Inhibition of ER stress by 2-Aminopurine treatment modulates cardiomyopathy in a murine chronic Chagas disease model

Janeesh A Plakkal[1], Kezia lizardo[1], Sean Wang[2], Edward Yurkow[2], Jyothi F Nagajyothi[1*]

[1] Department of Microbiology, Biochemistry and Molecular Genetics, Public Health Research Institute, New Jersey Medical School, Newark ,USA

[2] Rutgers Molecular Imaging Center, Piscataway, New Jersey, USA

*Author to whom correspondence should be addressed.

***Corresponding Address**:

Jyothi F Nagajyothi

225, Warren Street,

Newark, NJ- 07103

Fax: 973-854-3101

Telephone: 973-854-3450

Email: jfn31@njms.rutgers.edu

ABSTRACT:

Trypanosoma cruzi infection results in debilitating cardiomyopathy, which is a major cause of mortality and morbidity in the endemic regions of Chagas disease (CD). The pathogenesis of Chagasic cardiomyopathy (CCM) has been intensely studied as a chronic inflammatory disease until recent observations reporting the role of cardio-metabolic dysfunctions. In particular, we demonstrated accumulation of lipid droplets and impaired cardiac lipid metabolism in the hearts of cardiomyopathic mice and patients, and their association with impaired mitochondrial functions and endoplasmic reticulum (ER) stress in CD mice. In the present study, we examined whether treating infected mice with an ER stress inhibitor can modify the pathogenesis of cardiomyopathy during chronic stages of infection. *T. cruzi* infected mice were treated with an ER stress inhibitor 2-Aminopurine (2AP) during the indeterminate stage and evaluated for cardiac pathophysiology during the subsequent chronic stage. Our study demonstrates that inhibition of ER stress improves cardiac pathology caused by *T. cruzi* infection by reducing ER stress and downstream signaling of phosphorylated eukaryotic initiation factor (P-elF2α) in the hearts of chronically infected mice. Importantly, cardiac ultrasound imaging showed amelioration of ventricular enlargement, suggesting that inhibition of ER stress may be a valuable strategy to combat the progression of cardiomyopathy in Chagas patients.

Keywords: Chagas disease, cardiomyopathy, mitochondrial stress, endoplasmic reticulum stress, 2-aminopurine

 doi:10.20944/preprints201808.0059.v1

1. INTRODUCTION

Chagas disease (CD), caused by parasite *Trypanosoma cruzi*, is endemic in Latin America, where it is responsible for 12,000 deaths per year. CD has two main stages in patients – acute and chronic [1]. Acute infection causes mild symptoms, and mortality (approximately 5%) is reported predominantly in untreated children [2]. However, chronic Chagas disease, which is typically asymptomatic, may progress to the chronic cardiac form in approximately 30% of *T. cruzi* infected people [3]. The severity and manifestations of cardiac symptoms vary in these patients and can lead to death due to cardiomyopathy, arrhythmias and/or progressive heart failure [4]. The mechanism(s) underlying the transition between asymptomatic and cardiac form is not completely understood. Furthermore, there are no efficient drugs or vaccines to prevent the pathogenesis of Chagasic cardiomyopathy [5].

Murine CD models are suitable to investigate the pathogenesis of cardiomyopathy because they recapitulate the cardiac symptoms of Chagas patients [6, 7]. Acute and chronic stages of CD can be modeled in mice by manipulating the strain and number of *T. cruzi* parasites used in infection, and mouse diet [8, 9]. For example, we have demonstrated that infecting CD1 mice with 10^3 trypomastigotes of *T. cruzi* (Brazil strain) leads to acute infection with low mortality rate and parasitemia before 35 days post infection (DPI) and chronic cardiomyopathy after approximately 90 DPI [10, 11]. Between 35 and 90 DPI, these infected mice usually appear to be in the indeterminate (asymptomatic) stage, showing no significant change in serum inflammatory markers and parasitemia. Thus, these models of CD are suitable to investigate the molecular mechanism(s) of the pathogenesis of cardiomyopathy.

Earlier, using these murine CD models, we demonstrated that *T. cruzi* infection induces cardiac lipid accumulation, which causes oxidative stress and inflammation, leading to cardiomyopathy during the chronic stage of infection [10, 12]. In the present study, we investigated the role of ER stress in causing cardiac inflammation, mitochondrial dysfunction, apoptosis, fibrosis and cardiomyopathy in a *T. cruzi*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

infected murine chronic CD model. We also demonstrated that infected mice treated with an ER stress inhibitor, 2-Aminopurine (2AP), during indeterminate stage (after 40 DPI) significantly modifies cardiac dysfunction, including cardiomyopathy caused by *T. cruzi* infection [13]. The results shed light on the role of cardiac ER stress in the pathogenesis of Chagasic cardiomyopathy and suggest that developing drugs that inhibit cardiac ER stress may be a valuable strategy to combat cardiac pathology in chronic Chagas disease.

