Profiling gene transcription reveals a deficiency of mitochondrial oxidative phosphorylation in Trypanosoma cruzi-infected murine hearts: implications in chagasic myocarditis development

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Abstract

In this study, we report the host genetic responses that characterize Trypanosoma cruzi-induced myocarditis in a murine model of infection and disease development. The mRNA species from the myocardium of infected mice were assessed using cDNA microarray technology at immediate early, acute, and chronic stages of infection. The immediate early reaction of the host to T. cruzi infection was marked by up-regulation of transcripts indicative of proinflammatory and interferon-induced immune responses. Following acute infection, overexpression of transcripts for extracellular matrix (ECM) proteins, possibly initiated in response to myocardial injuries by invading and replicating parasites, was suggestive of active reparative and remodeling reactions. Surprisingly, progression to the cardiac disease phase was associated with coordinated down-regulation of a majority (>70%) of the differentially expressed genes. Among the most repressed genes were the troponins, essential for contractile function of the myofibrils, and the genes encoding components of oxidative phosphorylation (OXPHOS) pathways. Reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and biochemical assays confirmed the microarray results and provided evidence for the deficiency of OXPHOS complex IV in the chagasic murine heart. We discuss the apparent role of OXPHOS dysfunction in the cardiac hypertrophic and remodeling processes with the development of chagasic cardiomyopathy (CCM).

Keywords: Animal model of human disease; Chagasic cardiomyopathy; Cytochrome c oxidase; Gene expression analysis; Oxidative phosphorylation; Oxidative stress

1. Introduction

Chagasic cardiomyopathy (CCM), a major health problem in southern parts of the American Continent, is caused by Trypanosoma cruzi [1]. Following infection, the acute phase encompasses peak blood and tissue parasitemia. A significant number of infected individuals (~ 40%) then enter the quiescent phase, where hosts remain serologically positive, but disease-free for the rest of their lives. In others, the indeterminate phase is associated with a slow progression of myocarditis, eventually leading to heart failure and the patients' death [2].

The relationships among T. cruzi infection, host responses, and disease development are very complex. During the acute infection phase, frequent parasitic lesions associated with extensive inflammatory infiltrates, cellular injury, and cell death are observed in all organs and tissues of the infected host [3–9]. The heart, however, is the major target organ where progressive evolution of CCM is associated with profound structural and functional alterations of the myocardium [10]. Standard histological analyses of tissue biopsies obtained from chagasic patients undergoing heart transplantation or tissues from human cadavers that exhibited evidence of CCM usually reveal mild-to-severe inflammation, extensive tissue necrosis or fibrosis, and...
ventricular dilations. Despite decades of research, limited information is available on the cellular and molecular mechanisms by which cardiovascular structure and functions are adversely affected in *T. cruzi* infected patients and experimental models.

Recent studies have provided some details in elucidating the host cellular responses induced in response to acute *T. cruzi* infection. *T. cruzi* can replicate and survive in all cell types, and is in general considered a silent parasite that elicits few changes in the host fibroblasts during invasion and infection processes [11]. In macrophages, the NFκB signaling pathway is activated in response to phagocytosis of *T. cruzi* [12]. Though not directly demonstrated, NFκB-regulated transcription of cytokine genes is proposed to induce proinflammatory responses in infected macrophages [12]. Subsequent studies have demonstrated the importance of the proinflammatory cytokines (e.g. TNF-α, IL-1 and IL-6) and chemokines (e.g. IP-10, MCP-1, MIG, and RANTES) as key regulators of the innate and adaptive immune responses responsible for the control of acute infection [13,14]. Interestingly, glycosylphosphatidylinositol and mucins, abundantly expressed by the infective and intracellular stages of the parasite, have been characterized as the prime inducers of the proinflammatory cytokines and chemokines in the host macrophages [15–17].

Others have documented the induction of endothelin-1 (ET-1), a potent vasoconstrictor, during the acute infection phase and its adverse effects on the host myocardium. In a susceptible murine model of *T. cruzi* infection, where a majority of infected mice succumbed within 20 days post-infection (dpi), the plasma levels of ET-1 peaked just before the animal’s death [18,19]. Corresponding to this increase in ET-1 expression, *T. cruzi*-infected mice exhibited a substantial increase in cardiac tissue inflammation, fibrosis, and microvascular spasms. These responses could be reversed upon treatment with ET-1 inhibitors, suggesting the participation of ET-1 in stimulating hypertrophic and other reactions. ET-1 is proposed to mediate its effects through the activation of the mitogen-activated protein kinase (MAPK) pathway and subsequent proliferation of smooth muscle cells [19].

The host genetic elements that change with progression from the acute infection phase to the development of chagasic myocarditis have, however, remained largely unknown. Microarray technology has revolutionized the field of molecular biology and afforded the opportunity to profile the expression of thousands of genes. In this study, we have used cDNA microarrays and a murine model of *T. cruzi* infection to expand the repertoire of host genes that change during the course of *T. cruzi* infection and disease development. C3H/HeN (male) mice infected with *T. cruzi* (SylvioX10/4 strain) trypomastigotes mimicked the symptoms of human CCM and provide an excellent model system to monitor changes associated with the development of chronic disease. A total of 1176 cDNAs arrayed on nylon membranes were profiled for expression changes in the heart of mice at different stages of infection and disease development. The differentially expressed genes were sorted according to their functional characteristics in the context of the infection. Here, we report the down-regulation of several of the transcripts encoding components of the mitochondrial oxidative phosphorylation (OXPHOS) pathway in *T. cruzi*-infected murine hearts and discuss their possible role in the development of chagasic myocarditis.

