Institut Pasteur
Microbes and Infection 19 (2017) 55–61
www.elsevier.com/locate/micinf

Short communication

Decreased cruzipain and gp85/trans-sialidase family protein expression contributes to loss of *Trypanosoma cruzi* trypomastigote virulence

Juan San Francisco [a], Iván Barría [b], Bessy Gutiérrez [a], Iván Neira [a], Christian Muñoz [a], Hernán Sagua [a], Jorge E. Araya [a], Juan Carlos Andrade [a], Anibal Zailberger [c], Alejandro Catalán [a], Francisco Remonsellez [d], José Luis Vega [b], Jorge González [a,*]

[a] *Molecular Parasitology Unit, Medical Technology Department, University of Antofagasta, Antofagasta, Chile*
[b] *Experimental Physiology Laboratory (EPhyL), Antofagasta Institute, University of Antofagasta, Antofagasta, Chile*
[c] *National University of La Plata, La Plata, Argentina*
[d] *Applied Microbiology and Extremophiles Laboratory, Chemical Engineering Department, North Catholic University, Antofagasta, Chile*

Received 24 June 2016; accepted 10 August 2016
Available online 20 August 2016

Abstract

Two cell lines derived from a single *Trypanosoma cruzi* clone by long-term passaging generated a highly virulent (C8C3***hvir***) and a low virulent (C8C3***lvir***) cell line. The C8C3***hvir*** cell line was highly infective and lethal to Balb/c mice, and the C8C3***lvir*** cell line was three- to five-fold less infective to mouse cardiomyocytes than C8C3***hvir***. The highly virulent *T. cruzi* cell line abundantly expressed the major cysteine proteinase cruzipain (Czp), complement regulatory protein (CRP) and trans-sialidase (TS), all of which are known to act as virulence factors in this parasite. The *in vitro* invasion capacity and *in vivo* Balb/c mouse infectiveness of the highly virulent strain was strongly reduced by pre-treatment with antisense oligonucleotides targeting TS or CRP or with E64d. Based on these results, we conclude that decreased levels of TS, CRP and Czp expression could contribute to loss of *T. cruzi* trypomastigote virulence.

Keywords: *Trypanosoma cruzi*; Virulence; Genetically related cell lines; Cruzipain; Gp85/trans-sialidase family

1. Introduction

The virulence of the highly evolved parasite *Trypanosoma cruzi* involves its abilities to differentiate into the infectious stage, to penetrate the epithelium, invade cells, and proliferate while evading the host's immune response [1].

Upon invasion, metacyclic trypomastigotes (MTs) must resist the oxidative environment in macrophage phagosomes to establish an infection. After evading the phagolysosome, MTs differentiate into amastigotes, multiply intracellularly, and form blood trypomastigotes, which are then released into the bloodstream [2]. Subsequently, blood trypomastigotes must express a new set of molecules to resist the host immune response and to invade their target cells.

Several proteins involved in immune evasion have been described and are considered to be virulence factors. However, the true contribution of each factor remains unknown. Moreover, no experimental evidence has revealed the virulence of *T. cruzi* in a comprehensive and integrated manner [3].

Importantly, many reports have suggested that certain proteins, including cysteine proteases and members of the Gp85/trans-sialidase (TS) family, play a key role in *T. cruzi* virulence. For *T. cruzi*, cruzipain (Czp) is a major cysteine peptidase involved in immune evasion, host cell invasion and intracellular development [4]. This enzyme is present in lysosome-related organelles and is associated with the plasma membrane; some isoforms of Czp are secreted into the extracellular space [4].

* Corresponding author. Avenida Universidad de Antofagasta, 02800, Antofagasta, Chile.
E-mail address: jorge.gonzalez@uantof.cl (J. González).

http://dx.doi.org/10.1016/j.micinf.2016.08.003

Moreover, the TS family consists of distinct subfamilies that share a sialidase domain at the N-terminus. The TS family is composed of active TSs, complement regulatory proteins (CRPs), and proteins of unknown function [5,6]. TS in *T. cruzi* is a unique enzyme involved in the transfer of sialic acid from host glycoconjugates to mucins [5]. TS is involved in several virulence mechanisms, including invasion and immune evasion [7–12].

