Published in final edited form as:

Microbes Infect. 2014 April ; 16(4): 337–344. doi:10.1016/j.micinf.2014.01.001.

***Trypanosoma cruzi* infection results in an increase in intracellular cholesterol**

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Abstract

Chagasic cardiomyopathy caused by *Trypanosoma cruzi* is a major health concern in Latin America and among immigrant populations in non-endemic areas. *T. cruzi* has a high affinity for host lipoproteins and uses the low density lipoprotein receptor (LDLr) for invasion. Herein, we report that *T. cruzi* infection is associated with an accumulation of LDL and cholesterol in tissues in both acute and chronic murine Chagas disease. Similar findings were observed in tissue samples from a human case of Chagasic cardiomyopathy. *T. cruzi* infection of cultured cells displayed increased invasion with increasing cholesterol levels in the medium. Studies of infected host cells demonstrated alterations in their cholesterol regulation. *T. cruzi* invasion/infection via LDLr appears to be involved in changes in intracellular cholesterol homeostasis. The observed changes in intracellular lipids and associated oxidative stress due to these elevated lipids may contribute to the development of Chagasic cardiomyopathy.

Keywords

Chagas Disease; *Trypanosoma cruzi*; LDL; cholesterol; invasion; pathogenesis

1. INTRODUCTION

Chagas disease, also known as American trypanosomiasis is caused by the protozoan parasite *Trypanosoma cruzi* and is endemic to many countries of Latin America [1]. There are approximately 15 million people affected with Chagas disease worldwide [2]. Chagas disease has gone global and is now seen in North America, Europe and Asia which is outside its traditional endemic region [3]. Due to immigration from endemic countries it is

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estimated that ~300,000 people in the United States are infected with *T. cruzi*. In addition, studies have shown the presence of the insect vector as well as *T. cruzi* infection of domestic dogs and wild animals in the United States as well as transmission to humans [4–7].

It is noteworthy that even 100 years after Carlos Chagas discovery of the infection which bears his name, the mechanisms involved in *T. cruzi* invasion and host-parasite interaction are not fully understood. Furthermore, although there have been many studies on both experimental models of Chagas disease, as well on samples obtained from infected humans, the pathophysiological mechanisms that eventually result in Chagasic cardiomyopathy also remain uncertain. The etiology of Chagasic cardiomyopathy is clearly multifactorial and parasite persistence is an important contributory factor [8, 9]. *T. cruzi* infection results in the upregulation of the pro-inflammatory pathway which is essential for controlling acute parasitemia, but may also have adverse effects on the host [10–12].

T. cruzi invades a wide variety of mammalian cells [13–16]. We previously demonstrated that the low density lipoprotein receptor (LDLr) is involved in *T. cruzi* invasion and the subsequent fusion of the parasitophorous vacuole with host-cell lysosomes [17]. In addition, we have shown that this parasite-host cell interaction triggers an accumulation of LDLr and phosphatidylinositol phosphates in coated pits, which initiates a signaling cascade for lysosome recruitment [17–19]. We and others have demonstrated that this parasite has high affinity for lipoproteins [17, 20]. In *vivo* studies demonstrate a significant decrease in serum lipid levels (triglycerides and cholesterol) 15 to 30 days post infection (dpi) in a murine model of *T. cruzi* infection, as well as sequestered LDL and LDLr surrounding amastigotes in hearts of infected CD1 mice [17]. These observations suggest that host LDLr and LDL accumulation in the heart can alter intracellular lipid homeostasis contributing to the pathogenesis of Chagas disease.

T. cruzi infection dramatically affects the structure and function of adipose tissue at all stages of infection [21, 22]. Adipose tissue and adipocytes are early targets and reservoirs for this parasite in experimental animals and humans [21–23]. During acute infection, *T. cruzi* infection induces lipolysis and fat cell necrosis [12]. *T. cruzi*-infected CD1 mice display increased serum lipid levels during the early acute stage of infection followed by a reduction in serum lipid levels during the latent stage of infection [17]. Herein, we report the consequences of infection on cholesterol and LDL levels employing *in vitro* and *in vivo* models of *T. cruzi* infection. Additionally, we present data on the regulatory effects of parasite invasion/infection on host LDLr using shRNA knock-down and LDLr overexpressed cells.

