The Adipocyte as an Important Target Cell for *Trypanosoma cruzi* Infection

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Running title:
Trypanosoma cruzi Infection of Adipocytes

Abbreviations:
Acrp30: adipocyte complement-related protein of 30 kDa; PAI-1: plasminogen activator inhibitor type-1; IL-6: Interleukin –6; TNFα: Tumor necrosis Factor α; MCP-1: Monocyte Chemoattractant Protein-1; WAT: White Adipose Tissue; BAT: Brown Adipose Tissue;
Abstract

Adipose tissue plays an active role in normal metabolic homeostasis as well as in the development of human disease. Beyond its obvious role as a depot for triglycerides, adipose tissue controls energy expenditure through secretion of several factors. Little attention has been given to the role of adipocytes in the pathogenesis of Chagas’ disease and the associated metabolic alterations. Our previous studies have indicated that hyperglycemia significantly increases parasitemia and mortality in mice infected with T. cruzi. We determined the consequences of adipocyte infection in vitro and in vivo. Cultured 3T3-L1 adipocytes can be infected at high efficiency. Electron micrographs of infected cells revealed a large number of intracellular parasites that cluster around lipid droplets. Furthermore, infected adipocytes exhibited changes in expression levels of a number of different adipocyte-specific or adipocyte-enriched proteins. The adipocyte is therefore an important target cell during acute Chagas’ disease. Infection of adipocytes by T. cruzi, profoundly influences the pattern of adipokines. During chronic infection, adipocytes may represent an important long-term reservoir for parasites from which relapse of infection can occur. The acute infection manifests a unique metabolic profile, with a high degree of local inflammation in adipose tissue, hypoadiponectinemia, hypoglycemia and hypoinsulinemia, but relatively normal glucose disposal during an oral glucose tolerance test.
Introduction

Chagas’ disease caused by the protozoan parasite, *Trypanosoma cruzi*, is endemic in Mexico, Central and South America where it causes significant morbidity and mortality (1). In fact, it is a major cause of heart disease in endemic areas. Based on seroepidemiologic studies, it is estimated that there are at least a hundred thousand immigrants in the United States chronically infected with this parasite (2). In addition, Chagas’ disease is now appreciated as an opportunistic infection in immune-compromised individuals including those with HIV/AIDS (3). Some investigators have concluded that the cause of death in acute Chagas’ disease is a septic shock-like picture accompanied by hypoglycemia (4,5), but a more detailed metabolic characterization has not yet been reported.

For many years the association between human *T. cruzi* infection and diabetes has received little formal evaluation. Anecdotally, there had been a general belief that the incidence of diabetes is greater in the chagasic population. In recent years, there have been several reports suggesting that diabetes is indeed more common in the setting of *T. cruzi* infection (6,7). One study demonstrated a significant reduction in insulin among chronically infected individuals (8). Interestingly, our previous studies indicated that when mice with chemical-induced diabetes are infected with *T. cruzi*, they have a higher parasitemia and mortality (9). The same observation is seen when diabetic *db/db* mice are infected (9,10). The underlying reasons for these phenomena are unknown.

The adipocyte and its relationship to the pathogenesis of infection has only recently been explored (11,12). The fat laden cells of the skin are among the cells that are initially encountered by trypomastigotes of *T. cruzi* and may be one of the initial targets. The adipose tissue in the acute and chronic state may serve as one of the reservoirs for the parasite from which recrudescence may occur during immune suppression.

As no systematic approach has been undertaken to more precisely define the role of the adipocyte in the normal and diabetic state during infection with *T. cruzi*, we examined the direct effects of *T. cruzi* infection on adipocytes in vitro and in vivo. Our investigations indicate that the adipocyte is an important target for infection and profoundly changes its cellular homeostasis, both in vitro as well as in vivo as a consequence of infection. Such changes permit the adipocyte to function as an important reservoir host cell for chronic Chagas’ disease. Since
the adipocyte has found increasing appreciation in recent years as a highly active endocrine cell (11), the observation that these cells represent a target tissue for *T. cruzi* significantly changes our perspectives of the role of the adipocyte in the progression and re-activation of this disease.
Materials and Methods

Animals. Mice were maintained on a 12-hour light/dark cycle and standard chow diet. All animal experimental protocols were approved by the Institute for Animal Studies of the Albert Einstein College of Medicine. The Brazil strain of *T. cruzi* was maintained by passage in C3H/Hej mice (Jackson Laboratories, Bar Harbor, ME). Male CD1 mice (Jackson Laboratories) were infected at 8-10 weeks of age with $5 \times 10^4$ trypomastigotes and the various experiments were performed on the indicated days post infection. Parasitemia was evaluated by counting in a hemocytometer as previously described (9).

