

In vivo infection by *Trypanosoma cruzi*: The conserved FLY domain of the gp85/trans-sialidase family potentiates host infection

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(Received 14 June 2010; revised 16 August and 7 September 2010; accepted 13 September 2010; first published online 2 November 2010)

SUMMARY

Trypanosoma cruzi is a protozoan parasite that infects vertebrates, causing in humans a pathological condition known as Chagas' disease. The infection of host cells by *T. cruzi* involves a vast collection of molecules, including a family of 85 kDa GPI-anchored glycoproteins belonging to the gp85/trans-sialidase superfamily, which contains a conserved cell-binding sequence (VTVXNVFLYNR) known as FLY, for short. Herein, it is shown that BALB/c mice administered with a single dose (1 µg/animal, intraperitoneally) of FLY-synthetic peptide are more susceptible to infection by *T. cruzi*, with increased systemic parasitaemia (2-fold) and mortality. Higher tissue parasitism was observed in bladder (7.6-fold), heart (3-fold) and small intestine (3.6-fold). Moreover, an intense inflammatory response and increment of CD4⁺ T cells (1.7-fold) were detected in the heart of FLY-primed and infected animals, with a 5-fold relative increase of CD4⁺CD25⁺FoxP3⁺ T (Treg) cells. Mice treated with anti-CD25 antibodies prior to infection, showed a decrease in parasitaemia in the FLY model employed. In conclusion, the results suggest that FLY facilitates *in vivo* infection by *T. cruzi* and concurs with other factors to improve parasite survival to such an extent that might influence the progression of pathology in Chagas' disease.

Key words: *Trypanosoma cruzi*, Chagas' disease, CD4⁺CD25⁺FoxP3⁺ T cells, Tc-85, gp85/trans-sialidase glycoprotein family.

INTRODUCTION

Trypanosoma cruzi, a flagellate protozoan parasite, is able to infect a wide range of cell types. Parasite persistence in the nerve, cardiac and skeletal muscle tissues is characteristic of Chagas' disease. In most of the cases infection is not lethal in immunocompetent hosts, being asymptomatic throughout life, but 30–40% of the individuals will develop severe chronic symptoms. Chagas' disease remains a health problem, considering that 8–10 million people are infected. Chemotherapy is still problematic and no effective vaccine is available. Parasite adhesion to the host cell surface is one of the necessary steps leading to internalization of *T. cruzi*, with the parasite exploiting a series of its own molecules as the Tc-85 family members (Giordano *et al.* 1999; Marroquin-Quelopana *et al.* 2004), gp82 (Neira *et al.* 2003), and the active and inactive trans-sialidase (TS) (Previato *et al.* 1985; Schenkman *et al.* 1991; Dias *et al.* 2008)

that belong to a large superfamily of surface glycosylphosphatidylinositol (GPI)-anchored glycoproteins, the gp85/TS, encoded by more than 700 genes (El-Sayed *et al.* 2005).

Data in the literature show that members of gp85/TS superfamily are targets of the antibody response (Burns *et al.* 1992; Ribeirão *et al.* 2000; Pitcovsky *et al.* 2001), CD4⁺ and CD8⁺ T cells (Martin *et al.* 2006; Singh *et al.* 2007; Alvarez *et al.* 2008; Dias *et al.* 2008), being particularly prominent targets of *T. cruzi*-specific CD8⁺T cells (Martin *et al.* 2006; Tarleton, 2007). In addition, TS is known to (1) increase macrophage invasion by *T. cruzi* through mitogen-activated protein (MAP) kinase signalling (Chuenkova and Pereira, 2001), (2) induce lymphocyte apoptosis (Leguizamón *et al.* 1999), (3) increase cell responses *in vitro* through activation of accessory cell functions and, finally, (4) bind to and co-stimulate T cells through interaction with CD43 *in vivo* (Todeschini *et al.* 2002). Moreover, while surface TS is involved in the escape of the parasite from the parasitophorous vacuole (Lopez *et al.* 2002; Rubin-de-Celis *et al.* 2006), but not in the invasion step (Yoshida *et al.* 1997), soluble TS acts as a

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virulence factor increasing parasitaemia and mortality in *T. cruzi*-infected mice (Chuenkova and Pereira, 1995). TS and Tc-85 proteins are also released by the infective trypomastigotes as membrane vesicles. An increase in heart parasitism with an intense inflammatory response was observed in vesicle-primed mice before infection with *T. cruzi* (Torrecilhas *et al.* 2009).

Although the virulence-enhancing effect described for TS was associated to the sialic acid-binding site of the enzyme, the observed activity may also occur through a site distinct from the catalytic region. The conserved sequence (VTV×NV×LYNR, herein called FLY) localized upstream from the carboxyl terminus in all members of the gp85/trans-sialidase family is a potential good candidate for a virulence factor *in vivo*, since FLY-synthetic peptide activates ERK 1/2 signalling pathways *in vitro* leading to cytokeratin 18 dephosphorylation and potentiation of *T. cruzi* infection (Magdesian *et al.* 2001, 2007).

In order to clarify whether FLY has any role *in vivo* a well-studied mouse model was employed. Here it is shown that FLY increases parasitaemia and tissue parasitism, as well as the mortality rate of experimental animals (BALB/c). More intense cardiac inflammation, with the prevalence of CD4⁺ T cells and an increase of CD4⁺ T cells expressing the transcription factor FoxP3 were detected in FLY-primed animals. Depletion of CD4⁺CD25⁺ T cells with anti-CD25 antibodies resulted in a significant decrease of parasitaemia, suggesting the relevance of these cells to the establishment of *T. cruzi* infection in FLY-primed animals.