2. Results:

2.1. 2AP treatment during indeterminate stage reduces cardiac ER stress in chronic Chagas mice: Immunoblot analyses demonstrated significantly increased levels of cardiac ER stress markers such as BIP (Immunoglobulin Binding Protein), p-elF2α (phosphorylated eukaryotic translation initiation factor 2 alpha), and CHOP (C/EBP homologous protein) in chronic *T. cruzi* infected mice compared to uninfected mice (Figs. 1a and b). Treating infected mice with 2AP, an ER stress inhibitor, resulted in a significant reduction in the levels of cardiac ER stress markers (Figs. 1a and b) [13]. Immunohistochemical analyses of cardiac sections also demonstrated a significant decrease in the levels of BIP, pELF2a and CHOP in infected 2AP treated mice compared to untreated mice (Supplementary Fig. 1). Next, qPCR analysis was performed to analyze the effect of 2AP treatment on the mRNA levels of several genes involved in response to ER stress (Fig. 1c). We observed a significant decrease in the levels of BIP ($p < 0.05$), PRKR-like endoplasmic reticulum kinase (PERK) ($p < 0.001$), ER-residing protein endoplasmic oxidoreductin-1 alfa (Erol-α) (, $p < 0.05$), and CHOP($p < 0.05$) in the hearts of infected 2AP treated mice compared to infected untreated mice (Fig.1c). These data demonstrate that 2AP acts as a potent ER stress inhibitor and reduces cardiac ER stress in chronic *T. cruzi* infected mice.

2.2. Reducing cardiac ER stress results in decreased apoptotic signals during chronic infection: Irreversible ER stress induces several pro-apoptotic mechanisms to eliminate damaged cells [14]. ER stress-mediated cell death is executed by the canonical mitochondrial apoptosis pathway, where the BCL-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

2 (B-cell lymphoma/leukemia-2) family plays a crucial role [15]. Transcriptional and post-transcriptional mechanisms are activated to regulate pro-apoptotic members of the BCL-2 family that facilitate cytochrome c release from the mitochondria and calcium release from the ER to engage downstream apoptotic signaling events [16]. Our qPCR analysis demonstrated a significant increase in the cardiac mRNA levels of B-cell lymphoma-extra-large (BCL-Xl) ($p < 0.05$) and decrease in Tumor necrosis factor receptor 1 (TNF-R1) ($p < 0.01$) and BCL2 Antagonist/Killer 1(BAK) ($p < 0.01$) levels in infected 2AP treated mice compared to infected untreated mice (Fig. 2). BCL-X1 is an anti-apoptotic marker, while TNF-R1 and BAK are known apoptotic markers [17, 18]. These data indicate that 2AP induced inhibition of ER stress decreased the expression of pro-apoptotic markers in the hearts of infected mice during chronic infection.

2.3. 2AP improves mitochondrial function and reduces oxidative stress in the hearts of T. cruzi infected mice: ER stress and mitochondrial oxidative stress regulate each other and form a vicious cycle, resulting in apoptosis [19]. To evaluate the effect of reduced ER stress on the cardiac mitochondrial function and oxidative stress, we measured mRNA levels of the genes involved in mitochondrial function: cytochrome c oxidase subunit 3 (COX3), cytochrome b (CYTB), ATP synthase Fo subunit 6 (ATP6) and NADH dehydrogenase, subunit 1 (ND1). We also measured the expression of anti-oxidative stress genes: mitochondrial antioxidant manganese superoxide dismutase (MNSOD), catalase (CAT), glutathione peroxidase 1 (GPX1), glutathione peroxidase 2 (GPX2), peroxisome proliferator-activated receptor γ coactivator 1 alfa (PGC1α) and glycogen synthase kinase 3 beta (GSK3β) in the heart samples of mice from different experimental groups (Fig 3a, 3b). This qPCR analysis demonstrated a significant decrease in the mRNA levels of some of the genes involved in mitochondrial function and oxidative stress resistance in the hearts of infected mice compared to uninfected mice (Fig 3 a, 3b). Furthermore, 2AP treatment significantly increased mRNA levels of the genes involved in mitochondrial function and resistance to oxidative stress in the hearts of infected mice compared to infected untreated mice (Fig 3a and b).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

2.4. Reduced ER stress significantly decreases cardiac inflammation:

We have previously shown that *T. cruzi* infection induces increased infiltration of immune cells into the myocardium, leading to pro-inflammatory signaling [8, 10]. We used immunoblot analysis to quantify the levels of pro-inflammatory cytokine TNFα (tumor necrosis factor alpha) in the myocardium at 120DPI. We found that cardiac TNFα levels significantly increased in infected mice compared to uninfected mice at 120DPI (Fig. 4a, 4b). However, the treatment with 2AP in infected mice significantly reduced the levels of TNFα in the hearts compared to untreated mice at 120DPI (Fig. 4a and 4b), indicating that reducing ER stress also counteracts the inflammatory processes in the heart.