Within the TS superfamily, CRPs form one of three functionally distinct subfamilies that lack TS activity [7]. CRPs are surface-anchored glycoproteins that inhibit the alternative and classical complement activation pathways expressed by trypomastigotes but not by epimastigotes [10].

Despite this evidence, the study of *T. cruzi* virulence has been difficult, mainly due to the parasite's complex biology. On one hand, *T. cruzi* has unique mechanisms for gene expression, such as the constitutive polycistronic transcription of protein-coding genes, RNA editing and trans-splicing. Furthermore, in the absence of mechanisms controlling transcription initiation, organized subsets of *T. cruzi* genes must be post-transcriptionally co-regulated in response to extracellular signals [13]. On the other hand, *T. cruzi* has a complex life cycle that includes two different infective forms: MTs and blood trypomastigotes (and their counterparts tissue culture trypomastigotes, TCTs). Both infective forms are biochemically and antigenically distinct, and to invade their respective hosts, MTs and TCTs engage distinct sets of surface molecules that differentially interact with host components [14]. Therefore, studying the virulence among *T. cruzi* cell lines with opposing virulence phenotypes could reveal the molecular basis of these differences.

Here, using TCTs from two genetically related *T. cruzi* cell lines derived from the same clone with opposing characteristics of virulence and pathogenicity, we evaluated the contribution of these virulence factors to parasite virulence *in vivo* and *in vitro*. The relationship between the differential expression of these proteins and *T. cruzi* virulence or pathogenicity is also discussed.

2. Material and methods

2.1. Parasite cell lines and cell cultures

The *T. cruzi* H510 C8C3 clone was used throughout this study [15]. The clone was obtained from the House 510 *T. cruzi* strain, which was isolated from a Costa Rican *Triatoma dimidiata* [16]. This clone was treated in two different ways. One aliquot was passed weekly in mice for a period of 30 years, after which it was maintained cyclically in Balb/c mice and cell culture; this cell line was named C8C3***hvir***. The other aliquot was cultured over the same 30-year period in an axenic medium, after which it was maintained cyclically in liver infusion tryptose (LIT) broth [17] and Vero cells; this cell line was subsequently named C8C3***lvir***. For different studies, Vero cells were infected with TCTs as previously described [18]. Five days later, supernatants containing more than 95% TCTs were collected via centrifugation.

2.2. Genotyping of the C8C3*hvir* and C8C3*lvir* T. cruzi cell lines

TCTs from C8C3***hvir*** and C8C3***lvir*** cell lines was used to isolate DNA using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Typing was performed via PCR [19]. Briefly, the repetitive intergenic sequence fragment of the mini-exon SL gene was amplified using the Forward 5′-gggaagcttctgtactatattggta-3′ and the reverse 5′-gggaattcaatatagtacagaaactg-3′ primers. Once identified, the group through the intergenic regions of mini-exon (SL), the sub-group to which belonged both *T. cruzi* cell lines of our clone was identified as previously described [20]. For phylogenetic analysis, sequences were aligned using ClustalW [21] and a maximum likelihood analysis with a general time reversible substitution model (GTR) was conducted.

2.3. Cardiomyocyte culture and cell invasion assays

Ventricular cardiomyocytes from 1-to-3-day-old neonatal mice were prepared as previously described [22]. Cardiomyocytes were plated at a final density of 5×10^4 cells on 2% gelatin-coated 4-well Lab-Tek Chamber Slides (Nunc, ThermoFisher, Roskilde, Denmark).

Cell invasion assays were performed as previously described [18]. Cardiomyocytes were incubated with 5×10^5 TCTs from either the C8C3***hvir*** or C8C3***lvir*** cell line, either untreated or previously treated with the cell-permeable cysteine proteinase inhibitor E64d (Sigma Aldrich, St. Louis, Mo, USA) or with antibodies. After 3 h of incubation, the infected cultures were washed with PBS, fixed with methanol and stained with propidium iodide to visualize cell nuclei as well as parasite nuclei and kinetoplasts. The number of intracellular parasites in 500 cells was counted using an Olympus BX 51 epifluorescence microscope (Olympus Corporation, Tokyo, Japan).

