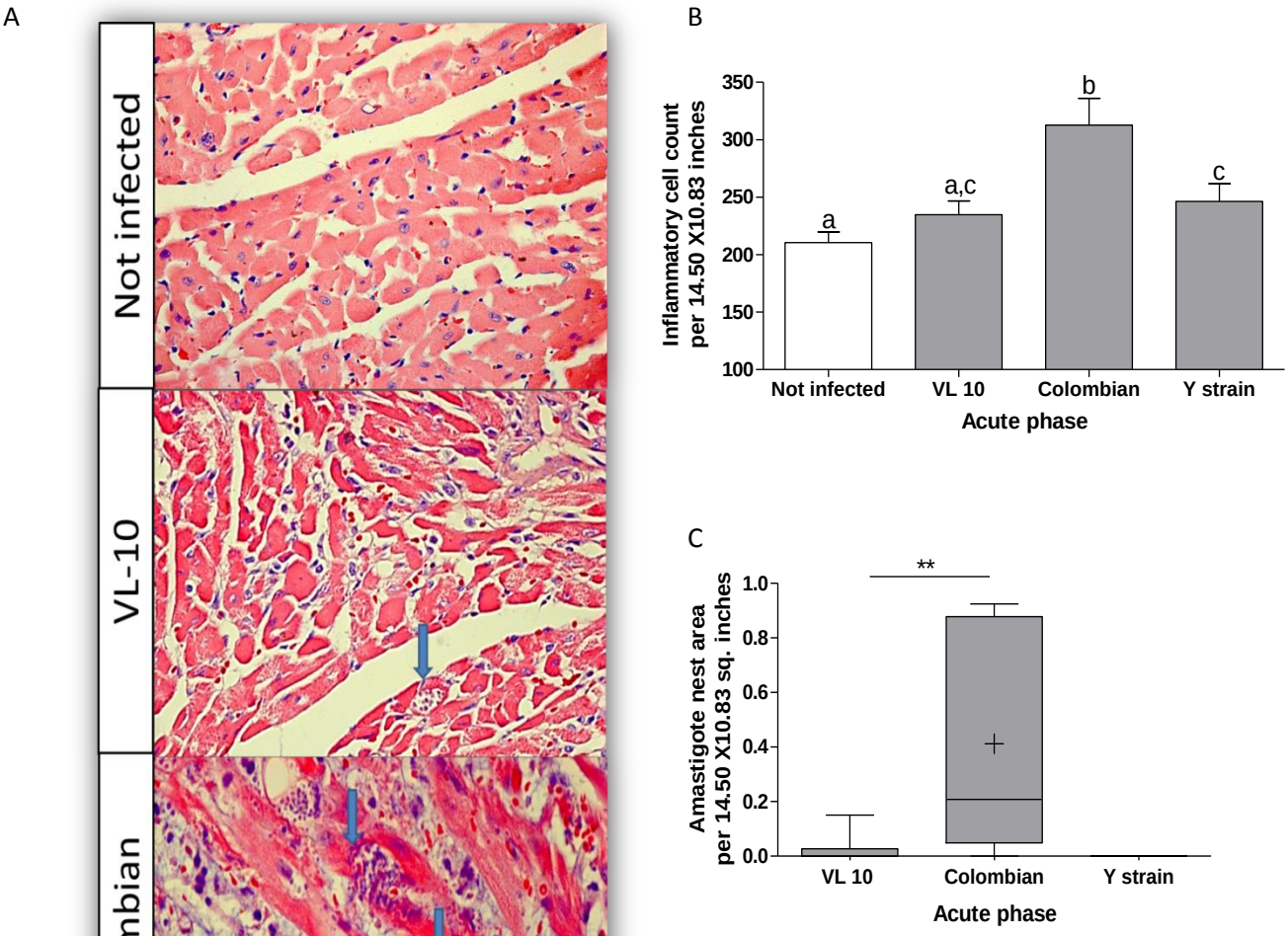


Figure 10. Inflammatory cell count and amastigote nest area in heart from C7BL/6 mice during acute phase of infection. Infiltration and amastigote nests in acute phase of animals infected with different strains of *T. cruzi* (100 parasites) (A), quantification of infiltration of cells (B) and amastigote nest area (C). Data are shown as a mean of 10 animals. (**) and different letters denote that difference in the expression is significant at $P<0.05$. Images were taken at magnifications of 40X .

5.2.4 Expression of angiogenic mediators in cardiac tissue infected by VL-10, Colombian and Y strains of *Trypanosoma cruzi*.

Following degree of inflammation and expression of inflammatory mediators in heart, subsequently, a quantitative real time PCR and ELISA to VEGF were performed. Mice infected with Colombian and Y strains of *T. cruzi* exhibited decrease VEGF mRNA (Fig 11A). It was very surprising to find that in mice infected with Colombian strain *T. cruzi* decreased expression of VEGF by 5 folds. The measurement of VEGF in heart, by ELISA (Fig 11 B), indicated that animals infected with Colombian strain showed decrease concentration of VEGF which can be compared with the expression of VEGF mRNA. The expression of Angiopoetin-1 (ANG 1) and angiopoetin-2 (ANG 2) (Fig 12 A and B) were also evaluated showing reduction in mice infected by Colombian strain of *T. cruzi* by 4.8 and 4.3 folds, respectively. It was also found that both TSP-1 and TSP-2 mRNA expression in heart was reduced by Y strain and only expression of TSP-2 mRNA was lowered in animals infected with Colombian (Fig 12 C and D).

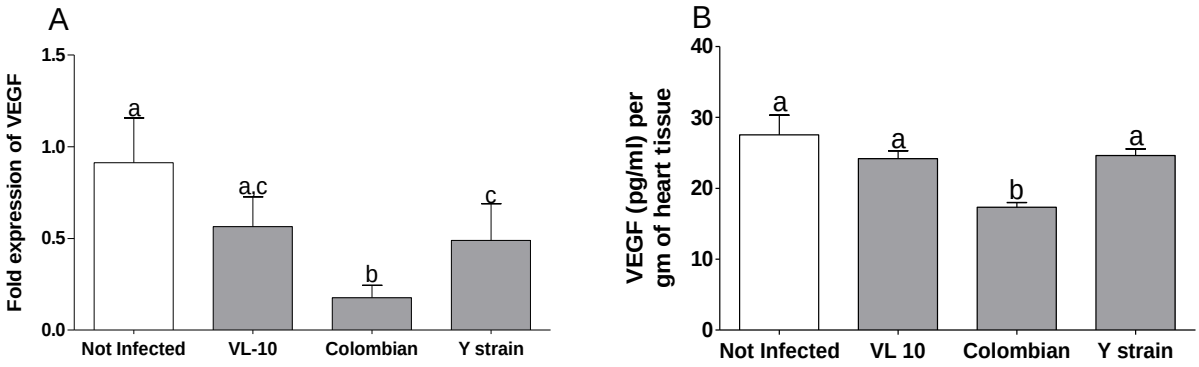


Figure 11. Concentration and relative expression of vascular endothelial growth factor (VEGF) mRNA in heart tissue from C7BL/6 mice in acute phase of infection. VEGF mRNA expression was measured by quantitative PCR (A) and concentration by ELISA (B) in heart from animals infected or not with different strains of parasites. Data are shown as a mean of 10 animals. Different letters denote that difference in the expression is significant at $P<0.05$.

