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Trypanosoma cruzi, the causative agent of Chagas disease, modulates interleukin-6-induced STAT3 phosphorylation via gp130 cleavage in different host cells

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ABSTRACT

Interleukin-6 mediates host defense and cell survival mainly through the activation of the transcription factor STAT3 via the glycoprotein gp130, a shared signal-transducing receptor for several IL-6-type cytokines. We have reported that the cardiotrophic parasite *Trypanosoma cruzi* protects murine cardiomyocytes from apoptosis. In agreement, an intense induction of the anti-apoptotic factor Bcl-2 is found in cardiac fibers during the acute phase of infection, establishing a higher threshold against apoptosis. We report here that inactive cruzipain, the main cysteine protease secreted by the parasite, specifically triggered TLR2 and the subsequent release of IL-6, which acted as an essential anti-apoptotic factor for cardiomyocyte cultures. Although comparable IL-6 levels were found under active cruzipain stimulation, starved cardiac cell monolayers could not be rescued from apoptosis. Moreover, cardiomyocytes treated with active cruzipain completely abrogated the STAT3 phosphorylation and nuclear translocation induced by recombinant IL-6. This inhibition was also observed on splenocytes, but it was reverted when the enzyme was complexed with chagasin, a parasite cysteine protease inhibitor. Furthermore, the inhibition of IL-6-induced p-STAT3 was evidenced in spleen cells stimulated with pre-activated supernatants derived from trypomastigotes. To account for these observations, we found that cruzipain enzymatically cleaved recombinant gp130 ectodomain, and induced the release of membrane-distal N-terminal domain of this receptor on human peripheral blood mononuclear cells. These results demonstrate, for the first time, that the parasite may modify the IL-6-induced response through the modulation of its cysteine protease activity, suggesting that specific inhibitors may help to improve the immune cell activation and cardioprotective effects.

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1. Introduction

Myocarditis caused by infection with the intracellular protozoan *Trypanosoma cruzi* still remains as the major infectious heart disease worldwide. After infection, the parasite invades and multiplies in the myocardium, leading to an acute myocarditis that kills around 5% of non-treated infected individuals. However, this acute episode in most cases self-resolves in about two months. During the subsequent chronic phase, parasitemia is reduced to virtually undetectable levels and the anti-parasite immune response recedes. Nonetheless, low levels of *T. cruzi* DNA can still be detected in the heart despite the absence of prominent tissue parasitism, illustrating that long term parasite persistence occurs in the presence of an intact immune system. In addition, cardiomyocytes are terminally differentiated non-dividing cells with minimal renewal capability, and it is therefore clear that the loss of cardiomyocytes due to parasite infection cannot occur in a timely manner and is detrimental to cardiac function. Consequently, the strategies evolved by the host in order to protect cardiomyocytes against initial or persistent parasite infection are critical for most of the infected individuals.

Innate immunity provides the first line of defense by detecting the infectious agent through pattern recognition receptors, including toll-like receptors (TLRs) [1,2]. Activation of TLRs on cardiac myocytes leads to a Nuclear Factor-κB (NF-κB)-mediated cardiomyocyte inflammatory response through cytokine release. Cumulative evidence

Abbreviations: CM, conditioned media; dnTLR2, dominant-negative TLR2; GMFI, geometric mean of fluorescence intensity

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highlights the importance of the interleukin-6 (IL-6)/gp130/STAT3 axis in the biology of cellular survival, particularly in cardiac cells [3,4]. Recently, we reported that TLR2-dependent signaling induces IL-6 production, which acts as an essential anti-apoptotic factor and plays a crucial role in murine cardiomyocyte protection during *T. cruzi* infection [5].

The cytokine IL-6 belongs to a cytokine family that shares the transmembrane glycoprotein gp130 as a common signal-transducing receptor in responsive cells. The N-terminal extracellular region of gp130 is formed by the immunoglobulin-like domain (D1), followed by the cytokine-binding domains (D2–D3) and three membrane-proximal fibronectin type-III ones (D4, D5, and D6). The gp130 together with the IL-6 receptor subunit-alpha (IL-6Rα) composes the functional IL-6 receptor. When IL-6 binds to the alpha subunit, the binary complex (IL-6–IL-6Rα) becomes competent to engage gp130 receptor, and then the trimolecular complex (IL-6Rα–IL-6–gp130) dimerizes via the gp130 D1 domain [6]. The Janus tyrosine kinases that are constitutively associated with gp130 become activated, resulting in tyrosine phosphorylation of the transcription factor STAT3, which migrates to the nucleus where it induces IL-6 target genes involved in defense, inflammation and cytoprotection. In addition, engagement of gp130 also leads to the activation of Ras-ERK1/2/MAPK and PI3K/AKT pathways [7].

We have previously demonstrated that the parasite cysteine protease, cruzipain, when devoid of enzymatic activity, improves the pro-survival effect elicited by the parasite in cardiomyocyte cultures [8]. Related to this, cruzipain itself induces cardiomyocyte survival through the activation of two signal transduction pathways, the PI3K/Akt and MEK1/ERK, with both pathways leading to a decrease in the activated caspase-3 [9]. However, the nature of the receptor that interacts with cruzipain and subsequently triggers this molecular mechanism still remains to be elucidated.

Cruzipain is the main *T. cruzi* papain-like cysteine protease and is one of the most extensively studied parasite antigens. This enzyme is expressed as a mixture of isoforms throughout all the developmental forms of the parasite and is constitutively secreted to the extracellular milieu as free cruzipain and as cruzipain–chagasin complexes by the trypomastigote infective stage. Its activity has been directly associated with intracellular amastigote survival [10,11], host cell invasion [12–14] and induction of inflammation [15,16]. Reinforcing previous results obtained in our laboratory [17,18], a more recent report has suggested that cruzipain activity drives immune evasion mechanisms by preventing macrophage classical activation [19]. Of further interest, huge cruzipain deposits have been observed in the cytoplasm of macrophages or free at the extracellular matrix during human chronic chagasic myocarditis [20].

Herein, we investigated the putative effects of shed cruzipain on cellular physiology, emphasizing its capacity to regulate IL-6 signaling and cardioprotective effect. We found that cruzipain enzymatically cleaved the IL-6 signal transducer gp130, and consequently abrogated STAT3 phosphorylation, leading to the inhibition of IL-6-induced cardioprotection. This strategy may be critical during natural infection, since the gp130 cleavage induced by the purified enzyme as well as by trypomastigotes supernatants was independent of the cell type analyzed.

2. Materials and methods

2.1. Animals

BALB/c and C57BL/6 mice were purchased from Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina. C57BL/10ScNJ mice which were lacking the Tlr4 gene (Tlr4lps-del) and C57BL/6 TLR2-knockout (TLR2KO) mice were purchased from The Jackson Laboratory, Bar Harbor, ME, USA. Animals were maintained at the Animal Resource Facility of the CIBICI-CONICET (National Institutes of Health-USA assurance number A5802-01) following the recommendations in the Guide for the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the CIBICI-CONICET committee.

All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI)-CONICET.

