Biochimica et Biophysica Acta 1832 (2013) 485–494

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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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ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form 19 November 2012
Accepted 9 December 2012
Available online 16 December 2012

Keywords:
Trypanosoma cruzi
TLR2
IL-6 signaling
gp130 Ig-like domain
Human leukocytes
Cardiomyocytes

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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http://dx.doi.org/10.1016/j.bbadis.2012.12.003

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

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