2.5. Inhibition of ER stress modulates cardiac morphology in T. cruzi infected mice:

Because the levels of TNFα in the hearts correlate with the levels of infiltrated immune cells into the myocardium during infection [8, 10], we analyzed the levels of immune cells in the myocardium by histological analysis. Photomicrographs of H&E stained heart sections demonstrated significantly damaged cardiac morphology during *T. cruzi* infection compared to uninfected mice at 120 DPI. *T. cruzi* infection significantly increased the levels of infiltrated immune cells, lipid droplets, degenerating cardiac fibers and fibrosis in the hearts (Fig. 5 and Supplemental Fig. 2). H&E stained cardiac sections of infected 2AP treated mice showed a significant decrease ($P \leq 0.01$) in cardiac damage (reflected by levels of infiltrated immune cells, lipid droplets, degenerating cardiac fibers, and cellular hypertrophy) compared to infected mice without treatment (Fig. 5a and Supplemental Fig. 2). We also observed significantly increased fibrosis and collagen deposition in the myocardium of infected mice compared to uninfected mice (Fig. 5b). However, infected mice treated with 2AP showed significantly reduced levels ($p \leq 0.01$) of fibrosis and collagen deposition in the heart sections compared to infected untreated mice (Fig 5b). This observation was further confirmed by analyzing the mRNA levels of collagen I and III – genes whose overexpression results in collagen deposition [20] – in the heart samples (Fig. 5c). qPCR analysis demonstrated a significant increase in the cardiac mRNA levels of collagen I and III in the infected mice

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

compared to uninfected mice. In contrast, the mRNA levels of collagen I and III in infected 2AP treated mice showed no significant difference compared to uninfected mice and were significantly reduced compared to infected untreated mice (Fig. 5c). These data demonstrate that 2AP treatment significantly improves cardiac morphology by reducing collagen deposition and fibrosis. Decreased cardiac collagen deposition improves contractile function of the myocardium [21]. The cardiac isoform of the sarco/endoplasmic reticulum Ca2+ATPase (SERCA2a) plays a major role in controlling excitation/contraction coupling [22]. qPCR analysis demonstrated a significant increase in the mRNA levels of SARCA 2 in infected 2AP treated mice compared to infected untreated mice (Fig. 5c). These data suggest that 2AP treatment decreases cardiac collagen deposition and improves cardiac contractile functions in infected mice.

2.7. 2AP treatment significantly diminishes ventricular dilation caused by T. cruzi infection:

Previously we demonstrated significant alterations in cardiac morphology in *T. cruzi* infected mice during the chronic phase of infection, including a reduction in the left ventricle internal diameter (LVID) and an increase in the right ventricle internal diameter (RVID) (at both diastole and systole) [x]. Here we evaluated whether inhibiting cardiac ER stress by treating infected mice with 2AP modulates *T. cruzi* infection caused LVID reduction and RVID dilation using a VisualSonics, Vevo2100 ultra-high frequency ultrasound system. As previously demonstrated [8,10], *T. cruzi* infected mice showed significantly reduced LVID and dilated RVID (measured at both systolic and diastolic phase) (Fig. 6). In contrast, 2AP treated infected mice showed significantly ameliorated LVID (systole) and RVID (both diastole and systole) compared to infected untreated mice (Fig. 6). These data demonstrated that inhibition of cardiac ER stress improves cardiac morphology in chronic *T. cruzi* infection.

3. Discussion

 doi:10.20944/preprints201808.0059.v1

We previously showed that acute *T. cruzi* infection in mice causes cardiac lipid accumulation, which in turn promotes mitochondrial oxidative stress and results in cardiac ventricular dilation and dysfunction [23, 24]. Other studies have reported that intracellular lipid accumulation results in ER stress and cell death [25]. In this study we tested the hypothesis that increased cardiac lipid accumulation may cause ER stress in the myocardium and form a vicious cycle with mitochondrial stress and exacerbate cardiac pathology during chronic infection. The most important findings of this report are (i) *T. cruzi* infection induces cardiac ER stress and results in ventricular dilation during chronic stages of infection, and (ii) oral feeding of the ER stress inhibitor 2AP to CD mice significantly reduced cardiac inflammation and pathology induced by chronic *T. cruzi* infection (Figs. 5 and 6).

The ER is the main intracellular organelle in the secretory pathway as well as the site of biosynthesis for steroids, cholesterol, and other lipids [26]. The main function of ER is to carry out appropriate protein folding, assembly, and disulfide bond formation, leading to production of functional, mature proteins in sacs called cisternae and the transport of synthesized proteins in vesicles to the Golgi apparatus. The accumulation of unfolded proteins in the lumen of ER causes ER stress characterized by increasing production of ER molecular chaperones and diminishing global protein synthesis, a process by which ER stress will be relieved under physiological conditions [27, 28]. Activation of the signaling network in response to ER stress is known as unfolded protein response (UPR). Recent reports have demonstrated that lipids/lipoproteins can also trigger UPR [29]. The pathophysiological insults caused by acute *T. cruzi* infection lead to cardiac accumulation of lipids and unfolded proteins in the ER and result ER stress. There are three distinct UPR signaling pathways triggered in response to ER stress, which are mediated by PERK, ATF6, and IRE1 [30]. PERK is the major protein responsible for decreasing the mRNA translation under ER stress, inhibiting influx of newly synthesized proteins into the already stressed ER compartment [31]. This translational attenuation is mediated by phosphorylation of eIF2α. The phosphorylation of eIF2α (P-elF2α) inhibits the recycling of eIF2α to its active GTP-bound form, which is required for the initiation phase of polypeptide chain synthesis. Paradoxically, eIF-2α