MATERIALS AND METHODS

Materials

Hoechst 33342 was purchased from Molecular Probes (Oregon, USA), Tissue-Tek[®] OCT compound from Sakura Finetek, and Superfrost Plus Micro Slide for frozen tissue sections were obtained from VWR (West Chester, PA, USA), and 3,3'-diaminobenzidine (D4418) and paraformaldehyde (P6148) from Sigma (St Louis, MO, USA). Normal goat serum (S-1000), biotinylated anti-mouse IgG (BA-1300), anti-goat IgG (BA-5000) or anti-rabbit IgG (BA-1000) and avidin (A-2000) were purchased from Vector Laboratories Inc., (Burlingame, CA, USA). Goat anti-mouse CD4 polyclonal antibody (RM4-5 sc-19643); goat anti-FoxP3 polyclonal antibody (N-12, sc 21072); rabbit anti-mouse CD8 monoclonal antibody (H-160, sc 7188); mouse mAb anti-CD14 (UCH-M1, sc-1182) and anti-iNOS polyclonal antibody (C-11 sc-7271) were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). RPMI medium was purchased from Life Technologies (Grand Island, NY, USA) and Opteia kit for Elisa assays from B&D (Franklin Lakes, NJ, USA).

Experimental animals and parasites

Eight-week-old wild-type BALB/c female mice were housed under barrier conditions at the animal care facility at the Instituto de Química, Universidade de São Paulo (IQUSP, Brazil). All procedures were in accordance with international guidelines for the use of animals and received prior approval from the animal ethics committee of IQ-USP. Mice were routinely anesthetized by intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg) plus xylazine hydrochloride (10 mg/kg). *Trypanosoma cruzi* parasites (Y strain) were routinely maintained by weekly i.p. infection of BALB/c mice with 500 parasites. Blood trypomastigotes were obtained from infected blood by cardiac puncture of anesthetized mice, as described (Torrecilhas *et al.* 2009).

Parasite inoculation and parasitaemia and mortality determination

BALB/c mice were intraperitoneally (i.p) inoculated with 1 µg/animal of the FLY peptide (GKKSVTVTNVFLYNRPLN), PBS or the control peptide FAY (SVTVTNVFAYNRPLN), 7 days before infection (i.p.) with 500 blood trypomastigotes (5 mice per group). Parasitaemia was evaluated under a phase-contrast microscope (40× objective) by counting motile parasites in 10 µl blood samples drawn from lateral tail veins from infected mice (Brenner, 1962). For the determination of parasitaemia in CD25-depleted animals, BALB/c mice were injected i.p. with 0.4 mg of a purified anti-CD25 (mAb) or GL113 (rat IgG1 anti-*Escherichia coli* β-galactosidase) 1 day before peptide injection, followed by parasite infection 7 days later. The anti-CD25 antibody (rat mAb, clone PC61, IgG1 isotype), purified by 45% ammonium sulfate precipitation from ascites liquid followed by protein G affinity chromatography, was kindly provided by Dr José Maria Alvarez Mosig (Universidade de São Paulo). Parasitaemia data were compared by ANOVA test and the Tukey's multiple comparison test. Mortality was monitored daily and was followed for up to 30 days after infection. Three independent experiments were performed, with 5 mice per experimental group. Statistical analysis of mortality was made using the Mantel-Cox test.

Histopathological analysis

Tissue specimens from anesthetized and infected mice previously inoculated with FLY or FAY peptides or PBS were collected on Day 15 after infection, fixed in neutral buffered formalin and paraffin-embedded for further processing. Three non-consecutive slides from tissue sections of each mouse (5 µm thick) were stained with haematoxylin-eosin (H&E) and analysed, in a blind manner, by optical microscopy for parasite nest quantification. The

degree of heart inflammation (atria and ventricles) was evaluated at the same experimental points where the tissue parasitism was quantified. Considering the presence of inflammatory infiltrates, inflammation was graded as 0 (1 inflammatory focus or absent); 1 (1–2 inflammatory foci) and 2 (more than 3 inflammatory foci).

Immunohistochemical staining

Half of the hearts from experimental and control mice (Day 15 after infection) were covered with Tissue-Tek[®] OCT compound, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Cryostat sections ($5\ \mu\text{m}$) were dried on Superfrost Plus Micro Slide glass slides and fixed in cold acetone. Subsequently, the sections were incubated in 3% normal goat serum, followed by one of the following antibodies: goat anti-mouse CD4 polyclonal antibody, goat anti-FoxP3 polyclonal antibody, rabbit anti-mouse CD8 mAb antibody, mouse mAb anti-CD14 or anti-iNOS diluted in phosphate-buffered saline (PBS). The sections were then incubated with biotinylated anti-mouse IgG, anti-rabbit IgG or anti-goat IgG, followed by incubation with avidin and then in a solution of 3,3'-diaminobenzidine ($0.7\ \text{mg/ml}$). After washing, the slides were counterstained with Mayer's haematoxylin. Negative controls were obtained by the omission of primary antibodies. The number of positively stained cells for each antibody was counted per 5 consecutive microscopic high-power fields ($20\times$).

Assessment of cytokine and nitrite production by resident peritoneal cells

Peritoneal-derived cells were harvested from normal BALB/c mice by washing the peritoneal cavity with cold FCS-free RPMI medium. The suspension was centrifuged, re-suspended in RPMI-10% FCS and cultured in 24-well flat-bottomed plates at 5×10^6 cells/ml. Thereafter, cultured cells were incubated in the presence or absence of the FLY peptide (1, 10 and $100\ \mu\text{g/ml}$) for 24 h. IL10 was quantified by chemiluminescent enzyme-linked immune-assay (ELISA) using the Opteia kit. Nitrite quantities were measured indirectly by the Griess reaction, as described (Chuenkova and Pereira, 2001).

Analysis of peritoneal cell infection with Trypanosoma cruzi

Peritoneal-derived cells plated onto cover slips were pre-incubated with 1, 10 or $100\ \mu\text{g}$ from the FLY or FAY synthetic peptides for 30 min and then infected for 2 h at 37°C with 10 trypomastigotes/cell. Cover slips were washed 10 times with PBS, the cells were fixed in 4% paraformaldehyde and the nuclei stained with Hoechst dye. For each experimental condition 20 photos of each cover slip were made enabling the

counting of approximately 500 cells in each sample. The data were compared for statistical significance using unpaired Student's *t*-test.