Reagents. DMEM (Dulbecco's modified Eagle's Medium) was purchased from Mediatech Inc (Herndon, VA). All other chemicals were purchased from Fisher (Pittsburgh, PA) and were of the highest purity.

Magnetic resonance imaging. Mice were anesthetized with ketamine/xylazine injection (intraperitoneal) and were placed in a custom built 35 mm inner diameter RF coil. Body temperature was maintained by keeping the NESLAB gradient water cooling system set at 30ºC. The 1H NMR spectra of the mice were acquired using a routine one pulse experiment with a 5 s relaxation delay and signal averaging of 4 transients and a GE Omega 9.4T vertical bore NMR system (Fremont, CA). The spectra were integrated using the standard vendor NMR software. All procedures were approved by our institutional animal care and use committee and are in accordance with accepted institutional and governmental policies.

Adipocyte differentiation in cell culture. 3T3-L1 murine fibroblasts (a generous gift of Dr. Charles Rubin, Department of Molecular Pharmacology, Albert Einstein College of Medicine) were propagated and differentiated to adipocytes as previously described (13). In brief, the cells were propagated in "FCS" (DMEM containing 10% fetal calf serum (JRH Biosciences) supplemented with Penicillin / Streptomycin (100U/ml each) and allowed to reach confluence (Day -2). After two days (Day 0), the medium was changed to "DM1" (containing "FCS" and 160nM insulin, 250nM dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine). Two days later
(Day 2), the medium was switched to "DM2" ("FCS" containing 160nM insulin). After another two days, the cells were switched back to "FCS". Cells were used between day 8 and 12 post induction of differentiation. Cells were infected at a multiplicity of infection of 2:1.

**Assay protocols.** Serum values for glucose was measured by FastBlue B glucose assay (Sigma-Aldrich) and adiponectin with a mouse adiponectin RIA kit (LINCO Research, St. Charles, MO). Other adipokines were measured using LINCOPLEX reagents (LINCO Research). All metabolic parameters measured in the fasted state: animals were assayed at 10 AM following a 16-hour fast. Oral glucose tolerance tests (OGTTs) were performed in animals without access to food for 2 hours prior to administration of 2.5mg/g body weight glucose load by oral gavage, and during the course of the study.

**Quantitative determination of parasite load in tissue:** Heart, spleen, brown adipose tissue (BAT) and white adipose tissue (WAT) tissues were collected from mice 15, 30, 60, 300 days post-infection and stored at -80°C. DNA was isolated from these tissues as well as *T. cruzi* epimastigotes using the Qiagen DNeasy tissue kit following the manufacturers protocol.

A standard curve in the range of 50 pg to 50 ng for the quantification of *T. cruzi* DNA by real time PCR was developed using the *T. cruzi* 195-bp repeat DNA specific primers TCZ-F (5’-GCTCTTGGCCACAAAGGG TGC-3’) and TCZ-R (5’CCAAGCAGCGGATAGTCCAGG-3’) (14) and genomic DNA purified from *T. cruzi* epimastigotes.

Quantitative PCR was performed using samples contained 50 ng genomic DNA, 0.5 µM of TCZF and TCZR primes (that amplify a 182-bp product), 1.6 µl MgCl2 (25 mM), light cycler-fast start master Syber green1 (Roche), and PCR grade water (Roche) to a final total volume of 20 µl. A parallel reaction was done for each sample using 50ng genomic DNA and 0.2 µM of the murine specific microglobulin primers β2F2 (5’-TGGAAGCCGAACATACTG-3’) and β2R2 (5’-GCAGGCTATGTATCGTCTCA-3’), designed by TIB Molbiol LLC, which amplify 190-bp product. These reaction mixes were loaded into Roche Light Cycler Capillaries, capped, centrifuged for 10s at 2000 rpm and placed in the Light Cycler (Roche). In the denaturation phase the capillary was heated to 95°C at 20°C/s ramp and held for 10 min. During the 45 cycles of the amplification phase there were three steps: 95°C at 20 °C/s ramp, for

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2 s, 57°C at 20°C/s ramp, for 5 s, and 72°C at 20°C/s ramp, for a 10 s hold. During extension the fluorescence intensity was acquired as single color detection (Syber green). The third phase was a 95°C at 20°C/s ramp for 0 s hold, followed by 65°C at 20°C/s ramp, for 15 s hold, and finally 95°C at 0.1°C/s ramp for 0 s hold. During the melting phase, the acquisition setting was set at “continuous”. The final phase was the cooling phase lasting 30 s at 40°C at 20°C/s. Data were acquired and analyzed with Light Cycler Version 3.0 software. Each run contained a negative control lacking template DNA.