 doi:10.20944/preprints201808.0059.v1

196 phosphorylation also enhances the autophagy gene transcription signaling inducing cell death [32]. Thus
197 elevation of PERK- P-elF2α signaling along with the other ER molecular chaperons inhibit global protein
198 synthesis. However, constant inhibition of global protein synthesis might suppress normal cellular
199 functions and cause cell death, and resulting in pathological conditions of the heart in Chagas disease
200 [33].

201 Reminiscent to our previous studies, histological analysis of the hearts of chronic *T. cruzi* infected
202 mice demonstrated significantly elevated lipid droplets and infiltrated immune cells (compared to
203 uninfected mice), which could be the main cause of cardiac pathology in the infected mice. Increased
204 cardiac lipid levels might have elevated UPR causing ER stress [12, 29]. We found that the cardiac ER
205 stress caused by *T. cruzi* infection upregulates BIP dissociation, resulting in high levels of PERK,
206 phosphorylation of elF2α, ATF4 and chaperone proteins (e.g. CHOP) in the hearts (Fig. 1 a-c). We
207 showed myocardial inflammation, apoptosis and fibrosis in the hearts of chronic infected mice as
208 demonstrated by increased levels of TNFα, apoptotic markers (CHOP, TNF-R1 and BAK) and collagen
209 levels in the hearts of infected mice at 120DPI. These findings suggest that increased eIF-2α
210 phosphorylation and its downstream signaling might have enhanced the levels of ER stress chaperones
211 and apoptotic response, and inhibited global protein synthesis leading to the pathological conditions of the
212 chagasic heart.

213 To evaluate the effect of cardiac ER stress and the downstream effect of P-elF-2α signaling on the
214 pathogenesis of cardiomyopathy in *T. cruzi* infected mice, we treated infected mice with 2AP, an inhibitor
215 of P-elF-2α after the acute infection (40 DPI) for 80 days and evaluated its effect on cardiac ER stress,
216 mitochondrial stress and inflammation. A consequence of eIF-2α phosphorylation is upregulation of
217 protein chaperons [28, 30, 31]. 2AP inhibits eIF-2α phosphorylation and protein chaperons' upregulation,
218 and prevents apoptosis, and induce protein synthesis which are required for the normal functioning of the
219 cells. Treatment of infected mice with 2AP significantly reduced ER stress by decreasing P-elF2α levels
220 and its downstream signaling. Inhibition of ER stress also significantly reduced cardiac inflammation,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

apoptosis and fibrosis, and improved contractile ability of the hearts during chronic Chagas infection. More importantly, cardiac inhibition of ER stress significantly modulated ventricular enlargements commonly observed in murine chronic Chagas disease models.

These results suggest that ER stress plays a major role in the pathogenesis of Chagas disease by elevating cardiac inflammation, apoptosis, and fibrosis during the long periods of indeterminate stages of infection. The persistence of ER stress in the heart could increase the pathological conditions and elevate the risk of ventricular dilations of the heart in *T. cruzi* infected mice.

4. MATERIALS AND METHOD

4.1 Animal model and experimental design

Mice were maintained on a 12-h light/dark cycle. The Brazil strain of *T. cruzi* was maintained by passage in C3H/Hej mice (Jackson Laboratories, Bar Harbor, ME). Male CD-1 mice (Jackson Laboratories, n=55) were infected intraperitoneally (i.p., n=35) at 6–8 weeks of age with 10^3 trypomastigotes of the Brazil strain and fed on Formulab diet #5008 (Lab diet). After 40 days post infection (DPI), both uninfected and infected mice were divided into two groups and one group gavaged with 2- Amino purine (**100mg/kg body weight**) and the other with vehicle alone for 80 days (120DPI) (Supplemental Figure 3). Cardiac imaging analysis was done at 100DPI and all the animals were sacrificed at 120DPI to collect heart and blood samples for the following studies. All animal experimental protocols were approved by the Institutional Animal Care and Use and Institutional Biosafety Committees of Rutgers University and adhere to the National Research Council guidelines.