Statistics

Statistical analysis and data representation were performed with GraphPad Prism 5.01 software (GraphPad Software, La Jolla, CA, USA) or Origin 8.0 software (OriginLab, Northampton, MA, USA). One-way ANOVA using Tukey's multiple comparison test was used for comparison of groups. Statistical analysis of mortality was made using the Mantel-Cox test. In all cases *P* values <0.05 were considered statistically significant.

RESULTS

FLY increases parasitaemia and mortality of Trypanosoma cruzi-infected mice

The effect of the peptide FLY on *in vivo* infection with *T. cruzi* was tested by monitoring the parasitaemia and the mortality of BALB/c mice pre-inoculated with $1\ \mu\text{g/animal}$ of FLY (GKKS-VTVTNVFLYNRPLN), FAY (SVTVTNV-FAYNRPLN) or PBS, and challenged, 7 days later, with trypomastigote bloodstream forms. The FAY peptide was used as a control since it does not bind to the LLC-MK₂ epithelial cell line nor increases parasitaemia *in vitro*, in contrast to FLY. The fact that the FLY peptide employed in the experiment (GKK-FLY) is 3 amino acids longer than FAY is not relevant since the effect of FLY is independent of the presence of GKK, as previously described (Magdesian *et al.* 2001). The peak of parasitaemia was detected on Day 9 after infection, independently of pre-inoculation with FLY, FAY or PBS (Fig. 1A). Consistently, a significantly 2-fold increase in the number of circulating trypomastigotes was quantified in the peripheral blood of mice inoculated with FLY when compared to the controls (ANOVA test, $P<0.003$) (Fig. 1A). Similar results were obtained with a 10-fold higher dose ($10\ \mu\text{g}$ FLY/animal) or when 1 or $10\ \mu\text{g}$ FLY/animal were inoculated 2 h before the challenge with *T. cruzi* (data not shown). Mortality also increased in mice inoculated with FLY ($1\ \mu\text{g/animal}$) with animals dying within 20 days post-infection (Fig. 1B) (ANOVA test, $P<0.03$).

FLY increases Trypanosoma cruzi tissue parasitism in vivo

We next examined whether FLY can potentiate the *in vivo* dissemination of *T. cruzi* infection. For that purpose, the number of parasite nests was determined in tissue sections from animals inoculated with FLY, FAY or PBS (3 animals per group) and

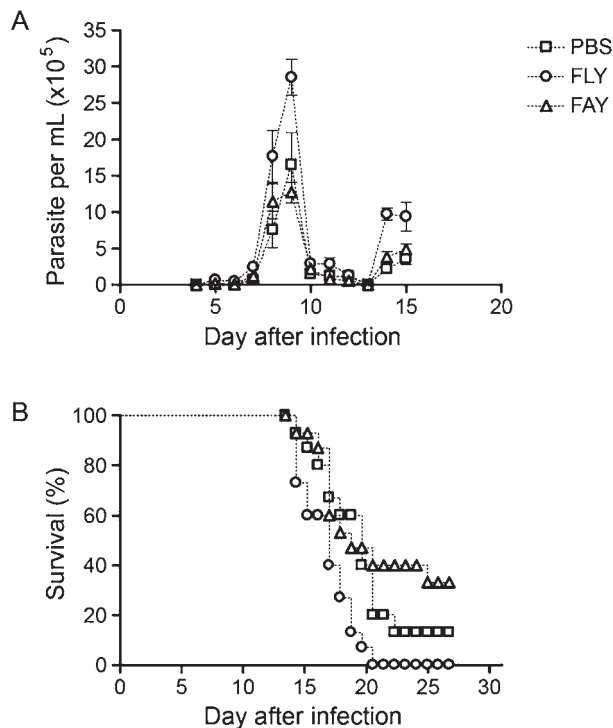


Fig. 1. Parasitaemia and mortality increases in FLY-primed mice infected with *Trypanosoma cruzi*. Parasitaemia and mortality assays were performed on BALB/c mice administered intraperitoneally (i.p) with PBS, FLY or FAY peptides (1 μ g/animal) 7 days before challenge with 500 bloodstream trypomastigote forms of *T. cruzi*, Y strain. (A) Parasitaemia level is represented by arithmetic means \pm S.E.M. of individual values from 5 mice. Differences are statistically significant when analysed by one-way ANOVA and the Tukey's Multiple Comparison test ($P < 0.003$). (B) Survival curves from 15 mice per group (Mantel-Cox test, $P < 0.05$). Data are representative of 3 independent experiments.

infected, 7 days later, with *T. cruzi*. On Day 15 after infection, in all experimental groups, mainly amastigotes were observed individually or in small nests in the bladder (epithelium, connective tissue and muscle cells), heart (atria and ventricles), small intestine (in the proximity of the Auerbach plexus) (Fig. 2) and in the red and white pulp of the spleen (not shown). Although FLY does not influence parasite dissemination, higher parasitism in FLY-treated animals was observed when compared to the controls, being approximately 7.6-fold higher in the bladder (Student's *t*-test, $P < 0.005$) (Fig. 2D), 3.6-fold higher in the small intestine (Student's *t*-test, $P < 0.003$) (Fig. 2G) and 3-fold higher in the heart (Student's *t*-test, $P < 0.0001$) (Fig. 2A). An approximately 10-fold higher number of amastigotes/nest was found in the heart when compared to the bladder and small intestine. Amastigote nests were consistently more abundant in the atria than in the ventricles in all experimental groups, although approximately 3-fold higher in FLY-primed mice, as pointed out previously (Fig. 2A–I).

The magnitude of the inflammation process in the experimental groups was then evaluated. Inflammatory foci were detected only in infected animals and were more intense in the atria than in the ventricles in all groups (Fig. 3). In keeping with the previous observations, inflammation was higher in infected mice pre-treated with FLY (mean scores: atria = 2.6, ventricles = 1.7) than in those pre-inoculated with FAY or PBS (mean scores: atria = 1.6, ventricles = 1).