2.2. Isolation of parasite molecules

Cruzipain was purified from Tulahuen epimastigotes, as described by Giordanengo [17] followed by a fast protein liquid chromatography (Amersham Pharmacia Biotech ÄKTAFPLC system) with a Superdex75 pre-packed column. For inactive cruzipain, the epimastigotes were previously resuspended in sucrose solution containing the irreversible protease inhibitors TLCK and PMSF. For active cruzipain, the enzyme was pre-activated with 2.5 mM dithiothreitol (DTT) (Bio-Rad) for 15 min at 37 °C immediately before being added to the cultures since these enzymes need to maintain the active-site cysteine in its reduced state to ensure activity. The Limulus amebocyte lysate assay (Charles River Laboratories) was used to show that there was no endotoxin presence. Recombinant chagasin was produced in *E. coli* and purified as described by dos Reis et al. [21], and recombinant cruzipain was obtained as described by Cazorla et al. [22].

2.3. T. cruzi supernatants

Trypomastigote forms (Tulahuen strain) obtained from the supernatant of infected LLC-MK2 cells (a cell line from kidney adult Rhesus monkey), were washed with HBSS, and 1×10^7 parasites/ml were incubated in culture medium for 120 min at 37 °C. Then, parasites were pelleted, and the conditioned media (CM) containing secreted trypomastigote molecules were collected and filtered. Aliquots of CM were incubated with 2.5 mM DTT for 15 min at 37 °C to activate the cysteine protease before use. Cultured cells were incubated with the CM diluted 1:2 with culture medium and kept in a 5% CO_2 incubator at 37 °C. After 120 min, cells were treated with 10 ng/ml bioactive recombinant IL-6 (eBioscience) for an additional 15 min.

2.4. TLR ligand screening

TLR ligand screening was performed by Invivogen (Genbiotech www.genbiotech.com.ar). Briefly, TLR stimulation was tested in HEK293 cells stably transfected with a plasmid that constitutively expresses a given functional mouse TLR (TLR2, 3, 4, 5, 7, 8 and 9) and a plasmid containing the SEAP reporter gene under the control of a promoter inducible by NF-κB. Inactive cruzipain (5 μg/ml) was tested and compared with the following control ligands: TLR2: HKLM (heat-killed Listeria monocytogenes) (10^8 cells/ml), TLR3: Poly(I:C) (1 μg/ml), TLR4: *E. coli* K12 LPS (1 μg/ml), TLR5: Salmonella typhimurium flagellin (1 μg/ml), TLR7: CL097 (1 μg/ml), TLR8: CL075 (1 μg/ml) + PolydT (10 μM), and TLR9: CpG ODN 1826 (1 μg/ml). After 16–20 h incubation, the OD at 650 nm was read on an Absorbance Detector. Cells transfected with control plasmid expressing the NF-κB inducible reporter system stimulated with TNF-α (100 ng/ml) were used as control.

2.5. Primary cardiomyocyte cultures

Cardiomyocyte cultures from neonatal BALB/c, C57BL/6, C57BL/10ScNJ (TLR4 deficient) and C57BL/6 TLR2-knockout mice were carried out as described previously [8]. The cultures were kept in a 5% CO_2 incubator at 37 °C to allow the cells to start beating. More than 85% of cells were found to be cardiomyocytes. After 24 h, the cells were washed and

stimulated with or without the following: inactive cruzipain (5 μg/ml), active cruzipain (5 μg/ml), active cruzipain–chagasin complexes (1:1), recombinant cruzipain (5 μg/ml), bioactive recombinant IL-6 (10 ng/ml) (eBioscience), LPS (1 μg/ml) (Invivogen), CM (Section 2.3), purified rat anti-mouse IL-6 (5 μg/ml) (BD Pharmingen) or IgG1 isotype control (5 μg/ml) (BD Pharmingen). The cultures were maintained in complete culture medium (10% FBS-DMEM) or in serum starved conditions (0.1% FBS-DMEM). When indicated in the figure legend, monolayers were pre-treated with the inhibitors: Ly294002 (25 mM) (Sigma-Aldrich), sulfasalazine (5 mM) (Sigma-Aldrich) or PD098059 (38 mM) (Calbiochem) for 1 h and then incubated in the medium containing the inhibitors in the presence or absence of inactive cruzipain. The optimal inhibitor concentration was selected in preliminary assays, based on their capacity to inhibit the kinase activity and not affect the cell survival rate. DMSO was used as a vehicle at a concentration of 0.2%. Control cultures received the vehicle alone.

2.6. Spleen cell cultures

Splenic cell cultures were performed as described previously [17]. The spleens from BALB/c mice were mechanically disintegrated, red blood cells were removed using lysis buffer (Sigma) and the remaining cells were then plated at a density of 2.6×10^5 cells/cm^2 in RPMI 1640 with gentamicin (both from Gibco). Cells were maintained in medium alone or stimulated with inactive cruzipain (5 μg/ml), active cruzipain (5 μg/ml) or active cruzipain–chagasin complexes (1:1). In another experiment, cultured cells were incubated with CM pre-activated with DTT (cruzipain activator) or without treatment. After 120 min of stimuli at 37 °C, cultures were treated with bioactive recombinant IL-6 (10 ng/ml) for 15 min.

2.7. Human peripheral blood mononuclear cell cultures

Peripheral blood was collected from healthy donors after obtaining their signed informed consent. The mononuclear cells (PBMC) were isolated on a Ficoll-Hypaque (GE Healthcare) density gradient by centrifugation. Then the PMBC were plated at a density of 2.6×10^5 cells/cm^2 in RPMI 1640 medium with gentamicin. Cultured cells were maintained in medium alone, or subjected to treatment with active cruzipain (5 μg/ml) or recombinant cruzipain (5 μg/ml) for 120 min at 37 °C and then incubated with 10 ng/ml of recombinant IL-6 for an additional 15 min. The procedure for such collection and details of informed consents were in accordance with a proposal approved by the Institutional Ethics Committee (Hospital Nuestra Señora de la Misericordia-Córdoba, Argentina).

2.8. Cardiomyocyte transfection

Neonatal cardiac cell cultures from BALB/c mice were transfected as was previously described [5]. After 2 days of culture, contracting cardiomyocytes were transfected with dominant negative forms of TLR2 (dn-TLR2) (pZERO-mTLR2) (0.5 μg/well) or the empty control vector (pZERO-mcs) (0.5 μg/well) (both from Invivogen) using Lipofectamine Plus Reagent (Invitrogen), following the manufacturer protocol. Twenty-four hours after transfection, the cells were incubated with inactive cruzipain for 48 h. The supernatants were collected for cytokine detection by ELISA and the transfection efficiency was monitored by flow cytometry (data not shown).

2.9. Measurement of apoptotic cell death

Following treatments for 48 h in serum starved conditions, the cardiomyocyte monolayers were labeled with 5 μl of FITC-Annexin V (BD Pharmingen) for 15 min on ice. Previous to the acquisition, the cells were stained with propidium iodide. A minimum of 30,000 events for each condition were analyzed by flow cytometry (FACSCanto II, Becton Dickinson), with cellular debris being excluded from the analysis. Data were analyzed using FlowJo software.