2.4. Detection of Czp protein expression

To investigate Czp activity, TCTs were lysed via sonication, and the protein concentration of the lysates was determined [23]. Then, 100 μg of protein from the C8C3***hvir*** and C8C3***lvir*** cell lines were immunoprecipitated using polyclonal antibodies against Czp and protein G Sepharose (General Electric Healthcare Life Sciences, Pittsburgh, PA, USA). After incubation, the pellets were washed three times with 1× PBS and resuspended in a final volume of 90 μL of the fluorogenic substrate Z-Phe-Arg-AMC (Sigma Chemical Co., St. Louis, MO, USA). The enzymatic reaction mixtures were incubated for 30 min at 37 °C, and fluorescence was recorded in a TECAN Infinite M200 PRO spectrofluorometer (Tecan, Männedorf, Switzerland) using an excitation wavelength of 353 nm and an emission wavelength of 442 nm. To investigate Czp activity in TCTs pretreated with E64d, the inhibition of cysteine proteinase activity was measured via zymography, as previously described [24].

For flow cytometry, live TCTs from either C8C3***hvir*** or C8C3***lvir*** (4×10^6 parasites) were fixed with 2% paraformaldehyde in PBS for 30 min. Then, the parasites were washed out and incubated with polyclonal antibodies against *T. cruzi* Czp for 1 h at room temperature. After washing in PBS, the parasites were incubated with Alexa Fluor 488-labeled goat anti-mouse IgG for 1 h at room temperature. The number of fluorescent parasites was estimated with a Becton Dickinson (BD) FACSJazz cell sorter (Becton Dickinson and Company, New Jersey, USA).

We identified Czp in the secretome of the C8C3***hvir*** and C8C3***lvir*** cell lines as previously described [25]. Then, the samples were centrifuged, and the supernatants were filtered through Puradisc 25-mm syringe filters (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The filtered samples were precipitated with TCA-acetone, and the protein concentrations were determined as described above. Finally, proteins were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. Immunoblots were developed using primary antibodies against Czp and peroxidase-labeled anti-mouse or anti-rabbit IgG secondary antibodies.

2.5. Generation of antisense complementary oligonucleotide (ASCO) morpholinos directed against CRP or TS and in vivo infection

Third-generation ASCO morpholinos were used. For CRP, the *sense p*rimer was *5′-ACA CAC ATA TTT ATA TGC TCT CAC G-3′*, and the antisense primer was *5′-CGT GAG AGC ATA TAA ATA TGT GTG T-3′*. For TS, the sense primer was *5′-*AAC CGT CGC CGC GTG ACC GGA T-*3′*, and the antisense primer was *5′-*ATC CGG TCA CGC GGC GAC GGT T-*3’*. The control was a standard unrelated oligonucleotide: *5′-CCT CTT ACC TCA GTT ACA ATT ATT-3′* (Gene Tools, LLC, Philomath, OR, USA). Aliquots of C8C3***hvir*** or C8C3***lvir*** were incubated for 18 h with each ASCO against CRP or TS at a concentration of 10 μM together with 6 μM Endo-Porter according to the protocol suggested by the manufacturer. Sense or antisense treated trypomastigotes from the C8C3***vir*** cell line were used in the *in vivo* infection experiments.

Four-to six-week-old female Balb/c mice were infected via intraperitoneal injection of TCTs (1×10^5 parasites per mouse) from the C8C3***hvir*** cell line, previously treated with ASCO or untreated. Beginning on day 2 post-inoculation, parasitemia was monitored every 2 days as previously described [26]. Experiments were stopped on day 18, as the control mice started to die on day 20. All experiments were conducted according to the regulations of the Institutional Ethics Committee for Animal Experimentation, and all protocols were approved by the Committee (CEIC REV/2013).