CD4⁺CD25⁺ T cells are involved in the potentiation of Trypanosoma cruzi infection of FLY-treated mice

Nitric oxide (NO) produced by macrophages and other cells is an important microbicide to many intracellular pathogens, including *T. cruzi*. To address the question of whether NO production could be impaired in cardiomyocytes from FLY-treated animals, hearts from mice inoculated or not with the synthetic peptides were harvested on Day 15 after infection and analysed by immunohistochemistry with an antibody against inducible nitric oxide synthase enzyme (iNOS). As shown in Fig. 4A–C animals infected with *T. cruzi* presented higher levels of iNOS expression when compared with non-infected control mice. Nevertheless, no quantitative differences regarding iNOS expression were observed among the experimental groups. These data suggest that mechanisms other than induction/repression of iNOS expression may be involved in the potentiation of infection and/or amastigote survival and replication in FLY-treated animals.

Evaluation of the infiltrating cell profile ($CD4^+$ T, $CD8^+$ T and $CD14^+$) in the hearts of infected mice was performed by immunohistochemistry. The same number of labelled fields for $CD8^+$ T and $CD14^+$ cells was detected in the cardiac tissue of infected mice in all experimental groups (data not shown). In contrast, $CD4^+$ T cells were approximately 1.7-fold more abundant in FLY- than in FAY- or PBS-treated and infected mice (Student's *t*-test, $P < 0.002$) (Fig. 4D).

Although the role of $CD4^+$ $CD25^+$ T cells (Treg) in the persistence of *T. cruzi* infection is controversial in the literature (Kotner and Tarleton, 2007; Mariano *et al.* 2008; Sales *et al.* 2008), the expression of FoxP3 (a transcription factor specifically expressed by Tregs in mice) was assessed in heart sections by immunohistochemistry. Approximately 10% of the $CD4^+$ T cell population in the cardiac tissue of the infected mice inoculated with FLY was positive for FoxP3 in comparison to 2% of positive cells in FAY or PBS pre-treated animals (Student's *t*-test, $P < 0.001$) (Fig. 4D–F). $CD25$ -inactivation by the administration of anti- $CD25$ mAb (PC61 clone) prior to FLY and *T. cruzi* inoculation reduces the number of circulating trypomastigotes to half of the control