The primer set TCZ-F::TCZ-R has been previously used (14) for routine PCR-based detection of T. cruzi (16). Primer set TCZ amplifies a 195-bp, tandemly repeated genomic sequence, e.g. satellite DNA, present in approximately 120,000 copies in the Y strain of T. cruzi (15). As an internal control β2-microglobulin, a double copy mouse gene, was used to normalize the amount of mouse DNA present, e.g. number of cells, in each analysis. A standard for murine β2-microglobulin concentration was developed from a serial dilution of DNA (PCR product murine genomic DNA). The number of parasites per cell was calculated by dividing the number of parasites (number of copies of T. cruzi DNA obtained by real time PCR) by number of cells (number of copies of β2-microglobulin obtained by real time PCR).

Immunoblot analysis. After infection, plates of differentiated 3T3-L1 cells were washed twice with phosphate-buffered saline and lysed in 1 ml SDS-PAGE sample buffer (0.75 % sodium dodecyl sulfate, 0.5 M Tris-HCl pH 6.8, 16 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride, and lysates were boiled for 5 minutes followed by brief sonication. Total protein (30 µg) protein was resolved by SDS-PAGE on 12% acrylamide gels, and transferred to BA83 nitrocellulose (Schleicher and Schuell). Blots were probed with various antibodies as indicated. The rabbit polyclonal antibodies to the guanine nucleotide dissociation inhibitor (GDI) was a generous gift from Dr. Perry Bickel (Washington University, St. Louis, MO). Primary and secondary antibodies were diluted in PBS or Tris-buffered saline with 0.1% Tween 20 and 1% bovine serum albumin. Bound antibodies were detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ). Immunoblots from a minimum of three independent experiments were scanned and the background-corrected signal from each band was quantitated by densitometry using an Alpha Innotech Multiimage Light.
Cabinet with Chemiimager 4400 software. Signal for each sample lane was normalized to the signals obtained for either GDI or β-actin. The normalized relative levels for each experimental group are represented as the mean +/- standard error of the mean.

**Immunohistochemistry.** Freshly isolated tissues were fixed with phosphate-buffered formalin overnight and then paraffin wax embedded. 5μm sections were incubated overnight with a monoclonal anti-F4/80 antibody. After washing in PBS, slides were incubated with biotinylated goat anti-rat or anti-rat IgG at 5 μg/ml (Vector Laboratories, Burlingame, CA 94010) for 1 hour at room temperature. Slides were developed using a peroxidase detection kit (Vector Laboratories) and counterstained with hematoxylin (Sigma-Aldrich).

**Transmission electron microscopy:** 3T3-L1 adipocytes were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% Osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington VT). Ultrathin sections (80nm) were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1200EX transmission electron microscope at 80kv. For EM on primary adipocytes, mice were anesthetized and both brown and white adipose tissue (epididymal), were carefully dissected. These samples were then fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. They were then postfixed with 1% osmium tetroxide followed by 1% uranyl acetate and dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington VT) and sectioned and imaged as indicated above.
**Scanning electron microscopy:** Fat cells were quick fixed in 1% Osmium Tetroxide, 0.1 M sodium Cacodylate, 0.2 M Sucrose, 5mM MgCl₂ pH.4 (SEM buffer) for five seconds, followed by two changes of 2.5% gluteraldehyde in SEM buffer. Then cells were fixed cells with 1% Osmium Tetroxide, in SEM buffer and dehydrated through a graded series of ethanol. Critical point drying was accomplished using liquid carbon dioxide in a Tousimis Samdri 795 Critical Point Drier (Rockville MD). They were then sputter coated with gold-palladium in a Denton Vacuum Desk-2 Sputter Coater (Cherry Hill NJ) and examined in a JEOL JSM6400 Scanning Electron Microscope (Peabody MA), using an accelerating voltage of 10 KV.

**Statistical analysis:** The results are shown as means ± SEM. Statistical analysis was performed by one- or two-way analysis of variance (ANOVA). Significance was accepted at p<0.05.
Results

Parasitology

CD1 mice had a 60% mortality by day 35-40 post infection. The peak parasitemia at day 35-40 post infection ranged from 5x10^5 to 1x10^6 trypomastigotes/ml of blood and the parasitemia waned so that by day 60 post infection parasites were not observed on routine blood examinations.