4.2. Cardiac Ultrasound imaging analysis

Cardiac geometry, systolic and diastolic function were evaluated by echocardiography using a VISUALSONICS high-resolution Vevo 2100 system ultrasound system (VISUALSONICS Inc., Toronto, Canada) equipped with a 30-MHz transducer. Briefly, mice were placed in supine position on a movable,

 doi:10.20944/preprints201808.0059.v1

heated platform maintained at 37°C, and anesthetized with 1.0%-1.5% isoflurane (Baxter Healthcare Corp, New Providence, RI, USA) to keep the heart rate stabilized at 400 to 500 beats per minute. Doppler ultrasound capabilities of the system was also used to determine the blood flow velocities of the aorta and pulmonary arteries as well as to profile mitral valve function. All imaging procedures was performed under inhalation anesthesia with isoflurane at a concentration of 4-5% for induction of anesthesia and 1 - 2% for maintenance. Scan time was approximately 1 hour/mouse. Conventional echocardiographic parameters, such as wall thickness and chamber dimensions, were obtained from M-mode images at the mid-papillary level in the parasternal short axis view, and also from B-mode images acquired in the parasternal long- and short-axis views, then internal diameters of the ventricles and wall thickness were calculated.

4.3 Immunoblot analysis

Heart lysates were prepared as previously described (8, 10). An aliquot of each sample (30µg protein) was subjected to SDS-PAGE and the proteins were transferred to nitrocellulose filters for immunoblot analysis. BIP- specific rabbit monoclonal antibody (1:1000 dilution, C50B12, Cell Signaling), TNF-alpha-specific rabbit polyclonal antibody (1:2000 dilution, AB6671, Abcam). HSP60 specific rabbit monoclonal antibody (1:1000 dilution, 12165, Cell Signaling), Phospho-eIF2α (Ser51) specific rabbit monoclonal antibody 1:1000 dilution, 3597, Cell Signaling) and CHOP-specific mouse monoclonal antibody (1:1000 dilution, L63F7, Cell Signaling), were used as primary antibody. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (1:2000 dilution, Thermo Scientific) or horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:2000 dilution, Thermo Scientific) were used to detect specific protein bands (explained in figure legends) using a chemiluminescence system (8, 10). GDI (1: 10,000 dilution, 71-0300, and rabbit polyclonal, Invitrogen, CA) and a secondary antibody horseradish peroxidase conjugated goat anti-rabbit (1:2000 dilution, Amersham Biosciences) was used to normalize protein loading.

4.4. Real time PCR quantification

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

Total host RNA from the heart of *T.cruzi* infected mice and matched uninfected control animals at day 120 p.i. was isolated, using the Trizol reagent (Invitrogen, Carlsbad, CA). Isolated RNA was purified by on-column digestion of the contaminating DNA using DNase I. The quality and quantity of the purified RNA were assessed by formaldehyde–agarose gel electrophoresis and a NanoDrop instrument (NanoDrop Products, Wilmington, DE), as previously described. RNA was reverse transcribed from 100 ng of total RNA using All-in-One cDNA Synthesis SuperMix (Biotool) according to the manufacturer's protocol. The primers used for the amplification of quantitative PCR (qPCR) of BIP, TNF-A, COX3 , CYTB , ATP6, ND1, MNSOD, Catalase (CAT), (GPX1, GPX2, PGC1α, GSK3 BETA,collagen isoform 1 (COL1), collagen isoform 3 (COLIII) , SERCA2, PERK, Erol Alfa,ATF4,GADD34, BCL2, BCL-XL, TNF-R1, BAK, CHOP and HPRT (Hypoxanthine-guanine phosphoribosyltransferase) genes. The qPCR was run using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) following the manufacturer's protocol. To normalize gene expression and to calculate fold change mRNA expression of the housekeeping gene, HPRT was measured. For each sample, both the housekeeping and target genes were amplified in triplicate using the reaction condition and analytic parameters.

4.5. Immunohistochemical analyses

Freshly isolated tissues were fixed with phosphate-buffered formalin overnight and then embedded in paraffin wax. Hematoxylin and eosin (H&E) staining was performed, and the images were captured as previously published (8, 10). Immunohistochemical analysis was performed on the formalin-fixed heart using BIP- specific rabbit monoclonal antibody 1:500 dilution, C50B12, Cell Signaling), Phospho-eIF2α (Ser51) specific rabbit monoclonal antibody 1:500 dilution, 3597, Cell Signaling) and CHOP-specific mouse monoclonal antibody (1:1000 dilution, L63F7, Cell Signaling) as demonstrated earlier (Combs et al. 2005).

4.6. Statistical analysis

Statistical analyses were performed using a Student's t-test as appropriate and significance differences were determined as p values between <0.05 and <0.001 as appropriate.

295

296 **5. Conclusions:** This report demonstrated that ER stress occurred in the hearts of infected mice, as
297 revealed by increased phosphorylation eIF-2α and increased expression of other ER chaperones. In
298 conclusion, these data provide clear evidence that chronic *T. cruzi* infection induced ER stress impairs
299 cardiac ventricular internal diameters and an early treatment to reduce ER stress modulate/prevent the
300 pathogenesis of cardiomyopathy in a murine Chagas model. A therapeutic strategy targeting cardiac ER
301 stress inhibition during asymptomatic stage may be a valuable tool to combat development and
302 progression of cardiomyopathy in Chagas patients.