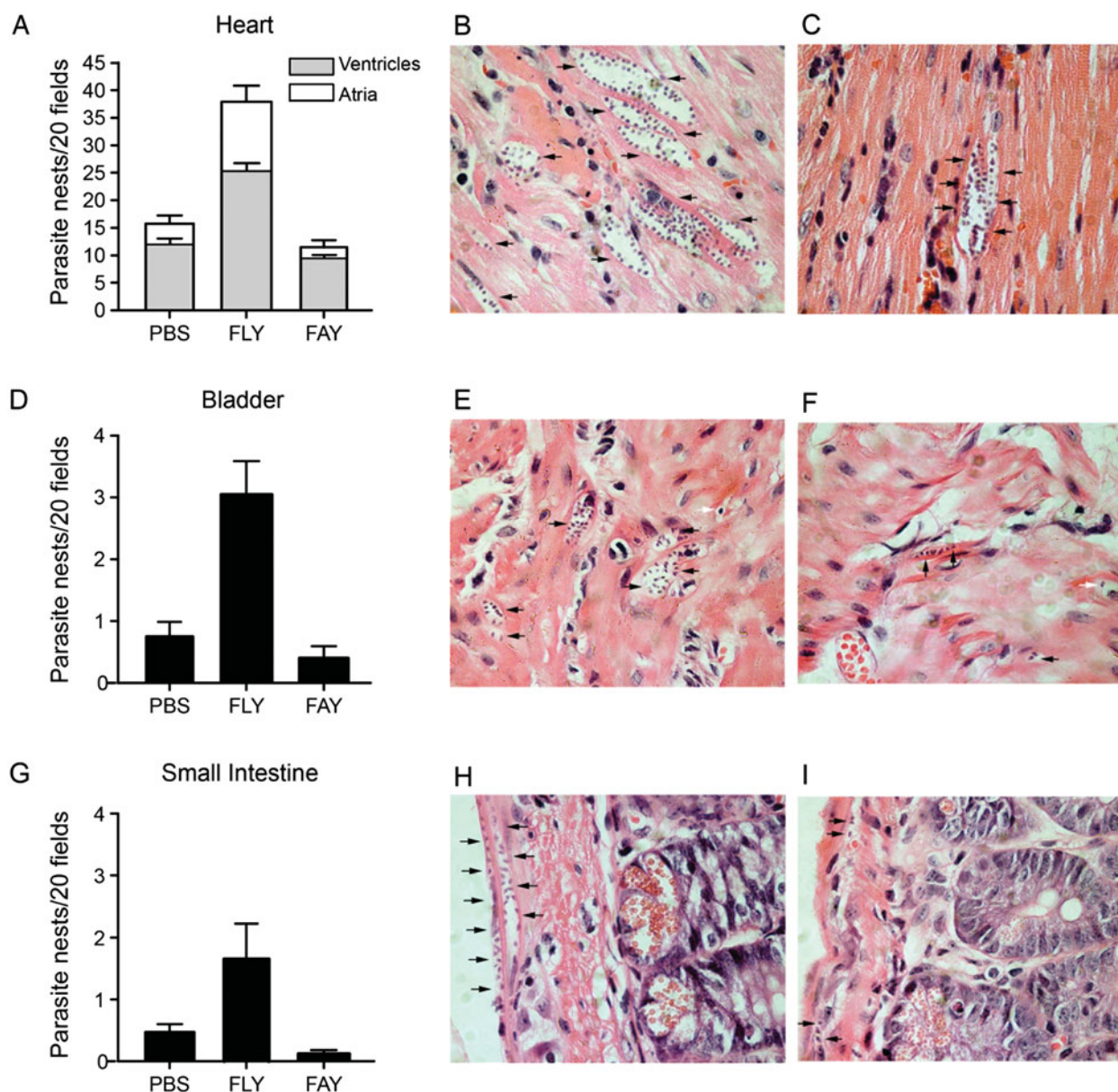


Fig. 2. Number of parasite nests increases in infected mice pre-administered with FLY. Thin sections of haematoxylin-eosin stained tissues from mice administered with FLY, FAY or PBS and infected with 500 bloodstream trypomastigotes from the Y strain were analysed on Day 15 for the determination of the number of parasite nests in the hearts (A–C), bladder (D–F) and small intestine (G–I). Results are arithmetic means \pm standard deviation of 20 HPF (High Power Fields, objective 40 \times). Arrows indicate parasite nests in organ sections from mice previously treated with FLY (B,E,H) or PBS (C,F,I).

parasitaemia, injected with an unrelated antibody (GL113 mAb) (Table 1), pointing to a possible role of CD4⁺CD25⁺FoxP3⁺ T cells in the FLY-induced increment of *T. cruzi* infection.

FLY peptide increases infection and IL10 production by resident peritoneal cells

Previous studies suggest that the suppressor activity of Treg cells may also be induced by the presence of soluble factors such as TGF β and IL10 (Murai *et al.* 2009; Scholzen *et al.* 2009) both produced during

infection by *T. cruzi* (Reed *et al.* 1994; Mariano *et al.* 2008).

The production of IL10 was then determined in the supernatant of resident-peritoneal cells (cells present in the locality of *T. cruzi* infection in our experimental model) incubated or not with FLY. As shown in Fig. 5A, the production of IL10 is dose dependent with levels of this cytokine being almost 1.8-fold higher in FLY-stimulated cells (at a concentration of 1 μ g/well) than in FAY-stimulated cells. Interestingly, when the levels of NO were determined in the supernatants, no differences were observed amongst the experimental groups (Fig. 5B),

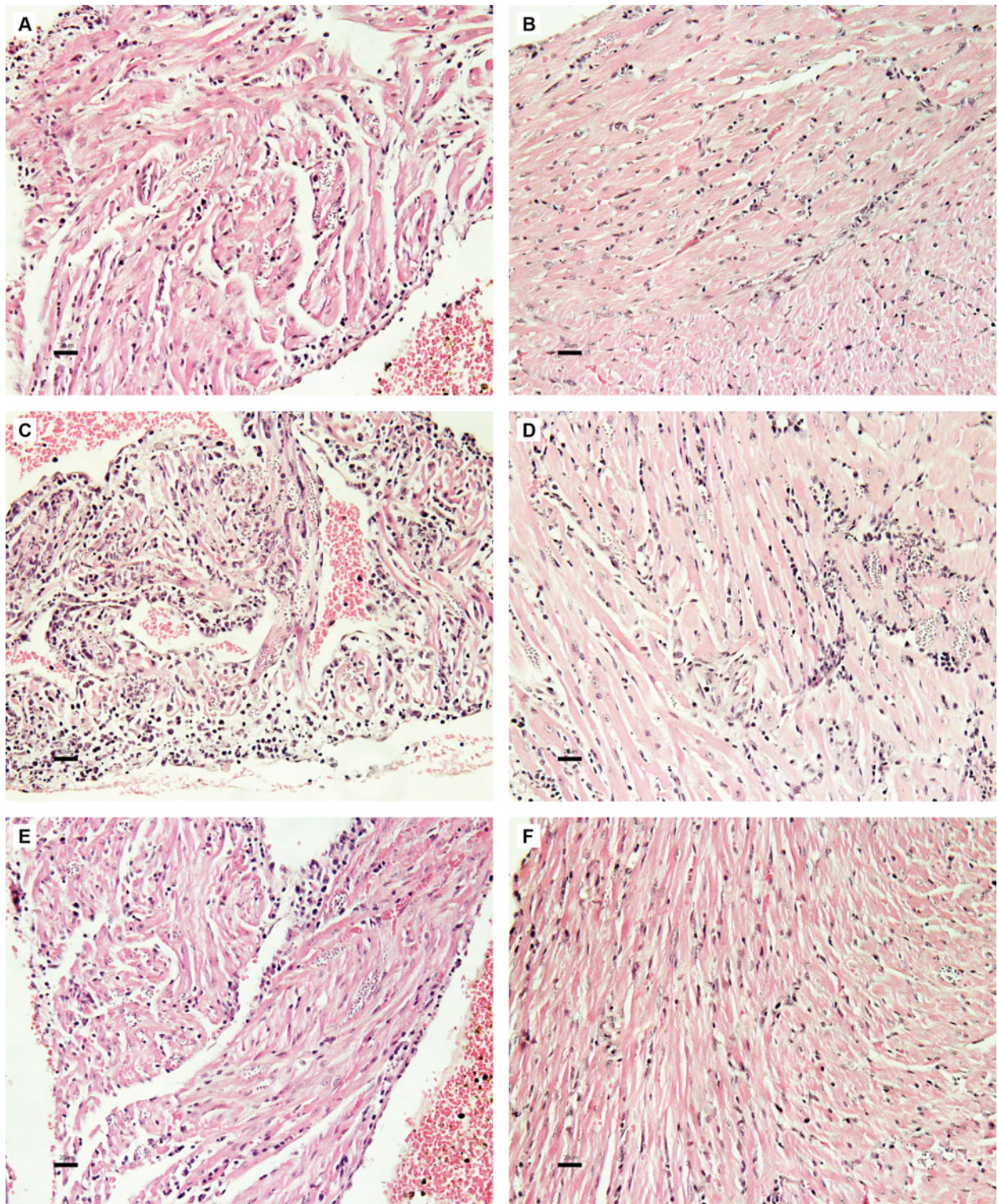


Fig. 3. Analysis of the inflammation in atria and ventricles. Haematoxylin-eosin sections from the atria (A, C, E) and ventricles (B, D, F) of hearts from BALB/c mice administered with PBS (A and B), FLY (C and D) and FAY (E and F) on Day 15 post-infection with 500 trypomastigotes from the Y strain. Scale bars = 20 μ m.

showing that FLY does not affect NO production, in line with our results on the quantification of iNOS synthase in the heart (Fig. 4A–C). In addition, FLY may contribute to the increment of mouse parasitaemia by the enhancement of local infection of peritoneal cells. Although the clearance of FLY in the peritoneal cavity was not determined in our

experimental conditions, it is worth noting that previous incubation of peritoneal cells with increasing concentrations of FLY *in vitro* resulted in a significant rise in the number of infected cells (Fig. 6), being 3.6 times higher at 10 μ g of FLY (Student's *t*-test, $P < 0.002$, $P < 0.0002$) in relation to FAY or PBS, respectively.