2.10. Cytokine assays

The ELISA assay was performed for quantification of cytokine levels. Briefly, ELISA plates were coated with anti-cytokine antibodies (BD Pharmingen and e-Bioscience) overnight and then washed and blocked. The culture supernatants obtained were incubated overnight, before the plates were subjected to biotinylated anti-cytokine antibody (BD Pharmingen and e-Bioscience) for 1 h. After washing, the plates were incubated with streptavidin–peroxidase (BD Pharmingen). The reaction was revealed using DakoCytomation TMB Substrate Chromogen (Dako), before being read at 490 nm in a Microplate reader (Bio-Rad). Standard curves were generated using recombinant cytokines (BD Pharmingen and e-Bioscience).

2.11. Flow cytometric analysis

For the study of TLR2 and TLR4 expression, 2×10^5 cardiac cells were stained with the following antibodies: PE-labeled monoclonal anti-mouse TLR2 (eBioscience) or with Alexa Fluor 488-labeled monoclonal anti-mouse TLR4 antibody (eBioscience). For detection of gp130 (CD130) expression and D1 domain expression, stimulated PBMC were harvested, fixed and stained with the following antibodies: mouse monoclonal anti-human gp130 (clone: B-T2, Abcam) followed by PE-labeled monoclonal anti-mouse IgG1 (Biolegend), together with Alexa fluor-647 conjugated polyclonal anti-human gp130 antibody (eBioscience) in concentrations recommended by the manufacturer. Stained samples were acquired using the FACSCanto II cytometer. Lymphocytes and monocytes were gated by FSC/SSC scatter characteristics. Data were analyzed using FlowJo software.

2.12. Western blot assays

Cardiomyocyte or spleen cell cultures were subjected to different stimuli for 120 min at 37 °C. Then, the cultures were treated with bioactive recombinant IL-6 (10 ng/ml) for 15 min and immediately harvested and mixed with loading buffer. Aliquots with equal amounts of protein were separated on SDS-PAGE and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories). After being blocked, the membranes were incubated with rabbit polyclonal phospho-STAT3 antibody (Cell Signaling). Then, the membranes were incubated with HRP-conjugated anti-rabbit antibody (Sigma) and assayed using the ECL chemiluminescent system (Amersham Pharmacia Biotech). The membranes were stripped and incubated with rabbit polyclonal anti-STAT3 (Cell Signaling), washed and revealed. The band intensity for p-STAT3 was semi-quantified by densitometric scanning and normalized with respect to the total STAT3 using ImageJ processing software (www.rsb.info.nih.gov/ij).

2.13. Colloidal silver staining procedure

Recombinant human gp130 (baculovirus-derived Leu24-Glu619, Accession number # P40189, R&D Systems) was maintained in PBS alone or incubated with active cruzipain at an enzyme/substrate ratio of 1/100 (w/w) for 120 min at 37 °C. The reaction was stopped by adding loading buffer to the mixture, and heated to 90 °C for 10 min. Samples were separated on SDS-PAGE and electrotransferred to nitrocellulose membranes as described above. The protein silver staining procedure was adapted from a technique published by Kovarik et al. [23]. The membranes were washed in deionized water and stained under gentle agitation with a colloidal silver staining solution prepared immediately before use. The staining was stopped after 10 min by washing with running deionized water.

2.14. Statistical analysis

To compare different experimental conditions, an analysis of variance (two-way or one-way ANOVA) with the Tukey post hoc test was performed. A two-tailed Student' *t*-test was used for comparison between control and experimental samples. A p-value <0.05 was considered significant with NS denoting non-significant.

3. Results

3.1. Cruzipain triggers TLR2-signaling

We have recently demonstrated that *T. cruzi* protects isolated cardiomyocytes from apoptosis through TLR2 triggering [5]. In addition, we previously reported that treatment of cardiomyocytes with enzymatically inactive cruzipain resulted in an anti-apoptotic effect on serum starved cultures [8].

In order to determine if inactive cruzipain was able to trigger TLR signaling, a TLR ligand screening assay was performed. We found that cruzipain had a selective stimulatory effect on TLR2-transfected cells, as shown by the expression levels of SEAP in cruzipain-treated cultures, which reached about 60% of the levels detected with the TLR2 ligand HKLM (Fig. 1A). On the other hand, cruzipain did not promote any significant activation of HEK293 cells transfected with other TLR. Interestingly, cells transfected with control plasmid expressing the NF-κB inducible reporter did not show NF-κB activation, either constitutively or in response to cruzipain, although they were strongly stimulated by TNF-α treatment when used as a positive control (Fig. 1A). Taken together, these results show that cruzipain by itself was able to trigger TLR2, resulting in the activation of NF-κB.

3.2. TLR2 is involved in cardiomyocyte protection induced by cruzipain

In order to investigate the potential role of TLR2 in the cruzipain-induced cytoprotective effect, cardiomyocyte primary cultures from C57BL/6 or C57BL/6 TLR2KO were incubated with inactive cruzipain for 48 h in serum starved conditions. In agreement with the results obtained with cultured cardiomyocytes derived from BALB/c (Fig. 1B), inactive cruzipain incubation decreased the percentage of apoptotic cardiomyocytes derived from C57BL/6 mice (Fig. 1C), showing that the survival effect was independent of the mouse strain. In contrast, in cultures from TLR2KO mice, the anti-apoptotic effect induced by cruzipain treatment was abrogated (Fig. 1D). These results strongly suggest that the TLR2 triggering was critical for the cardiomyocyte survival induction in our model.

We next evaluated cell surface expression of TLR2 and TLR4 in cultured cardiac myocytes at different time points post-stimuli with inactive cruzipain, by flow cytometry (Fig. 2). Cardiomyocytes were found to express low constitutive levels of TLR2 and TLR4, and while cruzipain treatment significantly increased the percentage of TLR2-expressing cells (Fig. 2A) and TLR2 surface expression (Fig. 2B), the rate of TLR4 positive cells was not significantly altered during the culture period, either in treated or untreated cultures (Fig. 2C). The up-regulation of TLR2 involved PI3K and NF-κB-signaling pathways, but not MEK1 activation, since the pre-treatment with Ly294002 (PI3K inhibitor) or with sulfasalazine (NF-κB inhibitor), but not with PD098059 (MEK1 inhibitor), abrogated the increase in TLR2 (Fig. S1). In the absence of cruzipain, the inhibitors had no effect on TLR2 expression (data not shown).

3.3. Inactivation of cruzipain is required to induce cardiomyocyte survival

Considering that the previous assays were performed with pharmacologically-inactivated cruzipain, we investigated whether the enzymatic activity could interfere with its cardioprotective properties. To carry this out, Annexin V labeling was evaluated in cultures treated with either active cruzipain or cruzipain devoid of enzymatic activity through pharmacological treatment or complexed with chagasin (Fig. 3). Surprisingly, we found that active cruzipain was unable to reduce the degree of serum deprivation-induced apoptosis in