303

304 **ACKNOWLEDGEMENTS:**

305 We thank Erika Shor at the Public Health Research Institute for a critical reading of the manuscript.

306 This study was supported by grants from the National Heart, Lung, and Blood Institute (National
307 Institutes of Health HL-122866) to Jyothi Nagajyothi

308

309 **CONFLICT OF INTEREST STATEMENT**

310 None of the authors have conflict of interest.

311

312 **Primer list:** supplementary Table.1

313

314 REFERENCES

315 1. Tanowitz, H.B.; Kirchhoff, L.V.; Simon, D.;Morris, S.A.; Weiss, L.M.; Wittner, M. Chagas'
316 disease. *Clin. Microbiol. Rev.* **1992**, 5, 400–419.
317 2. Memorial, C.C. Chagas' disease and its toll on the heart. *Eur. Heart. J.* **2009**, 30, 2063–2072.

3. Nunes, M.C.P.; Dones, W.; Morillo, C.A.; Encina, J.J.; Ribeiro, A.L. Chagas disease: an overview of clinical and epidemiological aspects. *J. Am. Coll. Cardiol.* **2013**, 62, 767–776.
4. Quijano-Hernandez, I.; Dumonteil, E. Advances and challenges towards a vaccine against Chagas disease. *Hum. Vaccin.* **2011**, 7, 1184–1191.
5. Jelicks, L.A.; Tanowitz, H.B.; Advances in imaging of animal models of Chagas disease. *Adv. Parasitol.* **2011**, 75, 193–208.
6. Machado, F.S.; Jelicks, L.A.; Kirchhoff, L.V.; Shirani, J.; Nagajyothi, F.; Mukherjee, S.; Nelson, R.; Coyle, C.M.; Spray, D.C.; De Carvalho.; et al. Chagas heart disease: report on recent developments. *Cardiol. Rev.* **2012**, 20, 53–65.
7. Nagajyothi, F.; Weiss, L.M.; Zhao, D.; Koba, W.; Jelicks, L.A.; Cui, M.H.; Factor, S.M.; Scherer, P.E.; Tanowitz, H.B.; High fat diet modulates *Trypanosoma cruzi* infection associated myocarditis. *PLoS. Negl. Trop. Dis.* **2014**, 8, p.e3118.
8. Soares, M.B.P.; De Lima, R.S.; Rocha, L.L.; Vasconcelos, J.F.; Rogatto, S.R.; Dos Santos, R.R.; Iacobas, S.; Goldenberg, R.C.; Iacobas, D.A.; Tanowitz, H.B.; et al. Gene expression changes associated with myocarditis and fibrosis in hearts of mice with chronic chagasic cardiomyopathy. *J. Infect. Dis.* **2010**, 202, 416–426.
9. Kezia L.; Janeesh P. A.; Cui, M.H.; Rashmi B.; Jelicks, L.A.; Nagajyothi, F. High Fat Diet Aggravates Cardiomyopathy In Murine Chronic Chagas Disease. *Microbes. Infect.* **2018.** https://doi.org/10.1016/j.micinf.2018.07.001.
10. Jelicks, L.A.; Chandra, M.; Shtutin, V.; Tang, B.; Christ, G.J.; Factor, S.M.; Wittner, M.; Huang, H., Douglas, S.A., Weiss, L.M.; et alPhosphoramidon treatment improves the consequences of chagasic heart disease in mice. *Clin. Sci. (Lond).* **2002**, 103, 48,267S–271S.
11. Johndrow, C.;Nelson, R.; Tanowitz, H.;Weiss, L.M.; Nagajyothi, F.; *Trypanosoma cruzi* infection results in an increase in intracellular cholesterol. *Microbes. Infect.***2014.** 16,337–344.