2.6. SDS-PAGE and immunoblot

C8C3***hvir*** and C8C3***lvir*** *T. cruzi* cells were lysed with 1× PBS containing 0.1% NP-40 and cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (ThermoFisher, Rockford, IL, USA), and the protein concentration of the lysate was determined [23]. Parasite extracts were resolved via SDS-PAGE on a 12% gel and electrotransferred to nitrocellulose membranes in a Trans-Blot Turbo System (Bio-Rad. Hercules CA, USA). Immunoreactivity was determined via incubation of the membranes with a polyclonal antibody against Czp (either prepared by us (JG) or provided by Dr. Juan José Cazzulo of the Universidad Nacional de San Martin, Buenos Aires, Argentina); mouse anti-recombinant CRP serum (provided by Dr. Karen Norris of the University of Pittsburgh, Pittsburgh, PA, USA), and monoclonal antibody 39 against TS (provided by Dr. Sergio Schenkman of the Federal University of Sao Paulo, Sao Paulo, Brazil). Horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoblots were developed using ECL reagent (ThermoFisher, Rockford, IL, USA). ECL images were captured in a DNR Bio-Imaging System (Mahale HaHamisha,
Jerusalem, Israel) using Gel-Capture software.

2.7. Statistics

For statistical analysis, Student's *t*-test and ANOVA were performed using Prism 5 software (GraphPad). In the *T. cruzi* experiments, the significance of differences in infectivity or cell invasion between the C8C3***hvir*** and C8C3***lvir*** cell lines was determined based on a significance level (α) of 0.05.

3. Results and discussion

Genetic variability in *T. cruzi* has been reported [27]. Thus, differences between pathogenic and non-pathogenic isolates might simply reflect inter-isolate variation rather than specific virulence-related differences. To circumvent this problem, two genetically related *T. cruzi* cell lines with 99% of identity and substantial differences in virulence were compared; this analysis constitutes a powerful method to determine the mechanisms of *T. cruzi* virulence.

Specifically, we used two genetically related *T. cruzi* cell lines derived from the H510 C8C3 clone that belong to the DTU TcIa as a model to answer fundamental questions regarding *T. cruzi* virulence.

Our results strongly suggest that one reason for the observed differences in the parasitemia curves and cell invasion capacities between the two cell lines could be the greater capacity of C8C3***hvir*** to invade tissues. To explore the molecular basis of the differences between C8C3***hvir*** and C8C3***lvir***, our focus was to study the *in vivo* roles of cysteine proteinases and members of the Gp85/TS family that have been widely recognized as relevant virulence factors in *T. cruzi* [3].

To study the differences in cell invasion ability between the C8C3***hvir*** and C8C3***lvir*** cell lines, in one set of experiments, cells were infected for 3 h with TCTs from either C8C3***hvir*** or C8C3***lvir*** that were previously incubated in the presence or absence of E64d. Here, we observed that the C8C3***hvir*** cell line was three- to fivefold more infective to mouse cardiomyocytes than the C8C3***lvir*** cell line (Fig. 1A).

We also observed a gradual decrease in the number of parasites within the cardiomyocytes when we increased the concentration of the inhibitor (Fig. 1A). When the cells were infected with TCTs pre-treated with 25 μM or 50 μM E64d, parasite invasion decreased by approximately 45% and 60%, respectively (Fig. 1A).

To confirm our results regarding the role of Czp in the cell invasiveness of C8C3***hvir*** and C8C3***lvir***, invasion assays in the presence or absence of antibodies against Czp were also performed. Incubating C8C3***hvir*** cells in the presence of anti-Czp antibodies significantly reduced the number of invading cells by 70% relative to the control cells incubated in normal rabbit serum (Fig. 1B). These observations show that cysteine proteinases, especially Czp, are involved in cardiomyocyte invasion by *T. cruzi* C8C3***hvir*** cells.