Infection of mice with T. cruzi caused transient hypoglycemia during the acute phase of the infection, but does not impair glucose tolerance

To determine the metabolic consequences of T. cruzi infection, we monitored basal glucose levels as well as insulin sensitivity by means of an oral glucose tolerance test prior to infection, during the acute phase of infection (day 30) as well as during the chronic stage (approximately 90 days post infection) and compared it with a cohort of mice that were injected with vehicle only. The acute phase of the infection was associated with severe hypoglycemia in mice (Fig.1A) in agreement with previously reported data (4,5). Clinically, similar observations have been reported for patients. Conventionally, the metabolic response to sepsis entails hyperglycemia with insulin resistance, profound negative nitrogen balance, and diversion of protein from skeletal muscle to splanchnic tissues. The underlying reasons for the hypoglycemia during acute T. cruzi infections that may be a major reason for mortality are not known. The oral glucose tolerance tests between the two groups were overlapping before infection (day 0) and in the chronic stage of infection (day 90) when the mice have fully recovered. Surprisingly, during the acute phase of infection (day 30), glucose levels in the infected animals remained at all stages below the levels measured for the control animals. Even though the base line glucose levels in the infected animals were lower, the oral glucose tolerance tests indicate relatively normal ability to clear the ingested glucose despite the high degree of inflammation associated with this state (Fig. 1B).
Hypoglycemia is a predictor of mortality in T. cruzi infected mice

To test whether the hypoglycemia may directly or indirectly be linked to mortality, we compared the fasting glucose levels and survival rate of infected mice (Fig. 1C). There was a strong correlation between the relative reduction in glucose levels and mortality. The average normal glucose levels of CD1 mice were 150mg/dl. During acute infection, glucose levels were reduced as low as 20mg/dl, a level that over a sustained period of time can be fatal, though there are rare examples of mice that spontaneously recovered from such a severe hypoglycemic condition. Even though the basal glucose levels correlate well, we failed to detect significant differences in glucose clearance during OGTTs between survivors and mice that died in the course of infection (Fig. 1B).

T. cruzi infection has a profound impact on systemic inflammation and adipokine levels

Prompted by the very low glucose levels, we decided to examine the plasma levels of two key adipokines released by adipocytes. Adiponectin has potent insulin-sensitizing properties, and decreased levels of this hormone tend to be associated with insulin resistance, hyperglycemia and obesity. In addition, decreased levels of adiponectin are observed with increased systemic inflammation associated with cardiovascular disease. However, acute inflammation triggered by endotoxemia does not have an effect on adiponectin levels (12). The hypoglycemia coupled with the potent pro-inflammatory profile associated with the acute phase of T. cruzi infection makes it difficult to predict how adiponectin levels would be affected. We therefore monitored the levels throughout the experimental period. Adiponectin levels were significantly reduced during acute infection (Fig. 2A), suggesting that the pro-inflammatory component of the infection is the main driving force for the expression of adiponectin. The hypoglycemia observed during that period cannot be explained by excess adiponectin levels. To our knowledge, this is the first example of a physiologically relevant condition that combines hypoglycemia and normal glucose tolerance with significantly reduced serum adiponectin levels. In contrast to the situation during infection of mice with rodent malaria, which is also accompanied by hypoglycemia triggered by a severe hyperinsulinemia (16), insulin levels were not significantly affected on day 15 and day 30 post infection. In fact, there was a trend towards lower levels of insulin on day 30 in the infected
group, but it failed to reach statistical significance (Fig. 2B, left panel). The decreased insulin levels are consistent with a physiological response to the very low glucose levels during that time. It is unlikely that this is a reflection of pathological changes at the level of the pancreatic β cells, since the β cell morphology is normal on day 30 of infection and insulin levels revert to normal levels at later stages (data not shown). Leptin levels were significantly decreased in infected mice compared to control mice (Fig. 2B, middle panel). Resistin, another fat cell-specific secretory factor with insulin-desensitizing properties, was unaffected by infection (Fig. 2B, right panel). Similarly, levels of PAI-1 which is also prominently expressed in adipocytes, are completely unaffected by infection (not shown). In contrast, pro-inflammatory markers such as IL-6 and MCP-1 (Fig. 2C) as well as TNFα (not shown) were markedly elevated in the infected mice in response to the high parasite load on day 15 and day 30. The significant decrease in leptin levels is surprising since the infected mice gained more weight than the control mice (Fig. 2C). To test whether the weight gain is due to an increase in fat mass or may be due to other reasons such as edema formation, we performed a detailed body composition analysis by MRI. Consistent with the decreased leptin levels, we found significantly reduced levels of adipose tissue during acute infection (Table I). Interestingly, the decrease in adipose tissue persisted even at later stages during chronic infection and was at that stage primarily due to a decrease in abdominal adipose tissue. Mice that suffered from cardiac dilation (used here as a surrogate marker for the increased severity of infection at the earlier stages) had an even more dramatic loss of both total and abdominal fat depots. The weight gain in infected animals appeared to be related to edema, which is a consequence of cardiac dysfunction in these animals.

