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Trypanosoma cruzi infection induces the expression of CD40 in murine cardiomyocytes favoring CD40 ligation-dependent production of cardiopathogenic IL-6

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Abstract The inflammatory response in the myocardium is an important aspect of the pathogenesis of Chagas' heart disease raised by *Trypanosoma cruzi*. CD40, a transmembrane type I receptor belonging to the tumor necrosis factor receptor (TNFR) family, is expressed in a broad spectrum of cell types and is crucial in several inflammatory and autoimmune diseases. Activation of CD40 through ligation to CD40L (CD154) induces multiple effects, including the secretion of proinflammatory molecules. In the present study, we examined the ability of *T. cruzi* to trigger the expression of CD40 in cardiac myocytes in vitro and in a murine model of chagasic cardiomyopathy. Our results indicate, for the first time, that *T. cruzi* is able to induce the expression of CD40 in HL-1 murine cardiomyocytes. Moreover, ligation of CD40 receptor upregulated interleukin-6 (IL-6), associated with inflammation. Furthermore, the induction of this costimulatory molecule was demonstrated in vivo in myocardium of mice infected with *T. cruzi*. This suggests that CD40-bearing cardiac

muscle cells could interact with CD40L-expressing lymphocytes infiltrating the heart, thus contributing to inflammatory injury in chagasic cardiomyopathy.

Keywords CD40 · Cardiomyocytes · *Trypanosoma cruzi* · Chagas' disease

Introduction

Cardiomyocytes are specialized cells whose main function is contraction. However, there is substantial evidence that this cell type can respond to danger signals with complex inflammatory and functional responses (Boyd et al. 2006). In this regard, cardiac muscle cells, a critical target for *Trypanosoma cruzi*, the causative agent of Chagas' disease, readily produce marked amounts of cytokines and chemokines upon infection with this protozoan parasite (Teixeira et al. 2002). Among them, interleukin-6 (IL-6) released by infected cardiac cells has been shown to mediate cardiomyocyte protection from apoptosis, a critical feature for establishment of persistent *T. cruzi* infection in the myocardium (Ponce et al. 2012). This pleiotropic cytokine was consistently observed in heart lesions of experimental mice with acute and chronic Chagas' disease (Sunnemark et al. 1996; Zhang and Tarleton 1996), being associated with endothelial dysfunction and inflammatory cardiomyopathy (Chandrasekar et al. 1996; Laucella et al. 1996; Powell et al. 1998; Sunnemark et al. 1996). The strong IL-6 expression in heart tissue and increased level of circulating IL-6 have also been linked to lethality upon mouse infection with a high parasite load (Sanoja et al. 2013). Further, the fibrogenic action of IL-6 has been suggested to contribute to the development of myocardial fibrosis, a hallmark of the advanced phase of this parasitic illness leading to heart failure (Tostes et al. 2005; Diaz et al. 2009).

Mariela Alejandra Moreno Ayala and Agustina Casasco contributed equally to this work.

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CD40 is a transmembrane type I receptor that belongs to the tumor necrosis factor receptor (TNFR) family and is expressed on the surface of B cells, dendritic cells, and monocytes/macrophages. Apart from immune cells, CD40 has also been identified on endothelial cells, epithelial cells, fibroblasts, myocytes, and hepatocytes (van Kooten and Banchereau 2000). The widespread expression of CD40 accounts for the key role of this costimulatory molecule in the regulation of immune response and host defense. Activation of CD40 through ligation to CD40 ligand (CD40L, CD154) induces multiple effects, including the secretion of interleukins, chemokines, and adhesion molecules, which results in recruitment and activation of immune cells. The CD40/CD40L interaction was found to be crucial in several inflammatory and autoimmune diseases such as lupus, nephritis, encephalitis, arthritis, and vasculopathies (van Kooten and Banchereau 2000). Previous studies demonstrated that the CD40/CD40L signaling pathway in *T. cruzi* acutely infected mice mediated protective effect related to upregulation of interleukin 12 (IL-12) and nitric oxide by a direct stimulation of IFN- γ activated macrophages (Chaussabel et al. 1999; Planelles et al. 2003; Chamekh et al. 2005; Habib et al. 2007). However, the involvement of CD40/CD40L in the pathogenesis of *T. cruzi*-elicited cardiomyopathy remains unexplored.