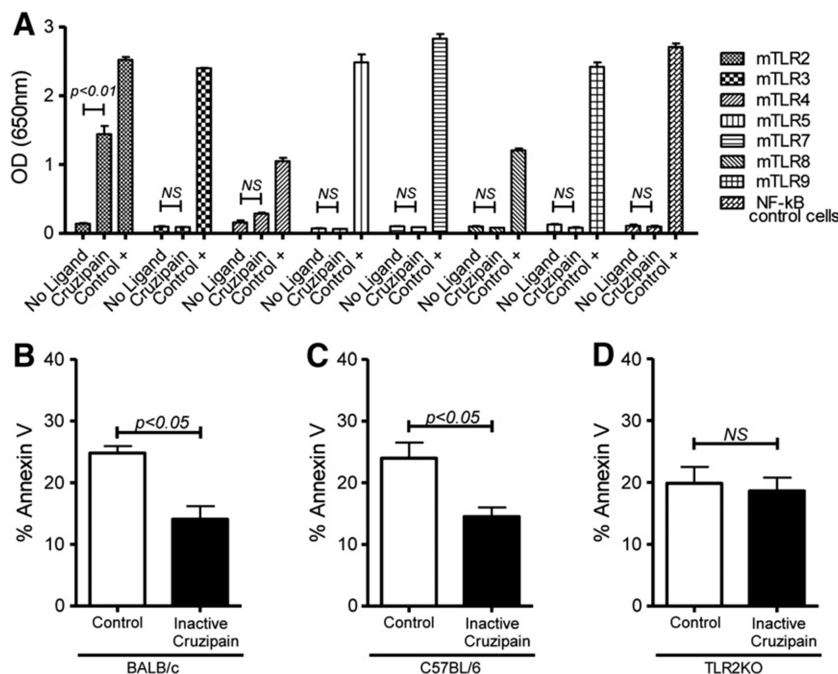

Fig. 1. Cruzipain triggers the TLR2 pathway and consequently the survival of cultured cardiomyocytes. (A) HEK293 cells transfected with plasmids expressing a given mouse TLR gene and the NF-κB-inducible SEAP reporter gene (mTLRs) were left untreated (no ligand), and subjected to treatment with inactive cruzipain or with specific control ligands (control +). The cells transfected with control plasmid expressing the NF-κB inducible reporter (NF-κB control cells) were used as control. Data represent mean ± SEM of two independent experiments carried out in duplicate with two different cruzipain samples. Cardiomyocyte monolayers from (B) BALB/c, (C) C57BL/6 and (D) C57BL/6 TLR2KO mice were treated with inactive cruzipain or untreated (control) and maintained in starved medium for 48 h. The apoptotic rate was evaluated by Annexin V-FITC labeling and FACS. Data in B, C and D are shown as mean ± SEM of a representative assay of three independent experiments performed in quadruplicate.

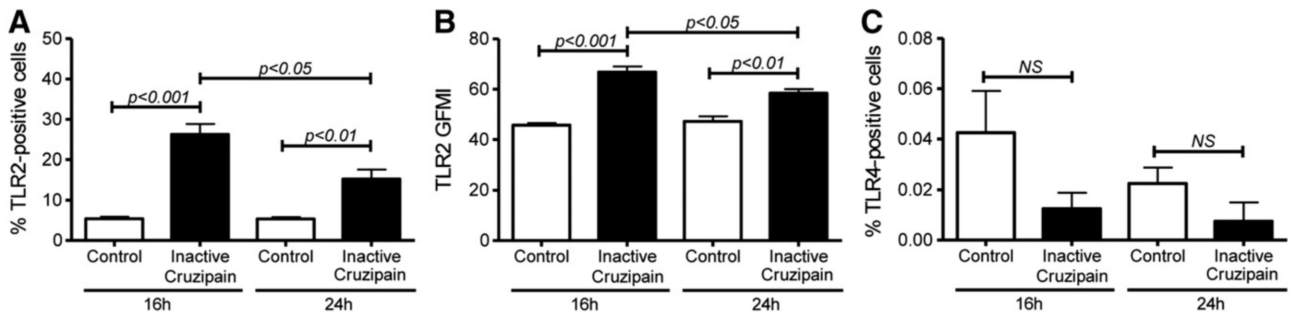

Fig. 2. Cruzipain increases the TLR2 expression on cardiomyocyte cultures. Primary cardiomyocyte cultures were treated with inactive cruzipain or maintained in medium alone (control). The percentage (A) and the geometric mean of fluorescence intensity (GMFI) for (B) TLR2 and (C) TLR4 expression were measured at 16 h and 24 h post-treatment. The bars represent mean ± SEM of three independent experiments performed in triplicate.

cultured cardiomyocytes, while its inactivation with recombinant chagasin restored its capability to promote cardioprotection (Fig. 3A). To rule out the possible participation of TLR4 in the observed results, we performed survival experiments with cardiomyocytes obtained from C57BL/10ScNJ mice lacking the TLR4 gene (Fig. 3B). In agreement with the results obtained with BALB/c cells, we found that inactive cruzipain (pharmacologically-inactivated or complexed with chagasin) significantly reduced the apoptotic rate compared with cells maintained in medium alone or treated with active cruzipain (Fig. 3B). In both models, chagasin alone did not show any effects on cell survival (data not shown). Furthermore, as we described previously for infected monolayers, pharmacologically-inactivated cruzipain or cruzipain-chagasin treated cultures remained beating after 48 h of serum starvation (Videos S2 and S4, respectively), in contrast to active cruzipain-treated or untreated control cultures, which did not beat at this time point (Videos S3 and S1, respectively).

3.4. Cruzipain stimulates the production of IL-6 through TLR2 and NF-κB activation

We next sought to determine the cytokine profile (IL-1β, TNF-α, IL-12, IL-6, IL-17 and IL-10) produced as a consequence of cruzipain stimulation in 16 h-culture supernatants. Among the cytokines tested, only a rapid and sustained production of IL-6 in stimulated cultures was detected (Fig. 4A), with IL-6 release being blocked by pre-incubation with sulfasalazine. These findings illustrate that cardiomyocyte cultures are able to respond to cruzipain efficiently by producing IL-6 in a NF-κB activation-dependent manner. Depletion of IL-6 by neutralizing antibodies in cruzipain-stimulated cultures was found to abrogate the observed cytoprotection (Fig. S2).

To investigate if inactive cruzipain-induced IL-6 secretion occurred as a result of TLR2 activation, cardiomyocytes were transfected with a dominant-negative TLR2 plasmid (dnTLR2). In comparison with untreated cells, significant levels of IL-6 were found in stimulated cultures transfected with control vector. Nevertheless, the amount of IL-6 released by cells bearing the dnTLR2 construct after stimulation dropped to levels similar to those secreted by unstimulated monolayers (Fig. 4B). In agreement with this, there were no significant differences in the IL-6 levels produced upon cruzipain treatment by cardiomyocytes derived from TLR2KO mice (Fig. 4D), in comparison with those produced by cultures derived from the C57BL/6 wild type (Fig. 4C). These results show a direct correlation between the activation of TLR2 and IL-6 production induced by inactive cruzipain. Next, we tested the impact of cruzipain enzymatic activity on IL-6 production, and the ELISA data showed that the secretion of IL-6 was comparable in cultures subjected to inactive or active cruzipain treatments (Fig. 4E).

3.5. Cruzipain activity prevents IL-6-induced STAT3 phosphorylation by means of gp130 cleavage

Taking into account that IL-6 promotes anti-apoptosis through the gp130 receptor predominantly via p-STAT3 [3,24], we next investigated if the cruzipain enzymatic activity interfered with gp130 signaling. To carry this out, the degree of STAT3 phosphorylation was measured in cardiomyocyte cultures pre-incubated for 120 min with inactive or active cruzipain before they were stimulated with recombinant IL-6, and it was found that enzymatically active cruzipain, but not the inactive one, completely abrogated the STAT3 phosphorylation and nuclear translocation induced by IL-6 (Figs. 5A and S3).