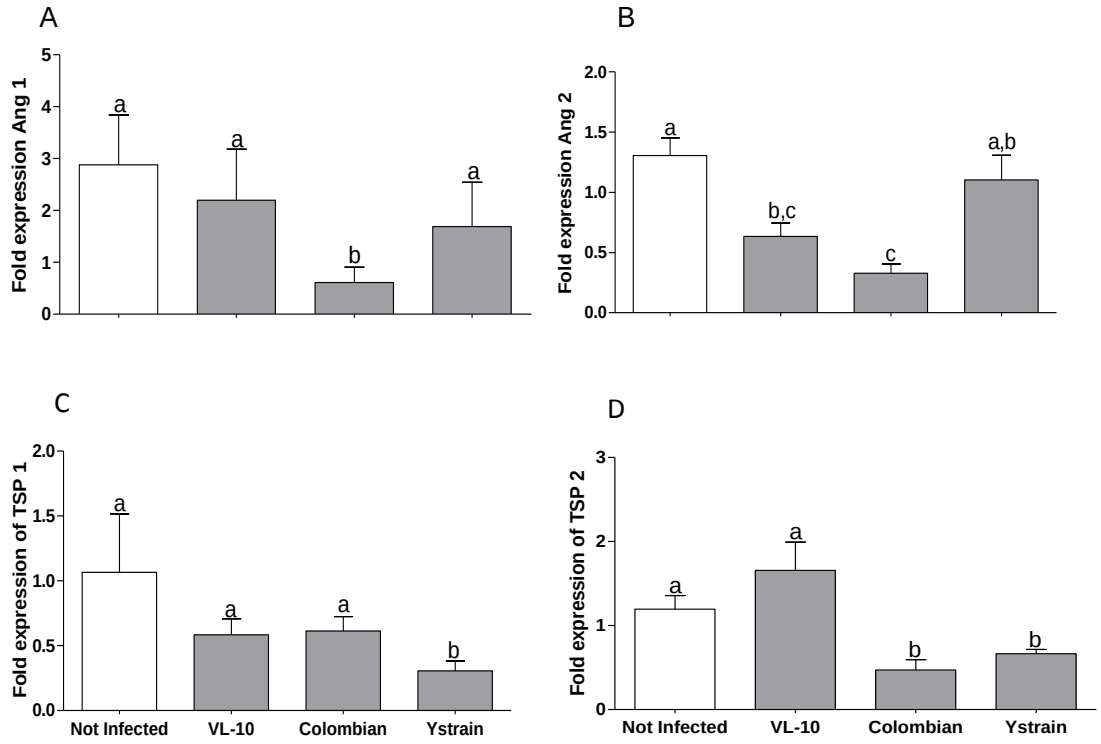
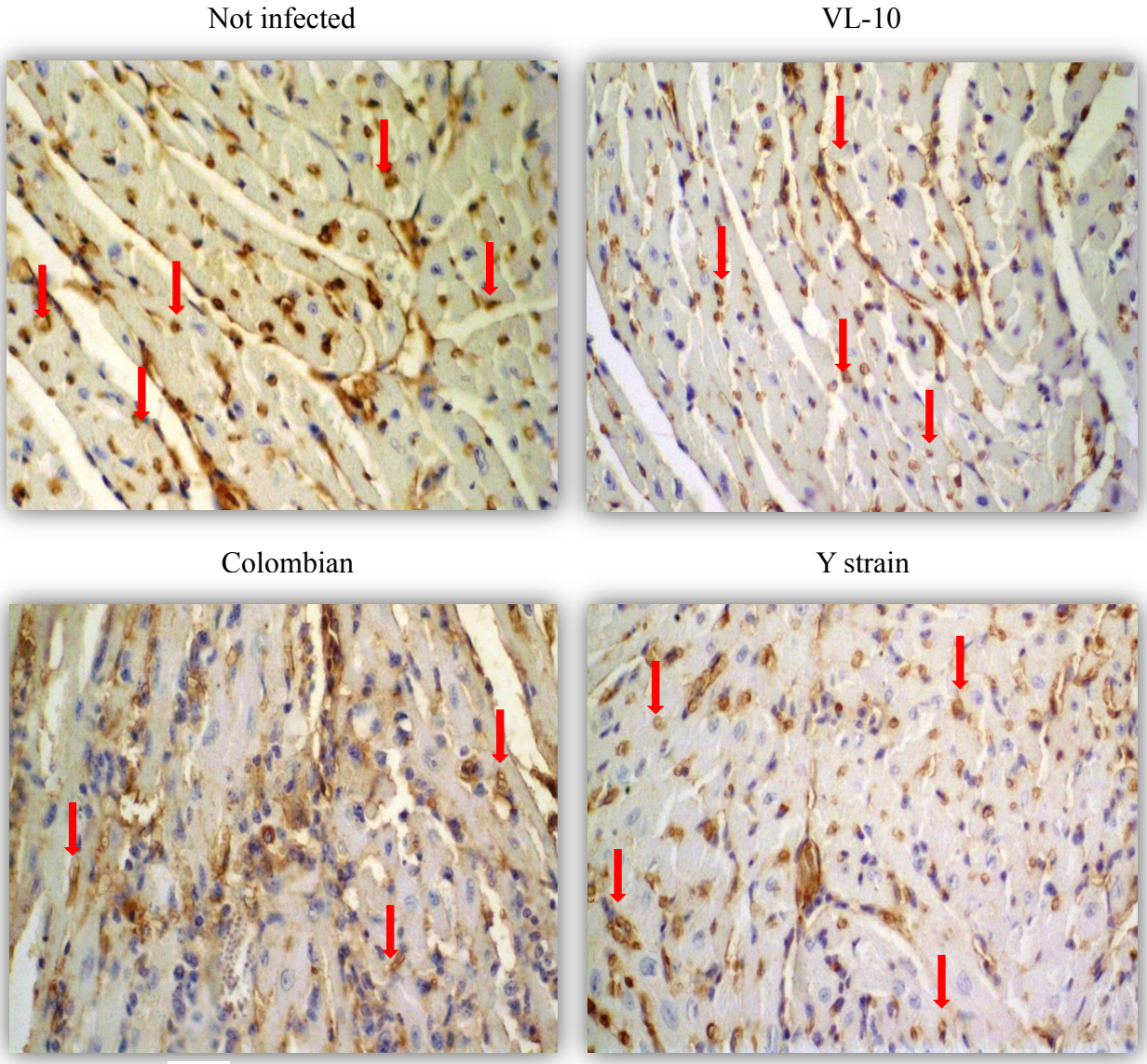


Figure 12. Relative mRNA expression of Angiopoeitin 1 and 2 and Thrombospondin 1 and 2 in heart from C57BL⁄6 mice in acute phase of infection. Ang-1 and -2 (A and B) and TSP-1 and -2 (C and D) mRNA in heart tissue were measured by quantitative PCR (qPCR) in mice infected or not with different strains of the *T. cruzi* parasite. Data are shown as a mean of 10 animals. Different letters denote that difference in the expression is significant at P<0. 05.

5.2.5 Quantification of blood vessel by immunohistochemistry

To quantify number of blood vessels in heart, all specimens were analyzed for the presence of endothelial marker CD31 (PECAM 1) by immunohistochemistry. 10 photos were taken from each heart section after immunostaining and a grid with 100 intersections was used to count the number of blood vessels. We found the number of blood vessels in animals infected with Colombian strain exhibited lesser blood vessel count than not infected groups but similar to Y and VL-10 strain infected groups (Fig 13 A and B).

A



B

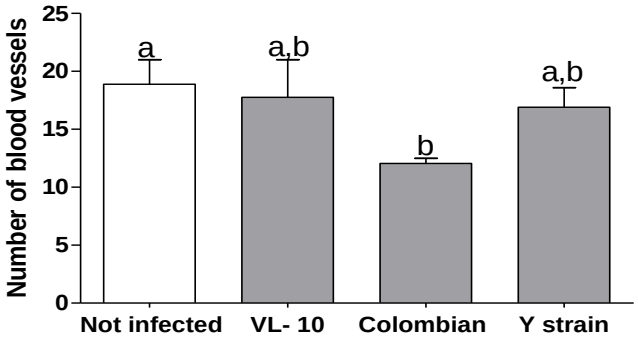


Figure 13. Quantification of blood vessel in heart section from infected and uninfected C57BL/6 mice in acute phase of infection. Blood vessels in heart section were evaluated by immunohistochemistry using anti CD-31 antibody. Red arrows show small blood vessels

after staining which are stained brown circular structures (A).Number of blood vessels are counted and represented in graphs in figure B. Data are shown as a mean of 10 animals. Different letters denote that difference in the expression is significant at $p<0.05$.

5.3 Pharmacological effects of different drugs on inflammation and angiogenesis.

Heart is the most affected organ among one third of the patients infected with *T. cruzi* which may lead to life threating Chagas heart disease (Andrade et al., 1994). Besides having side effects, Benznidazole is still one of the two available drugs given to Chagas disease patients. (Machado-de-Assis et al., 2013) and since it has already shown that Simvastatin and Enalapril can modulate immune response (Melo et al., 2011; de Paula et al., 2010), now to further address what can happen to angiogenesis parameters using these drugs, we analyzed the expression of angiogenic and inflammatory parameters both in plasma and in heart of those animals infected with Colombian strain of *T. cruzi*. We chose this strain owing to its excessive inflammation and prominent tropism to the heart.

In an independent experiment to count the number of circulating parasites until the death of animals, we found no difference in the pre-patent period and the parasitemia until day 22 in animals infected with Colombian strain of *T. cruzi* and receiving Enalapril, Simvastatin or vehicle (phosphate buffer). All animals treated with Simvastatin died within 26 days after infection while the animals receiving Enalapril and those not treated survived until 30 days. However, animals treated with Enalapril reduced the peak of circulating parasites (Fig 14 A) and maintained this pattern until 29th day of infection. With the decrease in circulating parasites, Enalapril treated infected animals also demonstrated decreased weight of heart to body ratio (Fig 14 B). Although the Colombian strain is considered as the drug resistant, Benznidazole treatment was able to maintain the weight of heart similar to uninfected groups (Fig 14 B). Parasite was not detected in blood until 50 days but when analyzed later, the number of circulating parasites was very low and animals stayed alive until 6 months and then were euthanized (Fig not shown).