In this study, we investigated the ability of *T. cruzi* to trigger the expression of CD40 on cardiac myocytes in vitro and in a murine model of chagasic heart disease and also analyzed the functional effect of CD40 ligation in cardiomyocytes.

Materials and methods

Cell culture and in vitro *T. cruzi* infection

Immortalized HL-1 cells display biochemical, morphological, and electrophysiological properties of adult cardiomyocytes (Claycomb et al. 1998). Murine HL-1 cardiomyocytes were cultured on a gelatin (0.02 % [wt/vol])/fibronectin (10 μ g/ml) matrix with Claycomb medium (JRH Biosciences, Lenexa, KS, USA) supplemented with 10 % (vol/vol) fetal bovine serum (FBS), glutamine (2 mM/l), norepinephrine (0.1 mM/l), penicillin (100 U/ml), and streptomycin (100 U/ml). Cells were seeded in 6- (1.5 \times 10⁶/well) or 24-well plates containing 13-mm coverslips (1 \times 10⁵/well) and infected with Vero cells culture-derived *T. cruzi* trypomastigotes (Tulahuen strain) at a 5:1 parasite-cell ratio for 3 h. When indicated, cells were stimulated with recombinant interferon- γ (rIFN- γ ; 150 ng/well; Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA). Plates were rinsed with a complete Claycomb medium to remove free parasites and then incubated in a fresh medium at 37 °C, 5 % CO₂ during 4 h for semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis or 24 h for immunostaining.

T. cruzi infection in mice

Six- to 8-week-old female C3H/He mice (15/group) were inoculated by intraperitoneal route with trypomastigote forms of Sylvio X10/4 strain of *T. cruzi* (10⁶ parasites/mouse, cell culture-derived forms). This model of in vivo *T. cruzi* infection is similar to the infection and disease in humans, including an acute phase characterized by low to undetectable parasitemia and reduced mortality, followed by a chronic phase with inflammation and tissue damage focused on the heart, with resulting cardiovascular dysfunction. *T. cruzi* localization is restricted to myocardial fibers, and parasites are found in a fewer number in all acutely infected mice and in \approx 50 % of chronically infected mice (Postan et al. 1986). Infected mice were euthanized at 7, 21 (acute infection), or 360 dpi (chronic phase). The hearts were removed and fixed in 10 % buffered formalin. All animal study protocols were conducted according to the National Research Council's guide for animal care and were approved by our internal ethics committee.

RT-PCR for CD40 expression

HL-1 cells were removed from 6-well culture plates, and total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's specifications. RT-PCR was performed from 5 μ g/sample of total RNA as previously described (Cutrullis et al. 2009). Primer sequences specific for murine CD40 have been published elsewhere (Krzesz et al. 1999). The β -actin gene was used as endogenous control. RAW 264.7 cells stimulated with LPS were used as positive control for CD40 expression. Amplified products were quantified with densitometric analysis software (SionImage version 1.54, National Institutes of Health, Bethesda, MD, USA). The densitometry value for each gene was corrected for the mouse β -actin value for each sample.

Immunostaining

Coverslips with adhered HL-1 cells were removed from culture plates, washed in phosphate-buffered saline (PBS), and fixed in formalin. Cardiomyocytes were washed with PBS and incubated with rabbit polyclonal antibody specific for murine CD40 (sc-975, Santa Cruz Biotechnology, CA, USA), further rinsed with PBS and incubated with biotinylated polyclonal swine anti-rabbit Ig (MultiLink, Dako, Glostrup, Denmark). The reaction product was revealed by streptavidin-horseradish peroxidase complex (LSAB System HRP, Dako) with diaminobenzidine tetrahydrochloride and hydrogen peroxide chromogen substrate (DAB, Dako). The cells were then counterstained with Mayer's hematoxylin previous mounting onto slides with Permount (Thermo Fisher Scientific, Inc.,

Rockford, IL, USA). Omission of the primary antibody and use of isotype-matched control antibody served as controls.

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded cardiac muscle specimens. Five-micrometer sections were placed onto xylene-coated slides and deparaffinized routinely. The proceeding for CD40 immunostaining was identical to that for HL-1 cells. Microscopic images were captured at $\times 400$ magnification by a CX31 microscope (Olympus, Tokyo, Japan) equipped with an Infinity 1-2 CMOS digital camera (Lumenera Corp., Nepean, Canada). The acquired digital images representing whole tissue sections were analyzed using the Image J software (NIH, USA). The tissue area stained for CD40 was calculated and expressed as a percentage of the total surface area of the tissue section.