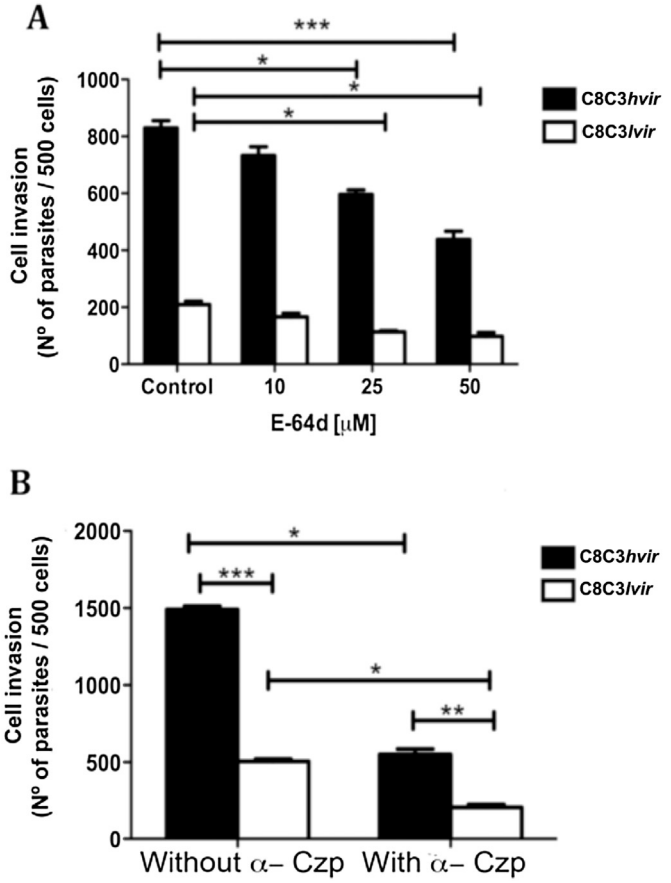

Fig. 1. The role of cysteine proteases in the cell invasiveness of *T. cruzi* C8C3***hvir*** and C8C3***lvir*** cell lines. (A) Cardiomyocytes were infected with 5×10^5 trypomastigotes of *T. cruzi* C8C3***hvir*** or C8C3***lvir*** C8C3 cells previously incubated in different concentrations of E64d. After 3 h, the cultures were washed with PBS, fixed, and stained with propidium iodide. Infectiveness was determined by counting the number of intracellular parasites in 500 cells. (B) Invasion assays were performed as described above using trypomastigotes from C8C3***hvir*** or C8C3***lvir*** *T. cruzi* cells preincubated in the presence or absence of polyclonal antibodies against cruzipain. After 3 h, the cultures were washed, fixed, and stained as described above. Infectiveness was determined by counting the number of intracellular parasites in 500 cells. The results are expressed as the means ± SD of experiments performed in triplicate. ***P < 0.001, **P < 0.01: two-way ANOVA for each group. *P < 0.05: one-way ANOVA for the variable in the corresponding column.

To understand if these differences in invasiveness could be due to the increased expression of Czp in the C8C3***hvir*** cell line, we studied Czp expression and secretion. We observed that Czp was highly expressed and secreted by C8C3***hvir*** cells (Fig. S1); specifically, C8C3***hvir*** cells displayed higher enzymatic activity the C8C3***lvir*** cells (Fig. S1A). Based on this finding, one reason why C8C3***hvir*** is more virulent than C8C3***lvir*** could be related to the fact that C8C3***hvir*** expressed and secreted more Czp. Several studies have demonstrated that *T. cruzi* virulence depends on Czp activity [28–31]. In *T. cruzi*, Czp is the main secreted lysosomal peptidase and appears to modulate the host immune response [32,33], affecting the susceptibility of the host to infection [34]. This function of Czp could explain why the strains and clones displaying higher Czp expression levels, such as the C8C3***hvir*** cell line, caused greater parasitemia than those displaying lower Czp expression. In addition, differences in Czp expression are correlated with the levels of cell invasion, differentiation, virulence and pathogenicity of different strains [32,35]. Elevated Czp expression in DTUs of TcI has also been reported [36], [37].