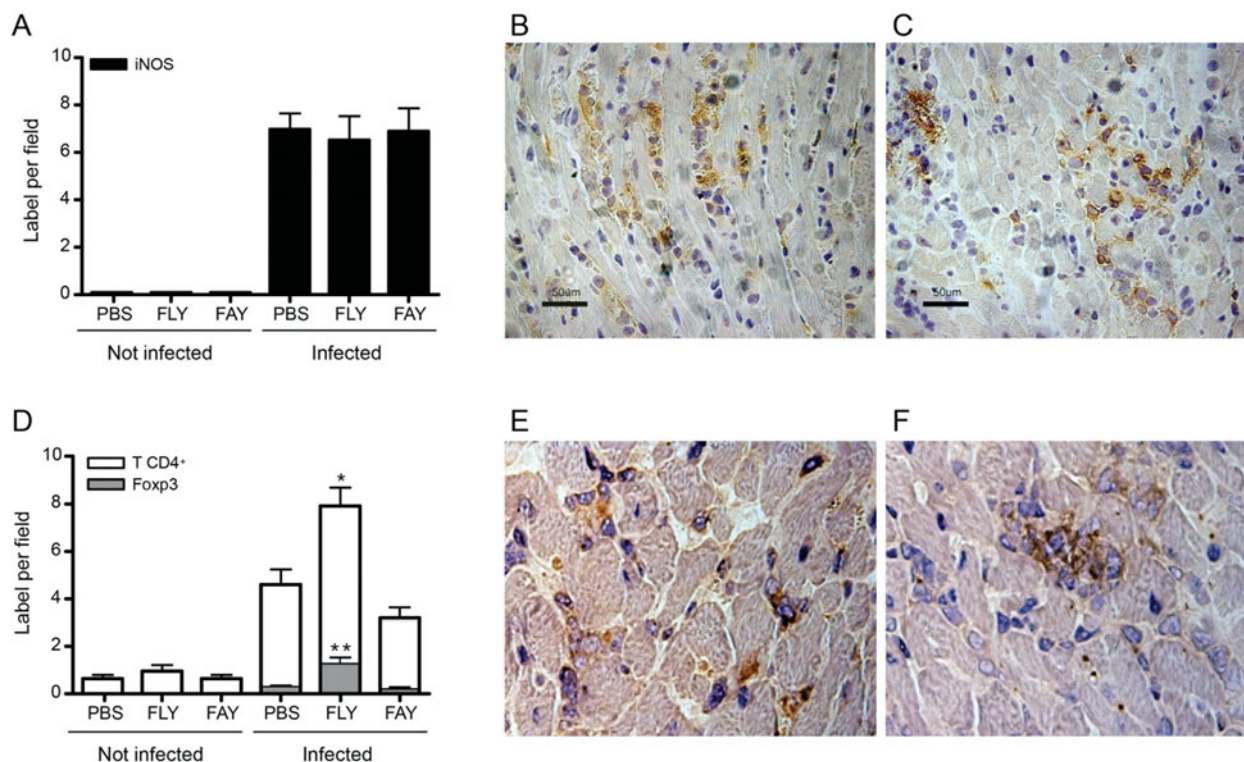


Fig. 4. Analysis of iNOS and CD4⁺FoxP3⁺ T cells in the hearts of FLY-primed and infected mice. Number of labelled fields for iNOS (A) CD4⁺ T (D, white bars), FoxP3 (D, grey bars) and positive cells in the hearts of mice administered with PBS, FLY or FAY, not infected or infected with *Trypanosoma cruzi* on Day 15 after infection. Examples of fresh-frozen heart sections from FLY (B, E) and PBS (C, F) labelled for iNOS (A, B) and FoxP3 (E, F). The sections were incubated with anti-iNOS mouse mAb or with polyclonal specific anti-CD4⁺ mouse IgG or anti-FoxP3 goat IgG and the reactivity was developed with biotinylated secondary antibody – avidin as described in the Materials and Methods section. Twenty fields were counted under the 40× objective in 2 independent experiments. Scale bars = 50 μm.

Table 1. Anti-CD25 mAb, but not control IgG (GL113), abolishes the FLY-induced rise in parasitaemia in *Trypanosoma cruzi*-infected mice

(**P* < 0.001 compared to control IgG. Data represent the mean ± S.E.M. of the parasitaemia peak.)

	Parasites per ml (× 10 ⁵)	
	GL113 (control mAb)	PC61 (anti-CD25)
PBS	28,9 ± 8,61	28,3 ± 13,8
FLY	47,8 ± 6,99	11,4 ± 5,91

DISCUSSION

It has previously been shown that the conserved VTVXNVFLYRP sequence (FLY), present on the gp85/TS glycoproteins contains a cell-binding motif and that substitution of the leucine residue for alanine (VTVXNVFAYRP, FAY sequence) abrogates its binding ability (Magdesian *et al.* 2001). Moreover, it was demonstrated that FLY, but not FAY, synthetic peptide phosphorylates ERK1/2, dephosphorylates cytokeratin 18 and increases infection of LLC-MK₂ cells by *T. cruzi* (Magdesian *et al.* 2007).