IL-6 production following ligation of CD40 on HL-1 cardiomyocytes

HL-1 cardiomyocytes were seeded in 96-well plates (1×10^5 /well) and infected with *T. cruzi* trypomastigotes as above. After replacement with fresh culture media supplemented with 0.5 % FBS, cells were pretreated with rIFN- γ . After 24 h, soluble rCD40L (Santa Cruz Biotechnology, CA, USA, 300 ng/well) which is known to stimulate CD40-expressing cells, was added for 24 h. Release of IL-6 from HL-1 cardiomyocytes was evaluated in culture supernatants using an ELISA kit according to manufacturer's instruction (BD Pharmingen, CA, USA).

Results

The widely documented participation of a local inflammatory response in the pathogenesis of chagasic heart disease, as well as the reported distribution of CD40 in

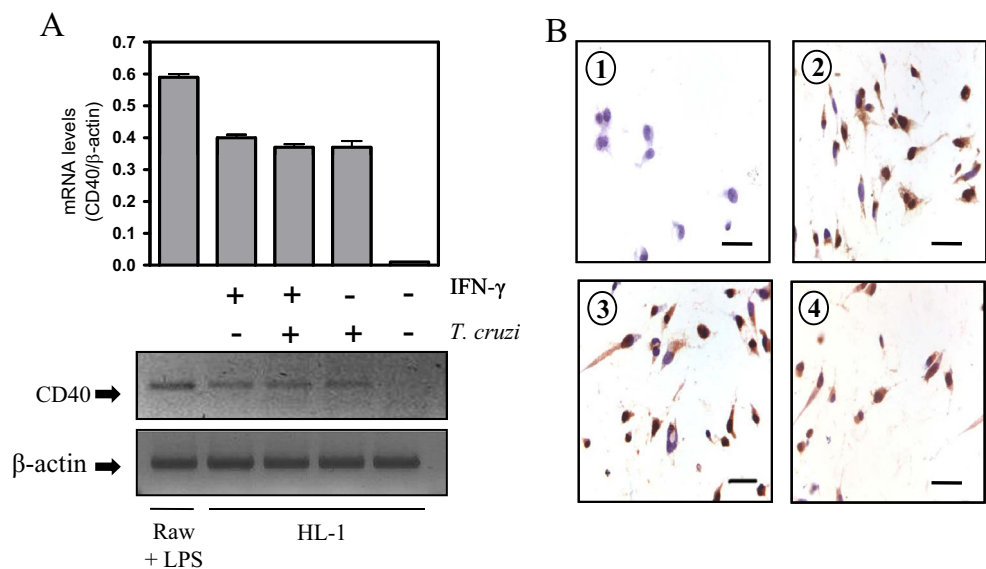
a broad range of cell types, prompted us to investigate whether adult cardiomyocytes express this receptor. As shown in Fig. 1a, RT-PCR analysis demonstrated that CD40 gene is not constitutively expressed in HL-1 cells. However, CD40 mRNA expression was induced after 4-h stimulation with IFN- γ and the protein produced upon 24-h stimulation as detected by immunostaining (Fig. 1b).

We next examined the impact of *T. cruzi* infection on CD40 expression by cardiomyocytes in vitro and in vivo. After 3 h of infection, cultured HL-1 cells displayed enhanced expression of CD40 at both the transcript and protein levels (Fig. 1a, b). As IFN- γ -mediated chronic myocardial inflammation is known to play a key role in the pathogenesis of chronic Chagas' cardiomyopathy (Teixeira et al. 2002), we asked whether it could contribute to parasite-dependent CD40 upregulation. No synergistic effect was observed when *T. cruzi* infection was combined with IFN- γ stimulation.