To comprehensively study the role of these different *T. cruzi* virulence factors, we used Balb/c mice to evaluate the *in vivo* infectivity of TCTs from the C8C3***hvir*** cell line pre-treated with E64d or with ASCO against TS or CRP. When mice were infected with TCTs pre-incubated with E64d, parasitemia was reduced by 50% (Fig. 2A), and the animals survived and appeared to be healthy (data not shown). Moreover, mice infected with TCTs not treated with the inhibitor developed higher levels of parasitemia (Fig. 2A) and died between days 20 and 25 (data not shown). Similarly, when mice were infected with TCTs from the C8C3***hvir*** cell line pre-treated with ASCO against CRP or TS, parasitemia was reduced by 60%. Furthermore, when mice were infected with TCTs from the C8C3***hvir*** cell line pre-treated with ASCO against CRP, TS and with E64d, parasitemia in the mice was reduced by 70% (Fig. 2B). Mice infected with TCTs pre-treated with sense oligonucleotides or control oligonucleotides and not treated with E64d developed greater parasitemia (Fig. 2B) and died between days 20 and 25 (data not shown). To demonstrate that treatment of TCTs with E64d or ASCO inhibited target enzymatic activity or reduced target protein expression, zymography and immunoblotting were utilized. In all cases, enzymatic activity and protein expression were reduced by E64d or ASCO treatment (Fig. 2C).

Based on these results, we concluded that decreased TS, CRP or Czp expression could contribute to the loss of virulence among *T. cruzi* trypomastigote forms. However, these molecules cannot exclusively be responsible for *T. cruzi* virulence because they are found in both pathogenic and non-pathogenic *T. cruzi* isolates. Moreover, simultaneously inhibiting CRP, Czp and TS dramatically reduced *T. cruzi* infectivity in mice (by 70%) but did not completely abolish host infection.

4. Conclusion

Different studies have reported that Czp, TS and CRP are relevant to *T. cruzi* virulence. This conclusion is also based on