As the signaling pathway IL-6/gp130/STAT3 is crucial in several immune cell compartments during the inflammatory response, we evaluated if the activity of cruzipain could interfere with IL-6 signaling in murine spleen cells. In fact, pre-incubation with active cruzipain significantly diminished the p-STAT3 expression induced by IL-6, but the inhibition was reverted when the enzyme was complexed with chagasin (Fig. 5B). Next, considering that cruzipain is released by trypomastigotes during natural infection [12], spleen cells were pre-incubated with CM treated with DTT (cruzipain activator) or without treatment, and then subjected to IL-6 stimulation (Fig. 5C). The

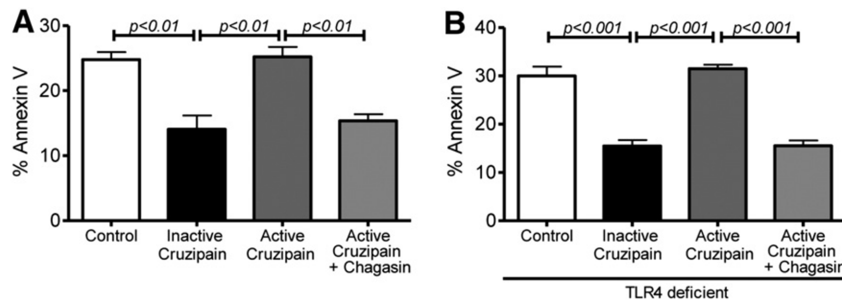

Fig. 3. Inactivation of cruzipain is required for cardiomyocyte survival induction. (A) Cardiomyocytes from BALB/c or (B) TLR4-deficient mice were incubated with inactive cruzipain, enzymatically active cruzipain, active cruzipain–chagasin complexes (active cruzipain + chagasin) or were untreated (control) and maintained in starved medium for 48 h. The apoptotic rate was measured and the data are shown as mean ± SEM of a representative assay of three independent experiments performed in quadruplicate.

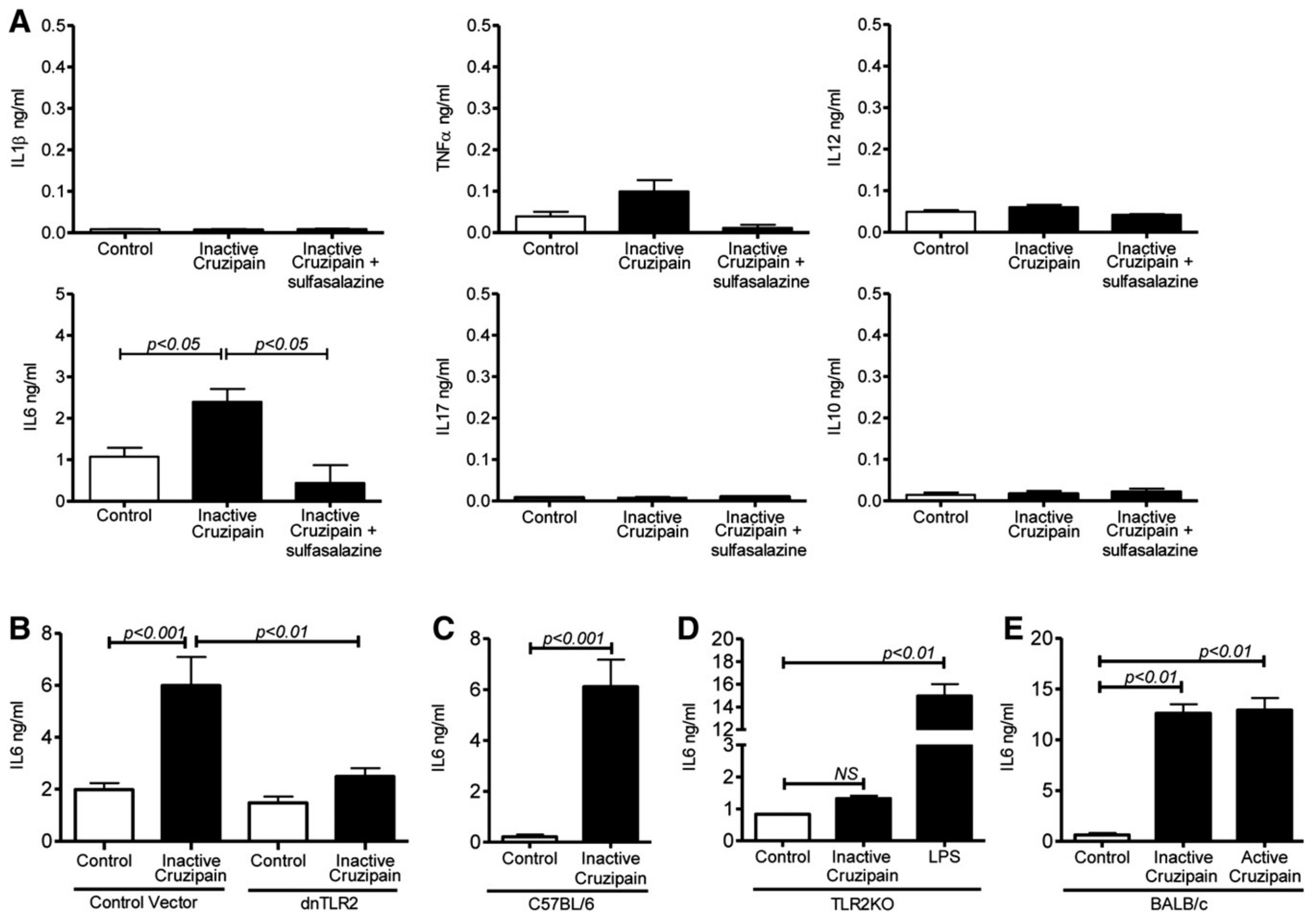

Fig. 4. Cruzipain induces the production of IL-6 by cardiomyocyte cultures. The cytokine production was quantified by ELISA in supernatants collected from the following cultures: (A) BALB/c primary cardiomyocyte cultures were left untreated (control) or incubated with inactive cruzipain for 16 h. Some monolayers were pre-incubated with sulfasalazine. (B) Transiently transfected cardiac myocytes with dnTLR2 or the empty control vector were incubated with inactive cruzipain for 48 h. (C) Cultures obtained from C57BL/6 or (D) TLR2KO mice were incubated with inactive cruzipain or LPS for 16 h. (E) Cultures from BALB/c mice were stimulated with inactive or active cruzipain for 48 h. The results are expressed as mean ± SEM of at least four independent experiments performed in triplicate.

DTT-activated CM completely abrogated IL-6-induced p-STAT3, while CM obtained from non-treated trypomastigotes partially allowed IL-6 signaling. Furthermore, spleen cells incubated with trypomastigotes (cell:parasite ratio 1:3) also permitted the fully STAT3 phosphorylation (Fig. 5C). In agreement, we found that starved cardiac cell cultures-treated with CM were rescued against apoptosis, while the supernatants activated with DTT partially lost the anti-apoptotic properties (Fig. 5D).