We attempted to verify if *T. cruzi* infection triggers myocardial CD40 expression in a murine model of severe Chagas' cardiomyopathy. Immunohistochemical staining of heart sections revealed patchy expression of CD40 by cardiomyocytes during the early acute phase of the infection (7 dpi), at the time scarce inflammatory cells infiltrate the myocardium (Fig. 2a). CD40 expression was observed at nuclear, cytoplasmic, and membrane levels (Fig. 2b). Once myocarditis (acute and chronic) was fully established, CD40 expression became more extensive and involved cardiomyocytes as well as inflammatory and endothelial cells (Fig. 2c–e). The area of myocardium positively stained for CD40 was significantly increased (≈ 40 %; $p < 0.05$, ANOVA and the post hoc Bonferroni test) at 21 and 360 dpi compared to the reactivity detected at 7 dpi (Fig. 2g). By contrast, CD40 expression in heart tissues of uninfected mice was weak or absent (Fig. 2f).

Fig. 1 CD40 costimulatory molecule is expressed in HL-1 cardiomyocytes infected with *T. cruzi*. **a** Graphical representation of CD40/ β -actin ratio (upper panel); representative RT-PCR of CD40 mRNA (lower panel). The figure is representative of two experiments. **b**

Immunocytochemical analysis of CD40 protein expression in HL-1 cardiomyocytes cultured in the absence (1) or presence of rIFN- γ (2), *T. cruzi* (3), and rIFN- γ +*T. cruzi* (4). Bar, 25 μ m