2. Materials and methods

2.1. Mice and parasites

Six- to eight-week-old male C3H/HeN mice were purchased from Harlan (Indianapolis, IN). *T. cruzi* (Sylvio X10/4 strain) and C2C12 cells (murine skeletal muscle cell line) were purchased from American Type Culture Collection (Manassas, VA). *T. cruzi* trypomastigotes were maintained and propagated by the continuous in vitro passage of parasites in monolayers of C2C12 cells [20]. Mice were infected by intraperitoneal injection of 1 million culture-derived trypomastigotes.

2.2. Preparation of DNA

Blood and tissue samples were harvested from normal and infected mice at weekly intervals (3 mice/time point/group), till 50 dpi and at monthly intervals thereafter. Blood samples were diluted 1:1 with GE buffer (6 M guanidine HCl, 0.2 M EDTA, pH 8.0), immediately boiled for 10 min, and stored at 4 °C [21]. Total DNA from blood-GE samples was extracted by phenol–chloroform method [22]. Briefly, 200 μl of blood-GE sample was mixed with an equal volume of phenol/chloroform/isooamylcohol (24:24:1), vortexed, and centrifuged at 12,000 × g for 5 min. The top aqueous phase was transferred to a clean 1.5-ml eppendorf tube, mixed with 1/10th volume of sodium acetate (3 M, pH 5.2) and 2.5 volume of ice-cold 100% ethanol. After centrifugation at 12,000 × g for 30 min at 4 °C, the pellet was washed with 70% alcohol and resuspended in 20-μl distilled water.

For the extraction of DNA from the heart and skeletal muscle, 50 mg of the finely chopped tissues were incubated in 250-μl lysis buffer (50 mM Tris HCl, pH 7.5 containing 0.5% SDS, 10 mM EDTA, 0.1 M NaCl, and 400 μg/ml Proteinase K) at 55 °C for 4 h. Tissue lysates were extracted with phenol/chloroform/isooamylcohol (24:24:1) and DNA purified by ethanol precipitation (as above). All genomic DNA samples were suspended in 100-μl distilled H2O.

2.3. Parasite detection by polymerase chain reaction (PCR)

To evaluate the parasite burden, 2 μl of the total DNA, isolated from blood or tissue samples, was amplified by PCR in a 50-μl reaction volume using *T. cruzi* 18S rDNA-specific
oligonucleotides (listed in Table 2). Denaturation, annealing, and elongation steps were performed for a total of 28 cycles, for 1 min each at 94, 58, and 72 °C, respectively. A 10-μl aliquot of each PCR reaction was electrophoresed on 1.2% agarose gels, stained with ethidium bromide, and densitometric analysis performed on a FluorChem Imaging System (Alpha Innotech, San Leandro, CA).

2.4. Histology

Some mice (three mice per group) were sacrificed at various time intervals post-infection for histological examination. The heart and skeletal muscle tissue were removed and fixed in 10% buffered formalin for 24 h, dehydrated in absolute ethanol, cleared in xylene, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin and evaluated by light microscopy. The parasite foci were quantitated, based upon the number of parasitic pseudocysts present in sections of the heart and skeletal muscle tissue were removed and processed with a tissue homogenizer, and the total RNA was isolated as described [25,26]. All RNA samples were stored at −80 °C until further use.

To remove the DNA that might be contaminating the RNA preparation, DNase treatment of total RNA was performed using a RNA purification kit (Ambion, Austin, TX). The [32P]-labeled cDNA probe was then generated by reverse transcription of 5 μg of total RNA using a gene-specific CDS primer mix (Clontech, Palo Alto, CA) and superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) in the presence of 5 μl of [α32P]dATP (3000 Ci/mmol, 10 μCi/μl, Amersham, Piscataway, NJ). The labeled cDNA probe was purified from unincorporated [32P]-labeled nucleotides and small (<0.1 kb) cDNA fragments using nucleospin extraction chromatography (Clontech), and the specific activity of the labeled probe was determined on LS 6500 liquid scintillation counter (Beckman, Fullerton, CA).

2.6. Microarray hybridization and data analysis

Atlas mouse 1.2 II array kits from Clontech were used for analysis of large-scale gene expression in control and T. cruzi-infected mouse hearts. The materials provided with the kit were used and the recommended protocol was followed for pre-hybridization, cDNA probe hybridization, and washing of the membranes. Membranes were exposed to a phosphorimaging screen for 3–5 days. The images obtained on the phosphorimaging screen were captured using a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed with AtlasImage 2.1 software (Clontech). Results from triplicate experiments were averaged, and clones with discordant or absent results were discarded. Genes that increased or decreased in infected murine hearts in comparison to the age-matched controls by at least 1.8-fold were considered differentially expressed. Hierarchical clustering of the differentially expressed genes was done using Spotfire (Spotfire Inc., Somerville, MA), and Cluster and TreeView software [27,28].

2.7. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses

To validate the microarray results, we examined the differential expression of selected genes by RT-PCR. First strand cDNA was synthesized by incubation of total RNA (2 μg) extracted from heart sections of normal and infected mice with 2.5-U MuMLV reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo-dT16 at 42 °C for 50 min in 20-μl reaction volume. Subsequently, 0.5 μl of the cDNA was amplified for 28 cycles by PCR using 2.5 U of Taq polymerase and 200-ng gene-specific primer pair (listed in Table 2), in a 50-μl reaction volume. Individual amplicons (10 μl) were electrophoresed in agarose gels and densitometric analysis conducted as above.