To determine if gp130 was substrate of cruzipain, we analyzed the expression of gp130 N-terminal domain (D1) on human PBMC subjected to cruzipain activity. To address this issue, it was employed the B-T2 mAb, which recognize an epitope on Ig-like domain (D1) of human gp130. The treatment resulted in a significant decrease in the amount of the D1 expression on lymphocytic (Fig. 6A) and monocytic populations (Fig. 7A). In contrast, the gp130 global expression, evaluated through the reactivity of a polyclonal antibody, remained unaltered (Figs. 6B and 7B, respectively). Comparable results were also obtained with pre-activated recombinant cruzipain (Fig. S4). To characterize further the gp130 cleavage resulting from cruzipain activity, the recombinant human gp130 ectodomain was incubated with active cruzipain, and the products released were run on SDS-PAGE and electrotransferred to nitrocellulose. In contrast to the untreated peptide that appeared as a single band corresponding to a 68 kDa molecular weight, the cruzipain-treated recombinant gp130 showed three additional bands with lower molecular weights (Fig. 8). These findings clearly demonstrate that cruzipain was able to cleave the human gp130 extracellular domain.

4. Discussion

Cruzipain is one of the *T. cruzi* molecules that have received the most attention by the scientific community. This parasite depends on its cysteine protease activity to invade and develop inside target mammalian host cells. Therefore, due to its crucial importance for parasite survival, inhibitors of cruzipain activity are attractive candidates for the therapeutic treatment of Chagas disease. The current study provides the first evidence that the parasite may manage the IL-6-induced response by means of gp130 cleavage, through the modulation of its cysteine protease activity.

We recently reported that *T. cruzi* induces cardioprotection through the activation of TLR2 and the subsequent secretion of IL-6 [5]. IL-6 released in response to parasite infection is able to induce STAT3 phosphorylation in cultured cardiomyocytes, a well-recognized mediator of cell survival [24]. Here, it is demonstrated that purified pharmacologically-inactivated cruzipain induced a strong TLR2 expression on cardiomyocytes. These results support our earlier observations in which the anti-apoptotic effect elicited by *T. cruzi* was significantly enhanced by pre-incubation with cruzipain [8]. We also demonstrated that inactive cruzipain was able to specifically trigger TLR2 signaling and NF-κB activation to promote IL-6 secretion. Thereby, the TLR2/NF-κB/IL-6 pathway may be critical in the previously reported cruzipain-cardioprotective effect [9].

Considering that the trans-sialidase (another *T. cruzi* enzyme) is able to induce the protection of Schwann cells from apoptosis [25] in a manner independent of its enzymatic activity, we investigated the

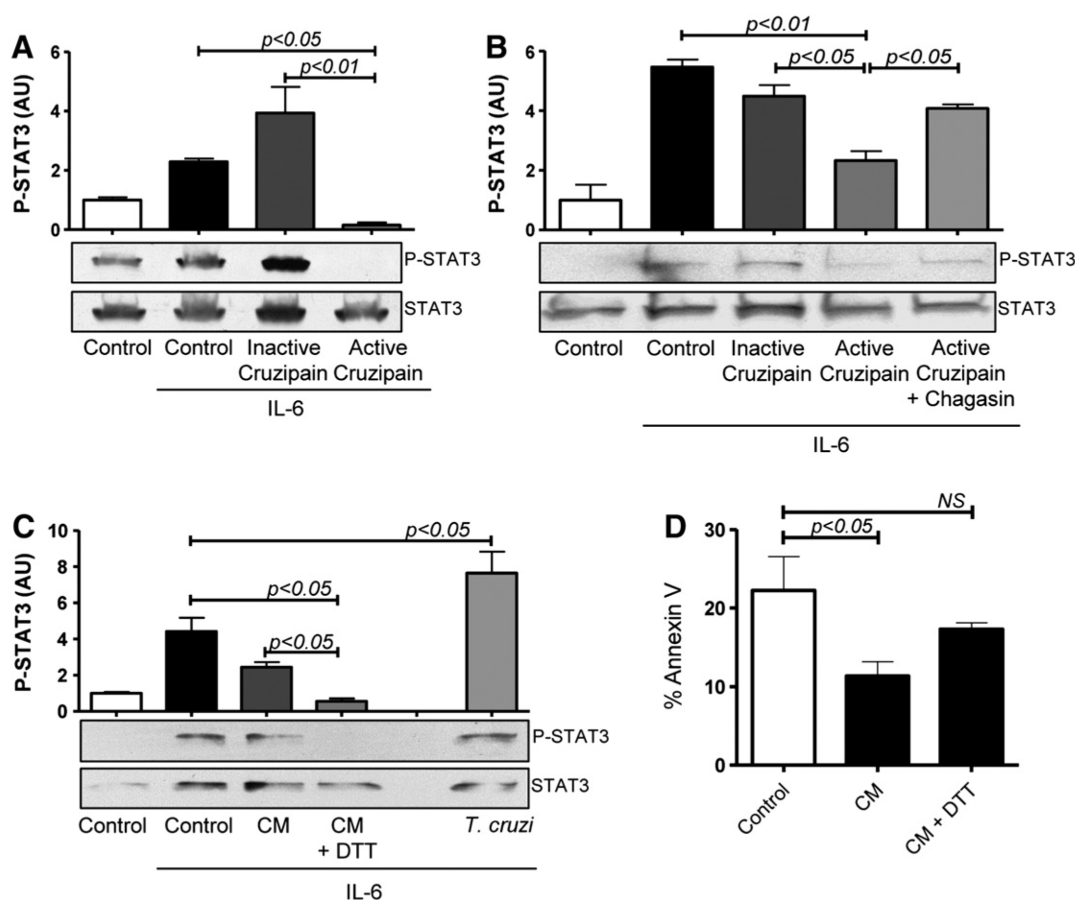

Fig. 5. Cysteine protease enzymatic activity abrogates STAT3 phosphorylation in different cell types. (A and D) BALB/c cardiomyocyte cultures and (B and C) spleen cell cultures, when indicated, were treated with inactive cruzipain, active cruzipain, active cruzipain–chagasin complexes (active cruzipain + chagasin), CM pre-treated with DTT (CM + DTT), untreated CM or with trypomastigotes (*T. cruzi*). (A, B and C) After 120 min, the cells were stimulated with recombinant IL-6 for 15 min. The STAT3 phosphorylation was estimated by immunoblotting with anti-p-STAT3 or anti-STAT3 antibodies. The band intensity for p-STAT3 was normalized with respect to the total STAT3. (D) After 48 h of starvation, the apoptotic rate of cardiomyocyte was evaluated by Annexin V-FITC labeling and FACS. The results are shown as mean ± SEM of at least three independent experiments.

impact of cruzipain activity on the cardioprotective effect. Strikingly, active cruzipain was unable to evoke significant cardiomyocyte protection against apoptosis, although this property was restored when the enzyme was complexed with its natural inhibitor chagasin. This different anti-apoptotic response was observed despite the fact that both stocks of cruzipain induced comparable levels of IL-6. We therefore hypothesize that cruzipain may promote downstream IL-6 signaling blockage by means of its enzymatic activity. Indeed, the pretreatment of cardiac cell monolayers with active cruzipain completely abrogated the phosphorylation and nuclear translocation of STAT3 induced by IL-6, thereby providing evidence that these cells became unresponsive to IL-6 stimulation. Moreover, CM obtained from trypomastigotes prevent apoptosis of starved cardiac cells but this effect was only partially reverted when the supernatants were activated with DTT, strongly suggesting that inactive enzyme stock released at the infective stage of the parasite is likely responsible for the rescue against apoptosis. Nevertheless, the cytoprotection was inversely correlated with the cysteine protease activity since the survival effect was not observed in a reducing environment, which ensures the catalytic activity of free cruzipain through the maintenance of the active-site cysteine in its reducing state.