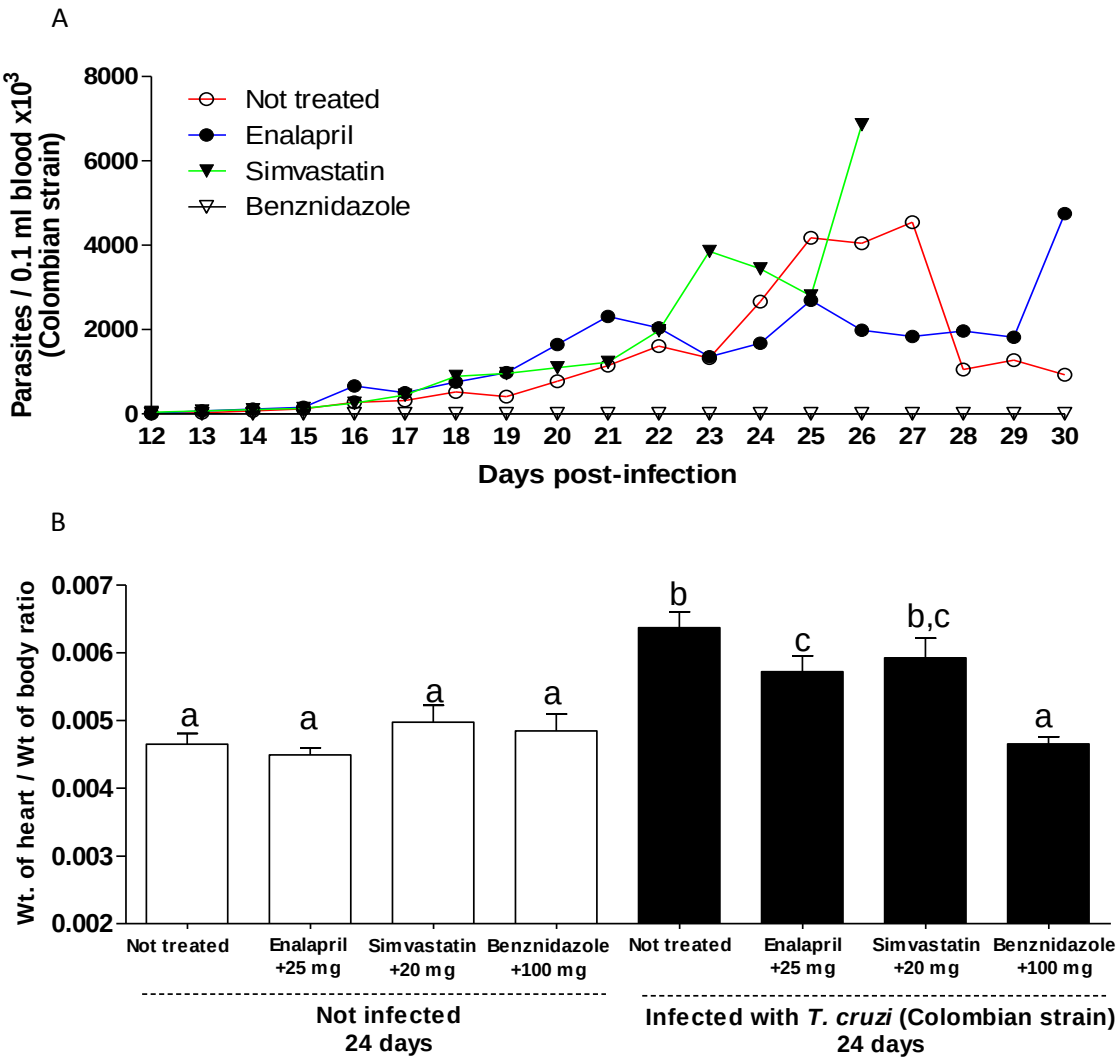


Figure 14. Parasitaemia curve and weight of heart to body ratio in C57BL/6 mice under different pharmacological treatments. C57BL/6 mice were infected with Colombian strain of *T. cruzi* and received Enalapril (25 mg ⁄ kg), Simvastatin (20 mg/kg), Benznidazole (100mg/kg) or vehicle (phosphate buffer) daily for 20 days. Parasitaemia was evaluated until 30 days of post infection (A). In parallel, animals were infected and treated with different drugs and were euthanized on day 24. Weight of heart and body ratio of mice was taken for infected and control groups (B). Data are shown as a mean of 10 animals and different letters denote significance difference at $p<0.05$.

In our study, we found that inflammatory chemokine, CCL5, was reduced in animals infected treated with Simvastatin and Enalapril while CCL2 was reduced by treatment with Simvastatin. In case of Benznidazole treated animals, chemokines CCL5 and CCL2 level were similar to uninfected animals (Fig 15 A and B).

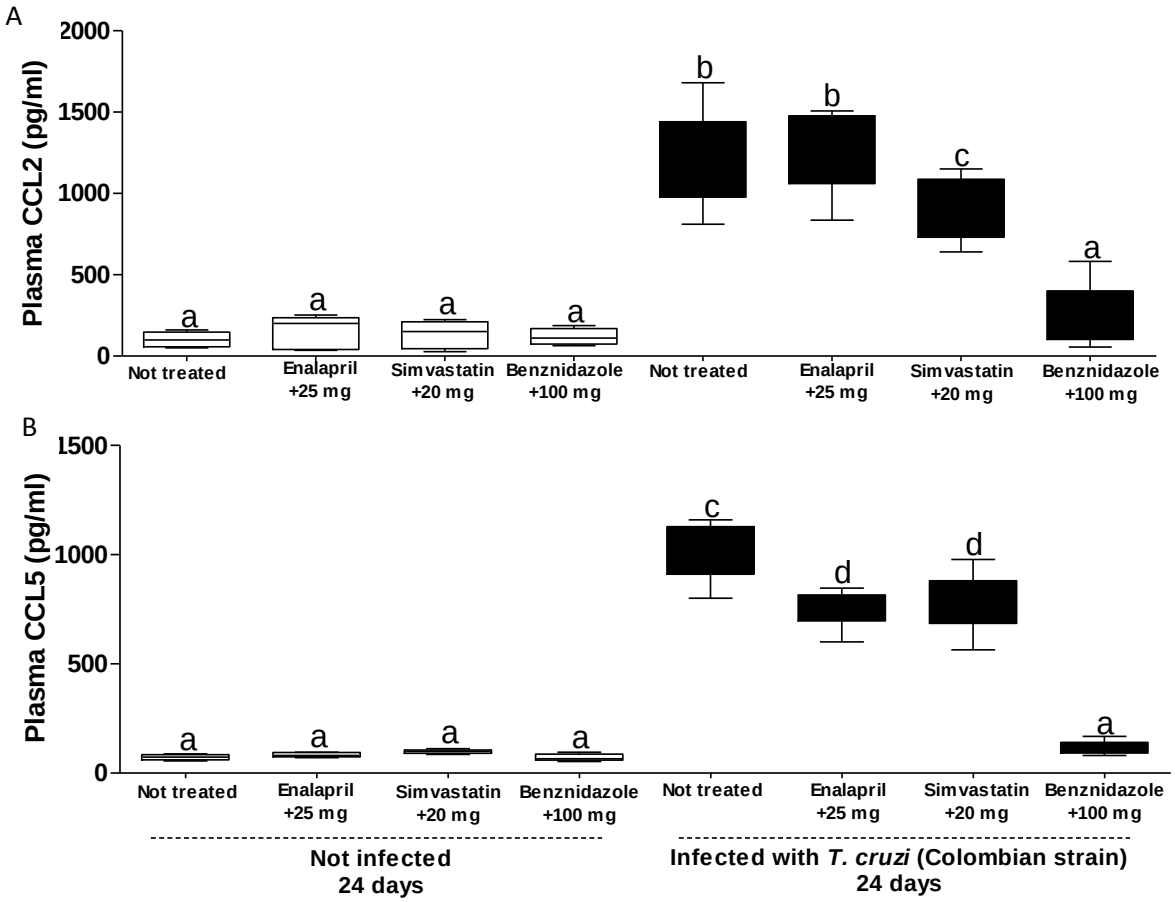


Figure 15. Production of inflammatory mediators in plasma in C57BL/6 mice under different pharmacological treatments. CCL2/MCP-1 (A) and CCL5/RANTES (B) were measured in plasma by ELISA in C57BL/6 mice infected with Colombian strain of *T. cruzi* and treated with Simvastatin, Enalapril, Benznidazole and phosphate buffer as vehicle. Data are shown as a mean of 10 animals and different letters denote significance difference at $p<0.05$.