2. MATERIALS AND METHODS

2.1 *T. cruzi* infection of cultured cells and mice

Human foreskin fibroblasts (HFF), Mouse NIH-3T3 embryonic fibroblasts and human embryonic kidney 293 (HEK 293 cells) were cultured and maintained in DMEM with 10% Bovine calf serum and Penicillin-Streptomycin solution as previously published [17]. Cells were infected with trypomastigotes of the Brazil strain at a multiplicity of infection (MOI) of 5:1 using the conditions described in sections 2.2 and 2.3.

The Brazil strain of *T. cruzi* was maintained by passage in C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME). Male CD1 mice (Charles River, Wilmington, MA) were maintained on a 12-hour light/dark cycle and fed standard chow diet.

Mice were infected at 8–10 weeks of age with 5×10^4 trypomastigotes. Samples of heart, pancreas and adipose tissues were collected at 30 and 120 dpi for histopathology and immunofluorescence analysis (n=5 per group). All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Albert Einstein College of Medicine.

2.2 shRNA induced LDLr knock-down cells

The pLKO.1-puro non-target shRNA control vector and the pLKO.1-puro vector containing shRNA against LDLr (TRCN clone 271877, 271881, 271926, 284544 and 284546 represented as sh 1–5 respectively) were purchased from Sigma (Sigma, MISSION shRNA, St. Louis, MO, USA). Fifty to 200ng of vectors was used to transfect 3T3 cells using Lipofectamin reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Stable cell lines were selected with puromycin (2 µg/ml).

2.3 LDLr over expression in host cells

The PCMV empty and PCMV6-LDLr transcription ready vector (SC110941, OriGene Technologies, Inc. Rockville, MD, USA) was used to transfect HEK293 cells as mentioned above.

2.4 Human heart sample

We examined heart tissues from a patient with end-stage Chagasic heart disease and a patient with ischemic heart disease (of similar age to the patient with Chagasic heart disease) who died at Montefiore Medical Center, Bronx, New York. The tissues were fixed in 4% formalin and frozen tissue sections were used for histology and immunofluorescence studies. The tissues were obtained under an Institutional Review Board (IRB) approved protocol.

2.5 RNA extraction and qPCR analysis of LDLr

Total RNA was extracted from the cells (infected and uninfected) using Trizol reagent (Invitrogen). RNA was then further purified using an RNeasy minikit (QIAGEN Sciences, Maryland) according to the manufacturer's instructions. LDLr mRNA levels were detected using qSTAR qPCR primer pairs designed by OriGene Technologies, Inc. (Rockville, MD, USA). Reverse transcription of total RNA and the quantitative PCR was carried out as previously published using iQ5 BioRad system [24].

2.6 DNA extraction and qPCR analysis of parasite load

Wild type, LDLr Knock-down cells and LDLr overexpression cells were incubated with trypomastigotes (5:1 MOI) for different time intervals (1h–96h) at 37°C. The cells were washed four times in PBS to remove unbound parasites. Parasite load in these cells was quantitated by qPCR as previously published [21].

2.7 Immunohistochemistry (IHC) and Immunofluorescence analysis (IFA)

Formalin fixed (4%) frozen tissue sections (10µm) were used for IHC and IFA. Cells were cultured on cover slips to 50 to 60% confluence and then infected with trypomastigotes (5:1 MOI) and cultured for 0 to 96h at 37°C. The cultures (infected and uninfected) were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton ×100 (30min) and stained with goat anti-human LDL (1:25, Sigma L 8016) and Alexa fluor 488 donkey anti goat IgG (1:500 dilution; Invitrogen, CA). The cells were stained with DAPI (blue) to detect nuclei following manufacturer's protocols (www.abcam.com/technical). IFA to detect LDLr was performed as previously published [17]. Images were obtained and analyzed by fluorescence microscopy using an inverted Olympus IX71 with a HQ2 CCD camera and a Nikon Microphot-FXA with Spot camera software. IFA of paraffin embedded tissues were performed as previously published [17]. Hematoxylin and eosin (H&E) staining was performed using standard methods for tissue sections [25]. Filipin staining was carried out on the frozen tissues embedded in OCT for cholesterol detection using 50ug/ml Filipin (Cayman Chemical, MI, USA) and following standard protocol [26]. The NIH-ImageJ program was used to quantify fluorescence intensities for a minimum of five images (images taken using a 20 x objective to analyze approximately a total of 100 cells) in each experiment. IFA of infected and uninfected HFF cells displayed no significant changes in the fluorescence intensity when incubated with the goat anti rabbit IgG (1:500) or donkey anti goat IgG (1:500) antibodies. The number of parasites per infected cell was determined for a total of 500 cells selected from 5 different areas of each culture plate (these results were replicated in three independent experiments).