2.8. Immunoblot analysis

Heart tissue from normal and infected mice was washed with ice-cold Tris–HCl (10 mM, pH 7.5), and homogenized in lysis buffer (10 mM Tris, pH 7.5, 1% Nonidet P40, 0.5% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 37 μg/ml n-alpha-tosyl-l-lysine chloromethyl ketone, and 10 μg/ml aprotinin). After incubation for 15 min on ice, homogenates were centrifuged at 5000 × g, 4 °C, for 20 min and the supernatants stored at −80 °C. Proteins were resolved on a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (BioRad, Hercules, CA). Membranes were washed with PBS (137 mM NaCl, 8 mM KCl, 2 mM KH2PO4, 10 mM Na2HPO4), blocked with PBS/0.1% Tween 20 (PBST) containing 5% nonfat dry milk (NFDM) for 1 h at room temperature, and incubated with mouse monoclonal antibodies to cytochrome c oxidase subunits 7aH, 7aL, or anti-β-actin antibody for 2 h in blocking buffer. After incubation with alkaline phosphatase conjugated secondary antibody, color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagents (BioRad).

2.9. Cytochrome c oxidase activity

Mitochondria were isolated from normal and infected murine hearts as described [29] with slight modifications.
Briefly, heart tissue (~100 mg) was washed with ice-cold isolation buffer (5 mM HEPES, pH 7.2 containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 0.5% bovine serum albumin (BSA)) and immediately homogenized (tissue/isolation buffer ratio, 1:10 w/v). The homogenate was centrifuged at 1000 × g, 4 °C, for 10 min to pellet the cell debris and blood cells. The supernatant was sequentially centrifuged at 1000 × g and 8000 × g, each for 10 min. The mitochondrial pellet so obtained was washed and suspended in isolation buffer (tissue/buffer, 1:1 ratio, w/v), and mitochondrial aliquots stored at −80 °C. For the determination of the mitochondrial proteins, mitochondrial pellet was suspended in isolation buffer without BSA, and protein concentration determined by the Bradford method [30].

Complex IV activity was measured following the oxidation of reduced cytochrome c at 550 nm (extinction coefficient 19.0 mM−1 cm−1) [31]. For this, mitochondria (2.5 μg protein) were permeabilized in 950 μl of assay buffer (10 mM Tris–HCl pH 7.0, 120 mM KCl) containing 2.5 mM n-dodecyl-D-maltoside for 2 min at 37 °C. The enzymatic reaction was started with 10 μM reduced cytochrome c, and a decrease in absorbance measured on an UltroSpec 2000 Spectrophotometer (Pharmacia, Peapack, NJ) at 550 nm for 2 min. The assay was repeated in the presence of 5 mM KCN to measure the nonspecific changes in the absorbance.

2.10. Electron microscopy (EM)

Following infection, heart tissue from infected mice was harvested and fixed in Ito’s fixative (1.25% formaldehyde, 2.5% glutaraldehyde, 0.03% CaCl2, and 0.03% trinitrophenol in 0.05 M cacodylate buffer, pH 7.3) at room temperature for 1 h and then overnight at 4 °C. After washing, samples were post-fixed in 1% osmium tetroxide for 1 h and en bloc stained with 1% uranyl acetate in 0.1 M maleate buffer. After dehydration in a graded series of ethanol, samples were embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Sorvall MT-6000 ultramicrotome (RMC, Tucson, AZ), stained with uranyl acetate and lead citrate, and examined in a Philips 201 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

3. Results

3.1. T. cruzi infection and disease development

*T. cruzi* ribosomal DNA (rDNA)-specific PCR was performed to evaluate the infection rate, and the blood and tissue parasite burden in C3H/HeN mice infected with *SylvioX10/4* strain. Mice infected with *T. cruzi* exhibited a course of infection and disease development similar to that observed in human and other species [2]. The acute phase was characterized by a consistent increase in blood and tissue parasitemia that peaked in intensity during 30–45 dpi. Parasite rDNA was detected as early as day 3 post-infection in blood and tissue samples (Fig. 1A and D, data not shown) obtained from all mice injected with *T. cruzi* trypomastigotes. The peak blood parasite load, indicated by an increase in parasite-rDNA, was noted at 30 dpi, after which the blood parasitemia was reduced to below detection level (data not shown). Maximum parasite burden in the cardiac tissue of infected mice was detected during days 37–45 post-infection (Fig. 1B and D). After 80 dpi, a very faint signal for parasite rDNA (Fig. 1C and D) was detected in infected murine hearts and was suggestive of control of acute parasite burden. A similar pattern of amplification of parasite rDNA was observed in the skeletal tissue of infected mice (data not shown). Specificity of the PCR reaction was confirmed by no amplification of the parasite rDNA upon incubation of *T. cruzi* or infected mouse DNA with one primer or normal mouse DNA with both the primers (data not shown).

Fig. 1. The course of *T. cruzi* infection and replication in mouse hearts. C3H/HeN mice were infected by intraperitoneal injection with culture-derived trypomastigotes of *T. cruzi* (*SylvioX10/4* strain, 1 × 10⁶ parasites/mouse). *T. cruzi* rDNA-specific PCR was performed for 28 cycles using total DNA isolated from whole heart tissue of normal and infected mice at various time intervals post-infection. PCR amplification of *T. cruzi* rDNA using total DNA isolated from heart tissue of 10 mice at day 3 (A), 37 (B), and 110 post-infection (C) is shown. (D) Quantitation of the parasite burden. The signal from PCR amplification of *T. cruzi* in heart tissues harvested during 3–110 dpi was quantitated using a FluorChem Imaging System.
The course of disease development in *T. cruzi*-infected mice was assessed on the basis of tissue parasite lesions, inflammation, and fibrosis. All infected mice exhibited parasite foci in the heart tissue (Fig. 2E, marked with arrow) and skeletal muscle during the acute phase of infection (up to 45 dpi). Intense infiltration of inflammatory cells either closely associated with the parasitic lesions or diffused throughout the tissue was also noticeable in the myocardium and skeletal muscle of the infected mice during the acute phase (Fig. 2C–F). After the acute parasite control, parasitic foci were not detected in all of the tissue sections examined by histological analysis. Accordingly, the extent of inflammation and associated tissue damage was remarkably reduced in skeletal muscle of infected mice at 120 dpi (Fig. 2H). However, infected mice (>100 dpi) continued to exhibit substantial inflammation and tissue fibrosis and necrosis in the heart tissue (Fig. 2G), the hallmarks of chagasic myocarditis. These results demonstrate that C3H mice infected with SylvioX10/4 strain of *T. cruzi* provide a suitable model system to study the molecular changes associated with chagasic myocarditis.