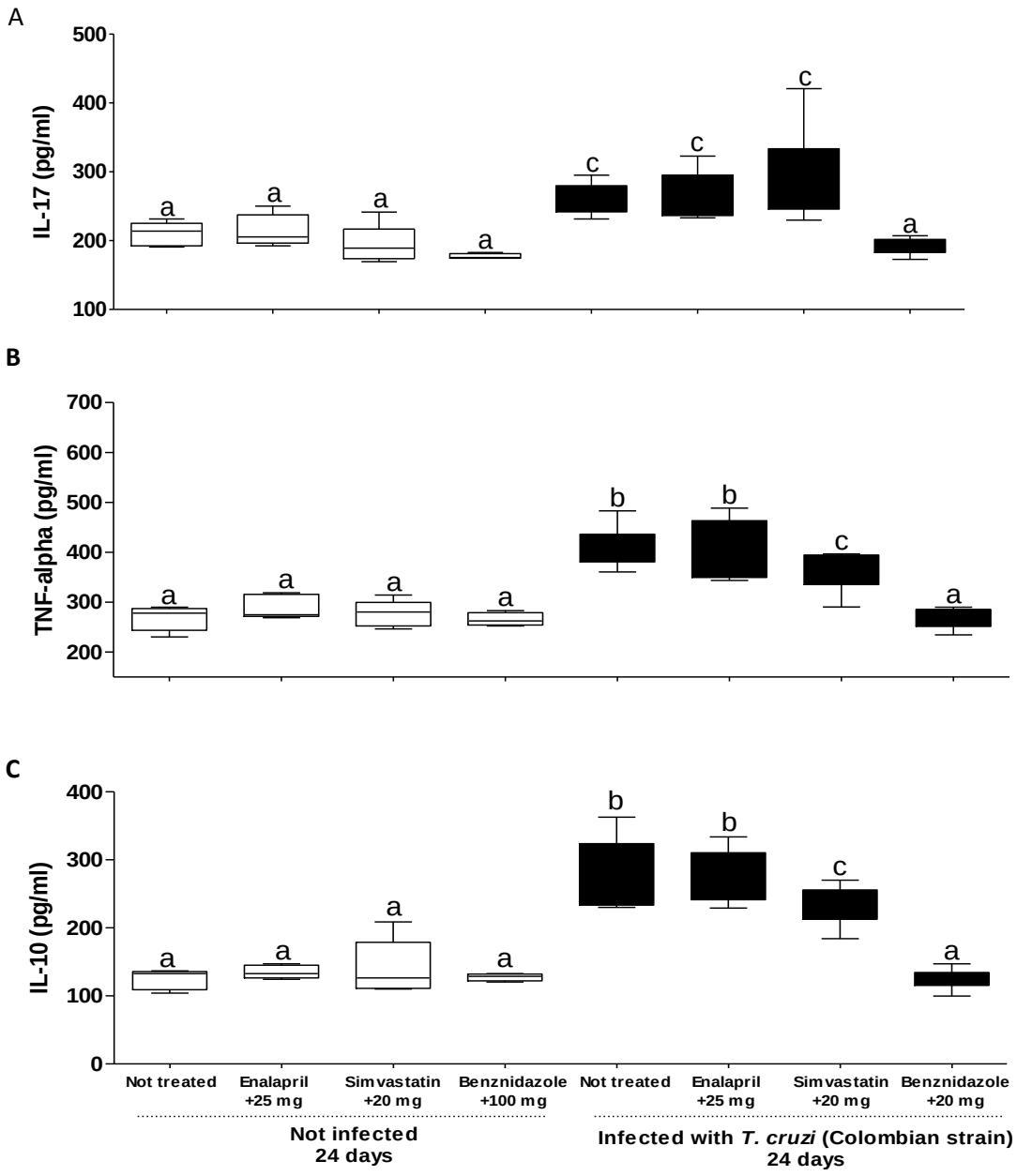


Figure 16. Concentration of inflammatory and regulatory cytokines in plasma in C57BL/6 mice under different pharmacological treatments. IL-17 (A), TNF-alpha (B) and IL-10 (C) were measured in plasma by ELISA in C57BL6 infected with Colombian strains of *T. cruzi* and treated with Simvastatin, Enalapril, Benznidazole and phosphate buffer as vehicle. Data are shown as a mean of 10 animals and different letters denote significance difference at $p<0.05$

Treatment with Simvastatin in infected animals reduced the level of inflammatory cytokines mainly TNF-alpha and regulatory cytokine IL-10. Benznidazole treatment in infected animals lowered the production of cytokines similar to the level of production in uninfected groups while there was no difference in the level of IL-17 in infected mice treated with Enalapril and Simvastatin (Fig 16 A, B & C).

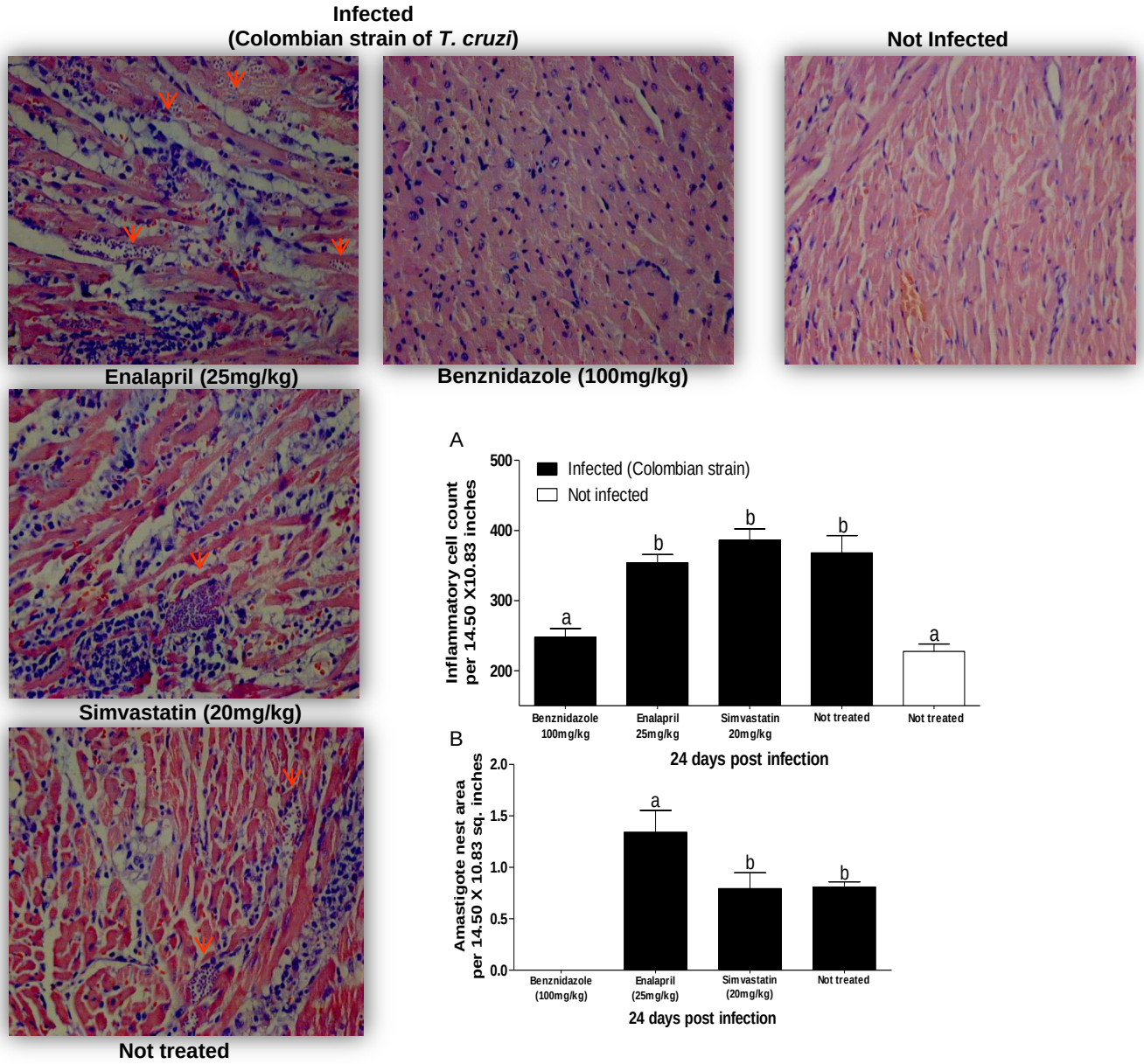


Figure 17. Inflammatory cell count and amastigote nest area in heart from C57BL/6 mice under different pharmacological treatments. The graph on the right shows comparison of inflammatory cell count (A) and amastigote nest area (B) in animals infected with Colombian strain of *T. cruzi* receiving Benznidazole, Enalapril, Simvastatin and

phosphate buffer. Images for infiltration and amastigote nests are illustrated here. Red arrows are marked to show amastigote nest. Data are shown as a mean of 10 animals and different letters denote significance difference at $p<0.05$

Mice infected treated or not with Simvastatin and Enalapril showed no difference in inflammatory cell count. Whereas, there was reduction in infiltration of inflammatory cells in animals infected and treated with Benznidazole, similar to uninfected groups (Fig 17 A). When we analyzed parasitism in heart, we noted that animals treated with Enalapril had almost two times more amastigote nest area than untreated or Simvastatin treated infected animals whereas no amastigote nest was found in Benznidazole treated animals (Fig 17 B).