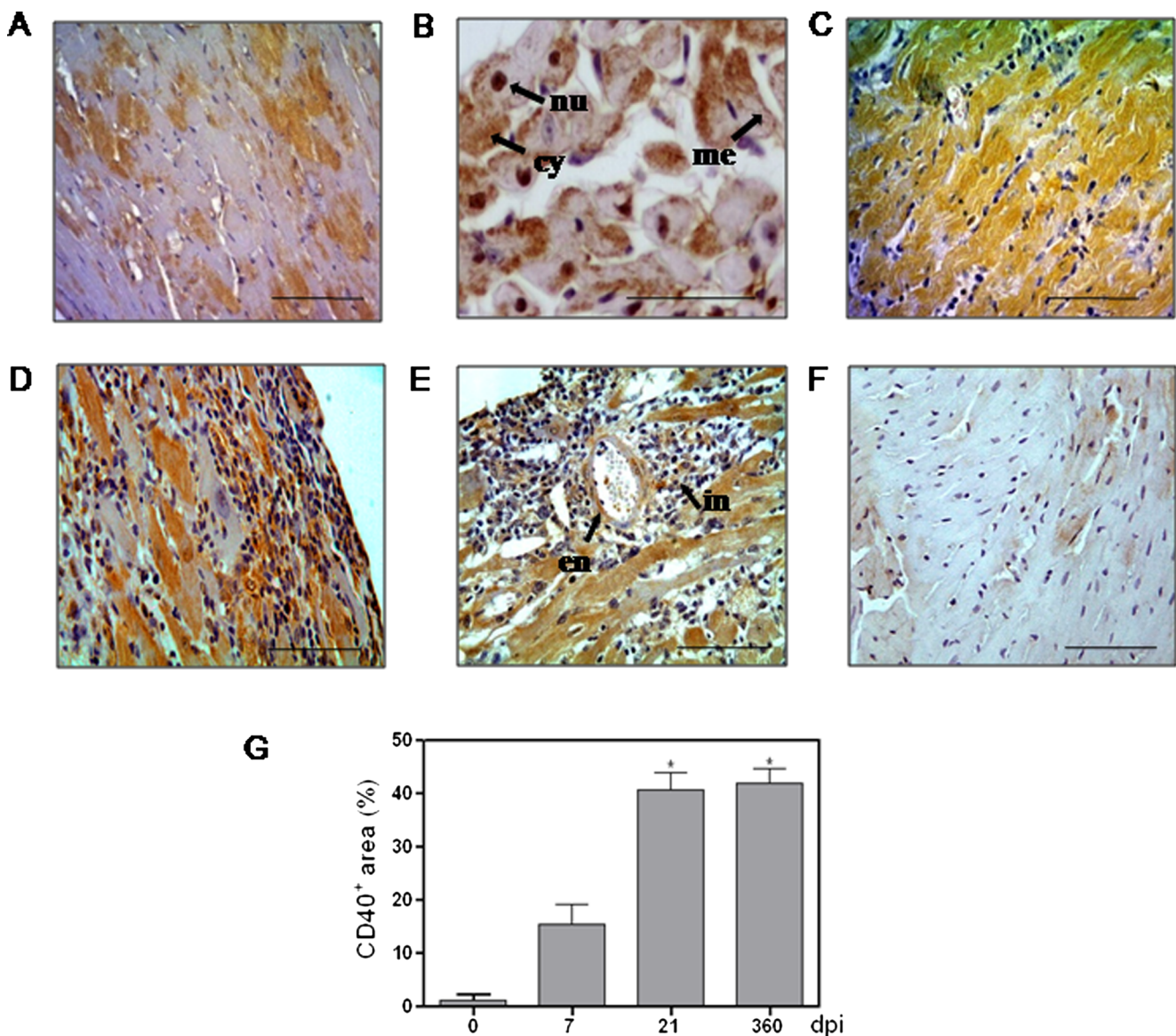


Fig. 2 CD40 costimulatory molecule is expressed in myocardium of *T. cruzi*-infected mice. Representative results of immunohistochemical analysis of myocardium from C3H/He mice infected with Sylvio X10/4 strain of *T. cruzi* at 7 (a, b), 21 (c, e), and 360 (d, e) dpi. Expression of CD40 was observed at cytoplasmic (cy), nuclear (nu), and membrane (me) levels (b). Note the intense mononuclear inflammatory infiltration (d) and

strong immunoreactivity in cardiomyocytes, infiltrating cells (in), and endothelial cells (en) from chronically infected mice (e). C3H/He-uninfected mice (f). Quantitative data of the immunohistochemical assay (g). Each bar represents the mean ± SEM; * $p < 0.05$. A value of $p < 0.05$ was considered to be significant. Bars a, c–f = 50 μ m; b = 25 μ m

The presence of CD40 was detected in cardiomyocytes as well as in endothelial cells and infiltrating leukocytes. Cardiac tissues from uninfected mice presented weak or lack of CD40 production (Fig. 2a, b). Infected myocardium stained with normal rabbit IgG (isotype control) showed no significant signals.

Finally, we examined whether CD40-CD40L interaction induces cardiac myocytes to produce IL-6, which may play a role in the development of myocardial inflammation during *T. cruzi* infection (Zhang and Tarleton 1996). While production of the cytokine was almost negligible in uninfected cultures, either stimulated or not with CD40L and/or IFN- γ

(data not shown), low and comparable levels of IL-6 were detected in supernatants from *T. cruzi*-infected HL-1 cells without treatment or treated with CD40L only.

In contrast, there was an increase in the amount of this cytokine released by *T. cruzi*-infected cells in response to IFN- γ ($p < 0.05$) or combined IFN- γ /rCD40L ($p < 0.001$) stimulation. Of note, we observed significant differences ($p < 0.05$, ANOVA and the post hoc Bonferroni test) between the IFN- γ - and the IFN- γ /rCD40L-stimulated myocytes (Fig. 3), suggesting a synergistic effect for these immune activators on IL-6 secretion from *T. cruzi*-infected heart cells.

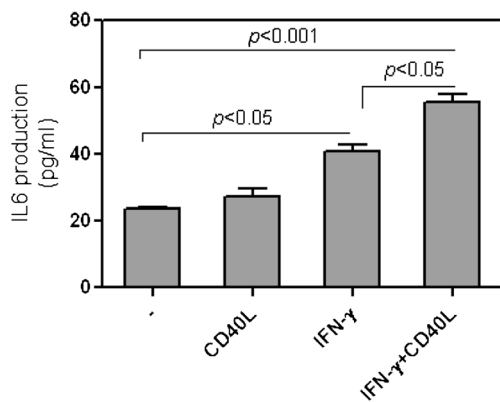


Fig. 3 CD40 ligation modulates IL-6 production in *T. cruzi*-infected HL-1 cardiomyocytes. Each bar represents the mean \pm SEM. A value of $p < 0.05$ was considered to be significant. Results were confirmed by two independent experiments

Discussion

The present study provides novel evidence of IFN- γ -mediated CD40 induction in adult HL-1 cardiomyocytes, as described for fetal ventricular myocytes (Seko et al. 1998). Remarkably, we also demonstrated for the first time that *T. cruzi* infection resulted in enhanced expression of this costimulatory molecule in cultured HL-1 cells. Previous reports showed that *T. cruzi* downregulates CD40 levels on the surface of spleen macrophages and dendritic cells (Planelles et al. 2003). It is likely that the parasite exerts a differential modulation of CD40 expression depending on the nature of the infected cell and target organ. More importantly, the induction of the costimulatory molecule CD40 in cardiac myofibers was confirmed in our murine model of Chagas' heart disease. In infected mice, progression of inflammatory cardiomyopathy was closely accompanied by increasing expression of myocardial CD40. Unlike previous reports describing the localization of CD40 on the sarcolemma of cardiac myocytes (Seko et al. 1998), our study revealed CD40 expression, not only in the membrane but also in the cytoplasm and nucleus of the cells, as described for other cell types (Lin-Lee et al. 2006).