3.2. Microarray analysis of gene expression revealed a repression of the murine transcriptome with the onset of the chagasic disease phase

Microarrays containing a substantial repertoire of known murine genes were used to evaluate changes in myocardial gene expression in *T. cruzi*-infected mice sacrificed at various time intervals post-infection. On the basis of parasite burden and pathological changes in the heart tissue of infected mice (Figs. 1 and 2), the three time points chosen for microarray analysis represented immediate early (3 dpi), acute (37 dpi), and chronic (110 dpi) phases of chagasic disease development. All arrays were normalized both globally and for the expression of a 40S rRNA and compared to arrays probed with RNA extracted from age-matched, normal murine hearts. Genes that increased or decreased in comparison to the controls by at least 1.8-fold in three independent experiments were considered differentially expressed.

Out of a total of 1176 genes printed on the arrays, 31, 89, and 66 genes were differentially regulated in the context of their expression trends at 3, 37, and 110 dpi, respectively (Fig. 3, Table 1). Noticeably, all of the differentially expressed transcripts in the myocardium at 3 dpi were upregulated and encoded immune-related or stress proteins. During the acute phase (37 dpi), mRNA species for 77 of the 89 differentially regulated genes were increased by at least twofold. Of these, 27 transcripts were increased by >10-fold, and 18 of the 27 transcripts encoded the immune-related proteins. Out of the 12 transcripts that were reproducibly repressed at 37 dpi, eight were characterized to encode proteins involved in mitochondrial energy...
metabolism. Surprisingly, a majority of the differentially expressed genes (>63%) in the myocardium of infected mice at 110 dpi were repressed relative to normal controls. The dramatic repression of the host transcriptional response at 110 dpi was confirmed in four independent experiments, carried out with RNA isolated from three mice per experiment. After discarding the sequences whose expression was changed in less than three experiments, we found 42 of the 66 differentially expressed mRNAs were repressed at 110 dpi. Of these, 26 (60%) transcripts have implications in sustaining the mitochondrial energy metabolism and maintaining the cytoskeletal and extracellular matrix (ECM) structure and function.

3.3. Expression of proinflammatory genes is up-regulated in response to T. cruzi infection

The histological data suggested the infiltration of the inflammatory infiltrate in T. cruzi-infected murine hearts as early as 20 dpi that was maintained throughout the infection and disease period, albeit at different levels (Fig. 2). The microarray studies provided a molecular imprint of the immune responses that might be responsible for maintaining the inflammation in the chagasic heart. IFN-γ, IL-1, IL-6, and TNF-α are well-characterized proinflammatory cytokines and are known to participate in control of infectious agents through different immune mechanisms [32]. IFN-γ regulates several aspects of the protective immune responses, including stimulation of the phagocytic activity of macrophages, enhancement of antigen presentation through class I and II MHC molecules, and synchronization of interaction between T cells and antigen-presenting cells [33,34], through the activation of a variety of cytokines, chemokines, and cell ligand and receptor molecules [34]. Accordingly, the immediate early response of the host to T. cruzi infection was marked with striking up-regulation of the IFN-inducible molecules (IIMs), including CC chemokines (e.g. MIG, SCY2), chemokine receptors (CCKR2 and CCKR5), interferon regulatory factors IRF1 and IRF2, and other IFN-dependent proteins (Fig. 3, Table 1A). The expression of IIMs was more pronounced at 37 dpi relative to 3 dpi. It is therefore likely that IFN-regulated cytokines and chemokines will function as important modulators of the innate immune responses against T. cruzi and mediate resistance to infection.

Further evidence for the elicitation of an immediate early response in infected murine heart was provided by the expression of transcripts for IL-1 (accession #U56773), IL-6 (accession #X62046) and TNF-α (accession #L24118, U37522, L26349) induced molecules (IITM). It is interesting to note that IITMs were consistently expressed throughout the infection and disease development period. In contrast, the expression of a majority of transcripts for IIMs subsided during the disease phase. Considering that the IFN-mediated immune reactions play an important role in control of the parasite [35,36], a decrease in the expression of IIMs is possibly an adaptation mechanism to control the destructive effects of the immune responses on the host cellular components. However, the consistent expression of IITMs provides molecular evidence for the persistence of the inflammation in infected hearts, also noted in histological studies (Fig. 2).

The stage-specific expression of the IIMs and the consistent up-regulation of the IITMs in T. cruzi-infected mice also suggest the differential composition of the proinflammatory responses exhibited at different stages of infection and disease development in the myocardium.

3.4. Gene expression pattern indicated cardiac remodeling in T. cruzi-infected murine hearts

Our gene expression studies indicated an imbalance in the expression of several of the genes that encode ECM proteins in the myocardium of infected mice relative to normal hearts (Fig. 3). At the acute infection stage (37 dpi), the transcripts for proteins that are structural components of the ECM, including several types of collagens, dystrophin, fibronectin, thrombospondin 2, osteoglycin, and drebrin, were elevated (Table 1C). Consequently, transcripts for the markers of the hypertrophic response [37,38], including serum amyloid, sialophorin, embryonic atrial natriuretic factor (ANF), and annexins, were also increased in T. cruzi-infected mice at 37 dpi (Fig. 3, Table 1B). Overexpression of these transcripts is indicative of active reparative and remodeling reactions that might be initiated in response to myocardial injuries by invading and replicating parasites.