Next to address what happens with the level and expression of angiogenic factor VEGF in plasma and in heart, we performed ELISA for VEGF in plasma and qPCR for mRNA expression in heart.

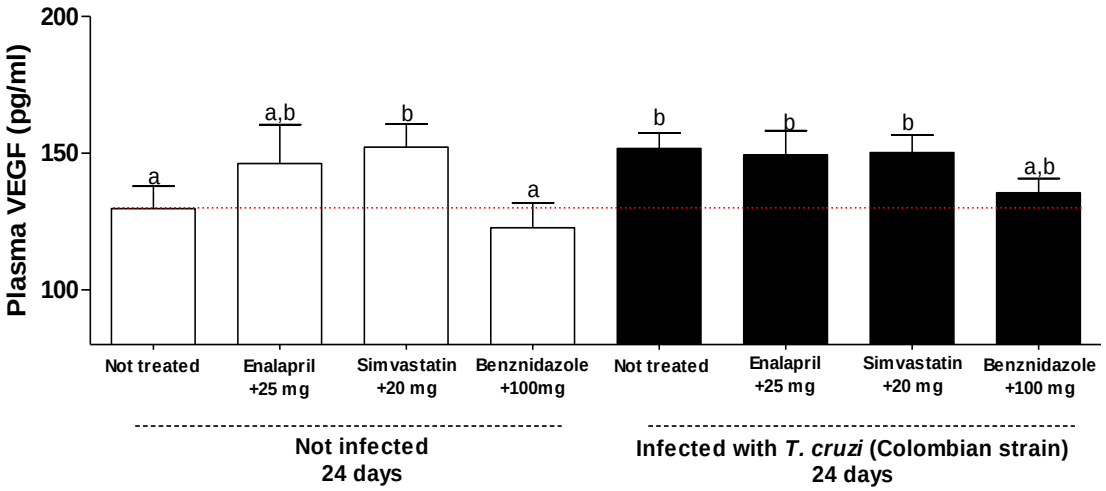


Figure 18. Concentration of vascular endothelial growth factor in plasma of C57BL/ mice under different pharmacological treatments. VEGF was measured in plasma by ELISA in all C57BL/6 mice infected with Colombian strains of *T. cruzi* and treated with Simvastatin, Enalapril, Benznidazole and phosphate buffer. Data are shown as a mean of 10 animals and different letters denote that difference in the production is significant at $p<0.05$.

Although in animals treated with Simvastatin and Enalapril displayed decrease in chemokine or cytokine levels in plasma, VEGF level in plasma was similar to infected but untreated animals. And as in previously shown in Colombian strain (Fig 8), the level of VEGF in plasma in infected groups except Benznidazole treated groups was higher than in uninfected untreated groups (Fig 18).

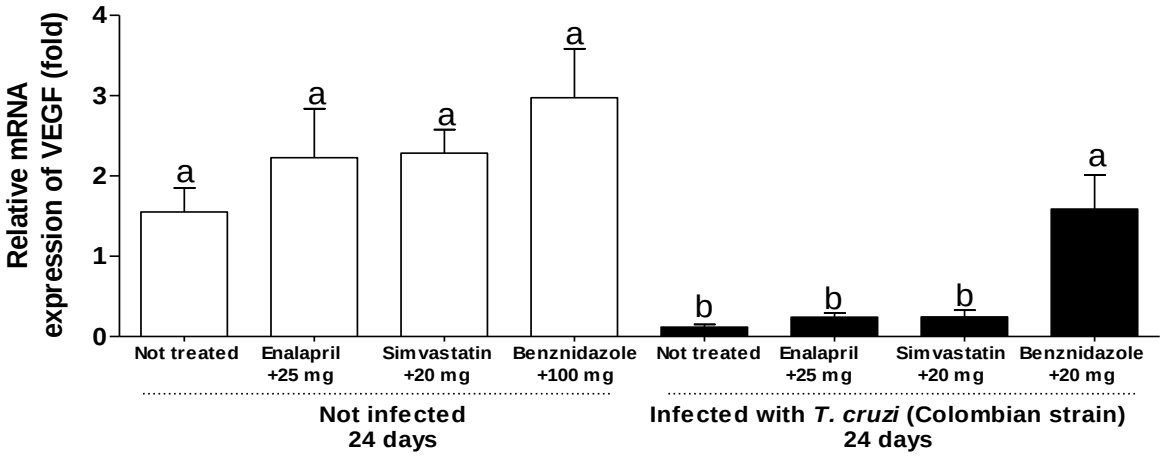


Figure 19. Relative expression of vascular endothelial growth factor mRNA in heart tissue from C57BL/6 mice under different pharmacological treatments. VEGF mRNA expression was measured by qPCR in animals treated with Enalapril, Simvastatin, Benznidazole and phosphate buffer infected or not with Colombian strain of *T. cruzi*. Data are shown as a mean of 10 animals. Different letters denote that difference in the expression is significant at $p<0.05$.

Likewise in figure 11A, there was diminished mRNA expression of VEGF in animals infected with Colombian strain of *T. cruzi* (Fig 19) compared to their uninfected control groups. However, we observed increased mRNA VEGF expression in Benznidazole treated infected groups.

5.4 Tabulated summary of results.

Table 2: Inflammation and angiogenesis parameter using sponge model on14 day (Y strain of *Trypanosoma. cruzi*)

Parameters	Sponge supernatant		Plasma	
	Uninfected	Infected	Uninfected	Infected
CCL2	+	+	X	X
CCL5	+	+++	+	+++
TNF-alpha	+	+	+	++
IL-10	+	+	++	+
VEGF	+++	+	+	+
Hb content	+++	+	X	X

Table 3: Inflammatory mediators and VEGF in plasma from mice with acute phase of *Trypanosoma cruzi* infection

Parameters in Plasma	Acute phase of infection			
	VL-10	Col	Y	NI
CCL2	+	++	+++	+
CCL3	+	++	+	+
CCL5	++	+++	++	+
TNF-alpha	++	+++	+++	+
IL-10	+	++	++	+
IL-17	++	++	++	+
VEGF	+	++	++	+

Table 4: Inflammatory cell infiltration and angiogenic parameters in heart from mice with acute phase of *Trypanosoma cruzi* infection

Parameters in heart	Acute phase of infection			
	VL-10	Col	Y	NI
Nuclei quantitation	+	+++	++	+
Amastigote nest	+	+++	-	-
VEGF	+++	++	+++	+++
Blood vessels (CD 31) count by immunohistochemistry	+++	++	+++	+++
Angiogenic/anti-angiogenic parameters (mRNA expression)				
VEGF	+++	+	++	+++
Ang-1	+++	+	+++	+++
Ang-2	++	+	+++	+++
TSP-1	+++	+++	++	+++
TSP-2	+++	++	++	+++

Table 5: Pharmacological effects on study of inflammation and angiogenesis in mice with acute phase of Colombian strain of *Trypanosoma cruzi* infection

	Uninfected				Infection with Colombian strain			
	Sim	Ena	Benz	NT	Sim	Ena	Benz	NT
Parameters in Plasma								
CCL2	+	+	+	+	++	+++	+	+++
CCL5	+	+	+	+	++	++	+	+++
TNF-alpha	+	+	+	+	++	+++	+	+++
IL-10	+	+	+	+	++	+++	+	+++
IL-17	+	+	+	+	+++	+++	+	+++
VEGF	++	+/++	+	+	++	++	+/++	++
Parameters in Heart								
Nuclei count	+	+	+	+	+++	+++	+	+++
Amastigote nest area count	-	-	-	-	+	++	-	+
VEGF mRNA	++	++	++	++	+	+	+++	+

X= Not measured
+= Present but at the lowest concentration/number/area
++= Present but at the concentration/number/area between + and +++
+++= Present but at the highest concentration/number/area
- = Not detected

VL-10=VL-10 strain
Col= Colombian strain
Y=Y strain
NI=Non-infected
NT=Non-treated

Sim= Simvastatin
Ena=Enalapril
Benz=Benznidazole

6. DISCUSSION

6. DISCUSSION

Angiogenesis has been well established as important component in various physical and pathological conditions. Angiogenesis, the re-establishment of a capillary network by endothelial cells, is important for cell function and survival. This process ensures adequate blood supply to developing tissue providing oxygen and nutrient to the tissue. VEGF is an important stimulatory signal that controls and maintains formation of new vessel (Leung et al., 1989; Lobov et al.; Jeansson et al., 2011). VEGF is produced by most tissues and its production is influenced by number of factors such as cytokines and hypoxic inducible factor 1 under hypoxic conditions. Since cytokines and hypoxic environment are obvious condition during inflammation and inflammatory cells are the major source of chemokines, cytokines and endothelial growth factors, there is well established association between angiogenesis and inflammation (Benelli et al., 2006; Naldini e carraro, 2005; Costa et al., 2007).