The role of cruzipain with enzymatic activity has been widely documented through in vitro [12,13,26] and in vivo cellular systems [27], using DTT as a reducing agent to ensure cruzipain activity. Nevertheless, its effect without activity has not been thoroughly investigated. The *T. cruzi* secretes significant amounts of free cruzipain, as well as of cruzipain complexed with chagasin [11], which could therefore provide the source of inactive cruzipain that is capable of promoting cytoprotection. Alternatively, cruzipain inactivated through interactions with mammalian inhibitors, such as the alfa 2-macroglobulin present in host plasma [28], may also mediate the anti-apoptosis during natural infection. In this sense, huge cruzipain deposits have been observed in the extracellular matrix of heart tissue during human chronic chagasic myocarditis [20]. Moreover, although in the myocardium of chagasic patients, apoptotic cells is found in high number, no apoptotic cardiomyocytes are detected and the majority of TUNEL-positive cells have macrophage markers [29]. In the light of the results described here, it is plausible to speculate that cruzipain remains in the myocardium under its inactive form, stimulating the cardiac cell survival in an unfavorable environment.

We were not able to determine whether inactive cruzipain promotes TLR2 activation directly by acting as a TLR2 ligand or by exerting its effect indirectly through interaction with additional cell surface molecules. Nevertheless, our results showed strong correlations among cruzipain and TLR2 activation, IL-6 production and cardioprotection, which suggest there are potential unforeseen roles for cruzipain-chagasin complexes. In line with this observation, in vivo kinetic study has revealed us that infected IL-6KO mice, rather than TLR2KO mice, showed significantly more TUNEL-positive cardiac cells than infected wild type mice (unpublished data).

The gp130-mediated STAT3 signals are involved in multiple steps of the immune response. Here, we also demonstrated that the blockade of gp130 downstream signaling was irrespective of the cell type analyzed. In fact, active cruzipain abrogated the IL-6-induced STAT3 phosphorylation in murine spleen cells, with this inhibition being mediated by its enzymatic activity, since it was reversed when cruzipain was

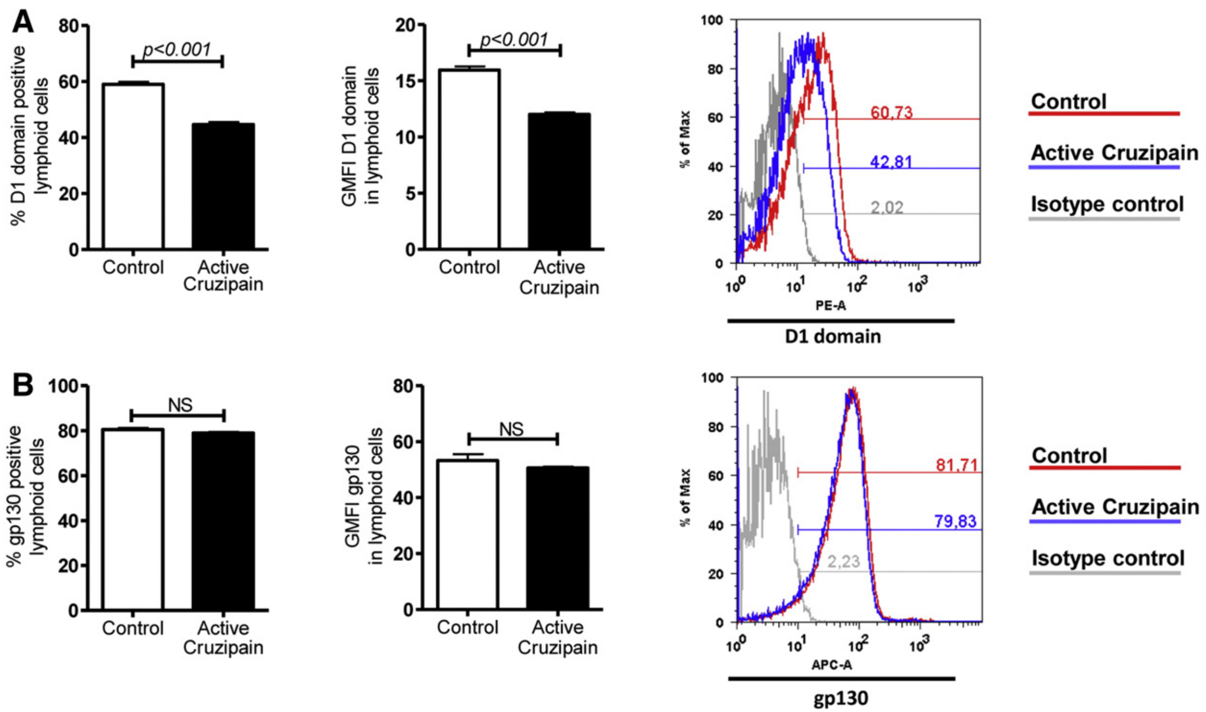

Fig. 6. Cruzipain activity reduces the gp130 D1 domain expression on human peripheral blood lymphocytes. Mononuclear cells were incubated with active cruzipain for 120 min. Then, the expression (percentage of positive cells and GMFI) of (A) gp130 D1 Domain and (B) global gp130 was determined on lymphocytes by flow cytometry. Peripheral blood lymphocyte subsets were gated by FSC/SSC scatter properties. Representative histograms (right) of D1 domain and gp130 expression on treated or untreated (control) cells are shown. The data (mean ± SEM) are representative of two independent experiments performed in quadruplicate.

complexed with chagasin. These data were reinforced by the fact that DTT-activated CM exerted the same effect as active cruzipain. Nevertheless, when the splenocytes were incubated with non-treated CM, the IL-6-induced p-STAT3 expression was diminished but not completely abrogated, suggesting that part of the soluble factor(s) released by the parasite itself was activated but coexisted with inactive stock of the enzyme. Under our experimental conditions, active infection with live parasites did not reduce the expression of p-STAT3 induced by IL-6.