It is worth noticing that with the onset of the disease phase (110 dpi) in the myocardium, mRNAs for collagens, fibronectin, and other ECM proteins were either elevated to a lesser extent than that observed at 37 dpi or were substantially repressed (Fig. 3, Table 1C). Additionally, transcripts for cardiac troponins (T1 and T2) and fast skeletal troponin were reduced at 110 dpi. The differential expression of the key sarcomere and cytoskeletal proteins, including alpha cardiac actin, β myosin, troponins, and alpha troponymosin, has previously been suspected in experimental heart failure models and inherited cardiomyopathies [39,40]. Our experiments may not have identified all of the CCM regulators due to the limited repertoire of the genes printed on the microarrays and possibly due to the stringency used in our experiments. However, down-regulation of the cardiac troponins, all of which are members of the contractile apparatus and essential for ventricular function, suggests the disruption of the sarcomeric filamentary system with the development of chagasic disease in infected mice.

3.5. Gene profiling suggested down-regulation of OXPHOS pathway in the myocardium of infected mice with disease development

The most striking feature of the host response to T. cruzi infection was, perhaps, the repression of a number of tran-
<table>
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<th>GenBank accession number</th>
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<td>A03e</td>
<td>Fas antigen; fasL receptor precursor; CD95 antigen</td>
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1B. Host defense proteins related to oxidative stress

A03n | Sialophor | X17018 | 1 30 1 |
B02b | Serum amyloid A 3 | X03479 | 1 33 1 |
B03f | Heat shock protein 2 (HSP2) | AF055664 | 2.8 4.2 1 |
B03i | Peripheral benzodiazepine receptor (PbDR) | D21207 | 1 27 1 |
C08b | Tyrosinase | M24560 | 1 –1.7 0 |
C10h | Heat shock 60-kDa protein (HSP60) | X55023 | 7.5 1.2 3.5 |
C11d | Anti-oxidant protein 1 | M28723 | 3.2 1.2 –3.3 |
E04d | Atrial natriuretic peptide precursor type B (ANF) | S58667 | 1 3.5 2 |
E08i | Mitogen-activated protein kinase kinase 1 (MAPKK1) | L02526 | 3.1 3 1.7 |
E12a | Annexin A2 | X17018 | 1 4 4.4 |
F11c | Jun zipper-associated 1 protein | AF069519 | 1 2.1 1.8 |

1C. Extracellular matrix/hypertrophy proteins

B08e | Fibronectin 1 | X93167 | 1 4 –1 |
B08j | Osteoglycin | D31951 | 1 2.6 –3.8 |
B08l | Procollagen 1 alpha 1 subunit (COL1A1) | U08020 | 1 3.6 –0.2 |
B08m | Procollagen 1 alpha 2 subunit (COL1A2) | X58251 | 1 4.7 –0.5 |
B08n | Procollagen 3 alpha 1 subunit (COL3A1) | X52046 | 1 4.4 1.7 |
B09b | Procollagen 5 alpha 2 subunit (COL5A2) | L02918 | 1 5 1 |
B09g | Thrombospondin 2 (THMB2) | L07803 | 1 23 18 |
F07m | Drebrin-like | U58884 | 1 3.4 1.4 |
F07a | Muscular dystrophin | M68859 | 4.2 3.4 2.4 |
F10e | Fast skeletal troponin C (FkTnC) | M57590 | 1 1 –1.8 |
F10f | Cardiac troponin T1 (cTnT1) | U09181 | 1 1 –1.8 |
F10g | Cardiac troponin T2 (cTnT2) | L47549 | 1 1 –3.3 |

1D. Mitochondrial/energy metabolism

B11k | Cytochrome b-558 beta polypeptide | M31775 | 2.7 11.1 1.6 |
B11l | Cytochrome b-558 alpha polypeptide | U43384 | 1 45 1 |
B11n | Cytochrome c oxidase polypeptide VilaH (COX7aH) | AF037370 | 1 1 –1.8 |
B12a | Cytochrome c oxidase, subunit VilaL (COX7aL) | AF037371 | 1 –1.7 –3.8 |
B12b | Cytochrome c oxidase polypeptide VilaC (COX7c) | X52940 | 1 1 –3.3 |
B12f | Cytochrome c oxidase polypeptide VilaH (COX6aH) | U08439 | 1 –2.3 –2 |
C02d | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 1 | Y07708 | 1 –1 –2.3 |

Values for repressed genes are log-transformed. UNIQID was assigned to individual genes in reference to the position on the array (Clontech).
with CCM development, albeit through activation of different cellular mechanisms.

3.6. Confirmation of microarray data

In independent experiments, the relative expression of 15 representative genes (listed in Table 2) in the myocardium of the infected mice was analyzed by RT-PCR analysis. These included COX7aH, COX7aL, cTnT1, cTnT2, and FskTnC, noted to be repressed with disease progression, in microarray studies; TNFIP2 and IRF1, induced at 3, 37 and 110 dpi; COL5A2, THMB2, and PBdzR up-regulated during the acute phase; and murine 18S rRNA, GPI8, and \( \beta\)-actin, which exhibited no change in expression in infected and normal mice. Expression data from nine of these transcripts, representative of each category, are shown in Fig. 4.