Trypanosoma cruzi infection is a classic example of the immune response driving the pathological condition in the infected mammalian hosts causing, in some cases, mild to severe heart disturbances (Andrade et al., 1994). This parasite-dependent inflammatory process co-exists in an environment with immune cells, inflammatory mediators, tissue destruction and local hypoxia culminating in a process of heart tissue repair or remodeling (Rossi & carobrez 1985; Machado et al., 2000). This type of inflamed environment is a prerequisite stimulus to initiate angiogenesis, therefore, it is plausible to propose the existence of an angiogenesis process in *T. cruzi* heart infection. To test this hypothesis previously we performed an experiment on sponge implant which was implanted subcutaneously in the neck pouch of Swiss mice and then antigen from *T. cruzi* was injected in the sponge. Here, we found that on 14 days post implantation there was increased number of blood vessels in sponge which was injected with *T. cruzi* antigen prior to formation of blood vessels on day 14. Also, there was increased level of CCL2, CCL5, TNF-alpha on day 7 in the sponge injected with antigen. These inflammatory mediators are proangiogenic and can be responsible for producing VEGF and later developing new vessels (da Silva et al., Unpublished data). This work with antigen suggested a possible association between antigens of *T. cruzi* and angiogenic process. Therefore, we performed other experiment to see host immune response and angiogenesis in sponge implant but infecting mice with *T. cruzi*. In this work, similar to studies performed in C57BL/6 (Barcelos et al, 2005), uninfected C57BL/6 mice showed the similar angiogenesis pattern where on day 14 there was "immense" vascularization in the sponge implant as demonstrated by Hb content and histological

staining. But interestingly, the sponge from infected mice on day 14 presented much reduced vascularization. It has also been demonstrated that TNF-alpha and CCL5 induces angiogenesis and are considered as proangiogenic factors (Barcelos et al 2005, Suffee et al, 2012). Increased TNF-alpha and CCL5 in plasma and CCL5 in supernatant from day 7 in our study indicated inflamed condition in infected mice. When we compared the pattern of chemokines and cytokines profile (day 1 to 14) between sponge supernatant from infected and uninfected mice, they were almost alike. However, unexpectedly, in the sponge implant we found there was no effect of inflammation to lead neovascularization in infected animals on day 14. The proangiogenic role of CCL5 has been well defined, but it has also demonstrated that when CCL5 is administered exogenously on 9, 10, 11 day after implantation, there was an antiangiogenic effect (Barcelos et al, 2009). In this study, in case of infected animals there was higher level of CCL5 on day 7 and 14 in sponge implant. Henceforth, we can assume that it is likely possible that CCL5 may be liberated from nearby vasculature to the sponge resembling an exogenous source for sponge implant later creating anti-angiogenic effect in the sponge. This anti-angiogenesis effect on sponge implant is yet to be understood if it's a host response when a host has a stronger stimuli for eg. *T. cruzi* parasite and such that all the immune cells are directed towards the stronger stimuli neglecting to weaker stimuli like sponge implant. However, these results with antigen and live parasites have opened new perspectives to study angiogenesis in tissue of animals in the experimental model of *T. cruzi* infection.

Inflammation is a key mechanism for the genesis and progression of lesions associated with *T. cruzi* infection, where the presence of parsite or its antigens triggers a humoral/cellular response which is not only capable of destroying the parasites, but also the host cells. During *T. cruzi* infection, angiogenesis can be an essential host mechanism to improve the tissue conditions however, may also be exploited by the parasites to guarantee their migration and survival in host cells (Ribatti et al., 2008). On the other hand, angiogenesis may not be interesting for parasite surveillance since this process can contribute, directly or indirectly, for its elimination by the increasing migration of inflammatory cells into infected tissue. Severity of tissue damage and fate of infected tissue and host depends on various factors that include genetics of parasite. We have used three different strains of *T. cruzi* parasites to study pattern of inflammation, angiogenesis and their mediators. Colombian and VL-10 strains are considered pathogenic and inflammatory strains and are resistant to standard drug Benznidazole. Y strain is partially sensitive to this drug and

induces huge inflammation in experimental animals. In general, we know that in the initial interaction of the parasite with the host cell, there will be intense release of inflammatory markers such as IL-12, TNF-alpha, IFN-gamma, CCL2, CCL3, CCL5 and nitric oxide (Coelho et al., 2002 Talvani & Teixeira 2011). This initial response is important to control parasite and encourage more migration of inflammatory cells to infected tissues. As this response is not sufficient to eliminate the parasite, its persistence will be a stimulus for maintenance of inflammation, making it a problem for the host. The high production of chemokines and influx of huge inflammatory cells would lead to the development of the cardiac form of the disease (Bonney & Engman, 2008; Guedes et al 2010) associated with a low expression of IL-10 that is related to the control of Th1 response (Costa et al, 2009). There is also the role of the chemokine IL-17 associated with protection from myocarditis by *T. cruzi* infection and exerts control over some chemokine CCL2 and CCL5 (Guedes et al, 2010). In this study, the intensity of inflammatory process together with its elements (soluble inflammatory factors) was evaluated systemically and in infected heart tissue using these three strains of *T. cruzi* and we obtained inflammatory pattern in the order of Colombian > Y > VL-10. This pattern was taken as a pre-condition to evaluated systemic and local inflammatory angiogenesis modulation.

Indeed, the single presence of parasite or its membrane glycoproteins is already sufficient to trigger a production of systemic inflammatory mediators such as cytokines, chemokines, lipid mediators and others (Golgher e Gazzinelli, 2004; Talvani e Teixeira, 2011; Rodrigues et al., 2012). But in this study, as a reinforcing of data from different authors (Talvani et al., 2002; de Paula et al., 2010; Silva et al., 2012; Borges et al 2013), Colombian strain was capable to induce high amounts of TNF-alpha, chemokines (CCL2 and CCL5), IL-17 and IL-10 in plasma and high expression of CCL2, CCL5 and their respective chemokine receptors (CCR2 and CCR5), TNF-alpha and IL 10 in heart. Colombian strain infected animals showed increased level of TNF- alpha, CCL2 and CCL5 level but not IL-10 in heart (data not shown). Colombian and VL-10 strain of *T. cruzi* are adaptive strains to infect heart tissue while Y strain is found homogeneous throughout the different organs, including heart (Oliveira et al., 2012). Possible, due to the characteristics of this Colombian strain, isogenic C5BL/6 infected mice show more inflammatory process, more amastigote nests and more cardiac disturbances (Talvani et al., 2000; Borges et al 2013). Interestingly, the genetic pattern of *T. cruzi* appears to be also dependent on the mammalian host. Previous study reported that VL-10 strain induced high inflammation, tissue destruction and disturbances in

dogs and Swiss mice (Caldas et al., 2013; Bahia et al., 2012). However, in our study VL-10 infected C57BL/6 mice demonstrated low parasite load and low production of inflammatory mediators in plasma and heart. This new inflammatory pattern of VL-10 observed in isogenic C57BL/6 is supported by previous studies involving variability of host genetic background and parasite interaction (Andrade et al., 2002; Freitas et al., 2009). Although Y strain showed higher parasite load and induced higher inflammation in plasma similar to Colombian strain, cellular infiltration and parasitism in heart was also reduced in our data, confronting the increased inflammation observed by our group in dog models (Guedes et al., 2010; Melo et al., 2011; Santos et al., 2012), once more reinforcing the importance of host genetic background in the pathogenesis of *T. cruzi* infection. Still presenting less inflammatory infiltration in comparison with Colombian strain, both Y and VL-10 strains of *T. cruzi* induced increasing expression of inflammatory cytokines, chemokines and their receptors in the isogenic mice infected heart suggesting the antigenic role of these parasites in a parasite genetic-dependent way.