Taken these data altogether, we hypothesize that the in vivo expression of CD40 on cardiomyocytes during *T. cruzi* infection could be induced by the parasite itself, proinflammatory cytokines such as IFN- γ released during the immune response elicited by the infection (Teixeira et al. 2002), or both. The pathophysiological implications of our finding of upregulated CD40 in cardiomyocytes are a matter of speculation. It is possible that CD40-bearing cardiac cells could interact with CD40L-expressing lymphocytes infiltrating the heart. This interplay, in turn, may induce the synthesis of other cytokines, chemokines, and adhesion molecules by cardiomyocytes that, in conjunction with those produced

by inflammatory cells, contribute to augment and perpetuate myocardial damage.

We further demonstrated that the production of IL-6 was enhanced by CD40 ligation on *T. cruzi*-infected HL-1 cardiomyocytes. A recent study by Manque et al. (2011) revealed that cardiomyocytes express several genes related to inflammation and cytokine production during early stages of *T. cruzi* infection. Moreover, IL-6 synthesis in cultured cardiac myocytes was shown to be induced by *T. cruzi* infection, and it was linked to a cardioprotective effect (Ponce et al. 2012). However, chronically elevated IL-6 levels may lead to chronic inflammation and fibrotic disorders in myocardium (Fontes et al. 2015). Accordingly, López et al. (2006) observed that IL-6 values were associated with the progressive phases of Chagas' disease, suggesting that this cytokine may contribute to the progression of the myocardial damage. In our study, the ability of IFN- γ alone to augment IL-6 production in *T. cruzi*-infected HL-1 cardiomyocytes was not surprising, as it is recognized as a key inducer of inflammatory cytokines, including IL-6, mostly by cross-regulation to additional activating mechanisms such as those mediated by TLRs (Hu and Ivashkiv 2009). Particularly, ligand activation of TLR4 on HL-1 cells has been documented to result in elevated NF κ B-dependent IL-6 production and decreased cardiomyocyte contractility (Boyd et al. 2006).

In addition, the cooperative effect of IFN- γ plus CD40L on IL-6 induction demonstrated in the infected cultures is similar to the synergism observed for these agents by other research groups (Chen et al. 2007).

Confirming previous findings from Boyd et al. (2006), we were unable to detect significant IL-6 secretion from non-infected HL-1 cardiomyocytes upon IFN- γ treatment. It is reasonable to speculate that this cell line may require additional stimulation by parasite components to efficiently trigger IL-6 release. In this sense, *T. cruzi* has been reported to display a set of TLR ligands (Campos et al. 2001; Ouaisi et al. 2002) including TLR2-targeting parasite molecules capable of inducing IL-6 production in cultured cardiomyocytes (Ponce et al. 2012).

In line with our findings, Seko et al. (1998) demonstrated CD40 expression in cardiomyocytes during Coxsackievirus B3-induced murine acute myocarditis. Moreover, the IL-6 production by cultured ventricular myocytes in response to CD40 signaling was verified. Interestingly, these investigators also achieved amelioration of myocardial inflammation by treatment of mice with anti-CD40L monoclonal antibody, suggesting the participation of CD40/CD40L interaction in the development of acute viral myocarditis.

In conclusion, we demonstrated herein that *T. cruzi* infection positively modulates the expression of CD40 by myocardial cells, whose ligation potentiates IFN- γ -induced IL-6 production in the infected myocytes. It is conceivable that this

receptor acts as a mediator of inflammatory response during chagasic myocardopathy. In this context, a recent report demonstrates that induction of proinflammatory cytokines by peripheral blood and heart-infiltrating T cells from chronic chagasic patients is critically dependent on CD40-CD40L interaction (Abel et al. 2014). Further studies will be necessary to determine the exact functional relevance of CD40 and its ligand in cardiopathogenic mechanisms associated with Chagas' disease.

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