3. RESULTS

3.1 Intracellular LDL/cholesterol accumulates in the tissues of *T. cruzi*-infected mice

During acute infection there is inflammation, necrosis and fibrosis in both heart and adipose tissue [21, 22, 27, 28]. There is increased lipolysis in white adipose tissue (WAT) and decreased levels of triglyceride [21, 22] (Figure 1a). IFA demonstrated a significant increase (2.5 fold, $p<0.005$) in cholesterol levels in WAT, a likely early target of *T. cruzi* infection (Figure 1b). IFA for LDL in frozen tissue sections from *T. cruzi* infected CD1 mice demonstrated an accumulation of LDL in tissues such as heart (2 fold, $p<0.005$) and pancreas [(3.2 fold, $p<0.005$) during chronic infection at 90 dpi] (Figures 1c and 1d) which persisted to 120 dpi (data not shown).

3.2 Tissue from a patient with chronic Chagasic cardiomyopathy also demonstrates an increase in LDL and cholesterol

Examination of heart tissue from a patient with Chagas disease revealed fibrosis between individual cardiomyocytes and bundles of cardiomyocytes, as well as cardiomyocyte hypertrophy which are characteristics of chronic Chagas heart disease [29] (Figures 2a H&E staining and 2b Trichrome staining). qPCR analysis confirmed the presence of *T. cruzi* (8 to 10 parasites/10,000 cells) in the heart and pericardial fat from this patient. IFA demonstrated increased intracellular cholesterol in this infected heart tissue and such changes were not observed in a patient of similar age who died of ischemic heart disease (Figure 2c, top panel). Interestingly, there was an increase in LDL in the vasculature of the patient with

Chagasic heart disease as well (Figure 2c, bottom panel). Quantification using NIH-image J demonstrated a 15-fold increase in cholesterol ($p<0.005$) and a 2.5-fold increase in LDL ($p<0.005$) in the heart of the patient with Chagas disease compared to heart tissue from the ischemic heart disease patient.

3.3 *T. cruzi* infection increases intracellular LDL and cholesterol levels and host cell hypertrophy

T. cruzi has a high affinity for host lipoproteins [17, 20]. An increased rate of invasion was observed with increased serum lipid concentrations *in vitro* (Figure 3a). The number of parasites per infected cell was determined for a total of 500 cells selected from 5 different areas of each culture plate. These studies demonstrated a significant difference in the invasion rate between infected cells incubated for 1h with varying concentrations of serum which provided the lipid source in the medium (these results were replicated in three independent experiments). Cells incubated in the absence of serum had mostly cell membrane attached extracellular parasites and approximately 142 invaded parasites (± 37) whereas, cells incubated with 10% and 30% serum had 978 (± 323) and 5732 (± 639) invaded parasites respectively ($p<0.005$). IFA demonstrated cell hypertrophy of the infected host cells incubated with 30% serum compared to uninfected cells (Figure 3b). At the same time, intracellular cholesterol concentrations were also increased in presence of increasing serum concentration in *T. cruzi* infected cells compared to uninfected cells (Figure 3c) consistent with the higher rates of invasion observed with higher concentrations of serum. We were unable to observe a significant difference in the images above 5% serum due to the quenching effect of filipin at higher concentrations of cholesterol (Cayman Chemical, USA). Accumulation of intracellular LDL/cholesterol level increased with the time of incubation during infection (Figures 3d and 3e).