12. Zhou, L.; Yang, D.; Wu, D.F.; Guo, Z.M.; Okoro, E.; Yang, H. Inhibition of endoplasmic reticulum stress and atherosclerosis by 2-aminopurine in apolipoprotein e-deficient mice. *ISRN Pharmacol.* **2013**, 2013,847310
13. Urra, H.; Dufey, E.; Lisbona, F., Rojas-Rivera, D.; Hetz, C. When ER stress reaches a dead end. *Biochim. Biophys. Acta*. **2013,** 1833,3507–3517.
14. Weber, G.F.; Menko, A.S. The canonical intrinsic mitochondrial death pathway has a non-apoptotic role in signaling lens cell differentiation. *J. Biol. Chem*. **2005,** 280, 22135–22145.
15. Carpio, M.A.; Michaud, M.; Zhou, W.;Fisher, J.K.; Walensky, L.D; Katz, S.G. BCL-2 family member BOK promotes apoptosis in response to endoplasmic reticulum stress. *Proc. Natl. Acad. Sci. USA*. **2015**, 112, 7201–7206.
16. Feng, C.Y.; Rise, M.L. Characterization and expression analyses of anti-apoptotic Bcl-2-like genes NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in Atlantic cod (Gadus morhua). *Mol. Immunol.* **2010**, 47, 763–784.
17. Westphal, D.; Dewson, G.; Czabotar, P.E.; Kluck, R.M. Molecular biology of Bax and Bak activation and action. *Biochim. Biophys. Acta*. **2011**, 1813, 521–531.
18. Cao, S.S.; Kaufman, R.J. 2014. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid. Redox. Signal.* **2014***,* 21, 396–413.
19. Li, X.; Zhao, D.; Guo, Z.; Li, T.; Qili, M.; Xu, B.; Qian, M.; Liang, H.; Xiaoqiang, E.;Gitau, S.C.; et al. Overexpression of SerpinE2/protease nexin-1 contribute to pathological cardiac fibrosis via increasing collagen deposition. *Sci. Rep*. **2016**, 6, 37635.
20. Van Kerckhoven, R.; Kalkman, E.A.; Saxena, P.R.; Schoemaker, R.G. Altered cardiac collagen and associated changes in diastolic function of infarcted rat hearts. *Cardiovasc. Res.*, **2000**, 46, 316–323.
21. Lipskaia, L.; Chemaly, E.R.; Hadri, L.; Lompre, A.M.; Hajjar, R.J. Sarcoplasmic reticulum Ca2+ ATPase as a therapeutic target for heart failure. *Expert. Opin. Biol. Ther.* **2010**,10, 29–41.

22. Gupta, S.; Wen, J.J.; Garg, N.J.; Oxidative stress in Chagas disease. *Interdiscip. Perspect. Infect. Dis.* **2009**, 2009, 190354.

23. Garg, N.; Popov, V.L.; Papaconstantinou, J. Profiling gene transcription reveals a deficiency of mitochondrial oxidative phosphorylation in *Trypanosoma cruzi*-infected murine hearts: implications in chagasic myocarditis development. *Biochim. Biophys. Acta.* **2003**,1638, 106–120.

24. Han, J.; Kaufman, R.J.; The role of ER stress in lipid metabolism and lipotoxicity. *J. Lipid. Res.* **2016**, 57, 1329–1338.

25. Jacquemyn, J., Cascalho, A.; Goodchild, R.E., The ins and outs of endoplasmic reticulum-controlled lipid biosynthesis. *EMBO. Rep.* **2017**, 18, 1905–1921.

26. Malhotra, J.D.; Kaufman, R.J.; The endoplasmic reticulum and the unfolded protein response. *Semin. Cell. Dev. Bio.* **2007**, 18, 716–731.

27. Rozpedek, W.; Pytel, D.; Mucha, B.; Leszczynska, H.; Diehl, J.A.; Majsterek, I. The role of the PERK/eIF2α/ATF4/CHOP signaling pathway in tumor progression during endoplasmic reticulum stress. *Curr. Mol. Med.* **2016 ,** 16, 533–544.

28. Volmer, R.; Ron, D.. Lipid-dependent regulation of the unfolded protein response. *Curr. Opin. Cell. Biol.* **2015** , 33, 67–73.

29. Gardner, B.M.; Pincus, D.; Gotthardt, K.; Gallagher, C.M.; Walter, P. Endoplasmic reticulum stress sensing in the unfolded protein response. *Cold. Spring. Harb. Perspect. Biol.* **2013** , 5, a013169.

30. Harding, H.P.; Zhang, Y.; Bertolotti, A.;Zeng, H.; Ron, D. Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol. Cell.* **2000**, 5,897–904.

31. B'chir, W.; Maurin, A.C.; Carraro, V.; Averous, J.; Jousse, C.; Muranishi, Y.; Parry, L.; Stepien, G.; Fafournoux, P.; Bruhat, A. The eIF2α/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic. Acids. Res.* **2013**, 41, 7683–7699.

391 32. Zhao, L.;Ackerman, S.L. Endoplasmic reticulum stress in health and disease. *Curr. Opin. Cell.*
392 *Biol*. **2006**, 18,444–452.

393

394 **Figures Legend**:

395 **Fig. 1: 2AP inhibits cardiac ER stress in chronic CD mice (n=10/group).**

396 (a) Immunoblot analysis demonstrated a significant decrease in the levels of ER stress markers BIP,
397 pELF2α and CHOP in the hearts of infected mice treated with 2AP compared to infected untreated mice
398 at 120DPI.
399 (b) Fold changes in the protein levels of BIP, pELF2α and CHOP were normalized to GDI
400 expression and represented as the bar graph.
401 (c) qPCR analysis demonstrated a significant decrease in the mRNA levels of ER stress response
402 genes such as BIP, PERK, EROlα, ATF4 and CHOP in the hearts of infected mice treated with 2AP
403 compared to infected untreated mice at 120DPI.
404 The error bars represent standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to
405 uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

406 **Fig. 2: Treatment with 2AP during indeterminate stage decreased apoptotic signaling in chronic *T.***
407 ***cruzi* infected mice (n=10/group).**

408 qPCR analysis demonstrated a significant increase in the cardiac mRNA levels of anti-apoptotic BCL-Xl,
409 and significant decrease in mRNA levels of pro-apoptotic markers TNF-R1 and BAK in the hearts of
410 infected mice treated with 2AP compared to infected untreated mice at 120DPI.
411 The error bars represent standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to
412 uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

Fig. 3: Inhibition of cardiac ER stress by 2AP modified mitochondrial function by upregulating anti-oxidant genes during chronic *T. cruzi* infection (n=10).