Similar to the microarray findings, a significant reduction in COX7aH and COX7aL transcripts was observed in the heart tissue of infected mice at 37 and 110 dpi, but not at 3 dpi when compared to control mice. Likewise, transcripts for cTnT1 and cTnT2 were reduced at 110 dpi. In agreement with the microarray results, a substantial increase in abun-

![Fig. 4. Confirmation of microarray expression data by RT-PCR analysis.](image)

Table 2

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank accession number</th>
<th>Forward primer sequence (5′ → 3′)</th>
<th>Reverse primer sequence (5′ → 3′)</th>
<th>Amplicon (bp)</th>
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<tr>
<td>Mouse</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac troponin T1 (cTnT1)</td>
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T. cruzi 18S rRNA

The infected mice were analyzed by RT-PCR analysis. These included COX7aH, COX7aL, cTnT1, cTnT2, and FskTnC, noted to be repressed with disease progression, in microarray studies; TNFIP2 and IRF1, induced at 3, 37 and 110 dpi; COL1A2, COL3A1, COL5A2, THMB2, and PbdzR up-regulated during the acute phase; and murine 18S rRNA, GPI8, and \( \beta\)-actin, which exhibited no change in expression in infected and normal mice. Expression data from nine of these transcripts, representative of each category, are shown in Fig. 4.

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![Fig. 5. Western blotting demonstrates a decrease in the expression of cytochrome c oxidase subunits in infected murine hearts.](image)

Total RNA isolated from whole hearts of normal and infected mice was used for RT-PCR analysis. First strand cDNA was synthesized from 2 

\( \mu \)g of the total RNA samples in a 20-

\( \mu \)l reaction. Subsequently, PCR was performed for 28 cycles using 0.5 

\( \mu \)l of the cDNA as template and the gene-specific primers. Abbreviations used: COX7aH, cytochrome c oxidase subunit 7aH; COX7aL, cytochrome c oxidase subunit 7aL; cTnT1, cardiac troponin T1; cTnT2, cardiac troponin T2; COL5A2, procollagen subunit 5A2; TNFIP2, TNF-induced protein 2; IRF1, interferon regulatory factor; 18S rRNA, 18S ribosomal RNA, GPI8, glycosylphosphatidylinositol-protein transamidase; and dpi, days post-infection.
dance of transcripts for COL5A2, TNFIP2, and IRF1 was noted in infected mice at 37 dpi; densitometric analysis suggested a ~ 2.5-fold increase in COL5A2 and TNFIP2, and a ~ 5-fold increase in IRF1 transcripts in infected mice at day 37 post-infection as compared to that detected at 3 and 110 dpi. Comparable amplification of murine 18S rRNA and GPI8 transcripts in the heart tissue of infected and normal mice confirmed the use of equal amounts of total RNA in cDNA generation and PCR reactions, and suggested no effect of T. cruzi infection on the expression of GPI8 and 18S rRNA in the myocardium of mice.

Next, we determined whether changes in transcript abundance correlated with protein expression. Considering the substantial decrease in transcripts of several complex IV polypeptides during the disease stage, we decided to examine the relative abundance of cytochrome c oxidase subunits in normal and infected murine hearts (Fig. 5). A significant decrease in COX7aH polypeptide was observed in the myocardium of infected mice at 110 dpi, the repression being amplified with chronic disease development (330 dpi). The protein level for COX7aL was decreased early during the acute phase. Densitometric analysis suggested >80% reduction in COX7aL levels during 45-330 dpi in infected murine hearts as compared to normal mice. Similar levels of the β-actin in all tissue lysates (Fig. 5) confirmed equal loading of the proteins in each lane. Overall, RT-PCR and Western blot analyses have provided independent confirmation of microarray data for the induction and repression of several of the cardiac genes in response to T. cruzi infection and disease development.

3.7. Cytochrome c oxidase activity is decreased in the myocardium of infected mice

Given the alterations in the expression of cytochrome c oxidase subunits in T. cruzi-infected murine hearts, we predicted that a deficiency of complex IV activity might ensue with the development of chagasic disease. To determine if such is the case, we estimated the enzymatic activity of cytochrome c oxidase in mitochondria isolated from the cardiac tissue of normal and infected mice, by spectrophotometric assay. As shown in Table 3, mitochondria isolated from infected mice exhibited substantial decline in complex IV activity. In comparison to normal controls, cardiac mitochondria isolated from infected mice showed 40% and 56% reduction in cytochrome c oxidase activity at days 37 and 110 post-infection, respectively. The decrease in the complex IV enzymatic activity in infected mice provides further evidence for the accuracy of microarray data and suggests that the infected murine hearts might be compromised in terms of mitochondrial OXPHOS capacity with CCM progression.

3.8. Mitochondrial collapse with the development of chagasic disease

To illustrate the consequences of the molecular changes in the expression of ECM and mitochondrial genes in

![Fig. 6. Transmission electron microscopy (EM) of heart tissue from T. cruzi-infected mice. C3H/HeN mice were infected with T. cruzi (as in Fig. 1). Heart tissue ultra-thin sections for EM analysis, obtained from infected mice at days 17 and 143 post-infection, are shown in panels A and B, respectively. EM image of an ultra thin heart tissue section from normal, uninfected mouse is shown in panel C. Representative micrographs showing parasites in a myocyte at early stage of infection (Panel A, parasites marked with letter p and arrow), and mitochondrial swelling (Panel B, marked with letter m) and lipid accumulation (Panel B, marked with arrow heads) in cardiac myocytes during disease phase (panel B) are presented. Note the absence of these features in cardiac myocyte of a normal mouse (C). Bar=1 μm. Original magnification, 9400 ×.](image-url)
infected murine hearts, we examined ultra-thin sections of the heart tissue by EM, and evaluated the extent of morphological alterations in the myocardium of infected mice at various time intervals post-infection. As expected from the results presented in Figs. 1 and 2, we detected parasites in the cardiomyocytes upon EM analysis of the tissue sections harvested from acutely infected mice (Fig. 6A, note the intracellular amastigotes marked with arrows). During this stage, a local depletion of the cell cytoplasm adjacent to the parasites was noted; however, no other injuries were evident: myofibrils were not damaged; mitochondria had normal appearance, and linear arrangement of myofibers and mitochondria was not disturbed (Fig. 6A). With progression of disease phase, parasites were not detected in all of the tissue sections obtained from mice sacrificed at 110 and 143 dpi. However, myocardial alterations characterized by large irregular nuclei, focal myofibrillar degeneration, and Z-line distortion were evident (Fig. 6B, data not shown). Additionally, mitochondria were swollen and displaced, and accumulation of lipid droplets alongside the irregular mitochondria (Fig. 6B, marked with arrows) was noticeable. These changes were particularly evident when compared with heart tissue sections obtained from uninfected, normal mice (Fig. 6C). Altogether, the results of the EM studies, suggestive of the loss of the myofibrils and mitochondrial structure and function in cardiac myocytes of the chronically infected mice, are consistent with the irregularities in the expression of ECM and mitochondrial genes, noted in microarray studies (Fig. 3).