Adipose tissue is an important target of T. cruzi

Since there was an alteration in systemic levels of one of the adipokines, we wanted to determine if the parasite directly invades adipose tissue. A real time PCR approach was utilized to evaluate the number of T. cruzi parasites host cell (using genomic copies of β2 microglobulin as a normalization standard). As shown in Fig. 3A, adipose tissue is a significant target tissue, harboring larger numbers of parasites than observed in more conventionally studied tissues, such as the heart and the spleen, with an average number of more than 7 parasites/cell (day 15 post infection) and 4 parasites/cell (day 30 post infection) during acute infection.
Particularly noteworthy is that in the chronic stage, on days 60 post infection, adipose tissue remains a prominent reservoir of parasites. Even 300 days post infection a comparable number of parasites were present in adipose and heart tissue when normalized on a per cell basis (Fig. 3B). This indicates that adipose tissue is a likely reservoir tissue for these parasites. This could be even more physiologically important in the setting of significant obesity.

Adipose tissue contains a number of different cell types. The stromal vascular fraction contains pre-adipocytes, macrophages as well as other cell types. An important role for local adipose tissue macrophages has recently been described (17). In the obese state, additional macrophages are recruited into adipose tissue and may significantly contribute towards local inflammation. The two cell types combined may be important contributors to systemic inflammation. Since there were numerous parasites in adipose tissue, we suspected that the massive local parasite count should be responsible for a considerable influx of macrophages. We wanted to test whether the high propensity to harbor and propagate *T. cruzi* in adipocytes also has an effect on macrophage levels. We therefore isolated white adipose tissue from two different fat depots of infected mice. As judged by an immunohistological stain for the macrophage-specific marker F4/80, white adipose tissue (WAT) there was a massive increase of F4/80 positive cells during the acute infection on day 18 (Fig. 3C). Even more pronounced changes were observed in brown adipose tissue (BAT) at the same time period (not shown) that was still be seen on day 30 post infection (Fig. 3D). The small insert demonstrates a higher magnification and highlights the intracellular location of the parasites within the adipocytes that in the case of brown adipocytes was characterized by multiple smaller intracellular lipid droplets as opposed to the densely packed white adipocytes that contained a single large lipid droplet. Despite these dramatic morphological changes in WAT and BAT during the acute infection that highlight the huge impact that the presence of large numbers of parasites exerts, the effects are at least morphologically fully reversible. Examining BAT (not shown) and epididymal WAT (Fig. 3E) during chronic infection (60 and 90 days post infection) there was a full recovery with reduced levels of macrophages that did not differ in number between infected and non-infected mice.
Primary adipocytes display vast numbers of intracellular parasites

To address the issue whether the parasites found in adipose tissue indeed have an intracellular location in adipocytes, we have systemically examined primary adipose tissue from infected mice from brown and white adipose tissue pads. Fig. 3F shows several micrographs from infected brown adipocytes. Note the characteristic multilocular small lipid droplet distribution and the abundant mitochondria. Similarly, a large number of parasites can be found in adipocytes isolated from white adipose tissue (Fig. 3G). It is interesting to note that the parasites are frequently found in close juxtaposition to the lipid droplet, suggesting that they may take advantage of local lipolysis at the surface of the lipid droplet to cover their energetic needs through the uptake and β oxidation of free fatty acids.

Infected adipocytes exhibited changes in the expression levels of a number of different adipocyte-specific or adipocyte-enriched proteins

The altered serum levels of adiponectin suggest that the invasion of adipocytes by parasites may also have direct consequences on additional proteins produced in adipose tissue. To directly address this issue, we performed immunoblot analysis for a number proteins that we and others previously demonstrated to be expressed in adipose tissue. Consistent with the reduction of plasma adiponectin, the levels of adiponectin in adipose tissue were reduced during acute infection in a number of different fat pads. Particularly perirenal and visceral adipose pads (both of which are important systemic sources of adiponectin) were significantly reduced on day 30 post infection, while the levels in brown adipose tissue were unaffected. (Fig. 4A). We extended our analysis of protein expression on brown and perirenal adipose tissue 30 days post infection to a number of additional inflammatory markers. The acute phase reactants α1 acid glycoprotein and serum amyloid A3, both of which we previously characterized to be expressed at high levels in adipocytes, are upregulated. Even more dramatic effects were observed for TNF-α, interferon-γ and IL-1β that were upregulated by at least 10-fold 30 days post infection (Fig. 4B). At day 90 post infection, a time when we could not detect any measurable metabolic or immunohistochemical differences in white adipose tissue, significant differences with respect to inflammatory markers such as TNF-α, interferon-γ and IL1-β persisted, suggesting that parasites in adipose tissue during chronic infection continued to affect the local inflammatory
state (Fig. 4C). Under all conditions, levels of a control protein, guanine nucleotide dissociation inhibitor (GDI), a marker that we have extensively used for normalization in the past, was unchanged.