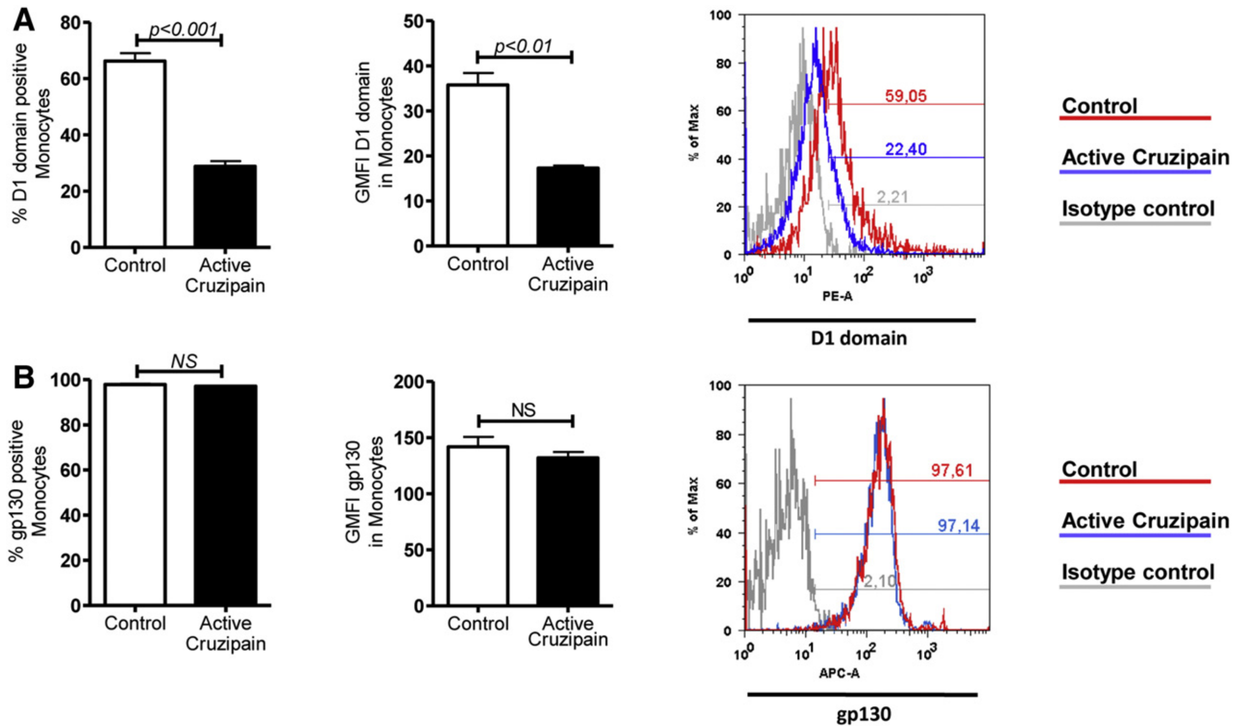

Fig. 7. Cruzipain activity diminishes the gp130 D1 domain expression on human peripheral blood monocytes. Mononuclear cells were incubated with active cruzipain for 120 min. Then, the expression (percentage of positive cells and GMFI) of (A) gp130 D1 domain and (B) global gp130 was determined on monocytes by flow cytometry. Peripheral blood monocyte subsets were gated by FSC/SSC scatter properties. Representative histograms (right) of D1 domain and gp130 expression on treated or untreated (control) cells are shown. The data (mean ± SEM) are representative of two independent experiments performed in quadruplicate.

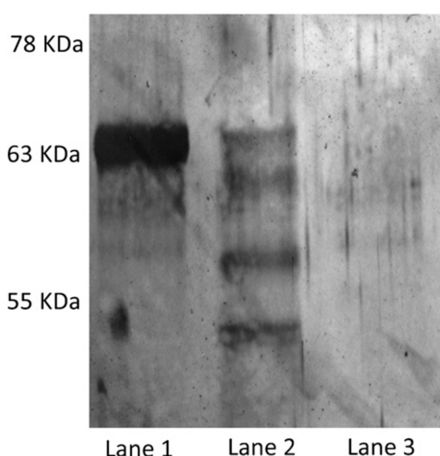

Fig. 8. Recombinant human gp130 is cleaved by cruzipain enzymatic activity. Recombinant gp130 ectodomain was incubated with active cruzipain for 120 min at 37 °C (lane 2) or maintained in PBS (lane 1). Samples were separated on SDS-PAGE, electrotransferred to the nitrocellulose membrane and the proteins were evidenced by silver staining. Cruzipain, at the concentration employed in the experiment, was run on lane 3.

Taking all these data into account, one can hypothesize that, in vivo, the parasites continuously release cysteine proteases which act in a systemic way by diffusing in the entire host's organism thus modulating the pleiotropic IL-6 downstream signaling. Since, in addition to IL-6, a large number of cytokines can signal through gp130 (IL-11, IL-27, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine), a decrease in the gp130-mediated signaling may have a broad impact on a number of immunological mechanisms.

The cytokine receptor gp130 requires the Ig-like domain (D1) in order to be functionally responsive to IL-6 cytokine [30,31]. It has been previously reported that DTT-activated cruzipain enzymatically cleaves the hinge regions of all human IgG subclasses [26]. Furthermore, considering that the B-T2 monoclonal antibody specifically recognizes the epitope in the D1 domain of human gp130, we performed a flow cytometry analysis of the expression of this domain on human peripheral blood mononuclear cells pre-treated with active cruzipain. Although the global expression of gp130 remained unaltered, the active enzyme induced a reduction in the D1 domain expression in different degrees of significance in the analyzed leukocyte subsets. This native cruzipain catalytic effect was reproduced by pre-activated recombinant cruzipain, thus demonstrating that the observed effect was specific to cruzipain. As was expected, the treatment with the inactive form of enzyme did not modify the D1 domain expression (data not shown). These data strongly suggest that the cruzipain enzymatic activity essentially involves the release of the Ig-like domain region on human gp130. Reinforcing these results, the recombinant human gp130 ectodomain was cleaved by the action of cruzipain. Taking into account that glycosylation protects protein against proteolytic degradation; it is plausible to think that under native conditions the gp130 extracellular domain on host cells underwent a lower digestion than the one observed in the gel. The identification of the amino acid sequences at the gp130 cleavage site is an aim of future investigations in our laboratory.

Interleukin-6 is a potent, pleiotropic, inflammatory cytokine that mediates a plethora of physiological functions, including cell proliferation, cell survival and amelioration of apoptotic signals. Depending upon the cell type, IL-6 is able to act through several classic protein kinase cascades such as MAPKs and PI-3 kinase. Administration of IL-6 induces the up-regulation of TLR2 mRNA but it does not affect the TLR4 mRNA levels on hepatocytes [32]. In agreement, in our model we found that cruzipain induced IL-6 secretion and the strong up-regulation of TLR2 but not of TLR4 surface expression. The increase in TLR2 positive population was dependent on NF-κB and PI3K/Akt, but independent of the MEK1/ERK signaling pathways. Moreover, cardiomyocytes stimulated with active cruzipain as well as IL-6 also increased the TLR2 expression (data not shown). Therefore, it is possible to speculate that IL-6 increased the TLR2 expressing cells through the activation of the PI3K/Akt/NF-κB signaling pathway. In this regard, it is important to stress that *T. cruzi* induces the expression of TLR2 in cardiac cells in the myocardium during experimental acute infection.

5. Conclusion

Our data suggest that the main parasite cysteine protease, devoid of enzymatic activity, triggers TLR2 up-regulation and signaling, leading to the subsequent release of pleiotropic IL-6. In this way, the parasite develops strategies that confer cardiomyocyte protection in response to such inflammatory environments. However, *T. cruzi* may modulate the enzymatic activity of the released proteases in order to maintain a stock of catalytically active cruzipain, which is capable of cleaving the cytokine receptor gp130. Considering that the signaling through gp130 by IL-6 family cytokines is involved in multiple steps of the immunological response, this might be a central mechanism for *T. cruzi* immune evasion.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2012.12.003.

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