After characterizing inflammation pattern systemically, then were evaluated factors fundamental for the "inflammatory angiogenesis" in isogenic mice infected with different *T. cruzi* strains. Vascular endothelial growth factor (VEGF)-A is secreted by various different types of cells including inflammatory cells and endothelial cells and, it is recognized as the best characterized and important angiogenic factor stimulating proliferation and migration of new endothelial cells (Noonan et al., 2008; Wang et al., 2008). VEGF production was high evidenced in this associative sequence (Colombian=Y> VL-10=not infected). When stimulus/injury remains for a long time, persistence of inflammation may cause oxygen reduced condition, hypoxia, and then trigger condition favorable for secretion of VEGF (Liu et al., 1995; Krock et al., 2011). The virulence observed to Y strain usually does not allow long time of surveillance in wild type and isogenic mice but the intensity of immune response observed after few days of infection reflected the equivalence of Y with Colombian strain, supported by high levels of TNF-alpha and CCL2 in plasma and heart tissue. Indeed, in a tissue repair environment, activated platelet uses to be the first vascular component to release VEGF after thrombin stimulation (Möhle et al., 1997) and monocytes expressing VEGF receptor Flt-1 exert chemotaxis to VEGF inducing angiogenesis (Clauss et al., 1996). Angiogenesis is due, in part, to the releasing of TNF-alpha which may in turn induce VEGF expression in local cells (Clauss et al., 2001; Lu et al., 2012). Systemic inflammatory

cytokines acting as paracrine factors of angiogenesis, stimulated by *T. cruzi* in infected animals, may be acting as triggers to secrete more VEGF in infected mice.

However, VEGF was also measured by immunoassay in macerated heart tissue and, surprisingly, there was low production of VEGF associated with those animals presenting high number of amastigote forms in hearts tissue from Colombian strain infected mice. The next step was to investigate the expression of VEGF, Angiopoetin-1 -2 (Ang-1 – 2) in those infected heart tissue. Consistent data was found among VEGF, Ang-1 and Ang-2 where their increased expression in heart tissue can be represented as Y = VL-10 > Colombian strains. A sustainable hypothesis for this decrease in expression could be based on *T. cruzi* released-calreticulin in heart tissue as a consequence of the high number of amastigote forms, in this case, associated with Colombian strain. *T. cruzi* molecule named calreticulin has been shown to inactivate classical and lectin complement pathways facilitating parasite infectivity and also induces both anti-tumor and anti-angiogenesis effects (Molina et al., 2005; Ramirez et al., 2011). This concept of *T. cruzi* inhibiting angiogenesis was pointed in some *in vitro* and *in vivo* experiments but did not take into high consideration on angiogenic factors and the genetic background of *T. cruzi.* Indeed, Colombian strain is able to promote increase of chemokines (CCL2, CCL5) and their receptors (CCR2 and CCR5) in the heart tissue as we have previously demonstrated (Talvani et al., 2000). The recruitment of inflammatory cells into the heart tissue to destroy parasites is mediated by a network of vessels. The reduction of angiogenic factors expression (eg. VEGF, Ang-1 and Ang-2) by *T. cruzi* Colombian strain might established an own "protective" mechanism in the cardiac tissue reducing its local vascularization. TSPs are the strong endogeneous antiangiogenic regulatory molecules (Lawler & Lawler, 2012). It has been shown that *T. cruzi* upregulates TSP 1 in mouse embryo fibroblast which along with calreticulin enhances cellular infection (Johnson et al., 2012). In the present study, in heart sections, TSP 1 and 2 expressions were reduced in Y strain infected mice and TSP 2 was reduced in Colombian infected mice. We further analyzed the number of blood vessels in the heart section performing immunohistochemistry using anti CD31 marker for endothelial cells. CD31 is an important and widely used marker to study in quantifying blood vessels in tumor vascularization (Berger et al., 1993; Wang S. et al., 2008). This marker is highly expressed in endothelial cells and in lesser content in platelets and leukocytes (Wang D. et al., 2008). CD31, or platelet endothelial cell adhesion molecule-1 (PECAM-1), is a surface marker of endothelial cells that plays an important role in adhesive interaction between endothelial cells and polymorphonuclear leukocytes, monocytes, and

lymphocytes during inflammation, and between adjacent endothelial cells during angiogenesis (Muller et al., 2002). The decreased number of blood vessels in heart from Colombian strain infected animals is much more related with the vascular destruction which is a common feature during *T. cruzi* infection. It is well understood that in acute phase of *T. cruzi* infection interaction of endothelial cells and lymphocytes leads to secrete mediators which not only destroy tissue but is also responsible for destruction of blood vessels (Andrade et al., 1994, Prado et al., 2011). Therefore in acute phase of infection by Colombian strain, decrease in VEGF expression cannot be correlated with decrease in blood vessel count. But still, the mechanism by which VEGF expression is down modulated need to be studied and we here propose a hypothesis of an inflammatory process induced by *T. cruzi* (in a strain-dependent way) and possible mechanism for antiangiogenesis which is summarized in Figure IV.

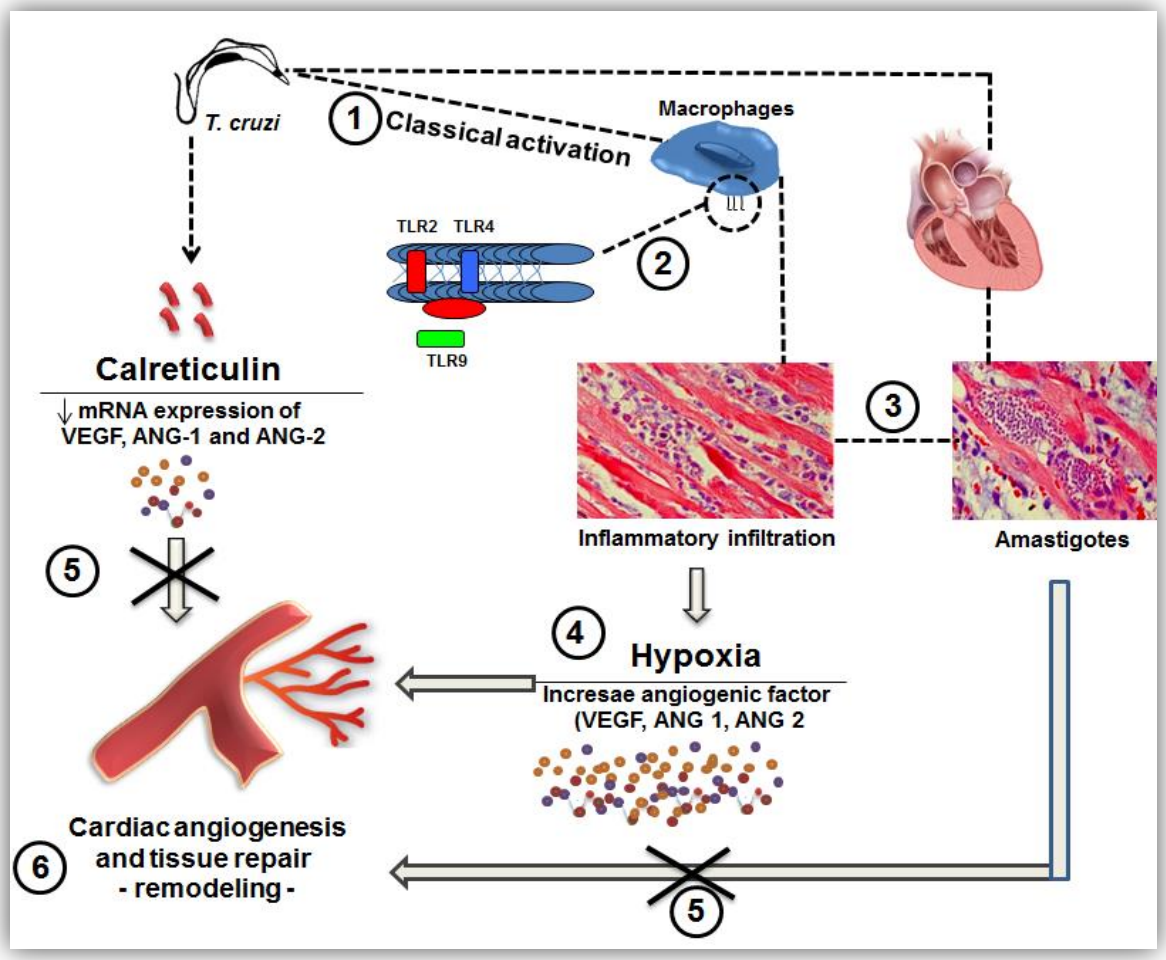


Figure III: Hypothesis of angiogenesis modulation in experimental *Trypanosoma cruzi* infection.

(1) In a mammalian host, *T. cruzi* acts as a potent trigger of immune response though, mainly, the activation of Toll-like receptors by membrane parasite-glycoproteins.