3.4 Infection induced accumulation of intracellular LDL/cholesterol alters the lysosome pH of infected cells

We examined the effect of accumulated LDL/cholesterol on lysosomal pH during *T. cruzi* invasion. Excessive cholesterol levels in lysosomes are known to alter the pH of the lysosomal compartment [30]. Fluorescence microscopic analysis using LysoSensor Green DND-153 which is brightly fluorescent at neutral pH (6-fold brighter than the uninfected cells) demonstrated that the pH of lysosome of infected HFF cells ranged from acidic to neutral at 24h post-infection, whereas uninfected cells revealed no change in pH ($p<0.005$).

3.5 *T. cruzi* infection induces expression of LDLr

Transient and stable shRNA transfected 3T3 fibroblasts displayed a significant decrease in the expression of LDLr (Figure 4). qPCR analysis demonstrated a 70 to 80% knockdown in the mRNA level of LDLr gene expression in five different shRNA transfected cells compared to control vector transfected 3T3 fibroblast cells (Figure 4a). These LDLr knockdown cells displayed a decreased rate of infection compared to control vector transfected cells (assayed at 12 h post-infection). However, prolonged incubation with trypomastigotes for 72 h demonstrated a significant increase in parasite load in these knockdown cells compared to the results seen at a 12h incubation (Figure 4b). qPCR

analysis demonstrated that these 72 h infected cells had 73% of the parasite load of the control vector transfected cells. This increase in rate of invasion is likely due to an increase in LDLr expression that occurs in these shRNA transfected cells as a result of infection (Figure 4c). qPCR analysis demonstrated a 68% increase in LDLr levels at 72h infection compared to 12h of infection in the knockdown cells. Immunofluorescence analysis confirmed an infection-induced expression of LDLr in knockdown cells (Figure 4d).

Transient transfection of HEK 293 T cells with the pCMV6-XL4huLDLr vector caused an overexpression of LDLr (3 fold, $p < 0.001$) in these transfected cells compared to empty pCMV vector transfected cells (Figure 4e). Infection of these cells demonstrated that overexpression of LDLr in HEK 293T cells results in a 8-fold increase ($p < 0.001$) in the parasite invasion rate (5:1 MOI; incubation for 1h) compared to endogenously expressed LDLr containing wild type HEK 293T cells. IFA of these pCMV6-XL4huLDLr HEK 293T cells incubated in presence of parasites demonstrated an altered LDLr distribution (Figure 4f), as we have observed previously with wild type cells [17].

4. DISCUSSION

The mechanisms by which *T. cruzi* invades and persists in host cells has been an active area of investigation [31, 32]. *T. cruzi* has a high affinity for lipoproteins and *in vitro* studies reveal an increased rate of invasion with increasing serum concentrations of lipoproteins. Invasion resulted in increased intracellular cholesterol levels and increased cholesterol levels were also observed in cultured cells incubated with parasites. Hypertrophied infected cells (after 48h pi) with increased intracellular cholesterol were observed when the cells were incubated in the absence of serum. It has been reported that cells that have been depleted of sterols synthesize endogenous cholesterol to counteract the depletion [33, 34]. The significantly increased cholesterol in infected host cells compared to uninfected host cells after 48h of incubation, in the absence of serum, suggests that replicating amastigotes interfere with the host lipid/cholesterol biosynthetic mechanisms altering intracellular cholesterol homeostasis. Recently, it has been reported that replicating amastigotes depend on host fatty acid metabolism [35]. We have observed such alterations in host lipid/cholesterol biosynthetic mechanisms in experimental infection using mRNA profiling (manuscript in preparation). Collectively, these observations indicate that parasite invasion and subsequent infection induces alterations in the lipid/cholesterol levels which would contribute to host cell/tissue lipotoxicity and the pathophysiology of infection.