4. Discussion

In this study, we profiled the alterations in host gene expression to gain insight into the molecular mechanisms underlying the development of chagasic myocarditis. For this, an experimental murine model of *T. cruzi* infection was employed and the changes in cardiac gene expression over the time period spanning from early infection to the onset of the disease phase was identified. As recognized in humans, the course of disease development in C3H/HeN mice infected with SylvioX10/4 strain of *T. cruzi* was divided into (a) the acute infection phase (up to 45 dpi) when a significant increase in the blood and tissue parasite burden was noted (Fig. 1), (b) the progressive disease phase marked by minimal parasite burden in association with extensive inflammation and cellular fibrosis, and (c) tissue degeneration in the myocardium (Figs. 2 and 6). Considering that the RNA for microarray hybridization was prepared from whole heart, consisting of infected and uninfected myocytes and other cell types, the true changes in gene expression in infected cardiomyocytes may be underestimated in the present study. Nevertheless, a total of 114 genes was found to be differentially expressed in the myocardium of infected mice over the course of disease development. The clustering of the genes exhibiting similar expression pattern and related functional characteristics revealed the molecular print of the biological processes that are perturbed in the myocardium of mice during the course of *T. cruzi* infection and onset of the disease phase.

The overall expression data suggested two important phenomena with respect to myocardial composition and function. One, the coordinated up-regulation of transcripts for ECM proteins, the increased expression of fetal ANF mRNA, and the abundance of mRNAs for several transcription and translation factors (Fig. 3, Table 1) occurred at 37 dpi. All of these changes are indicative of increased matrix deposition and protein synthesis, and suggest the instigation of hypertrophic response in the myocardium of acutely infected mice. Second, with the onset of the disease phase, the infected mice displayed a general deterioration of the myocardial structure as was evident by the repression of myofibril transcripts (Figs. 3 and 4) and accumulation of deformed myofibers separated by distorted Z-lines (Fig. 6). The data presented in this study provide insights into the molecular mechanisms that might be responsible for invoking the differential expression of ECM and sarcomeric genes leading to a multi-faceted outcome, i.e. tissue fibrosis and hypertrophy, cardiac remodeling, and myocardial degeneration of the *T. cruzi*-induced CCM.

The earliest response detected in *T. cruzi*-infected mice was the substantial increase in transcripts representative of the pro-inflammatory reactions that continued to persist throughout the infection and disease period (Fig. 3, Table 1A). The prime purpose of the proinflammatory cytokines in infected mice appears to be related to the control of the infectious agent. However, TNF-α and IL-1 can also exert similar biological effects on the host cellular components through regulation of different autocrine and paracrine mechanisms. Of note is the role of TNF-α and IL-1 in NF-κB activation, which evokes cell survival/cell growth responses through down-regulation of the apoptotic pathways [41]. Additionally, TNF-α is also known to induce the activation of caspases leading to apoptotic cell death [42]. Systemic administration of TNF-α causes irreversible cardiac depression in both human and experimental models [43,44]. It is thus reasonable to suggest that the extent of activation of the pro-apoptotic reactions by inflammatory responses and the NF-κB-induced anti-apoptotic pathways would decide the ultimate fate of the myocardial cells in infected mice; as long as the NF-κB pathway is functional and active, cells may survive and grow. However, if NFκB is blocked or the anti-apoptotic pathway is not activated, then cell death would prevail.

Recent studies have documented the silencing effects of interferon-regulated factor IRF1 and IRF2 on NFκB-induced transcriptional activity [45,46]. The demonstration of up-regulation of IRF1 and IRF2 in our studies (Fig. 3, Table 1A) suggests that NFκB might be depressed in *T. cruzi*-infected mice. Others have shown the cell-specific effect of *T. cruzi* infection on NFκB activation. In isolated macrophages exposed to *T. cruzi*, induction of NFκB tran-
scriptional activity is detectable [12]. However, cells derived from skeletal, smooth, and cardiac muscle are the prime site for intracellular replication of T. cruzi in infected host, and all of these cell types exhibit no induction of NF-κB in in vitro infection studies [47]. Whether NFκB inhibition in cells is mediated through direct interaction of NFκB with parasite molecules or with IRFs (induced in response to infection) is not known. Either way, the long-term inhibition of NFκB would be detrimental to the host, primarily because of the inability to control cell death as a consequence of parasite infection or inflammation. Up-regulation of apoptosis-related Fas antigen, in our system, supports this notion. Taken together, our results imply that persistent expression of IITMs would play an important role in promoting apoptotic cell death in chagasic myocarditis.