(2) This activation promotes release of distinct inflammatory mediators (TNF-alpha, IL-12, PAF and others) and chemokines (CCL2, CCL3, CCL5 and others) promoting recruitment of neutrophils, monocytes and latter, lymphocytes to cardiac inflammatory site.

(3) This leukocyte recruitment is coordinated by intrinsic characteristics of parasites and their ability to install in the cardiac tissue increasing the magnificence of the inflammation and the production of more inflammatory mediators.

(4) Cardiac cell destruction is a consequence of the persistence of the immune response trying to eliminate amastigote forms into the cardiac cell and, consequently, there will be a production of innumerous oxidative products which promote (i) destruction of cells/tissue, (ii) deposition of collagen (fibrosis) with change of the morphology and (iii) loss of original function. Together, this process will promote reduction of oxygen to the cardiac cells and, supported by the increase of local levels of other inflammatory proteins (eg. TNF-alpha), soluble angiogenic mediators will be released promoting neovascularization aiming the supply of nutrients to the heart cells but also increasing the ways from where leukocytes migrates into the heart to eliminate parasites.

(5) In parallel, *T. cruzi* in the circulation (trypomastigote forms) or in tissues (amastigote forms) perhaps, or using its molecule calreticulin, reduces mRNA to VEGF and angiopoetins mainly in the cardiac tissue promoting a coexistence, in the same environment, of pro-angiogenic and of anti-angiogenic factors driven by a parasite genetic-dependent way.

After studying inflammatory and angiogenic parameters in acute phase of infection, we selected the Colombian strain of *T. cruzi* to evaluate involvement of different drugs to ameliorate inflammation and angiogenic factors. In Chagas heart disease proper pharmacological therapies for the treatment is still in need although various drugs have been developed but all have some limitations. For the patient in chronic phase of infection with Chagas heart disease, existing anti *T. cruzi* therapies is not satisfactory. Therefore treatment with other pharmacological treatment that can reduce progression of heart failure and can improve heart functions. Recently, the focus has been diverted to combination therapies using antitrypanosomal drugs or with the drugs to improve cardiovascular functions. Benznidazole is the widely used drug for the treatment of chagas disease and as well as in experimental

model. Enalapril and Simvastin are drugs used for the treatment of cardiovascular disease. Enalapril and Simvastatin, besides their known physiological actions, have also modulatory effects in diseases of different etiologies and, part of this pleiotropic effect is related to angiogenic effects (Nishimoto-Hazuku et al., 2008; Sharma et al., 2010). Enalapril is an ACE-inhibitors used in the treatment for hypertension and Simvastatin is an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase that aids in reducing cholesterol. It has been shown that anti-inflammatory effect of Enalapril and Simvastatin was able to reduce parasite and reduced immune mediated cardiac damage. Our results confirms observation with Simvastatin that reduced CCL2, CCL5, TNF-alpha and IL-10 in plasma (Silva et al., 2012). In the study performed by de Paula, Enalapril reduced CCL5 and TNF-alpha levels in plasma and cell infiltration and amastigote nest in heart (de Paula et al., 2010). But in our study we could not find similar results except for CCL5 possibly because our studies were performed after 24 days post infection and their studies were performed after 35 days post infection. Although characterizing anti-inflammatory properties of Simvastatin, we did not find any change in expression of VEGF in heart may be because in heart there was no change in cellular infiltration and parasitism in Simvastatin treated animals. However, in plasma VEGF level in Simvastatin treated infected mice, in spite of reduced inflammation, is similar to not treated but infected mice which may be due to of angiogenic properties of Simvastatin. This can also be explained when we compared between control (uninfected) groups where Simvastatin treatment has increase VEGF level. It has been demonstrated that Simvastatin can stimulates VEGF production by upregulating HIF-1 alpha (Nishimoto-Hazaku et al., 2008). It is noteworthy that, we used the Benznidazole as the control treatment. It was able to match all the assessed parameters with uninfected animals. Even though this drug lacks efficacy against Colombian strain of *T. cruzi*, but yet proved adept to reduce blood and tissue parasitism, maintaining basal levels of circulating inflammatory marker, inflammatory cell migration and expression of VEGF in the heart

In conclusion, our study involving intertwined processes has opened new promising questions for mechanisms related to host-*T. cruzi* interactions, a process that parasite may induce in the mammalian cells/tissues in order to sustain within it. Therefore, our study will be helpful to evaluate unique properties of different *T. cruzi* strains to evolve *T. cruzi* infection associated with inflammation and angiogenesis.

7. SUMMARY

7. SUMMARY

1. In sponge implantation experiment, there was no difference in inflammatory (CCL2, TNF-alpha) and regulatory cytokines (IL-10) except CCL5 in sponge supernatant from mice infected or not with Y Strain of *Trypanosoma cruzi*. In plasma, inflammatory mediators CCL5 and TNF-alpha were higher in infected animals on day 14.

2. Hemoglobin (Hb), VEGF and blood vessels were significantly low on day 14 in sponge implants from infected mice. VEGF in plasma was found higher in uninfected animals on day 7 but was same on day 14 when compared with infected mice.

3. Number of circulating parasites in blood was higher in animals infected with Colombian strain and Y strain of *T. cruzi*. Whereas, only Colombian strain infected animals showed increased weight of heart to body ratio.

4. Colombian strain infected mice showed increased production of inflammatory chemokines and cytokines. The levels of CCL2, CCL3, CCL5, TNF-alpha, IL-17 and IL-10 were higher in animals infected with Colombian strain. Following Colombian, Y strain exhibited augmented production of CCL2, CCL5, TNF-alpha, Il-17 and IL-10 whereas VL-10 strain showed increase in CCL5 and IL-17 but relatively low level when compared with Colombian and Y strain in mice. In parallel, Colombian and Y strain demonstrated increased plasma VEGF level in mice.

5. Increased infiltration of inflammatory cells in heart was evidenced in Colombian strain infected animals followed by Y strain and VL-10. As well as greater amastigote nest area was found in heart from animals infected with Colombian strain followed by VL-10. No amastigote nest was seen in animals infected with Y stain. Number of blood vessels was also reduced in heart from Colombian infected mice.

6. Messenger RNA expression of VEGF, Ang-1, Ang-2 and TSP-2 and concentration VEGF was greatly reduced by Colombian strain while Y strain showed reduced expression of VEGF, TSP-1 and TSP-2 and VL-10 showed reduced expression of ANG-2 in heart from mice

7. Colombian strain infected animals, independent of the treatment, showed increased VEGF level when compared to uninfected untreated animals. Although Simvastatin reduced CCL2, CCL5 and TNF-alpha in infected animals VEGF level was similar to infected untreated

animals. Animals infected with Colombian strain and treated with Benznidazole, the level of chemokines, cytokines and VEGF levels were similar to uninfected animals.

8. Enalapril treated animals presented higher area of amastigote nest in heart though the influx of inflammatory cells was equivalent to other infected groups. Infected animals except Benznidazole treated showed reduced expression of VEGF mRNA in heart.

8. CONCLUSION

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Our results indicate following conclusions based on three independent experiments:

1. In sponge experiment, we found inflammatory induced angiogenesis was abrogated in sponge implant under the presence of stronger stimuli (*T. cruzi*)
2. In this study we also demonstrated that *T. cruzi* (Colombian) strain is highly adaptive to cardiac tissue inducing high inflammation as demonstrated by high expression of chemokines (CCL2, CCL5) and its receptor (CCR2, CCR5), cytokines (IL-17, TNF-alpha) and infiltration of inflammatory cells in heart tissue. However, this strain which is adaptive to heart tissue showed decreased production and expression of VEGF as well as expression of Angiopoeitin-1 and -2. Therefore, our results suggest an important mechanism for the survival of parasite in host tissue which can broaden our understanding of host parasite interaction and cardiac pathology during *T. cruzi* infection.
3. Furthermore, we found that Enalapril and Simvastin were not able to increase the expression of these important angiogenic parameters in infected mice but anti-*T.cruzi* drug Benznidazole is not only capable to reduce parasite and inflammation but also was able to maintain angiogenic parameters (VEGF, ANG-1 &-2, TSP-1 & -2) similar to control groups.

9. PERSPECTIVES

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After studying inflammation and angiogenesis in acute phase of infection, our future perspectives are follows:

1. Evaluating expression and production of hypoxia inducing factor in heart from infected animals

2. Studying inflammation and angiogenesis in chronic phase of *T. cruzi* infection.

3. Performing in-vitro studies by infecting different types of cells (macrophages, cardiomyocytes, endothelial cells) with *T. cruzi* to show expression and production of angiogenic factors.

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ANNEXES

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 thet alombian 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 thet alombian 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

PLOS Neglected Tropical Diseases

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