Previously, we reported that *T. cruzi* utilizes the host LDLr internalization complex system to invade host cells and traffic to lysosomes [17]. Therefore, in order to more completely understand the role of LDLr in this process we genetically manipulated cells so that LDLr was either knocked-down or over-expressed. Although shRNA transfected 3T3 fibroblasts displayed significantly reduced LDLr levels (~20%) compared with control vector transfected cells, infection of these cells by trypomastigotes resulted in increased LDLr levels. These data indicate that *T. cruzi* alters host LDLr levels at a transcriptional level thereby providing additional LDLr to facilitate invasion. Overexpression of LDLr enhanced parasite invasion which resulted in an 8-fold increase in parasite load compared to empty vector transfected cells. We found that invasion results in the accumulation of a large

amount of LDL/cholesterol into cells resulting in an elevated intracellular cholesterol levels. Additionally, this elevation in intracellular lipid levels was dependent on the concentration of serum in the culture medium and the time of incubation.

Previously, we reported that infection with *T. cruzi* leads to a significant decrease in serum lipid levels and that LDL concentrates around intracellular amastigotes in heart tissue [17] indicating that invasion results in increased tissue levels of LDL. In our current studies employing infected CD1 mice, we found that there was an accumulation of cholesterol in the heart, pancreas and WAT. This cholesterol accumulation, especially in necrotic cells with depleted triglycerides, strongly suggests a role for these accumulated cholesterol in the lipolysis seen during acute infection. These changes in the intracellular cholesterol levels persisted into the chronic phase. Similar findings were observed in heart tissue obtained at autopsy from a case of chronic Chagasic cardiomyopathy, in which the myocardium displayed an increase in LDL/cholesterol compared to a heart sample from a patient that died from ischemic heart disease. We appreciate the limitations that this is an observation in one human sample, but nevertheless it confirms what is observed in the infected mouse model. We believe that these alterations in intracellular LDL/cholesterol results in lipotoxicity eventually leading to metabolic and pathologic dysfunction.

Our current observations demonstrate that the rate of parasite invasion and progression of infection is dependent, in part, on host lipid and cholesterol levels and trafficking. Infection-induced modulation of cholesterol/lipid intracellular homeostasis persists during chronic infection and may lead to alterations in cardiac function. We hypothesize that infection-mediated elevated intracellular lipid levels and the increased demand for cholesterol biosynthesis during chronic infection alters membrane and lysosomal function and exhausts mitochondrial oxidative capacity thus, contributing to the development of Chagasic cardiomyopathy. The mechanism of invasion and host-pathogen mediated cellular responses can also lead to host cell metabolic alterations and immune-mediated cytotoxic reactions such as the production of reactive oxygen species.

Overall, the observations reported by our laboratory group and others suggest that a detailed examination of the metabolic and nutritional status of the host is important in evaluating the consequences of infection. This can have significant public health implications for *T. cruzi*-infected persons as endemic regions are transitioning to a “western-diet.” In addition, immigrants from endemic areas are also exposed to new diets.

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Figure 1. Infection results in the accumulation of LDL/cholesterol in the tissues of acute and chronic chagasic murine model
(a) Loss in triglyceride levels in the adipose tissue of infected mice (15dpi) (right panel) compared to uninfected mice (left panel) analyzed by Oil-red-O staining (red colored lipid globules). Fat loss and necrosis is seen in the WAT of infected mice (arrow). Images were taken using a 10X objective.

(b) Increased intracellular cholesterol levels (blue fluorescent) in the adipose tissue of infected mice (15dpi) (right panel) compared to uninfected mice (left panel) demonstrated by Immunofluorescence analysis (IFA) using fillipin (50µg/ml) staining. Images were taken using a 10X objective.

(c and d) IFA demonstrated increased LDL (increased green fluorescence, arrow) in the myocardium (c) and pancreas (d) of chronic (d120pi) infected mice (right panel) using polyclonal LDL antibody (green fluorescence) [17]. Plot profiles of the intensities were quantified using NIH-Image J program for b, c and d, and presented in the result section. (n=5, p< 0.005).

Images were taken at using a 20X objective.

Figure 2. Human chronic chagas heart tissue has an accumulation of LDL and cholesterol

(a) H&E staining and (b) trichrome staining of myocardium of Chagas heart demonstrated cardiomyocyte hypertrophy and severe fibrosis. Images were taken using a 20x (a) and 10x (b) objective.