**Infection by trypomastigotes of 3T3-L1 adipocytes in vitro**

To determine whether the *in vivo* observations extend to an isolated cell system 3T3-L1 adipocytes, a cell line widely used model for adipocytes was utilized. These cells are propagated as fibroblasts and can conveniently be differentiated into adipocytes over a period of 8 days (13). Isolated adipocytes cultured *in vitro* display a similar expression pattern with respect to the induction of specific pro-inflammatory markers upon infection as the primary adipocytes. Forty-eight hours post infection, adiponectin production was reduced, while the levels of Toll-like receptor 2 (TLR-2), an inflammatory marker, was upregulated compared to β-actin. Similarly, levels of TNF-α were increased 48 hours post infection. Interferon-γ and IL1-β were similarly upregulated under those conditions, albeit to a lesser extent than observed *in vivo* (Fig. 5A, quantitation provided in Fig. 5B).

3T3-L1 adipocytes faithfully reproduce the biological properties of primary adipocytes although they differ with respect to lipid droplet number and average lipid droplet size. Scanning electron micrographs of 3T3-L1 adipocytes revealed a large number of trypomastigotes actively invading cells (Fig. 5C). The smaller lipid droplet size makes the structural analysis of the intracellular morphology much more approachable. Comparing electron micrographs of uninfected and infected 3T3-L1 adipocytes revealed numerous intracellular amastigotes in the infected cells, clustered around the lipid droplets (Fig. 5D), similar to the observations made in primary adipocytes. These observations suggest that the parasite may have an affinity for events taking place on the surface of the lipid droplet such as lipolysis mediated by hormone sensitive lipase (HSL). This may provide an explanation for the high affinity of parasites for this cell type. It is noteworthy that other intracellular parasites, e.g. *T. gondii*, have also been demonstrated to have a significant need for host cell lipids and a similar phenomenon may explain the increased number of *T. cruzi* per cell in adipocytes.
**Discussion**

This is, to our knowledge, the first detailed analysis of the consequences of *T. cruzi* infection on adipose tissue and adipocytes both *in vitro* and *in vivo*. Our data indicate that adipocytes play an important role in the pathogenesis of Chagas’ disease. During acute infection, there are profound changes in the transcriptional program of adipocytes and as a result changes at the level of adipokines released by adipose tissue. Our data suggests that adipocytes serve as a reservoir for parasites from which recrudescence may occur during immunosuppression.

Previous observations from several laboratories have indicated that adipose tissue may serve as a prominent target tissue for *T. cruzi*, even though more quantitative assessments were lacking in these studies. Buckner et al (18) demonstrated a high level of *T. cruzi* infection of mesenteric fat pad using a genetically engineered strain of the parasite expressing β-galactosidase. In addition, Lenzi et al. (19) found large numbers of parasites in adipose tissue 15 days post infection. These observations are important and needed to be re-evaluated with our recent increased understanding of adipose tissue as both an endocrine organ and contributor to systemic inflammation.

There has been an increase in Type II diabetes in developing countries. However, there has been little attention given to the role of infectious diseases in the pathogenesis of diabetes including the role of the adipocyte. We believe that Chagas’ disease lends itself particularly well to the study of the interaction of infection and the diabetic state because of the critical involvement of the adipocyte as demonstrated in by the data in this paper. Using real time quantitative PCR adipose tissue can be demonstrated to continue to harbor a significant number of parasites 300 days post infection. This could be in part due to the unique metabolic conditions that the parasites find inside the adipocyte. Another important aspect to this persistence is the extremely slow turn-over of adipocytes. While various methods yield different results regarding the lifespan of adipocytes under normal physiological conditions, there is a general consensus that adipocytes are very long-lived and do not significantly turn over in 6 to 12 months. The vast majority of adipocytes present 300 days post infection may therefore represent cells that were already present during acute infection. Weisberg and co-workers recently reported a significant infiltration of additional macrophages into adipose tissue in obese mice (17). The increased local
concentration of macrophages is thought to act in concert with the adipocytes to increase local and systemic inflammatory levels. The dramatic increase in local adipose tissue macrophages during the acute phase of the infection not only highlights the large number of local parasites, but may also be responsible for a significant amount of systemic inflammation in light of the high pro-inflammatory potential of both macrophages and adipocytes. Additional clinical studies will be required to assess the correlations of disease outcome with both the acute and chronic phases of the infection and adipose tissue mass. Depending on the level of obesity, adipose tissue can account for greater than 40-60% of total body weight. Local phenomena observed in adipose tissue can therefore have a profound systemic impact.