The most striking response of the host to T. cruzi infection was the repression of the transcripts for various components of OXPHOS pathway (Figs. 3 and 4). Subsequently, a reduction in the expression of the complex IV polypeptides (Fig. 5) and a decline in the cytochrome c oxidase activity (Table 3) were also noted with disease development in the myocardium of infected mice. Our results suggest an overall loss of mitochondrial OXPHOS capacity in infected mice that can have an adverse influence on global energy production and subsequent cardiac performance. This suggestion is supported by the observations of morphologic abnormalities in mitochondrial structure associated with a decrease in OXPHOS capacity in cardiomyocytes of cardiomyopathy patients and experimental models of heart failure [48]. A reduction in high-energy phosphates has also been reported in the hypertrophied and failing hearts [49–51]. Our study is, however, the first to demonstrate the abnormalities of mitochondrial morphology and the OXPHOS pathway in chagasic myocarditis.

Several lines of evidence indicate that the myocardium of the infected mice might be exposed to excessive oxidative stress. A sub-optimal functionality of the OXPHOS complexes causes the release of electron energy to molecular oxygen and reactive oxygen production. Considering the high density of mitochondria in cardiomyocytes, OXPHOS dysfunction can be the major source of reactive oxygen species (ROS) in myocardium. The immense decrease in the expression and activity of cytochrome c oxidase (Figs. 3–5, Table 3) observed in this study suggests that uncoupling of the OXPHOS pathway and production of increasing amounts of ROS would occur with cardiac disease progression in infected mice. Other genes related to ROS production were also modulated in the myocardium of infected mice. For example, the levels of cytochrome b-558 α and β, the subunits of NADH oxidase, were elevated in infected mice (Table 1D). NADH oxidase is the main enzyme involved in ROS generation in epithelial tissues. The transcripts for HSP60, antioxidant protein 1, and tyrosinase (Table 1B) that participate in different oxidative stress defense mechanisms were also increased in the myocardium of infected mice. Thus, the distinct expression profile of the pro- and antioxidant genes might result in an imbalance between ROS production and free radical scavengers. As a result of the impaired redox state, direct oxidative damage of the cellular components (e.g. proteins, lipids, and DNA) may accelerate the cardiac injuries [52]. This notion is supported by the observations that acute injuries resulting in an imbalance between pro- and antioxidants induce apoptosis in cardiomyocytes [53]. The up-regulation of apoptosis-related Fas antigen transcripts and the alterations in the expression of ECM and sarcomeric genes in infected murine hearts can also be related to cell death. Taken together, our results imply that oxidative stress is induced in the myocardium of infected mice, and it may contribute to the pathogenesis of CCM through tissue injuries and cell death. The detection of a few, if any, parasites during chronic disease phase suggests that oxidative insult of the infected cells or the intracellular parasite may also contribute to control of T. cruzi in the infected myocardium.

Recent studies have suggested that oxidative stress contributes to cardiovascular diseases through activation of a variety of intracellular signaling pathways. One such relay system is the superfamily of MAPK. There are at least three distinct subfamilies (ERK, SAPK/JNK, and p38) of the MAPK superfamily, all of which can be induced by oxidative stress [54]. The ERKs, in general, activate a cytoprotective, anti-apoptotic effect, leading to an increase in protein synthesis and cell growth [55]. The JNK and p38 cascades can regulate both cell-growth and cell-death programs, depending upon the activation of downstream transactivating/DNA-binding transcription factors [56,57]. In this model of chagasic myocarditis, depression of the OXPHOS pathway in the myocardium correlated with alterations in transcripts for the members of ERK-MAPK super-family (MAPKK1) and downstream transcription factor c-Jun (Table 1B). Our results are consistent with others documenting the phosphorylation and activation of ERK-MAPKs and expression of c-jun/c-fos transcription factors upon T. cruzi infection in experimental models [58], and suggest that although ROS might be important in initiating tissue injury, it may also exert protective effects through an ERK-mediated increase in protein synthesis and cell growth in infected myocardium.

In microarray studies, validation of the results with methods such as RT-PCR or Northern blotting is necessary. We have used RT-PCR to confirm the microarray analysis of expression levels of several of the ECM- and OXPHOS-related genes during the CCM development. Additionally, Western blotting and biochemical assays were employed to further confirm the deficiency of complex IV of OXPHOS pathway during CCM progression.

In conclusion, the alterations in gene expression observed in our study provide a new framework for understanding the initiation and progression of chagasic myocarditis. Our data suggest the disorders of mitochondrial
OXPHOS with CCM development in infected mice. Oxidative stress, produced due to OXPHOS dysfunction or the up-regulation of other ROS-related genes, may be one of the key events in progression of CCM, either due to its injurious effects on cardiac cellular components or through redox activation of the signaling cascades involved in cardiac hypertrophy and remodeling responses. Further studies with mice given antioxidants (e.g. Vitamins A, C, and E cocktail) in combination with benznidazole (anti-parasite drug) will determine if the OXPHOS dysfunction-mediated oxidative stress is a consequence of mitochondrial injuries by the parasite infection, and whether oxidative stress is the causative mechanism in the development of CCM. Alternatively, it is possible that mitochondrial OXPHOS dysfunction and subsequent oxidative stress is a secondary response caused by increased expression and activity of the pro-inflammatory mediators. Studies with mice given anti-inflammatory agents during the course of disease development will determine if mitochondrial dysfunction associated oxidative stress is the downstream effect of cardiac pathology.

Finally, it is worth mentioning that, in comparison to isolated fibroblasts that exhibited few changes in gene expression in response to T. cruzi infection [11], significant alterations were observed in the expression profile of the whole heart in this study. Several factors including the molecular and biochemical changes in infected cardiomyocytes, infiltration of macrophages and inflammatory cells, and vascular impairment are expected to influence the overall expression profile of the whole heart. Studies that would assess the gene expression profile of individual cells or cell populations isolated from infected hearts will be needed to deduce the true changes in myocyte gene regulation in response to T. cruzi infection, and to distinguish the stimuli or signaling factors originating from infected vs. noninfected cells that might exert their effects in an autocrine or paracrine manner in formatting the disease outcome.

Acknowledgements

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References