(a) qPCR analysis demonstrated a significant increase in the cardiac mRNA levels of genes involved in mitochondrial functions such as COX3, CYTB, ATP6 and ND1in infected mice treated with 2AP compared to infected untreated mice at 120DPI.

(b) 2AP treatment significantly upregulates mRNA levels of anti-oxidant genes such as MNSOD, CAT, GPX1, GPX2, PGC1α and GSK3 in the hearts of infected mice treated with 2AP compared to infected untreated mice at 120DPI as demonstrated by qPCR analysis.

The error bars represent standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

Fig. 4: Treatment with 2AP during the indeterminate stage reduced cardiac inflammation in chronic *T. cruzi* infected mice (n=10).

(a) Immunoblot analysis demonstrated a significant decrease in the level of TNFα in the hearts of infected mice treated with 2AP compared to infected untreated mice at 120DPI.

(b) Fold changes in the protein levels of TNFα were normalized to GDI expression and represented as the bar graph.

(c) qPCR analysis demonstrates a significant decrease in the mRNA levels of TNF-A in the hearts of infected mice treated with 2AP compared to infected untreated mice at 120DPI.

The error bars represent standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

Fig. 5: Amelioration of myocardial damage by 2AP in mice during chronic infection at 120DPI (n=8, minimum five images/section were analyzed).

(a) H&E staining displayed significantly more damage (inflammation – long black arrow, fibrosis – read arrow head, degenerating cardiac muscle fibre – black arrow head (See supplemental figure 3) and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 August 2018 doi:10.20944/preprints201808.0059.v1

presence of adipocytes or lipid granules – read long arrow (See supplemental figure 3)) in infected mice hearts compared to the hearts of uninfected mice. However, infected 2AP treated mice displayed significantly reduced damage ($p \leq 0.01$) compared to infected untreated mice (bar -100um). Additional images are presented as supplemental Figure 3.

(b) The photomicrographs of trichrome Masson stained hearts sections demonstrated significant ($p \leq 0.01$) increase in cardiac fibrosis and collagen deposition in infected mice compared to uninfected mice. Infected 2AP treated mice showed significantly reduced damage ($p \leq 0.01$) compared to infected untreated mice (bar-100um).

(c) qPCR analysis demonstrated a significant increase in the cardiac mRNA levels of collagen I and III, and decrease in SERCA 2 in the infected mice compared to uninfected mice. Whereas, the mRNA levels of collagen I and III in infected 2AP treated mice were significantly reduced compared to infected untreated mice.

The error bars represent standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

Fig. 6: Treatment with 2AP during indeterminate stage improved the morphology of the heart during murine chronic CD at 100DPI (n=5/group).

Ultrasound analysis of the hearts both at diastole (d) and systole (s) condition showed a significant decrease in the left ventricle internal diameter (LVID) and significant increase in the right ventricle internal diameter (RVID) in the infected mice compared to uninfected mice at 120 DPI. However, the infected mice treated with 2AP displayed significantly modified LVID (s) and RVID (both d and s) compared to infected untreated mice at 100 DPI.

The error bars represent standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

 doi:10.20944/preprints201808.0059.v1

Supplementary Figures

Supplemental Figure 1: Treatment with 2AP during indeterminate stage significantly reduced ER stress in the myocardium of infected mice.

(a) IHC analysis demonstrated a significant decrease in the levels of ER stress markers BIP, pELF2α and CHOP in the hearts of infected mice treated with 2AP compared to infected untreated mice at 120DPI.

(b) IHC images of BIP, pELF2α and CHOP staining were quantified and represented as bar graph. Five images from each section were quantified using NIH image-J program. Bars represent mean values of the data with SEM as vertical lines.

Significance represent mean values of the data with Standard Error of the mean (SEM) as vertical lines. (The error bars represent standard error of the mean * $p \leq 0.05$, ** $p \leq 0.01$ or *** $p \leq 0.001$ compared to uninfected untreated mice. # $p \leq 0.05$, ## $p \leq 0.01$ or ### $p \leq 0.001$compared to infected untreated mice).

Supplemental Figure 2: **Histology of the myocardium of mice during chronic stages of infection (additional images, n=8).**

H&E staining displayed significantly more damage (degenerating cardiac muscle fibre – black arrow head and presence of adipocytes or lipid granules – read long arrow) in infected (untreated) mice compared to infected 2AP treated mice at 120 DPI (bar -25um, 40X magnification).

Supplemental Figure 3: Schematic explanation of Experimental design