In the present study, the potentiating effect of the peptide FLY on the infection by *T. cruzi* previously

described *in vitro*, is now confirmed *in vivo*. BALB/c mice administered intraperitoneally with a single dose of FLY have increased parasitaemia, higher mortality and an increment in tissue parasitism in the bladder (7.6-fold), small intestine (3.6-fold) and heart (3-fold), suggesting that FLY may be partially responsible for the virulence of trans-sialidase, previously described in the literature (Chuenkova and Pereira, 1995), which is explained by the sialylation of CD8⁺ T cell surface, dampening the antigen-specific response and favouring the persistence of the parasite in the host (Freire-de-Lima *et al.* 2010). However, the virulence of FLY has to occur by a different mechanism, since it lacks trans-sialidase activity. It is tempting to speculate that the host immune response to different segments of distinct members of the gp85/TS superfamily expressed by the parasite, may be relevant for the final outcome of the parasitaemia – inhibition or enhancement – during infection. In this context, it is important to mention that protection against *T. cruzi* infection was described for other members of the gp85/TS superfamily, such as the gp82 and gp90 proteins (Santori *et al.* 1996), devoid of trans-sialidase activity.

Since the heart is a main target organ in Chagas' disease, this study was concentrated on that organ. In agreement with the increment of tissue parasitaemia,

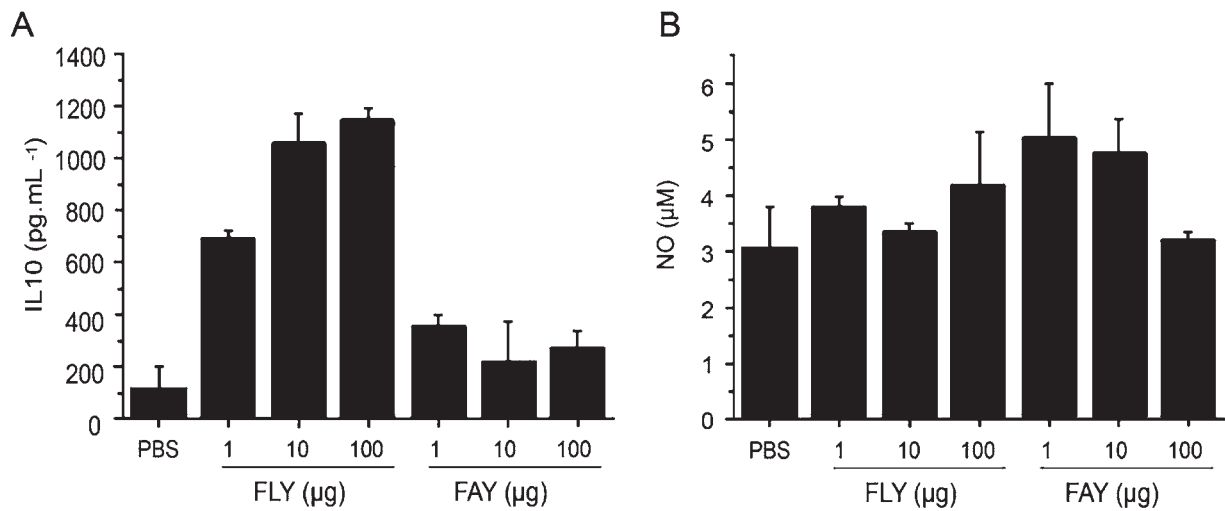


Fig. 5. IL10 and NO production by peritoneal cells stimulated with different concentrations of FLY. Peritoneal cells were treated with the indicated amounts of FLY, FAY or culture medium (control) for 24 h. After incubation, the supernatants were removed and used to measure IL10 (A) and NO (B) by ELISA. Results represent mean \pm standard deviation (S.D.) from triplicate experiments.

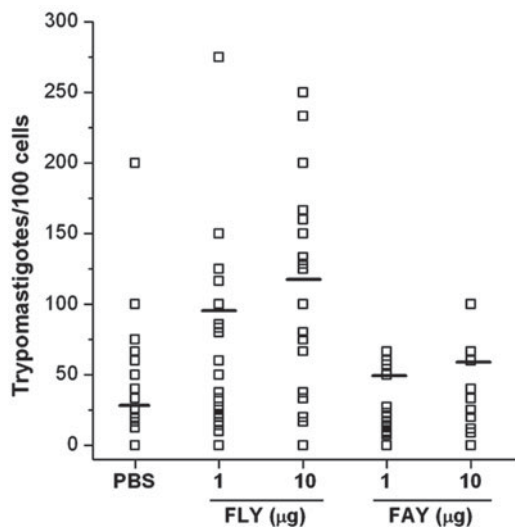


Fig. 6. Resident peritoneal cells were incubated for 30 min with PBS, FLY or FAY peptides and then infected with 100 trypanomastigotes/cell for 1 h. Non-associated trypanomastigotes were removed, the cells were fixed with methanol and DNA stained. Fifty photos of each experiment were made, enabling the counting of parasites/100 cells. Each open square represents the number of parasites/100 cells present in each photo. Black lines represent the average number of parasites/100 cells. Significant differences were obtained between the infection in the presence of 1 μ g of FLY or FAY ($P < 0.0002$) or 10 μ g of FLY and FAY ($P < 0.002$). There were no significant differences between the controls ($P > 0.5$).