Similar to the clinical disease progression reported for humans, we also observed severe hypoglycemia in mice during the acute phase of the infection. Metabolically, it is not known what accounts for this dramatic drop in glucose levels, which is in sharp contrast to the situation observed in septic patients who experience insulin resistance (20,21). Insulin resistance was not present, since the mice exhibited normal glucose tolerance tests and did not have elevated insulin levels. The hypoglycemia could be a consequence of an increased glucose uptake in peripheral tissues such as muscle or fat or due to a massive uptake of glucose by *T. cruzi* (22). However, we believe a more likely explanation is a failure of the liver to adjust hepatic glucose production, resulting in decreased hepatic gluconeogenesis and the massive hypoglycemia during acute *T. cruzi* infection. In fact, while *T. cruzi* does not directly infect cultured hepatocytes, it will infect sinusoidal cells in the liver resulting in hepatic inflammation (H. Tanowitz, unpublished results). Hypoglycemia could also be due to insulinomimetic properties of an inositol phosphate glycan derived from a parasite glycosyl phosphatidylinositol (23,24). The precise mechanism of hypoglycemia in the setting of acute Chagas’ disease will be addressed in the future using techniques that will allow an assessment of glucose fluxes during the acute phase of this infection.

Hypoglycemia has also been reported in the context of malaria, both in patients (25) as well as in mice (16). However, in this case, the hypoglycemia is primarily attributed to either treatment with quinine that triggers hyperinsulinemia or a generalized hyperinsulinemia that is present even in the absence of treatment (26). The underlying reasons for the hypoglycemia must be fundamentally different in the case of Chagas’ disease, since such a hyperinsulinemia is not
observed. These observations highlight a rather unique metabolic fingerprint caused by Chagas’
disease that has not been reported for any other infectious disease to date.

The prominent role of the adipocyte during *T. cruzi* infection may also have important
clinical implications in the context of specific disease states, such as HIV infections. HIV
represents an immune compromised state which can predispose an infected host to recrudescent
infections. Additionally, highly active antiretroviral therapy and HIV itself are associated with
lipodystrophy effects (27). This HIV associated lipodystrophy can result in a reduction of
subcutaneous tissue with the redistribution of fat depots towards a more central location. During
this process changes in adipocytes could lead to the release of intracellular parasites from these
adipocytes into the systemic circulation providing another mechanism (in addition to immune
suppression) for the development of recrudescent Chagas’ disease in the setting of HIV infection
(3).
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References


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Table I
Total fat and abdominal fat in control and infected mice.

Figure Legends

Figure 1: *T. cruzi* infection causes severe hypoglycemia, but does not have an effect on glucose clearance during an oral glucose tolerance test. (A) Fasting plasma glucose levels before and during infection of CD-1 mice with $10^4$ *T. cruzi* (Brazil strain). Plasma glucose levels were determined in the fasted state prior to infection and on days 15, 30, 60 and 90 post infection. Note the marked hypoglycemia on day 30. (B) Oral glucose tolerance tests at various stages of infection. Note that despite hypoglycemia, the rate of glucose clearance remains unaltered compared to control animals. Uninfected mice, infected mice that survived and infected mice that died during infection are plotted separately. Data for days 15 and 60 is not shown and looks similar to day 0 and day 90. (C) Hyperglycemia is associated with increased mortality. Plasma glucose levels have been plotted for individual mice at various stages of infection. Uninfected mice are displayed in closed circles, infected mice that survived until day 90 as open circles, and mice that died during the infection are represented with an x. N=10 for each group. In all cases, significance differences (p<0.05) are indicated with an asterisk (*).

Figure 2: Infection with *T. cruzi* is associated with transient changes in plasma adipokine levels. (A) Adiponectin levels during the indicated stages of infection. (B) Plasma insulin, leptin, resistin levels on day 15 and 30 post infection. (C) IL-6 and MCP-1 levels on day 15 and 30 post infection. All values were obtained using Lincoplex assays. (D) Weight of the mouse cohorts at different stages of infection.