(c) IFA displayed streaks of cholesterol (blue fluorescence) in the chagasic heart tissue which were not observed in an ischemic heart from a same age group patient using fillipin (50 μ g/ml) staining (c, top panel). IFA demonstrated increased LDL (green fluorescence) in the vessels and capillaries of chagasic heart (c, bottom panel). IFA was performed using human LDL polyclonal antibody as previously published [17]. Images were taken at 20x magnification. Plot profiles of the intensities were quantified using NIH-Image J program for c and presented in the result section. (5 images were quantified for each case, $p < 0.005$)

Figure 3. *T. cruzi* infection increases intracellular LDL and cholesterol levels and leads to cell hypertrophy

- (a)** HFF cells incubated with parasites for 1h at different concentrations of serum had an increase in their invasion rate with the increase in serum concentration. The number of parasites per infected cell was counted for total of 500 cells selected from 5 different areas (repeated for three independent experiments) using a 20X objective.
- (b)** Cell hypertrophy was observed in HFF cells incubated with parasites for 1h with 30% FBS (images taken using a 40X objective).
- (c)** Increased intracellular cholesterol (blue fluorescence) concentrations were observed in presence of increasing serum concentrations in *T. cruzi* infected cells compared to uninfected cells. We cannot quantify a significant difference in the images above 5% due to the quenching effect of filipin at higher concentrations of cholesterol (Cayman Chemical, MI, USA). Cholesterol levels (blue fluorescence) were determined by using fillipin (50µg/ml) staining (Images taken using a 20X objective).
- (d and e)** Intracellular LDL and cholesterol levels (red and blue fluorescence respectively) increased with increase in time of incubation during infection. ($p < 0.001$) (Images taken using a 20X objective, except for Figure d 72h which is a 100X objective).
- (f)** No significant changes in fluorescence intensities were observed when the uninfected and *T. cruzi* infected cells (72h pi) were incubated with either goat anti rabbit IgG (1:500) or donkey anti goat IgG (1:500) antibodies as demonstrated by IFA (Images taken using a 20X objective). DAPI staining demonstrates the presence of amastigotes (arrow) in the infected cells.

Figure 4. *T. cruzi* regulate mRNA expression of LDLr in shRNA knock-down cells

- (a) qPCR analysis of transfected 3T3 fibroblasts with 5 different LDLr-shRNA (sh 1–5) demonstrated a significant (*) fold reduction ($p < 0.005$) in the expression of LDLr.
- (b) Incubation of shRNA knock down cells (grey bars) with trypomastigotes for 12h displayed a decreased rate of infection compared to control vector cells (white bars) as measured by qPCR analysis. However, shRNA knock down cells showed a significant increase (**) in parasite load when the cells were incubated with parasites for 72h compared to 12h of incubation ($p < 0.005$).
- (c) qPCR analysis demonstrated significantly increased LDLr expression (***) in shRNA knock down cells in presence of trypomastigotes (72h post infection) ($p < 0.005$).
- (d) shRNA knock down 3T3 fibroblasts displayed increased LDLr levels (red fluorescence) in presence of trypomastigotes compared to uninfected knock down cells by IFA using monoclonal LDLr antibody (bottom panel, images taken using a 60 x objective ($p < 0.01$)). Quantitation of the fluorescence intensities were measured for images (images taken at 20 x magnification for the total of approximately 100 cells) using NIH-Image J program is represented as a bar graph (top panel, Uninfected – white bar, Infected – grey bar).
- (e) IFA of pCMV6-XL4huLDLr vector transfected HEK293T cells (LDLr OEX cells- LDL receptor over expressed cells) using monoclonal LDLr antibody demonstrated increased LDLr levels (red fluorescence) compared to control vector transfected cells (WT- wild type cells) (left panel). Quantification of data presented in quantified and represented as bar graph (right panel, Uninfected – empty bar, Infected – shaded bar) ($p < 0.001$) (Images taken using a 20X objective).
- (f) IFA demonstrated an altered LDLr distribution (arrow) in presence of parasites (DAPI staining –small blue dots, LDLr- red fluorescence) in the LDLr over expressed pCMV6-XL4huLDLr vector transfected HEK293T cells (Images taken using a 20X objective).