a more intense inflammatory response was detected in the heart of both FLY-primed and infected animals. The higher parasitaemia in FLY-treated animals, as compared to controls, could not be explained by differences in NO production, since similar amounts of labelled fields for NO synthase were detected

among the groups. Interestingly, a 5-fold increment in the number of $CD4^+CD25^+FoxP3^+$ T cells (T regulatory cells) was detected in the hearts of both FLY-primed and infected animals, which could be due to either local expansion of Tregs or their preferential homing at the inflammatory site. The latter possibility may be favoured by the more intense inflammation observed in FLY-treated mice and by the previous observation that the production of CCL4, a potent chemoattractant of $CD4^+CD25^+$ T in mice, overlaps with maximum tissue parasitism in *T. cruzi* (Sales *et al.* 2008). Additionally, FLY-induced IL10 production by resident peritoneal cells, in association with the inflammatory environment, may contribute to the increment of Tregs in heart tissues. It is now established that immune mediators (such as IL10 and $TGF\beta$) drive the induction and expansion of Tregs, albeit with quite distinct phenotypes (Collison *et al.* 2009). IL10 produced by cells other than T cells was required to maintain the expression of FoxP3 in Treg cells, despite their own ability to produce IL10, as shown recently in a murine model of colitis (Murai *et al.* 2009). Also, IL10, IL2 and $TGF\beta$ mediate Treg cell induction displaying high expression of FoxP3, independently of direct TCR stimulation, in infection by *Plasmodium falciparum* (Scholzen *et al.* 2009). Whatever the mechanism might be, the increment of the Treg population may contribute to the local tolerance to infection by *T. cruzi*, as described also for *Plasmodium yoelii* (Hisaeda *et al.* 2004; Chen *et al.* 2009) and *Leishmania major* (Belkaid *et al.* 2002a,b; Rouse and Suvas, 2004; Belkaid and Rouse, 2005). Moreover, depletion of $CD4^+CD25^+$ T with anti-CD25 antibody abolished the FLY-induced rise in parasitaemia.

The role of Treg in the infection by *T. cruzi* is controversial, with data suggesting that depletion of

Treg cells by anti-CD25 antibodies does not alter the outcome of the immune response (Kotner and Tarleton, 2007) or confer more resistance to infection in mice (Sales *et al.* 2008). In contrast, the involvement of Treg cells in the acute phase of *T. cruzi* infection has been described, with migration of Treg cells to the heart of infected mice and an increased mortality of anti-CD25-treated animals (Mariano *et al.* 2008). The conflicting results may be due to experimental protocols, distinct strains of mice and/or differences in the parasites employed, as pointed out (Mariano *et al.* 2008). In addition, Tregs were also implicated in the human immune response against *T. cruzi*. A higher frequency of circulating Tregs was found in patients with the indeterminate form in relation to the cardiac form of the disease, with the majority of CD4⁺CD25⁺ cells expressing FoxP3 (Araújo *et al.* 2007; Sathler-Avelar *et al.* 2009).

Taken together, our data suggest the importance of Treg cells in relation to the specific effect of FLY in the exacerbation of the infection by *T. cruzi*, although other activated CD25-expressing CD4⁺ T cells (Curotto de Lafaille and Lafaille, 2009) may concur to the same effect.

How FLY potentiates *T. cruzi* infection *in vivo* is not clear. FLY may act locally and/or systemically by independent, and not exclusive, mechanisms. A possible mechanism involves the dissemination of highly infected peritoneal cells to other organs/tissues, allowing the parasite to subvert the host immune system and to propagate. One example of this strategy was described for *Toxoplasma gondii*, where the adoptive transfer of *Toxoplasma*-infected dendritic cells in mice resulted in more rapid dissemination of the parasites to distant organs and exacerbation of infection when compared with intraperitoneal incubation with free parasites (Lambert *et al.* 2006).

Phagocytic removal of apoptotic cells, which appear in the course of the immune response, promotes the secretion of cytokines that increase, in the particular case of *T. cruzi*, intracellular replication of the parasite and parasitaemia in infected mice (Freire-de-Lima *et al.* 2000). In addition, the uptake of the parasites is enhanced in the presence of cytokines (Aliberti *et al.* 1999) and the binding to *T. cruzi* of CCL2, one of the murine β -chemokines produced by macrophages and cardiomyocytes infected with the parasite, induces chemoattraction and morphogenesis of the infective form of *T. cruzi*. In this model, chemokines could attract and facilitate *T. cruzi* uptake, with implications for the migration of the parasite to different tissues (Yamauchi *et al.* 2007).

Incubation of resident peritoneal cells with FLY results in a higher number of cells infected by *T. cruzi*. This is a similar biological effect to that previously obtained employing an epithelial cell line, where an increased number of infected cells, as well as a higher number of parasites/cell, were observed, suggesting that FLY facilitates infection of the same cell, by a

mechanism not fully understood (Magdesian *et al.* 2007).

The FLY peptide can exert its function through a cell surface receptor as previously suggested (Magdesian *et al.* 2001) and/or inside the cells after internalization by the same mechanism described for the cell-penetrating peptides (CPPs) (Patel *et al.* 2007a,b), since FLY contains a small cluster of 2 lysines and 1 arginine, essential residues for the internalization of CPPs.

One point to be addressed relates to how the FLY domain is exposed to the host in a natural infection. We hypothesized that membrane vesicles shed by *Trypanosoma cruzi in vitro* and *in vivo* (Gonçalves *et al.* 1991) may act as cargo for the delivery of the FLY sequence. FLY is a conserved domain of the gp85/TS glycoproteins, abundant in membrane vesicles, which are internalized by cells, including macrophages, enhancing *T. cruzi* infection *in vitro* and heart parasitism in mice and IL10 production (Torrecilhas *et al.* 2009). However, since FLY is not apparently exposed on the surface of these molecules, as judged by the structure of TS (Buschiazzi *et al.* 2002) and Tc-85 (Marroquin-Quelopana *et al.* 2004) a molecular modification by proteolysis or conformational changes have to be hypothesized in order to envisage a role for FLY *in vivo*.

In summary, the results herein presented suggest that the *T. cruzi*-borne FLY domain, may act as a virulence factor in the animal model by altering the host cell-mediated immune response critical to combat the infection by the parasite.

ACKNOWLEDGEMENTS

The helpful critical comments on the manuscript by Drs José Maria Mosig and Marcello Barcinski are deeply acknowledged. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grant #2004/03303-5) and CNPq. RRT and ACT were Post-doctoral Fellows supported by FAPESP.

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