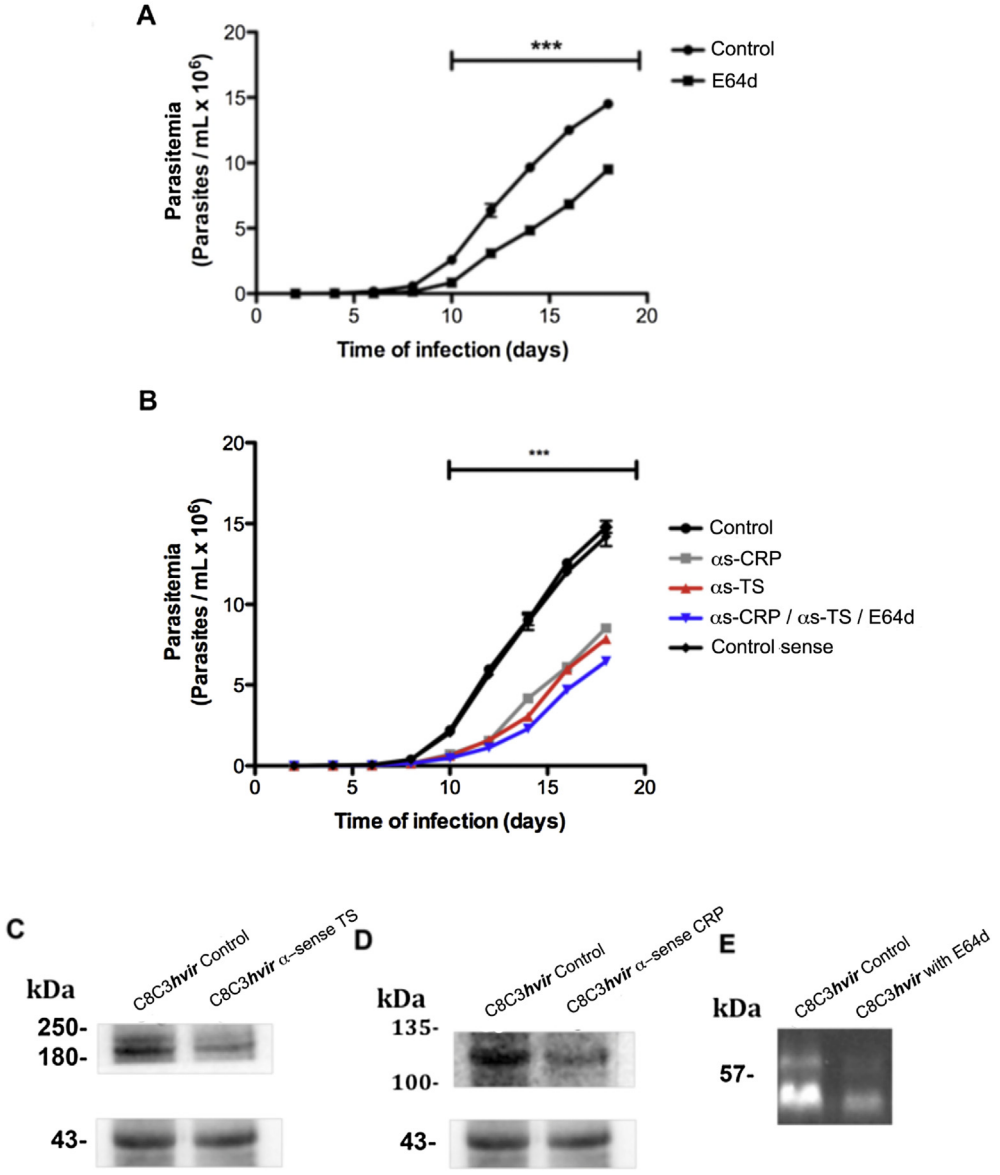

Fig. 2. Study of the *in vivo* roles of cruzipain, trans-sialidase and complement regulatory protein (CRP). Groups of 5 female Balb/c mice were infected with 1×10^5 trypomastigotes of C8C3***hvir*** cells treated with the cysteine protease inhibitor E64d (A), with sense or antisense oligonucleotides against trans-sialidase or CRP, or with both antisense oligonucleotides and E64d (B). Parasitemia curves were generated by counting (every 2 days) the number of parasites in a blood sample obtained from the mouse tail vein. Results are expressed as means ± standard error of the mean of at least 3 experiments performed in triplicate. **P < 0.01, ***P < 0.001: two-way ANOVA. C) The inhibition of CRP expression (C) and TS expression (D) was evaluated via western blot. β-actin was used as the parasite loading control. Inhibition of cysteine proteinase activity was evaluated via zymography (E).

the finding that inhibiting each of these proteins results in a marked reduction in either parasitemia or cell invasiveness.

Here, we concluded that decreased TS, CRP or Czp expression could contribute to the loss of *T. cruzi* trypomastigote virulence. However, these molecules cannot exclusively be responsible for *T. cruzi* virulence because they are found in both pathogenic and non-pathogenic *T. cruzi* isolates. Moreover, simultaneously inhibiting CRP, Czp and TS dramatically reduced *T. cruzi* infectivity in mice (by 70%) but did not completely abolish host infection. This observation should be taken into account when designing chemoprophylaxis and immunoprophylactic strategies. Then, it seems that, only the use of multi-antigenic or multi-target chimeras could have real success in assessing control strategies for Chagas disease.

According to Atayde et al. [1], a single glycoprotein, the gp82, is a key molecule in promoting efficient MT infection. In TCTs, the differential expression profile appears to be different. Then, we conclude that a combination of proteins rather than a single gene product may be responsible for the differences in virulence between the C8C3***hvir*** and C8C3***lvir*** cell lines.

Finally, further proteomic and transcriptomic investigations are needed to obtain a comprehensive understanding of the molecular basis of C8C3***hvir*** cell virulence.

Conflict of interest

The authors declare that they have no competing interest concerning this specific manuscript.

Acknowledgements

We are thankful for the valuable support from FONDECYT grants Nº1131007 (to J.G.) and Nº11130013 (to J.L.V.). Flow cytometry was performed using equipment purchased from funding provided by FONDEQUIP grant EQM120137. J.S.F. is a recipient of a University of Antofagasta (Basal Funds Program) Fellowship. I.B. holds a CONICYT PhD Fellowship. We thank Dr. Marta M. Teixeira and Luciana Lima (Parasitology Department, ICB, University of São Paulo, São Paulo, Brazil) for genotyping the C8C3***hvir*** and C8C3***lvir*** cell lines and Marlene Zuñiga for providing assistance in flow cytometry assays.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2016.08.003.

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