Figure 3: Quantitative assessment of parasite load at different stages of infection. (A) Assessment of parasite load by quantitative RT-PCR in heart, spleen, brown adipose tissue and white, epididymal adipose tissue after 15, 30, 60 and 300 days post infection. (B) Data for days 60 and 300 is shown again on a different scale. (C) Immunohistochemical analysis for the presence of macrophages in white adipose tissue on day 18 post infection using antibodies against the macrophage marker F4/80. Perirenal WAT is shown in the top two panels, epididymal
WAT is shown in the bottom two panels. A monoclonal control antibody of the same isotype was used at the same concentration in the panels on the left. (D) Immunohistochemical analysis for the presence of macrophages in brown adipose tissue on day 30 post infection using antibodies against F4/80. The small insert in the middle highlights the intracellular presence of the parasites in adipocytes. BAT was isolated from infected (top two panels) or uninfected mice (bottom two panels). A monoclonal control antibody of the same isotype was used at the same concentration in the panels on the left. (E) Analysis of macrophage infiltration in epididymal WAT on days 60 (top four panels) and day 90 post infection (bottom 4 panels) using F4/80 stains. (F) EM analysis of brown adipocytes at different magnifications. LD indicates lipid droplet. Arrows indicate parasites. Parasites are about 4 to 5 µm in diameter. (G) EM analysis of white adipocytes (epididymal) at different magnifications. LD indicates lipid droplet. Arrows indicate parasites.

**Figure 4: Effects of *T. cruzi* infection on protein expression in adipose tissue.** (A) Western blot analysis of adiponectin expression in brown, perirenal and visceral adipose tissue in control and infected animals 30 days after infection. (B) Protein expression in brown and perirenal adipose depots in control and infected animals 30 days after infection for α1 acid glycoprotein, serum amyloid A3, TNFα, interferon-γ and IL-1β. All signals were normalized for GDI levels and represent averages from measurements of 4 independent mice. (C) Samples as described in (B) except tissue was harvested 90 days after infection. In all cases, significance differences (p<0.05) are indicated with an asterisk (*).

**Figure 5: Effect of *T. cruzi* infection on 3T3-L1 adipocytes.** (A) 3T3-L1 adipocytes were infected at a multiplicity of infection of 2:1 with *T. cruzi* from the Brazil strain. 48 hours later, protein extracts were prepared and analyzed by Western blot analysis for levels of adiponectin, TLR-2, TNFα, IL-1β and β-actin. (B) Quantitative representation of data shown in (A) (Data are means ± SD, n = 4). In all cases, significance differences (p<0.05) are indicated with an asterisk (*). (C) Four representative scanning electron micrographs of 3T3-L1 adipocytes infected with *T. cruzi*. (D) Representative transmission electron micrographs of 3T3-L1 adipocytes 48 hours
post infection. Note the close proximity of parasites to lipid droplets indicated by arrowheads. The picture on the top left corresponds to an uninfected cell.
<table>
<thead>
<tr>
<th>Table I:</th>
<th>Total Fat</th>
<th>Abdominal Fat</th>
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<tbody>
<tr>
<td><strong>Acute Infection (30 days)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice (n=3)</td>
<td>12.2 +/-1.5%</td>
<td></td>
</tr>
<tr>
<td>Infected mice (n=6)</td>
<td>4.7 +/-0.7%</td>
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</tr>
<tr>
<td><strong>Chronic infection (90 days)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice (n=2)</td>
<td>12.0 +/-2.5%</td>
<td>8.0 +/-3.3%</td>
</tr>
<tr>
<td>Infected mice with enlarged right ventricles (n=2):</td>
<td>4.1 +/-1.2%</td>
<td>2.4 +/-0.3%</td>
</tr>
<tr>
<td>Infected mice with normal sized hearts (n=2):</td>
<td>8.9 +/-1.5%</td>
<td>6.0 +/-2.6%</td>
</tr>
</tbody>
</table>
Combs et al.
Fig. 3

A

Parasites / Cell

- Heart
- Spleen
- BAT
- EWAT

15 Days | 30 Days | 60 Days | 300 Days

B

Parasites / Cell

- Heart
- Spleen
- BAT
- EWAT

60 Days | 300 Days
Combs et al.
Fig. 3

D

Brown Adipose Tissue (BAT)

Day 30
Infected

Control IgG
F4/80 Stain

Day 30
Uninfected

Control IgG
F4/80 Stain
Combs et al.
Fig. 3

F
Combs et al.
Fig. 3

G

LD

LD

LD

LD

LD

LD

LD
Fig. 4

A

Day 30 Post Infection

- - + + - - + + Infection

Adiponectin

BAT Perirenal Visceral

B

Day 30 Post Infection

\( \alpha 1 \)-Acid Glycoprotein

Serum Amyloid A3

TNF\( \alpha \)

INF\( \gamma \)

IL1\beta

C

Day 90 Post Infection

TNF\( \alpha \)

INF\( \gamma \)

IL1\beta
Combs et al.
Fig. 5

A

CTRL INF

ADIPONECTIN

TLR-2

TNFα

IL1 β

INF γ

β-ACTIN

B

RELATIVE SIGNAL INTENSITY

CONTROL INFECTED

ADIPON TLR-2 TNFα IL1 β INF γ β-ACTIN
Combs et al.
Fig. 5

D
The adipocyte as an important target cell for trypanosoma cruzi infection

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